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Pre-TCR Signaling and Inactivation of p53 Induces Crucial Cell Survival Pathways in Pre-T Cells

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Summary

Signaling through the pre-TCR is essential for early T cell development and is severely impaired in mice lacking the CD3γ chain of the pre-TCR. We here address the molecular mechanisms underlying this defect. Impaired pre-TCR signaling is shown to be associated with a profound increase in the number of apoptotic CD4$^-$CD8$^-$ (DN) thymocytes. Introduction of p53 deficiency into CD3γ-deficient mice completely reverses the cell survival defect in CD3γ-deficient DN thymocytes and rescues the block in pre-T cell differentiation. In addition, the CD4$^+$CD8$^+$ (DP) compartment is expanded to its normal size. These findings suggest that the pre-TCR regulates progression through the DNA-damage checkpoint of the DN to DP transition by inactivating p53.

Introduction

The successive developmental stages of intrathymic T cell differentiation can be extraordinarily well defined on the basis of sequential loss and gain of cell surface expression of a number of key markers (reviewed in Fehling and Von Boehmer, 1997; Borst et al., 1996; Haks et al., 1999). The most immature thymocytes reside within the CD4$^-$CD8$^-$ double-negative (DN) population, comprising only about 1%–3% of all cells in the adult thymus. This DN population can be further subdivided into at least four distinct developmental stages that are defined by the differential expression of CD25 (IL-2 receptor α chain), CD44 (Pgp-1), and CD117 (c-kit) (Wu et al., 1991, 1996b; Godfrey et al., 1993, 1994; Jacobs et al., 1994; Wilson et al., 1994; Shortman and Wu, 1996). One of the first major checkpoints thymocytes encounter during their developmental program is the transition, within the CD117$^-$ DN population, from the CD44$^-$CD25$^+$ to the CD44$^-$CD25$^-$ pre-T cell stage, and we here address how that transition is regulated.

Only cells that produce a functional TCRβ protein expand and proceed from the CD44$^-$CD25$^+$ to the CD44$^-$CD25$^-$ DN stage. This process is referred to as "β-selection" and is mediated by the pre-TCR (Dudley et al., 1994; reviewed in Levelt and Eichmann, 1995; Fehling and Von Boehmer, 1997). This pre-TCR is a multi-subunit receptor complex consisting of a heterodimer of a conventional TCRβ chain and a nonrearranging pTα chain, as well as noncovalently linked CD3 subunits (Groettrup et al., 1993; Saint-Ruf et al., 1994; Van Oers et al., 1995; reviewed in Borst et al., 1996; Malissen and Malissen, 1996; Berger et al., 1997; Haks et al., 1998). Thymocytes from mice that cannot produce an essential component of the pre-TCR (Mombaerts et al., 1992a, 1992b; Shinkai et al., 1992; Liu et al., 1993; Love et al., 1993; Malissen et al., 1993, 1995; Ohno et al., 1993; Fehling et al., 1995; DeJarnette et al., 1998; Haks et al., 1998; Wang et al., 1998) are arrested at the CD44$^-$CD25$^+$ DN stage of their developmental program (reviewed in Fehling and Von Boehmer, 1997; Haks et al., 1999).

Functioning of the pre-TCR critically depends on the signal transduction capacity of the CD3 complex. All CD3 subunits involved in the formation of a mature TCRαβ-CD3 complex (CD3γ, CD3δ, CD3ε, and CD3ζ) have now been demonstrated to also be present in the pre-TCR, and CD3γ and CD3ε perform essential functions in this receptor complex (Liu et al., 1993; Love et al., 1993; Malissen et al., 1993, 1995; Ohno et al., 1993; Van Oers et al., 1995; Berger et al., 1997; Haks et al., 1998; DeJarnette et al., 1998; Wang et al., 1998). Signaling through the pre-TCR is associated with a complex cluster of differentiation events including downregulation of CD25, induction of CD4 and CD8 expression, and cell cycle entry followed by intense cell proliferation (Hoffman et al., 1996; reviewed in Fehling and Von Boehmer, 1997; Haks et al., 1999). In addition, further rearrangements at the TCRβ locus are terminated, resulting in allelic exclusion and ensuring the generation of DP thymocytes with a single productive TCRβ chain (Aifantis et al., 1997; Krotkova et al., 1997; Ardouin et al., 1998).

An important issue that still needs to be addressed is whether the primary role of the pre-TCR in early T cell development is enabling or inductive. While it is clear that pre-TCR derived signals trigger crucial cues for further development, it is not clear whether the pre-TCR itself actually transmits differentiative signals or whether it, by providing survival and/or expansion signals, allows differentiation steps triggered by other pathways to occur. Nevertheless, the signal transduction pathways used by the pre-TCR to control thymocyte differentiation involve the activation of Src-protein tyrosine kinases (PTKs) (reviewed in Eichmann, 1998; Tybulewicz, 1998; Haks et al., 1999), and several lines of evidence have highlighted in particular the importance of the Src-family PTK p56^{lck} in pre-TCR signaling (Molina et al., 1992; Anderson et al., 1993; Levin et al., 1993; Mombaerts et al., 1994; Levelt et al., 1995; Wallace et al., 1995; Groves et al., 1996; Van Oers et al., 1996; Fehling et al., 1997; Schmedt et al., 1998). In addition, some suggestions for the involvement of p53 in the transition from the DN to the DP stage have been generated. The importance of the p53 tumor suppressor gene as a regulator of the cell cycle and apoptosis in response to DNA damage arising

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from treatment with genotoxic mediators has been well established. Transcriptional activation of the cyclin-dependent kinase inhibitor $p21^{waf-1/cip-1}$ appears to mediate p53-induced growth arrest (El-Deiry et al., 1993; Harper et al., 1993), and apoptosis is promoted by repressing Bcl-2 and upregulating death genes such as Bax (Livingstone et al., 1992; reviewed in Hunter, 1993; Miyashita et al., 1994; Miyashita and Reed, 1995; Ko and Prives, 1996; White, 1996; Agarwal et al., 1998). Whether p53 also plays a role in response to DNA damage arising under physiological conditions, such as site-specific V(D)J recombination of the TCRβ locus in T cell precursors, is only beginning to be addressed. SCID × p53-deficient mice are permissive for the generation of low numbers of DP thymocytes, supporting the notion of a correlation between inactivation of the p53 gene and progression beyond the DN stage (Bogue et al., 1996; Guidos et al., 1996; Nacht et al., 1996). Since p53 deficiency also induces some DP thymocytes in RAG-deficient mice (Jiang et al., 1996) (although at a much lower frequency compared to SCID × $p53^{-/-}$ mice), while RAG-deficient thymocytes do not undergo TCR gene rearrangements, it is difficult to interpret these findings.

We here examine the role of the pre-TCR in suppressing a dominant programmed cell death pathway operating in DN thymocytes. First, we show that mice lacking a CD3γ chain have a pre-TCR signaling defect and a survival defect at the DN stage of development. We then investigated the possible influence of Bcl-2 and p53 genes on promoting differentiation to the DP stage and the survival potential of DN thymocytes that are defective in pre-TCR-mediated signaling. Although transgenic Bcl-2 had no impact on exit from the $CD44^{-}CD25^{+}$ DN stage and transition to the DP compartment in mice lacking a functional pre-TCR, loss of function of p53 restored pre-T cell development in such a setting. In addition, DN thymocytes in mice lacking a functional pre-TCR are rescued from apoptotic cell death when a p53 deficiency is introduced. These data implicate p53 as a candidate downstream target molecule of the pre-TCR signaling cascade.

Results

In CD3γ-Deficient Mice, Impaired Pre-T Cell Development Is a Consequence of Defective Signaling through the Pre-TCR

Transition from the $CD44^{-}CD25^{+}$ to the $CD44^{-}CD25^{-}$ DN stage of T cell development is under the control of the pre-TCR and severely impaired in CD3γ-deficient mice, with only very few cells progressing to the DP and SP stage (Haks et al., 1998; Figure 2A). We asked whether the few DP cells that are generated reflect aberrant signaling through the pre-TCR. In normal mice, thymocytes undergo a maturational program during the DN to DP transition that includes upregulation of CD5 and downregulation of CD24 (HSA) and CD25 (Hough et al., 1994; reviewed in Fehling and Von Boehmer, 1997). In CD3γ-deficient mice, DP thymocytes express higher levels of CD24, lower levels of CD5, and approximately ~55%–70% of the DP thymocytes still express CD25.

