V(D)J recombination activates a p53-dependent DNA damage checkpoint in *scid* lymphocyte precursors

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Double-stranded DNA breaks (DSBs) trigger p53-mediated cell cycle arrest or apoptosis pathways that limit the oncogenic consequences of exposure to genotoxic agents, but p53-mediated responses to DSB generated by normal physiologic events have not been documented. "Broken" V(D)J coding ends accumulate in *scid* lymphocyte precursors as a consequence of a mutation in DNA-dependent protein kinase (DNA-PK). The ensuing failure to rearrange efficiently antigen receptors arrests lymphoid development. Here we show that *scid* thymocytes express high levels of p53 protein, attributable to recombinase activating gene (RAG)-dependent generation of DSB adjacent to V, D, and J gene segments. To examine the functional importance of p53 expression in vivo, we bred p53^{-/-} *scid* mice. The absence of p53 facilitated production of in-frame V(D)J coding joints and developmental progression of *scid* thymocytes, in addition to a dramatic accumulation of pro-B cells. All mice developed disseminated pro-B or immature T cell lymphoma/leukemia by 7–12 weeks of age. We present evidence that p53 deficiency prolongs the survival of *scid* lymphocyte precursors harboring broken V(D)J coding ends, allowing the accumulation of aneuploid cells. These results demonstrate that a p53-mediated DNA damage checkpoint contributes to the immune deficiency characteristic of the *scid* mutation and limits the oncogenic potential of DSBs generated during V(D)J recombination.

[Key Words: DNA repair; *scid*; V(D)J recombination; protein kinase; p53 protein]

Received April 10, 1996; revised version accepted July 8, 1996.

The p53 tumor suppressor gene is a tetrameric transcription factor that plays a critical role in transducing a signal from damaged DNA to genes that control the cell cycle and apoptosis. Treatment of cells with DNA-damaging agents, such as ionizing or UV radiation, causes rapid accumulation of p53 by a post-transcriptional mechanism involving stabilization of p53 protein (for review, see Cox and Lane 1995). In addition to DNA damage, both types of radiation activate a wide spectrum of stress response pathways. However, recent studies show that small numbers of double-stranded DNA breaks (DSBs) provide a sufficient signal for p53 induction (Lu and Lane 1993; Nelson and Kastan 1994; Huang et al. 1996). DNA damage-induced accumulation of p53 protein can cause cell-cycle arrest through p53-mediated transcriptional activation of the cyclin-dependent kinase inhibitor p21^{waf-1/cip-1} (for review, see Ko and Prives

1996). In addition, DNA damage can induce p53-dependent apoptosis by transcriptional activation of death genes such as BAX, as well as by transcription-independent mechanisms (for review, see White 1996).

The importance of p53 functions in regulating the cell cycle and apoptosis in response to DNA damage arising from normal physiological processes, rather than from treatment with genotoxic mediators, has not been established. Embryonic and postnatal development proceeds normally in p53^{-/-} mice (Donehower et al. 1992; Jacks et al. 1994), suggesting that p53 does not perform essential functions during DNA replication and cell differentiation. However, the crucial role of p53 in regulating DNA damage-induced cell cycle checkpoints and apoptosis is underscored by the observation that the p53 gene is mutated or deleted in the majority of spontaneous human malignancies, as well as in the inherited Li-Fraumeni cancer syndrome (Malkin et al. 1990). In addition, mice lacking functional p53, because of overexpression of a dominant negative p53 transgene or to germ-line disruption of both p53 alleles, are highly tumor-prone

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(Lavigne et al. 1989; Donehower et al. 1992; Jacks et al. 1994). The vast majority (70–90%) of spontaneous malignancies in $p53^{-/-}$ mice are lymphomas (Donehower et al. 1992; Harvey et al. 1994, 1995; Jacks et al. 1994), suggesting that absence of p53 tumor suppressor function is particularly devastating in the lymphoid lineage.

A unique feature of lymphocyte development that may explain the sensitivity to p53 action is the site-specific DNA recombination process that assembles antigen receptor variable genes from dispersed coding segments. During this process, two recombinase-activating genes, RAG-1 and RAG-2, generate DSB at conserved recombination signal sequences (RSS) that flank V, D, or J coding segments (Mombaerts et al. 1992b; Shinkai et al. 1992; McBlane et al. 1995). Accumulating evidence suggests that lymphocyte precursors undergoing V(D)J recombination are arrested in the G_0/G_1 phase of the cell cycle (Lin and Desiderio 1995), presumably to minimize the chance of aberrant V(D)J joining events during the proliferative burst that occurs during lymphocyte development. It is not known, however, whether this cell cycle regulation occurs as a consequence of p53-mediated detection of V(D)J-specific DSB. Chromosomal translocations that juxtapose antigen receptor gene segments and proto-oncogenes are a common feature of lymphoid malignancies, attesting to the oncogenic potential of abnormal V(D)J recombination (Rabbitts 1994).