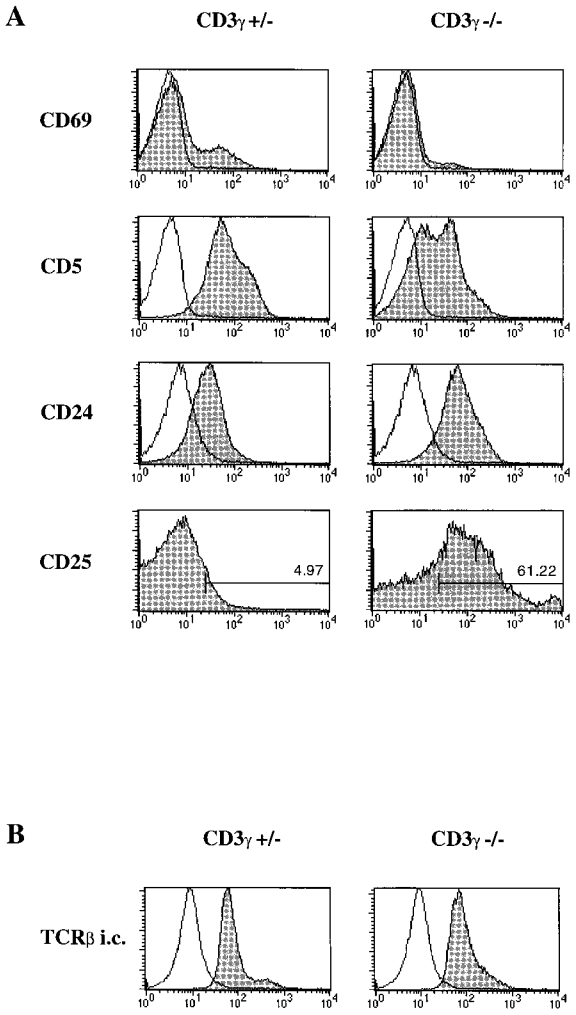
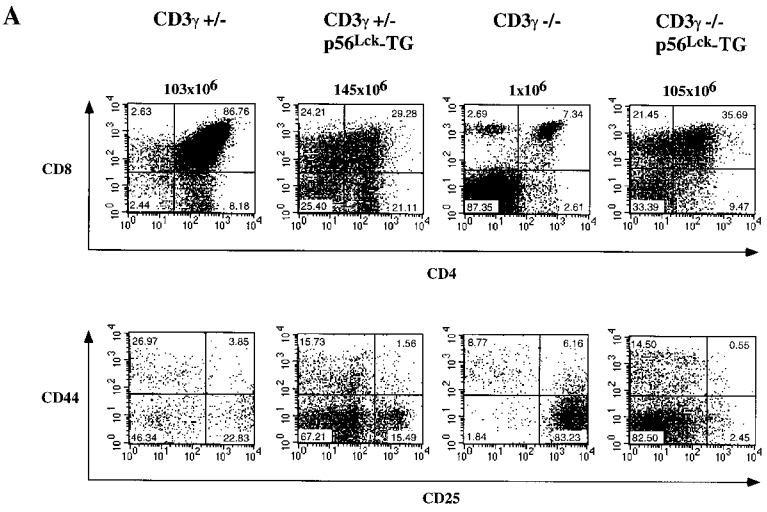


Figure 1. DP Thymocytes of CD3γ-Deficient Mice Display an Abnormal Phenotype

Electronically gated DP thymocytes from 6- to 8-week-old $CD3\gamma^{+/-}$ and $CD3\gamma^{-/-}$ mice were analyzed by three-parameter flow cytometry for (A) cell surface expression of CD69-FITC, CD5-FITC, CD24-PE, and CD25-Biotin plus SA-Tricolor (shaded areas) or an irrelevant mAb (open areas) and (B) intracellular expression of TCRβ-FITC (shaded areas) or an irrelevant mAb (open areas).

In heterozygous mice, this latter $CD25^{+}$ population comprises only 1%–5% (Figure 1A). These data indicate that DP thymocytes in CD3γ-deficient mice have been generated by aberrant signaling through the pre-TCR complex. It should be noted that the DP thymocytes generated in the absence of CD3γ do belong to the αβ T cell lineage, since all DP thymocytes express high levels of intracellular TCRβ chains (Figure 1B). Importantly, the percentage of CD69-positive DP thymocytes in CD3γ-deficient mice is reduced (~4% compared to ~20% in $CD3\gamma^{+/-}$ mice), suggesting inefficient positive selection in the absence of CD3γ.

To further address whether impaired progression beyond the DN stage is a consequence of defective pre-TCR signaling in CD3γ-deficient mice, mice transgenic



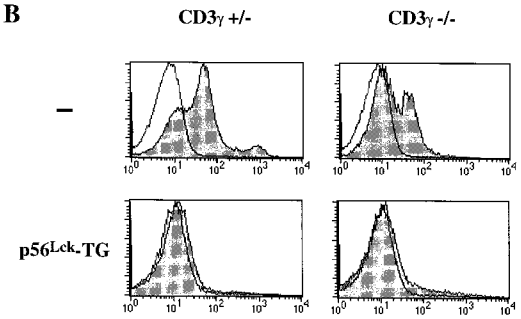


Figure 2. Transgenic p56^{lck} Can Rescue the Defect at the Pre-T Cell Stage in the Absence of CD3γ

Flow cytometry analysis of thymocytes from 6- to 8-week-old CD3γ$^{+/-}$, CD3γ$^{+/-}$p56^{lck}-TG, CD3γ$^{-/-}$, and CD3γ$^{-/-}$p56^{lck}-TG mice. (A) Total thymocytes were monitored for the expression of CD4-PE versus CD8-Biotin plus SA-Tricolor (top panel), while the expression of CD44-PE versus CD25-Biotin plus SA-Tricolor was analyzed within the CD4$^-$CD8$^-$ DN thymocyte subset (bottom panel). The percentage of cells within each quadrant is indicated. The absolute number of thymocytes detected in the different genotypes is depicted above the corresponding dot display. (B) Cell surface expression of TCRβ-FITC (shaded areas) or an irrelevant mAb (open areas) on electronically gated CD4-PE/CD8-Biotin plus SA-Tricolor DP thymocytes.

for constitutively active p56^{lck} were crossed to CD3γ-deficient mice (Figure 2). It should be noted that augmented expression of p56^{lck} results in some thymic maturational abnormalities, in that increased expression of p56^{lck} delays thymocyte development (Abraham et al., 1991a). Nevertheless, introduction of a catalytically active p56^{lck} transgene into the CD3γ mutant background restored the absolute number of thymocytes to wild-type (WT) levels and accounted for an approximately 500-fold expansion of the DP compartment (Figure 2A, top panel). More detailed analysis of the DN subset present in p56^{lck}-transgenic CD3γ$^{-/-}$ mice by monitoring for cell surface expression of CD44 and CD25 revealed that expression of a transgene encoding activated p56^{lck} in mice lacking CD3γ completely restores the transition from the CD44$^-$CD25$^+$ to the CD44$^-$CD25$^-$ DN stage (Figure 2A, bottom panel).

Another maturational event associated with signaling through the pre-TCR is termination of further rearrangements of the TCRβ genes, resulting in allelic exclusion at the TCRβ locus (Aifantis et al., 1997; Krotkova et al., 1997; Ardouin et al., 1998). It has been demonstrated that activated p56^{lck} inhibits V(D)J rearrangements, and, as a consequence, a drastic reduction in DP thymocytes expressing cell surface TCRβ is observed in p56^{lck}-transgenic mice (Anderson et al., 1992). In the absence of CD3γ, this effect of a constitutively active p56^{lck} transgene is still operational (Figure 2B), suggesting that at least in this setting induction of signals leading to allelic exclusion is independent of CD3γ. Taken together, a catalytically active p56^{lck} transgene can rescue pre-T cell development in the absence of CD3γ. This indicates that the observed developmental block in CD3γ-deficient mice is primarily due to defective pre-TCR signaling.

Lack of Pre-TCR-Mediated Signaling Correlates with Apoptotic Death

A possible primary function that has been ascribed to the pre-TCR is providing survival and/or expansion signals to DN thymocytes, allowing their differentiation to DP thymocytes triggered by other pathways to occur. A prediction based on this view is that DN thymocytes derived from mice defective in pre-TCR-mediated signaling would exhibit an increase in apoptotic cells. To explore this possibility, DN thymocytes of CD3γ$^{+/-}$, CD3γ$^{-/-}$, RAG-1$^{-/-}$, and SCID mice were compared by staining with both Annexin V and propidium iodide (PI), allowing discrimination of viable (Annexin V$^-$, PI$^-$), apoptotic (Annexin V$^+$, PI$^-$), and secondary necrotic (Annexin V$^+$, PI$^+$) cells (Vermes et al., 1995). Indeed, almost all (~95%) DN thymocytes derived from CD3γ$^{+/-}$ mice were

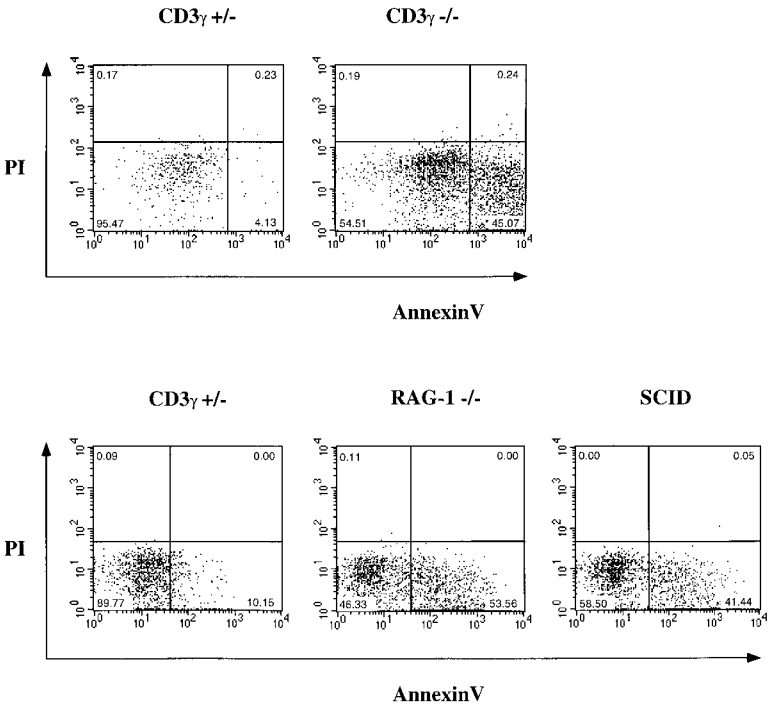


Figure 3. DN Thymocytes Defective in Pre-TCR Signaling Display an Increased Number of Apoptotic Cells

Electronically gated CD4-PE/CD8-APC DN thymocytes from 6- to 8-week-old CD3γ$^{+/-}$, CD3γ$^{-/-}$, RAG-1$^{-/-}$, and SCID mice were analyzed for the expression of Annexin V-FITC versus PI by flow cytometry. The percentage of cells within each quadrant is indicated.

alive, whereas a large proportion (~40%–55%) of DN thymocytes derived from pre-TCR signaling defective CD3γ$^{-/-}$, RAG-1$^{-/-}$, and SCID mice displayed an apoptotic phenotype (Figure 3). The correlation between lack of pre-TCR signaling and decreased cell survival suggests that blocking apoptosis is an essential function of the pre-TCR in thymocyte differentiation.

Loss of Function of p53 Rescues the Pre-T Cell Developmental Block Imposed by the Absence of CD3γ

Recently, the tumor suppressor gene product p53 has been suggested to be involved in the transition from the DN to the DP stage of thymocyte development (Bogue et al., 1996; Guidos et al., 1996; Nacht et al., 1996). A direct link between pre-TCR function and p53, however, remains to be established. To investigate this possibility, CD3γ-deficient mice that lack a functional pre-TCR were crossed to p53-deficient mice, to assess whether loss of function of p53 can overcome the defect imposed by the absence of CD3γ (Figure 4). Remarkably, inactivation of p53 in the CD3γ mutant background restored the absolute number of thymocytes to WT levels, induced the efficient generation of DP thymocytes (Figure 4A), and released the block at the CD44^{-}CD25^{+} DN stage (Figure 4B). Since the DP compartment now generated displays a diverse Vβ repertoire, as determined both by flow cytometry (Figure 4E) and RT-PCR analysis using Vβ6^{-} and Vβ8-specific primers in combination with a Jβ2.5 primer (data not shown), it does not reflect formation of tumors. In addition, the rescue imposed by p53 deficiency does not simply reflect more leakage to the DP stage, since the DP thymocytes generated in CD3γ$^{-/-}$ p53$^{-/-}$ mice, at least with respect to the expression of CD25, resemble DP thymocytes derived from heterozygous control mice (Figures 1A and 4C).

A number of observations indicate that, although inactivation of p53 rescues pre-T cell development in CD3γ-deficient mice, positive selection is not restored (Figures 4A, 4C, and 5C). First, expression of CD69 is normally upregulated by DP thymocytes that have been positively selected. In CD3γ-deficient × p53-deficient mice, however, an almost complete absence of CD69-positive DP thymocytes can be noted (Figure 4C). In addition, only very few SP thymocytes are generated in CD3γ$^{-/-}$p53$^{-/-}$ mice, compared to control mice (Figures 4A and 5C), indicating that positive selection is controlled by a p53-independent pathway.

Most importantly, comparison of the DN thymocytes derived from CD3γ$^{-/-}$ and CD3γ$^{-/-}$p53$^{-/-}$ mice by staining with both Annexin V and PI revealed that inactivation of p53 significantly increased the percentage of viable DN thymocytes from ~55% to ~95% (Figures 3 and 4D). The number of viable DN cells in CD3γ-deficient mice thus becomes equivalent to the percentage of viable DN thymocytes found in control mice by introduction of p53 deficiency. Only DN thymocytes that have produced a TCRβ chain are rescued from apoptotic cell death by loss of function of p53, since all DP thymocytes present in CD3γ$^{-/-}$p53$^{-/-}$ mice express high levels of intracellular TCRβ chains (Figure 4C). Taken together, these data are in accordance with a model implicating inactivation of p53 as a downstream event of pre-TCR signaling.

Besides Providing Survival Signals, the Pre-TCR Provides Additional Differentiative Signals

Besides the established role of p53 as a regulator of apoptosis (Livingstone et al., 1992; Miyashita et al., 1994; Miyashita and Reed, 1995), p53 also features as a possible mediator of cell cycle arrest (El-Deiry et al., 1993; Harper et al., 1993). The present finding of a rescue of

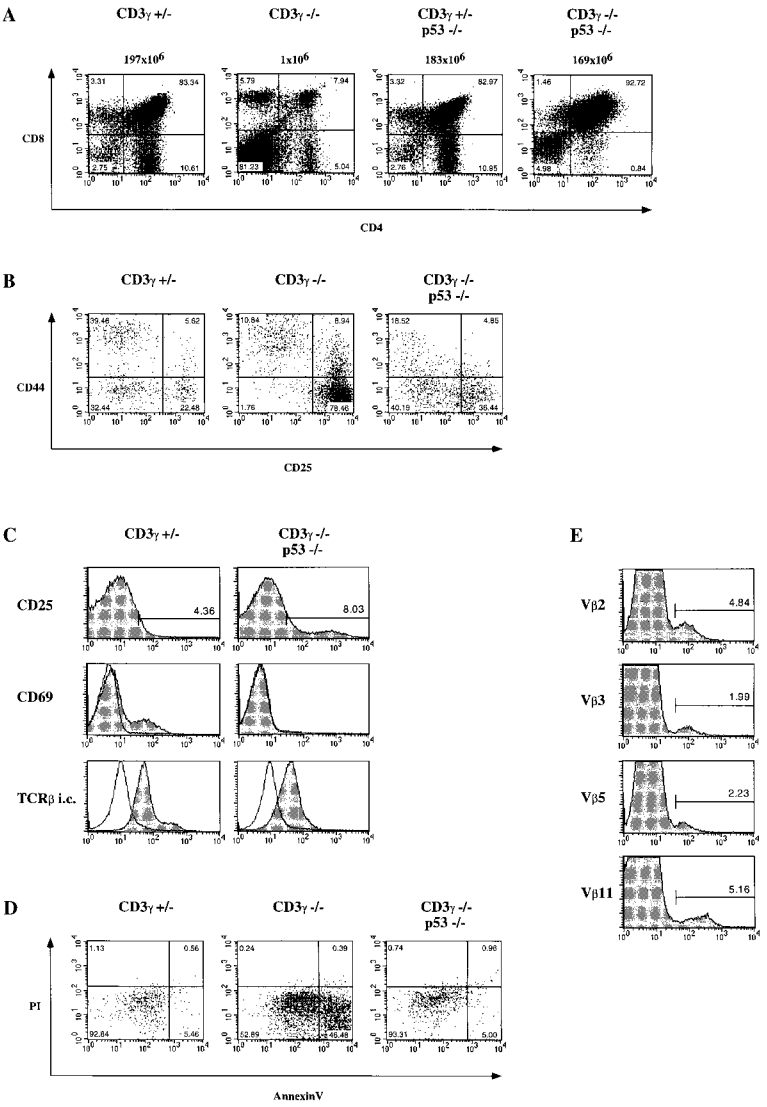


Figure 4. Inactivation of p53 Rescues the Pre-T Cell Developmental Block Imposed by the Absence of CD3γ

(A) Total thymocytes from 6-week-old CD3$\gamma^{+/-}$, CD3$\gamma^{+/-}$p53$^{-/-}$, CD3$\gamma^{-/-}$, and CD3$\gamma^{-/-}$p53$^{-/-}$ mice were monitored for the expression of CD4-PE versus CD8-Biotin plus SA-Tricolor. The percentage of cells within each quadrant is indicated. The absolute number of thymocytes detected in the different genotypes is depicted above the corresponding dot display.