The DNA-dependent protein kinase (DNA-PK) regulates a DSB repair activity required for site-specific V(D)J recombination. Recent studies have shown that DNA-PK belongs to a subfamily of PI-3 kinase-related genes (Hartley et al. 1995) known to regulate DNA damage-induced cell cycle checkpoints (Keith and Schreiber 1995; Zakian 1995). These include *RAD3* and *MEC1* in yeast, *mei-41* in *Drosophila*, and the ataxia telangiectasia (*ATM*) gene in humans. DNA-PK was first identified biochemically as a nuclear serine/threonine kinase with the unusual property of requiring the presence of DNA ends for catalytic activity (Lees-Miller and Anderson 1989; Carter et al. 1990; Lees-Miller et al. 1990). The large (~460 kD) catalytic subunit, DNA-PK_{cs}, is targeted to DNA ends by association with the Ku70/Ku80 heterodimer (Gottlieb and Jackson 1993). Recent studies have mapped the murine *scid* mutation (Bosma and Carroll 1991) to the DNA-PK_{cs} gene (Blunt et al. 1995; Kirchgessner et al. 1995; Peterson et al. 1995). This mutation confers a generalized DSB repair defect that renders all cell lineages more sensitive to the effects of DSB-inducing agents (Fulop and Phillips 1990; Biedermann et al. 1991; Hendrickson et al. 1991) and also impairs V(D)J recombination (Lieber et al. 1988; Malynn et al. 1988).

In contrast to normal lymphocyte precursors, V(D)J coding ends accumulate in *scid* lymphocyte precursors (Roth et al. 1992a,b; Zhu and Roth 1995) and are rejoined inefficiently (Lieber et al. 1988; Malynn et al. 1988; Blackwell et al. 1989). The profound immunodeficiency in *scid* mice (Bosma and Carroll 1991) is thought to reflect the absence of immunoglobulin μ (Ig μ) and T cell receptor β (TCR β) containing pre-B and pre-T cell receptor complexes that transmit essential signals for devel-

opmental progression of B or T cell progenitors (von Boemer 1994). This is consistent with the demonstration that genetic disruption of either RAG-1 or RAG-2 arrests T and B cell development at the same stage as the arrest caused by the *scid* mutation (Mombaerts et al. 1992b; Shinkai et al. 1992). Introduction of functionally rearranged TCR β transgenes, however, completely restores normal lymphocyte development in RAG-1- or RAG-2-deficient mice (Mombaerts et al. 1992a; Shinkai et al. 1993; Spanopoulou et al. 1994; Young et al. 1994), whereas introduction of receptor transgenes into *scid* mice only partially restores lymphocyte development (Reichman-Fried et al. 1990; Kishi et al. 1991; Shores et al. 1993). A recent study suggests that transgene-mediated rescue of lymphocyte development is incomplete in *scid* mice because of the potentially lethal effects of continuing attempts to rearrange endogenous receptor loci (Chang et al. 1995).

These considerations prompted us to investigate whether p53 might be operative in detection and management of DSB that arise during the process of V(D)J recombination, as this site-specific DNA damage potentially could contribute to oncogenesis. We have addressed the role of p53 in the context of V(D)J recombination in normal and *scid* lymphocyte precursors, which differ in their ability to resolve rapidly V(D)J-specific DSB into coding joints. We report that p53 protein is undetectable in normal thymocytes undergoing V(D)J recombination, and is dispensable for the cell cycle arrest that occurs in these cells. In contrast, we show that defective DNA-PK function in *scid* thymocytes results in high levels of p53 protein expression as a consequence of V(D)J-specific DSB. To examine the functional importance of p53 expression in *scid* lymphocyte precursors, we bred $p53^{-/-}$ *scid* mice. Analysis of these double-mutant mice indicates that a p53-mediated DNA damage checkpoint can be activated during site-specific V(D)J recombination to limit survival of precursors with unresolved V(D)J breaks, thus minimizing the oncogenic potential of abnormal V(D)J recombination.