(B) Expression of CD44-PE versus CD25-Biotin plus SA-Tricolor was analyzed within the CD4$^-$CD8$^-$ DN thymocyte subset of the indicated mice. The percentage of cells within each quadrant is indicated.

(C) Electronically gated DP thymocytes from CD3$\gamma^{+/-}$ and CD3$\gamma^{-/-}$p53$^{-/-}$ mice were analyzed by three-parameter flow cytometry for cell surface expression of CD25-Biotin plus SA-Tricolor and CD69-FITC (shaded areas) and intracellular expression of TCRβ-FITC (shaded areas) or an irrelevant mAb (open areas).

(D) Electronically gated CD4-PE/CD8-APC DN thymocytes from CD3$\gamma^{+/-}$, CD3$\gamma^{-/-}$, and CD3$\gamma^{-/-}$p53$^{-/-}$ mice were analyzed for the expression of Annexin V-FITC versus PI by flow cytometry. The percentage of cells within each quadrant is indicated.

(E) Analysis of intracellular Vβ chain expression on electronically gated DP thymocytes from CD3$\gamma^{-/-}$p53$^{-/-}$ mice. The percentage of positive cells is indicated.

development in mice lacking a functional pre-TCR by introduction of p53 deficiency could therefore also be explained by an effect on expansion. To investigate whether absence of CD3γ inhibits cell cycle progression at the transition from the DN to the DP stage, the cellular DNA content of CD44$^-$CD25$^-$ DN thymocytes derived from CD3$\gamma^{+/-}$ and CD3$\gamma^{-/-}$ mice (Figure 5A) and DP thymocytes derived from CD3$\gamma^{+/-}$, CD3$\gamma^{+/-}$p53$^{-/-}$, CD3$\gamma^{-/-}$, and CD3$\gamma^{-/-}$p53$^{-/-}$ mice (Figure 5B) was analyzed by staining with Hoechst 33342. In the CD44$^-$CD25$^-$ DN subset as well as the DP compartment of CD3$\gamma^{+/-}$ and CD3$\gamma^{-/-}$ mice, a comparable percentage of cycling cells (~2.5% in S phase and ~5.5% in G_2 + M phases) could be observed, indicating that cell cycle progression is not affected by the aberrant signaling through CD3γ-deficient pre-TCRs (Figures 5A and 5B, top panel). In addition, loss of function of p53 promotes cell cycle progression in CD3$\gamma^{+/-}$ as well as CD3$\gamma^{-/-}$ mice to a similar extent. Both mouse strains showed a reduction of cells in the G_1/G_0 phase of the cell cycle and a relative increase in cells in S (~9%) and G_2 + M (~12%) compared to control mice (Figure 5B).

Together with the effects of p53 on cell survival (Figure 4D), the above findings document that the rescue of pre-T cell development by p53 inactivation in pre-T cells lacking CD3γ is primarily a consequence of a release from an apoptotic pathway. We next asked whether yet additional pre-TCR-induced signals are required for the DN to DP transition, and argued that a kinetic analysis of the rescue imposed by loss of function of p53 would be informative in this regard. If p53 deficiency, by releasing the apoptotic block, was required and sufficient for triggering the DN to DP transition, its effect would be immediate. Remarkably, however, the release of the pre-T cell development block in CD3γ-deficient mice by inactivation of p53 is a time-consuming event (Figure 5C). Comparison of total thymocytes of 10-day-, 3 week-, and 6-week-old CD3$\gamma^{-/-}$ and CD3$\gamma^{-/-}$p53$^{-/-}$ mice showed that at 10 days of age, thymic cellularity as well as the CD4/CD8 profile was indistinguishable between both genotypes (Figure 5C). Starting at 2–3 weeks of age, loss of function of p53 clearly results in a progressive increase in the absolute cell number and percentage of DP thymocytes in thymi derived from

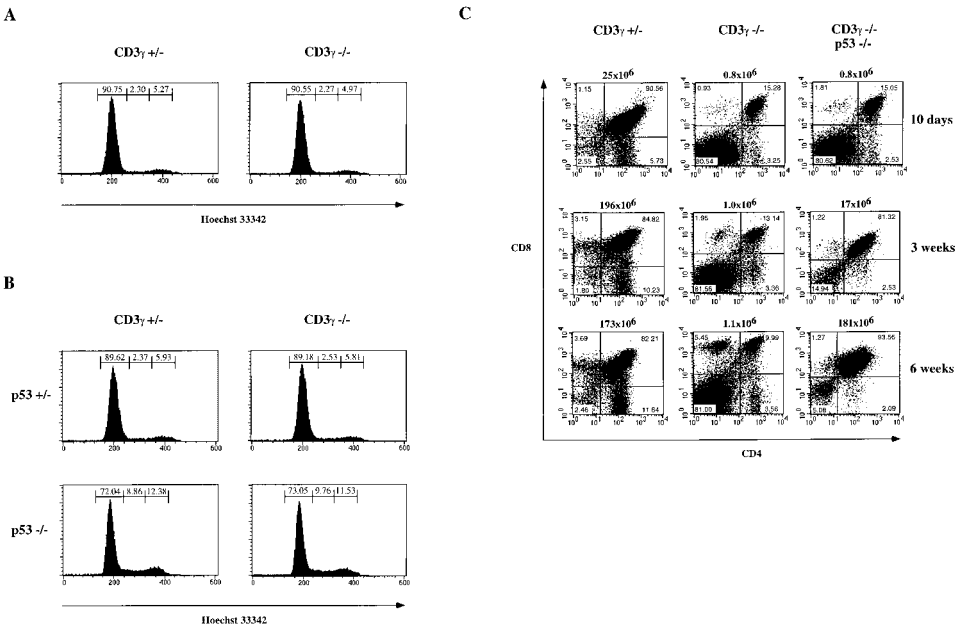


Figure 5. CD3$\gamma^{-/-}$p53$^{-/-}$ Mice Display a Delay in the Generation of a Full-Size DP Compartment that Cannot be Ascribed to a Defect in Cell Cycle Progression to the DP Stage

Cell cycle analysis of (A) CD44$^-$CD25$^-$ DN thymocytes from embryonic day 14 (E14) CD3$\gamma^{+/-}$ and CD3$\gamma^{-/-}$ mice or (B) CD8-FITC/CD4-PE DP thymocytes from 6-week-old CD3$\gamma^{+/-}$, CD3$\gamma^{+/-}$p53$^{-/-}$, CD3$\gamma^{-/-}$, and CD3$\gamma^{-/-}$p53$^{-/-}$ mice by staining cellular DNA content using Hoechst 33342. The percentage of cells within the G_1/G_0 versus S and G_2 + M stages of the cell cycle is indicated.

(C) Total thymocytes from 10-day, 3-, and 6-week-old CD3$\gamma^{+/-}$, CD3$\gamma^{-/-}$, and CD3$\gamma^{-/-}$p53$^{-/-}$ mice were monitored for the expression of CD4-PE versus CD8-Biotin plus SA-Tricolor. The percentage of cells within each quadrant is indicated. The absolute number of thymocytes detected in the different genotypes at the different ages is depicted above each corresponding dot display.

CD3$\gamma^{-/-}$p53$^{-/-}$ mice, compared to CD3$\gamma^{-/-}$ mice. Ultimately, both parameters reach wild-type levels at 5–6 weeks of age (Figures 4A and 5C). The most likely explanation for these findings is that inactivation of p53, while rescuing survival of CD3γ-deficient pre-T cells, does not rescue other pre-TCR-induced developmental signals. This strongly argues that the pre-TCR, besides providing crucial survival signals, provides additional differentiative signals that can be separated from those regulating cell cycle progression.

Transgenic Bcl-2 Does Not Rescue Pre-T Cell Development in CD3γ-Deficient Mice

Since the results outlined above document an essential role for the pre-TCR in blocking apoptosis at the pre-T cell stage, we next investigated whether Bcl-2, a protooncogene known to prolong cell survival by inhibiting programmed cell death in response to a variety of apoptotic stimuli (Korsmeyer, 1992; Oltvai and Korsmeyer, 1994), may participate in the transition from the DN to the DP stage. To address whether overexpression of Bcl-2 can rescue pre-T cell development in the absence of CD3γ, Bcl-2-transgenic mice were crossed to CD3γ-deficient mice (Figure 6). Expression of transgenic Bcl-2 had no effect on thymocyte development in mice lacking CD3γ. Thymic cellularity as well as the CD4/CD8 profile were comparable between nontransgenic and Bcl-2-transgenic CD3$\gamma^{-/-}$ mice (Figure 6A). Further analysis of the expression of CD44 and CD25 in DN thymocytes revealed also that thymocyte differentiation remained arrested at the CD44$^-$CD25$^+$ DN stage in CD3γ-deficient mice transgenic for Bcl-2 (Figure 6B). Consistent with these data, equivalent expression levels of intracellular Bcl-2 were detected in DN thymocytes derived from CD3$\gamma^{+/-}$, CD3$\gamma^{-/-}$, and RAG-1$^{-/-}$ mice, suggesting that apoptosis of DN thymocytes in the absence of pre-TCR signaling is not caused by reduced Bcl-2 expression levels (Figure 6C). In conclusion, these results suggest that pre-TCR signaling blocks a pathway to apoptosis that is insensitive to Bcl-2.

Discussion

Maturation of thymocyte precursors from the CD4$^-$ CD8$^-$ DN to the CD4$^+$CD8$^+$ DP stage of development requires productive rearrangement of the gene segments encoding the TCRβ chain (Dudley et al., 1994; Levelt and Eichmann, 1995; Hoffman et al., 1996; Fehling and Von Boehmer, 1997). Whether a functional TCRβ chain has been produced is assessed using the pre-TCR complex. One of the components of this pre-TCR complex is the CD3γ chain, and mice lacking CD3γ, due to targeted gene disruption, display dramatic defects in αβ T cell development (Haks et al., 1998). The transition from the CD44$^-$CD25$^+$ to the CD44$^-$CD25$^-$ DN stage, which is under the control of the pre-TCR, is severely impaired in these mice, resulting in a reduction in the number of DP and SP thymocytes and a concomitant drastic reduction in total thymic cellularity (Haks et al., 1998). Using these CD3γ-deficient mice, we here address which downstream effectors are involved in pre-TCR signaling.

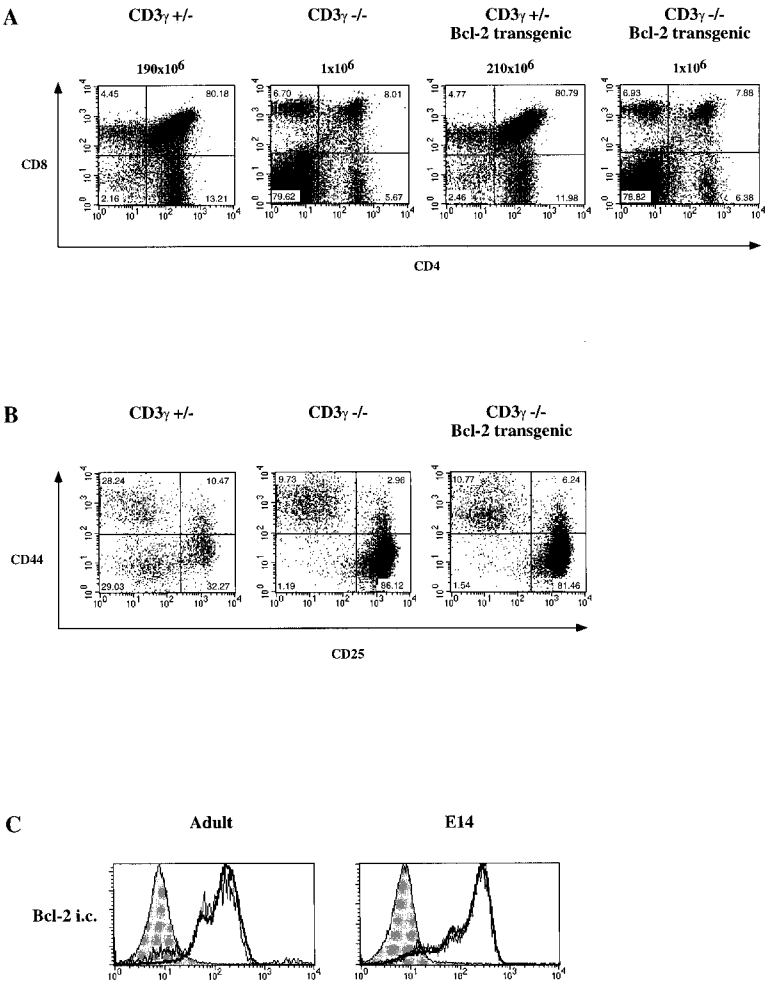


Figure 6. Transgenic Bcl-2 Does Not Rescue Pre-T Cell Development in CD3γ-Deficient Mice

(A) Total thymocytes from 6- to 8-week-old CD3$\gamma^{+/-}$, CD3$\gamma^{+/-}$Bcl-2-TG, CD3$\gamma^{-/-}$, and CD3$\gamma^{-/-}$Bcl-2-TG mice were monitored for the expression of CD4-PE versus CD8-Biotin plus SA-Tricolor. The percentage of cells within each quadrant is indicated. The absolute number of thymocytes detected in the different genotypes is depicted above the corresponding dot display.

(B) Expression of CD44-PE versus CD25-Biotin plus SA-Tricolor was analyzed within the CD4$^-$CD8$^-$ DN thymocyte subset from CD3$\gamma^{+/-}$, CD3$\gamma^{-/-}$, and CD3$\gamma^{-/-}$Bcl-2-TG mice. The percentage of cells within each quadrant is indicated.

(C) Expression of intracellular Bcl-2 (open areas) or an irrelevant mAb (shaded areas) was analyzed in CD4$^-$CD8$^-$- DN thymocytes derived from adult CD3$\gamma^{+/-}$ and CD3$\gamma^{-/-}$ mice or embryonic day 14 (E14) CD3$\gamma^{+/-}$ (thin solid line), CD3$\gamma^{-/-}$ (fat solid line), and RAG-1$^{-/-}$ (dotted line) mice.

Since we observed (Figure 3) that a large proportion of DN thymocytes in CD3γ-deficient mice have entered apoptosis, we investigated the possible connection between p53 and the pre-TCR defect of CD3γ-deficient mice. The transcription factor p53 plays a critical role in controlling expression of genes that regulate cell cycle arrest and apoptosis, and we postulated that it may also operate in the control of pre-T cell development. A first indication for a possible role of p53 in thymocyte development came from the observation that p53-deficient mice develop a high incidence of T cell lymphomas (Donehower et al., 1992; Purdie et al., 1994; reviewed in Jacks, 1996; Almog and Rotter, 1997). We found that introduction of the p53 deficiency by cross-breeding into CD3γ-deficient mice rescues DN cells from apoptosis and releases the pre-T cell developmental block, implicating p53 as a candidate downstream target molecule of the pre-TCR signaling cascade.

Our findings are consistent with a model (Figure 7) in which inactivation of p53 is placed downstream from pre-TCR signaling. p53 has been demonstrated to be involved in induction of growth arrest at the G_0/G_1 cell cycle checkpoint in response to genotoxic agents that cause DNA double-stranded breaks (DSBs) (reviewed in Jacks, 1996). Since thymocytes undergoing V(D)J recombination also slow down in cell proliferation (Pénit et al., 1995; Hoffman et al., 1996), activation of p53 may initially induce a growth arrest of pre-T cells. Presumably, in the event that a pre-T cell does not produce a functional pre-TCR, p53 accumulates further as a consequence of lack of pre-TCR signaling, and this ultimately results in apoptosis. In support of this view, it has been demonstrated that the nature of the effect of p53 (cell cycle arrest versus apoptosis) in certain cell types is controlled in a quantitative manner (Chen et al., 1996). Following productive TCRβ rearrangements and composition of the pre-TCR, pre-TCR-mediated signaling inactivates p53, resulting in a release from the apoptotic pathway, cell cycle entry, and differentiation. This hypothesis is consistent with the finding that cells with a functional pre-TCR induce activity of the mitotic kinase complex p34^{cdc2} (Hoffman et al., 1996). Since phosphorylation of p53 by p34^{cdc2} results in a dramatic reduction of its half-life (Lin and Desiderio, 1993), pre-TCR signaling can be predicted to result in p53 inactivation. In agreement with this prediction, defective pre-TCR signaling can be overcome by introduction of p53 deficiency. Signaling through the pre-TCR may thus regulate progression through the DNA-damage checkpoint of the DN to DP transition by phosphorylating p53. It is clear that subsequent expansion and differentiation signals are then free to proceed, and one particular topic for

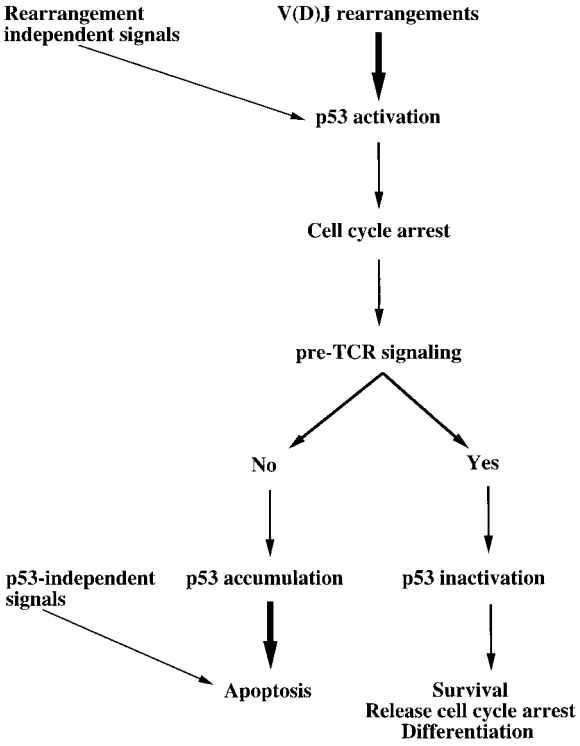


Figure 7. Model for p53-Dependent Pre-TCR Signaling

future research is to define the pathways that regulate these events.