Results

RSS-specific DSB induce p53 expression in scid lymphocyte precursors

We first sought to determine whether introduction of DSB adjacent to V(D)J coding segments causes accumulation of p53 protein in wild-type (WT) or *scid* thymocytes. Thymocyte nuclear extracts were evaluated for p53 protein levels by Western blotting. Freshly isolated, untreated *scid* thymocytes expressed substantial levels of p53 protein (Fig. 1). In contrast, p53 protein was not detected in RAG-2 $^{-/-}$ thymocytes (Fig. 1). Failure to rearrange TCR genes causes similar developmental arrest at the CD25 $^{+}$ CD4/CD8 double-negative (DN) stage in both *scid* and RAG-2 $^{-/-}$ mice, indicating that p53 up-regulation in *scid* thymocytes was not simply a consequence of developmental arrest. Freshly isolated *scid*

Figure 1. Western blot analysis of p53 protein expression in thymocyte nuclear extracts. Fifty micrograms of nuclear protein from each sample were resolved by SDS-PAGE, transferred to nitrocellulose filters and incubated with anti-p53 antibody, followed by anti-mouse horseradish peroxidase (HRP), and visualized by enhanced chemiluminescence (ECL). (A) Nuclear extracts prepared from fresh ex vivo thymocytes from the indicated mouse strains were analyzed for p53 protein expression by Western blotting. The last two lanes contain nuclear extract protein from purified WT (B6) CD25⁺ and CD25⁻ DN thymocytes. (B,C) p53 protein Western blot analysis of nuclear extracts prepared from fresh ex vivo thymocytes (lanes marked 0), or from thymocytes exposed to 100, 300, or 500 cGy from a ¹³⁷Cs source and cultured cells for 90 min at 37°C. Note that the TCRβ transgene results in greater thymus cellularity because of development and expansion of DP thymocytes, thereby providing a more similar populations for comparison with WT thymocytes, which are 75–85% DP. After evaluation with anti-p53 antibody, all membranes were stained with amido black to confirm equal protein loading (not shown).

splenocytes, which contain mostly myeloid cells and are largely devoid of V(D)J-specific DSB, did not express detectable amounts of p53 (data not shown), demonstrating that the *scid* mutation does not cause a generalized increase in p53 protein levels. To examine whether p53 expression in *scid* thymocytes was dependent on the generation of V(D)J-specific DSB, we bred RAG-2^{-/-} *scid* double-mutant mice in which RAG-2 deficiency prevents cleavage at RSS adjacent to V, D, and J coding ends. In contrast to *scid* thymocytes, RAG-2-deficient *scid* thymocytes lacked detectable p53 protein expression (Fig. 1A, lane 3), demonstrating that up-regulation of p53 in *scid* thymocytes requires RAG-2 gene function. Purified immature CD25⁺ DN thymocytes from WT mice, however, which are actively engaged in TCR rear-

angement (Pearse et al. 1989; Godfrey et al. 1994), did not express detectable p53 (Fig. 1A, lane 5), suggesting that V(D)J recombination is not accompanied by high-level p53 protein expression in a high frequency of WT thymocytes.

In *scid* thymocytes, rearrangements are attempted at the TCRβ, TCRγ, and TCRδ, whereas TCRα remains in germ-line configuration, presumably because of the developmental arrest (Schuler et al. 1988; Carroll and Bosma 1991; Roth et al. 1992b). Introduction of a TCRβ transgene into *scid* mice prevents rearrangement of endogenous TCRβ genes and promotes development to the CD4/CD8 double positive (DP) stage, where TCRα rearrangement begins (Kishi et al. 1991; Shores et al. 1993; Livak et al. 1996). We also observed high levels of p53 protein in TCRβ-transgenic *scid* thymocytes (Fig. 1B, lane 4; 1C, lane 5), suggesting that attempted rearrangements at TCRγ, TCRδ, or TCRα can also provoke p53 accumulation in *scid* thymocytes. In contrast, p53 protein was undetectable by Western blot analysis of freshly isolated WT thymocytes (Fig. 1B,C), although they consist predominantly of DP thymocytes known to be engaged actively in TCRα rearrangement. However, p53 protein was up-regulated 90 min after exposure of WT and TCRβ-transgenic *scid* thymocytes to low doses of γ-irradiation (Fig. 1B,C). Collectively, these results demonstrate that detection of V(D)J-specific DSB *in vivo* induces p53 up-regulation in *scid* thymocytes, and that radiation can induce further up-regulation of p53 in these cells.