Consistent with the view that activation of p53 may have been triggered in response to DSBs resulting from site-specific TCR gene recombinations occurring in early pre-T cells, Western blot analysis of DN thymocytes derived from SCID mice revealed substantial amounts of p53 protein (Guidos et al., 1996). Introduction of the RAG mutation into the SCID background decreased the expression of p53 protein below detection level, demonstrating that activation of p53 in SCID thymocytes requires RAG function (Guidos et al., 1996), presumably because generation of DSBs is mediated by the RAG-1 and RAG-2 proteins. Nevertheless, some rearrangement-independent activation of p53 may occur in the thymus, as immunoprecipitates of thymocyte lysates from RAG-deficient mice did reveal low levels of p53 protein (Jiang et al., 1996), and introduction of the p53 mutation into the RAG-deficient background is permissive for the development of some DP thymocytes (Jiang et al., 1996). The very fact that there is clearcut apoptosis in the DN compartment of RAG-deficient mice, despite the very marginal p53 expression, suggests that also p53-independent apoptotic pathways operate at this stage. The low protein level of p53 in RAG-deficient thymocytes, and therefore probably also of Bax, whose expression is positively regulated by p53 (Miyashita et al., 1994; Miyashita and Reed, 1995), may explain the observation that transgenic Bcl-2 does have some effect on pre-T cell development in RAG-deficient mice (Linette et al., 1994), while transgenic Bcl-2 does not affect pre-T cell development in CD3γ-deficient mice (Figure 6). Since Bcl-2 survival signals can be countered by the dominant inhibitor of Bcl-2, Bax (Oltvai et al., 1993; Yin et al., 1994; reviewed in Oltvai and Korsmeyer, 1994), the balance in Bcl-2-transgenic RAG-deficient mice is somewhat in favor of Bcl-2. However, in CD3γ-deficient mice, there is presumably a more intense activation of p53 and Bax in response to DSBs, and transgenic Bcl-2 is unable to counter their proapoptotic effects.

Since a number of findings point toward a role for the CD3 components as potential substrates and/or attachment sites for $p56^{lck}$ in the pre-TCR, we also addressed whether CD3γ is required in this regard. Our data clearly show that introduction of a constitutively active $p56^{lck}$ transgene in CD3γ-deficient mice efficiently restores pre-T cell development, documenting that CD3γ is not required for proper function of $p56^{lck}$ in pre-TCR-mediated signaling. These findings also exclude a mandatory role for the CD3γ-ITAM in recruitment of $p56^{lck}$ into the pre-TCR complex. Consistent with these findings, we previously found that the defect in pre-T cell development in CD3γ-deficient mice can be overcome by anti-CD3ε-mediated cross-linking (Haks et al., 1998), a phenomenon that has been demonstrated to be largely $p56^{lck}$ dependent (Levelt et al., 1995; Wu et al., 1996a).

In summary, the present study shows a complete rescue of the DN to DP transition by introduction of a p53 deficiency into a setting with a defined pre-TCR signaling defect. Moreover, in such a setting p53 deficiency rescues survival of DN thymocytes. Taken together, these results provide strong evidence for the notion that p53 inactivation is a crucial, pre-TCR triggered event in early T cell development. In addition, the finding that the DN to DP transition in p53-deficient mice with a pre-TCR signaling defect is a time-consuming event leads us to conclude that the search for pre-TCR triggered events is not over yet: clearly, additional differentiative signals, only poorly generated by the CD3γ-deficient pre-TCR studied here, are required for efficient differentiation to occur. Nevertheless, p53 inactivation is likely to represent the first step in the sequence of events that accompanies the DN to DP transition.

Experimental Procedures

Mice

Mice were maintained under specific pathogen-free conditions in the animal colony of the Netherlands Cancer Institute and analyzed at 6 to 8 weeks old unless indicated otherwise. C.B-17 scid (SCID) mice, mice deficient for CD3γ, RAG-1, or p53, and Bcl-2-Ig transgenic mice have been described in detail elsewhere (Bosma et al., 1983; McDonnell et al., 1989; Donehower et al., 1992; Mombaerts et al., 1992b; Haks et al., 1998).

Constitutively active $p56^{lck}$-transgenic mice were generated using the pLGF transgene described by Abraham et al. (1991a). Expression of this transgene is directed by the thymus-specific $p56^{lck}$ proximal promoter. Transgene-positive animals were identified by hybridization to a probe derived from the human growth hormone 3′ region (Abraham et al., 1991a, 1991b). Transgenic lines were maintained by crossing transgenic-positive $CD3\gamma^{+/-}$ males to $CD3\gamma^{+/-}$ female mice.

Flow Cytometry

Preparation of samples for flow cytometry analysis was performed as described (Haks et al., 1998). Cells were analyzed on a Becton-Dickinson FACScan with lysis II software. Forward- and side-scatter gating and/or propidium iodide (PI) gating was used to exclude dead cells from the analysis.

For an intracellular staining, cells were first stained for cell surface markers. Subsequently, cells were washed twice in PBA (1 × PBS, 1% BSA, and 0.02% NaN_3) containing 0.1% Saponin (Merck) and then incubated with mAb diluted in PBA containing 0.25% Saponin for 20–30 min at 4°C. Cells were washed twice in 100 μl of PBA containing 0.1% Saponin and if applicable incubated with second-step reagent in PBA containing 0.25% Saponin. Finally, all cells were washed twice and resuspended in 100 μl of PBA. Cells were analyzed on a Becton-Dickinson FACSCalibur.

Biotinylated, FITC-, PE-, or APC-conjugated antibodies specific for murine CD5 (clone 53-7.3), CD8α (clone 53-6.7), CD8β (clone 53-5.8), CD24 (HSA; clone M1/69), CD25 (clone 7D4), CD44 (clone IM7), CD69 (clone H1.2F3), TCRβ (clone H57-597), Vβ2 (clone B20.6), Vβ3 (clone KJ25), Vβ5.1,5.2 (clone MR9-4), Vβ11 (clone RR3-15), and purified hamster anti-mouse Bcl-2 (clone 3F11) were obtained from PharMingen. R-PE anti-mouse CD4 (clone CT-CD4) was purchased from Caltag. Where appropriate, streptavidin (SA)-Tricolor (Caltag) or goat anti-hamster IgG-FITC (Caltag) were used as second-step reagents.

Apoptosis Assay

Flow cytometry analysis of apoptotic cells was performed using an APOPTEST-FITC kit according to the manufacturer's protocol (Nexins Research). In brief, after staining of the cells for cell surface markers, cells were washed twice in PBA and resuspended in 1× binding buffer at 1×10^5 to 1×10^6 cells/ml. Annexin V-FITC and PI (2.5 μg/ml final concentration) were added and incubated in the dark for 10 min at 4°C. Thereafter, cells were immediately analyzed on a FACSCalibur.

Cell Cycle Analysis

Cellular DNA content was determined by Hoechst 33342 (Sigma) staining. Total thymocytes were cell surface stained with FITC- and PE-conjugated mAbs. Cells were then washed twice in 100 μl PBA and fixed in PBS containing 1% paraformaldehyde (PFA) for 30 min at 4°C. After washing twice in 100 μl PBA and once in 100 μl PBA containing 0.3% Saponin, cells were incubated with Hoechst 33342 (10 μg/ml diluted in PBA containing 0.3% Saponin) for 30 min at 37°C. Finally, cells were washed once in 100 μl PBA containing 0.3% Saponin, resuspended in 100 μl PBA, and analyzed on a FACStar^{plus} using an ultraviolet light source measuring blue fluorescence from Hoechst 33342 between 390 and 489 nM.

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References

Abraham, K.M., Levin, S.D., Marth, J.D., Forbush, K.A., and Perlmutter, R.M. (1991a). Delayed thymocyte development induced by augmented expression of p56*lck*. J. Exp. Med. *173*, 1421–1432.

Abraham, K.M., Levin, S.D., Marth, J.D., Forbush, K.A., and Perlmutter, R.M. (1991b). Thymic tumorigenesis induced by overexpression of $p56^{lck}$. Proc. Natl. Acad. Sci. USA *88*, 3977–3981.

Agarwal, M.L., Taylor, W.R., Chernov, M.V., Chernova, O.B., and Stark, G.R. (1998). The p53 network. J. Biol. Chem *273*, 1–4.

Aifantis, I., Buer, J., Von Boehmer, H., and Azogui, O. (1997). Essential role of the pre-T cell receptor in allelic exclusion of the T cell receptor β locus. Immunity *7*, 601–607.

Almog, N., and Rotter, V. (1997). Involvement of p53 in cell differentiation and development. Biochim. Biophys. Acta *1333*, F1–F27.

Anderson, S.J., Abraham, K.M., Nakayama, T., Singer, A., and Perlmutter, R.M. (1992). Inhibition of T-cell receptor β-chain gene rearrangement by overexpression of the non-receptor protein tyrosine kinase p56*lck*. EMBO J. *11*, 4877–4886.

Anderson, S.J., Levin, S.D., and Perlmutter, R.M. (1993). Protein tyrosine kinase $p56^{lck}$ controls allelic exclusion of T-cell receptor β-chain genes. Nature *365*, 552–554.

Ardouin, L., Ismaili, J., Malissen, B., and Malissen, M. (1998). The CD3-γδε and CD3-ζ/η modules are each essential for allelic exclusion at the T cell receptor β locus but are both dispensable for the initiation of V to (D)J recombination at the T cell receptor-β, -γ, and -δ loci. J. Exp. Med. *187*, 105–116.

Berger, M.A., Davé, V., Rhodes, M.R., Bosma, G.C., Bosma, M.J., Kappes, D.J., and Wiest, D.L. (1997). Subunit composition of pre-T cell receptor complexes expressed by primary thymocytes: CD3δ is physically associated but not functionally required. J. Exp. Med. *186*, 1461–1467.

Bogue, M.A., Zhu, C., Aguilar-Cordova, E., Donehower, L.A., and Roth, D.B. (1996). p53 is required for both radiation-induced differentiation and rescue of *V(D)J* rearrangement in scid mouse thymocytes. Genes Dev. *10*, 553–565.

Borst, J., Jacobs, H., and Brouns, G. (1996). Composition and function of T-cell receptor and B-cell receptor complexes on precursor lymphocytes. Curr. Opin. Immunol. *8*, 181–190.

Bosma, G.C., Custer, R.P., and Bosma, M.J. (1983). A severe combined immunodeficiency mutation in the mouse. Nature *301*, 527–530.

Chen, X., Ko, L.J., Jayaraman, L., and Prives, C. (1996). p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells. Genes Dev. *10*, 2438–2451.

DeJarnette, J.B., Sommers, C.L., Huang, K., Woodside, K.J., Emmons, R., Katz, K., Shores, E.W., and Love, P.E. (1998). Specific requirement for CD3ε in T cell development. Proc. Natl. Acad. Sci. USA *95*, 14909–14914.

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Jr., Butel, J.S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature *356*, 215–221.

Dudley, E.C., Petrie, H.T., Shah, L.M., Owen, M.J., and Hayday, A.C. (1994). T cell receptor β chain gene rearrangement and selection during thymocyte development in adult mice. Immunity *1*, 83–93.

Eichmann, K. (1998). The *Lck* paradox: because of inconsistent experimental evidence, the role of the protein tyrosine kinase $p56^{lck}$ in early thymic development remains poorly defined. Dev. Immunol. *6*, 19–24.

El-Deiry, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W., and Vogelstein, B. (1993). *WAF1*, a potential mediator of p53 tumor suppression. Cell *75*, 817–825.

Fehling, H.J., and Von Boehmer, H. (1997). Early αβ T cell development in the thymus of normal and genetically altered mice. Curr. Opin. Immunol. *9*, 263–275.

Fehling, H.J., Krotkova, A., Saint-Ruf, C., and Von Boehmer, H. (1995). Crucial role of the pre-T-cell receptor α gene in development of αβ but not γδ T cells. Nature *375*, 795–798.

Fehling, H.J., Iritani, B.M., Krotkova, A., Forbush, K.A., Laplace, C., Perlmutter, R.M., and Von Boehmer, H. (1997). Restoration of thymopoiesis in $pT\alpha^{-/-}$ mice by anti-CD3ε antibody treatment or with transgenes encoding activated Lck or tailless pTα. Immunity *6*, 703–714.

Godfrey, D.I., Kennedy, J., Suda, T., and Zlotnik, A. (1993). A developmental pathway involving four phenotypically and functionally distinct subsets of $CD3^-CD4^-CD8^-$ triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. J. Immunol. *150*, 4244–4252.

Godfrey, D.I., Kennedy, J., Mombaerts, P., Tonegawa, S., and Zlotnik, A. (1994). Onset of TCR-β gene rearrangement and role of TCR-β expression during $CD3^-CD4^-CD8^-$ thymocyte differentiation. J. Immunol. *152*, 4783–4792.

Groettrup, M., Ungewiss, K., Azogui, O., Palacios, R., Owen, M.J.,

Hayday, A.C., and Von Boehmer, H. (1993). A novel disulfide-linked heterodimer on pre-T cells consists of the T cell receptor β chain and a 33 kd glycoprotein. Cell *75*, 283–294.

Groves, T., Smiley, P., Cooke, M.P., Forbush, K., Perlmutter, R.M., and Guidos, C.J. (1996). Fyn can partially substitute for Lck in T lymphocyte development. Immunity *5*, 417–428.

Guidos, C.J., Williams, C.J., Grandal, I., Knowles, G., Huang, M.T.F., and Danska, J.S. (1996). V(D)J recombination activates a p53-dependent DNA damage checkpoint in *scid* lymphocyte precursors. Genes Dev. *10*, 2038–2054.

Haks, M.C., Krimpenfort, P., Borst, J., and Kruisbeek, A.M. (1998). The CD3γ chain is essential for development of both the TCRαβ and TCRγδ lineages. EMBO J. *17*, 1871–1882.

Haks, M.C., Oosterwegel, M.A., Blom, B., Spits, H., and Kruisbeek, A.M. (1999). Cell-fate decisions in early T cell development: regulation by cytokine receptors and the pre-TCR. Sem. Immunol. *11*, 23–37.

Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K., and Elledge, S.J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell *75*, 805–816.

Hoffman, E.S., Passoni, L., Crompton, T., Leu, T.M.J., Schatz, D.G., Koff, A., Owen, M.J., and Hayday, A.C. (1996). Productive T-cell receptor β-chain gene rearrangement: coincident regulation of cell cycle and clonality during development in vivo. Genes Dev. *10*, 948–962.

Hough, M.R., Takei, F., Humphries, R.K., and Kay, R. (1994). Defective development of thymocytes overexpressing the costimulatory molecule, heat-stable antigen. J. Exp. Med. *179*, 177–184.

Hunter, T. (1993). Braking the cycle. Cell *75*, 839–841.

Jacks, T. (1996). Lessons from the *p53* mutant mouse. J. Cancer Res. Clin. Oncol. *122*, 319–327.

Jacobs, H., Vandeputte, D., Tolkamp, L., De Vries, E., Borst, J., and Berns, A. (1994). CD3 components at the surface of pro-T cells can mediate pre-T cell development in vivo. Eur. J. Immunol. *24*, 934–939.

Jiang, D., Lenardo, M.J., and Zuniga-Pflücker, J.C. (1996). p53 prevents maturation to the $CD4^+CD8^+$ stage of thymocyte differentiation in the absence of T cell receptor rearrangement. J. Exp. Med. *183*, 1923–1928.

Ko, L.J., and Prives, C. (1996). p53: puzzle and paradigm. Genes Dev. *10*, 1054–1072.

Korsmeyer, S.J. (1992). Bcl-2: a repressor of lymphocyte death. Immunol. Today *13*, 285–288.

Krotkova, A., Von Boehmer, H., and Fehling, H.J. (1997). Allelic exclusion in pTα-deficient mice: no evidence for cell surface expression of two T cell receptor (TCR)-β chains, but less efficient inhibition of endogeneous Vβ→(D)Jβ rearrangements in the presence of a functional TCR-β transgene. J. Exp. Med. *186*, 767–775.

Levelt, C.N., and Eichmann, K. (1995). Receptors and signals in early thymic selection. Immunity *3*, 667–672.

Levelt, C.N., Mombaerts, P., Wang, B., Kohler, H., Tonegawa, S., Eichmann, K., and Terhorst, C. (1995). Regulation of thymocyte development through CD3: functional dissociation between p56*lck* and CD3ζ in early thymic selection. Immunity *3*, 215–222.

Levin, S.D., Anderson, S.J., Forbush, K.A., and Perlmutter, R.M. (1993). A dominant-negative transgene defines a role for p56*lck* in thymopoiesis. EMBO J. *12*, 1671–1680.

Lin, W.-C., and Desiderio, S. (1993). Regulation of V(D)J recombination activator protein RAG-2 by phosphorylation. Science *260*, 953–959.

Linette, G.P., Grusby, M.J., Hedrick, S.M., Hansen, T.H., Glimcher, L.H., and Korsmeyer, S.J. (1994). Bcl-2 is upregulated at the $CD4^+CD8^+$ stage during positive selection and promotes thymocyte differentiation at several control points. Immunity *1*, 197–205.

Liu, C.-P., Ueda, R., She, J., Sancho, J., Wang, B., Weddell, G., Loring, J., Kurahara, C., Dudley, E.C., Hayday, A., et al. (1993). Abnormal T cell development in CD3-$\zeta^{-/-}$ mutant mice and identification of a novel T cell population in the intestine. EMBO J. *12*, 4863–4875.

Livingstone, L.R., White, A., Sprouse, J., Livanos, E., Jacks, T., and Tisty, T.D. (1992). Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. Cell *70*, 923–935.

Love, P.E., Shores, E.W., Johnson, M.D., Tremblay, M.L., Lee, E.J., Grinberg, A., Huang, S.P., Singer, A., and Westphal, H. (1993). T cell development in mice that lack the ζ chain of the T cell antigen receptor complex. Science *261*, 918–921.

Malissen, B., and Malissen, M. (1996). Functions of TCR and pre-TCR subunits: lessons from gene ablation. Curr. Opin. Immunol. *8*, 383–393.

Malissen, M., Gillet, A., Rocha, B., Trucy, J., Vivier, E., Boyer, C., Köntgen, F., Brun, N., Mazza, G., Spanopoulou, E., et al. (1993). T cell development in mice lacking the CD3-ζ/η gene. EMBO J. *12*, 4347–4355.

Malissen, M., Gillet, A., Ardouin, L., Bouvier, G., Trucy, J., Ferrier, P., Vivier, E., and Malissen, B. (1995). Altered T cell development in mice with a targeted mutation of the CD3-ϵ gene. EMBO J. *14*, 4641–4653.

McDonnell, T.J., Deane, N., Platt, F.M., Nunez, G., Jaeger, U., McKearn, J.P., and Korsmeyer, S.J. (1989). *bcl-2*-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. Cell *57*, 79–88.

Miyashita, T., and Reed, J.C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human *bax* gene. Cell *80*, 293–299.

Miyashita, T., Krajewski, S., Krajewska, M., Wang, H.G., Lin, H.K., Liebermann, D.A., Hoffman, B., and Reed, J.C. (1994). Tumor suppressor p53 is a regulator of *bcl-2* and *bax* gene expression in vitro and in vivo. Oncogene *9*, 1799–1805.

Molina, T.J., Kishihara, K., Siderovski, D.P., Van Ewijk, W., Narendran, A., Timms, E., Wakeham, A., Paige, C.J., Hartmann, K.-U., Veillette, A., et al. (1992). Profound block in thymocyte development in mice lacking p56*lck*. Nature *357*, 161–164.

Mombaerts, P., Clarke, A.R., Rudnicki, M.A., Iacomini, J., Itohara, S., Lafaille, J.J., Wang, L., Ichikawa, Y., Jaenisch, R., Hooper, M.L., et al. (1992a). Mutations in T-cell antigen receptor genes α and β block thymocyte development at different stages. Nature *360*, 225–231.

Mombaerts, P., Iacomini, J., Johnson, R.S., Herrup, K., Tonegawa, S., and Papaioannou, V.E. (1992b). RAG-1 deficient mice have no mature B and T lymphocytes. Cell *68*, 869–877.

Mombaerts, P., Anderson, S.J., Perlmutter, R.M., Mak, T.W., and Tonegawa, S. (1994). An activated *lck* transgene promotes thymocyte development in *RAG-1* mutant mice. Immunity *1*, 261–267.

Nacht, M., Strasser, A., Chan, Y.R., Harris, A.W., Schlissel, M., Bronson, R.T., and Jacks, T. (1996). Mutations in the *p53* and *SCID* genes cooperate in tumorigenesis. Genes Dev. *10*, 2055–2066.

Ohno, H., Aoe, T., Taki, S., Kitamura, D., Ishida, Y., Rajewsky, K., and Saito, T. (1993). Developmental and functional impairment of T cells in mice lacking CD3ζ chains. EMBO J. *12*, 4357–4366.

Oltvai, Z.N., and Korsmeyer, S.J. (1994). Checkpoints of dueling dimers foil death wishes. Cell *79*, 189–192.

Oltvai, Z.N., Milliman, C.L., and Korsmeyer, S.J. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programed cell death. Cell *74*, 609–619.

Pénit, C., Lucas, B., and Vasseur, F. (1995). Cell expansion and growth arrest phases during the transition from precursor ($CD4^-8^-$) to immature ($CD4^+8^+$) thymocytes in normal and genetically modified mice. J. Immunol. *154*, 5103–5113.

Purdie, C.A., Harrison, D.J., Peter, A., Dobbie, L., White, S., Howie, S.E.M., Salter, D.M., Bird, C.C., Wyllie, A.H., Hooper, M.L., et al. (1994). Tumour incidence, spectrum and ploidy in mice with a large deletion in the p53 gene. Oncogene *9*, 603–609.

Saint-Ruf, C., Ungewiss, K., Groettrup, M., Bruno, L., Fehling, H.J., and Von Boehmer, H. (1994). Analysis and expression of a cloned pre-T cell receptor gene. Science *266*, 1208–1212.

Schmedt, C., Saijo, K., Niidome, T., Kühn, R., Aizawa, S., and Tarakhovsky, A. (1998). Csk controls antigen receptor-mediated development and selection of T-lineage cells. Nature *394*, 901–904.

Shinkai, Y., Rathbun, G., Lam, K.-P., Oltz, E.M., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A.M., et al. (1992).

RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell *68*, 855–867.

Shortman, K., and Wu, L. (1996). Early T lymphocyte progenitors. Annu. Rev. Immunol. *14*, 29–47.

Tybulewicz, V.L.J. (1998). Analysis of antigen receptor signaling using mouse gene targeting. Curr. Opin. Cell. Biol. *10*, 195–204.

Van Oers, N.S.C., Von Boehmer, H., and Weiss, A. (1995). The pre-T cell receptor (TCR) complex is functionally coupled to the TCR-ζ subunit. J. Exp. Med. *182*, 1585–1590.

Van Oers, N.S.C., Lowin-Kropf, B., Finlay, D., Connolly, K., and Weiss, A. (1996). αβ T cell development is abolished in mice lacking both Lck and Fyn protein tyrosine kinases. Immunity *5*, 429–436.

Vermes, I., Haanen, C., Steffens-Nakken, H., and Reutelingsperger, C. (1995). A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J. Immunol. Methods *184*, 39–51.

Wallace, V.A., Kawai, K., Levelt, C.N., Kishihara, K., Molina, T., Timms, E., Pircher, H., Penninger, J., Ohashi, P.S., Eichmann, K., et al. (1995). T lymphocyte development in $p56^{lck}$ deficient mice: allelic exclusion of the TcR β locus is incomplete but thymocyte development is not restored by TcR β or TcR αβ transgenes. Eur. J. Immunol. *25*, 1312–1318.

Wang, B., Wang, N., Salio, M., Sharpe, A., Allen, D., She, J., and Terhorst, C. (1998). Essential and partially overlapping role of CD3γ and CD3δ for development of αβ and γδ T lymphocytes. J. Exp. Med. *188*, 1375–1380.

White, E. (1996). Life, death, and the pursuit of apoptosis. Genes Dev. *10*, 1–15.

Wilson, A., Held, W., and MacDonald, H.R. (1994). Two waves of recombinase gene expression in developing thymocytes. J. Exp. Med. *179*, 1355–1360.

Wu, L., Scollay, R., Egerton, M., Pearse, M., Spangrude, G.J., and Shortman, K. (1991). CD4 expressed on earliest T-lineage precursor cells in the adult murine thymus. Nature *349*, 71–74.

Wu, G., Danska, J.S., and Guidos, C.J. (1996a). *Lck* dependence of signaling pathways activated by γ-irradiation and CD3ε engagement in RAG-$1^{-/-}$ immature thymocytes. Int. Immunol. *8*, 1159–1164.

Wu, L., Li, C.-L., and Shortman, K. (1996b). Thymic dendritic cell precursors: relationship to the T lymphocyte lineage and phenotype of the dendritic cell progeny. J. Exp. Med. *184*, 903–911.

Yin, X.-M., Oltvai, Z.N., and Korsmeyer, S.J. (1994). BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. Nature *369*, 321–323.