Absence of p53 partially restores TCRβ rearrangement and T cell development in scid mice

To examine the biological importance of p53 expression in *scid* lymphocyte precursors, we bred and analyzed p53^{-/-} *scid* mice. The phenotype of p53^{-/-} *scid* thymocytes was examined at multiple time points after birth. Three-week-old p53^{+/-} *scid* and p53^{+/+} *scid* (p53WT *scid*) thymocytes were homogeneously large (high forward scatter), CD25⁺ DN cells lacking intracellular TCRβ (iTCRβ) protein (Fig. 2A), an immature phenotype characteristic of arrested thymocyte development conferred by the *scid* mutation. In contrast, age-matched p53^{-/-} *scid* animals contained substantial populations of small DP thymocytes, a high frequency of which expressed iTCRβ protein. In six of seven 3-week-old p53^{-/-} *scid* mice, 10–60% of thymocytes expressed iTCRβ protein (Fig. 2B). Maturation of p53^{-/-} *scid* DP thymocytes in the absence of iTCRβ protein also occurred, similar to observations illustrating a TCRβ-independent developmental pathway in TCRβ-deficient animals (Mombaerts et al. 1992a; Zuniga-Pflucker et al. 1994; Guidos et al. 1995). Between 10 days and 5 weeks of age, the maturation and expansion of DP cells accounted for progressive increases in thymus cellularity (Fig. 2B). Thus, p53 deficiency partially overcame developmental arrest conferred by the *scid* mutation, allowing TCRβ protein production along with development and expansion of DP thymocytes.

Figure 2. Kinetics of thymocyte development in $p53^{-/-}$ *scid* mice. Thymocytes were obtained from mice of different ages and analyzed for forward scatter (FSC) and expression of CD4, CD8, and intracellular TCR β chain expression by multiparameter flow cytometry. (A) Two-parameter contour plots (7% probability) display CD4 versus CD8 expression (far left). Single parameter histograms show the expression of intracellular TCR β protein (clear histograms, middle) as compared with control *scid* intracellular TCR β expression (shaded histograms). Surface TCR β^{+} cells were found only in BALB/c mice (not shown). Also shown are single parameter histograms of the forward light scatter (an indication of cell size) of the total thymocyte population (shaded histogram, right) overlaid with the FSC of the DP thymocytes alone (dark outline). Control *scid* and BALB/c mice were 3 weeks old. Three- and 5-week-old $p53^{-/-}$ *scid* mice are shown for comparison. (B) (top) Number of viable thymocytes recovered from individual $p53^{-/-}$ *scid* mice at the indicated ages (open circles) is shown. The arrow on the y-axis indicates the average 3- to 6-week-old *scid* thymus value. The percentage of DP thymocytes (middle) or iTCR β^{+} thymocytes (bottom) in individual mice was determined by flow cytometry. Note that two animals in the 5-week group had <1% DP or iTCR β^{+} cells in their thymi.

TCR β protein or mRNA is detected rarely in p53WT *scid* animals (Bosma and Carroll 1991; Danska et al. 1994), owing to impaired V(D)J recombination. Intracellular TCR β protein expression in p53^{-/-} *scid* thymocytes could result from diverse V(D)J rearrangements in many precursors, or the expansion of relatively few, oligoclonal precursors. To examine the heterogeneity of these rearrangements in individual animals, reverse transcriptase coupled-polymerase chain reactions (RT-PCR) were performed with a series of seven TCRV β -specific primers in conjunction with an antisense C β primer (Danska et al. 1994). In BALB/c thymocytes, each V β -specific reaction yielded TCR β products, reflecting the use of multiple V-region gene segments in the WT thymocyte repertoire (Fig. 3A, top). At 3 weeks of age, transcripts containing all seven V β -regions were detected in three of four p53^{-/-} *scid* littermates, suggesting the heterogeneous use of V β gene segments. One 3 week-old p53^{-/-} *scid* thymus lacked both V β 7- and V β 14-containing transcripts, suggesting more limited TCR β diversity in this animal (Fig. 3A, second row). Using a V α -consensus/C α primer set for RT-PCR amplification (Danska et al. 1990), we found that two of four 3-week-old littermates contained low level TCR α transcripts of normal length (Fig. 3A, bottom). These results demonstrate that productive TCR β rearrangement and DP thymocyte maturation in p53^{-/-} *scid* animals was sometimes associated with progression to TCR α rearrangement and transcription. However, we did not detect surface TCR β ⁺ thymocytes in p53^{-/-} *scid* mice (data not shown). Because efficient TCR β transport to the cell surface is TCR α -dependent (Mombaerts et al. 1992a; Philpott et al. 1992), this observation suggests that few, if any, thymocytes harbored in-frame rearrangements at both TCR β and TCR α loci. Accordingly, cloning and sequence analysis of TCR α transcripts from double mutant animals demonstrated a low frequency of normal, productive VJ α coding joints (data not shown).

Previous studies have shown that rare V(D)J coding joints can be isolated from *scid* cells, often with characteristic structural features, including extensive P-nucleotide addition, homology-directed joining, and extensive coding sequence deletions (for review, see Lewis 1994). To examine the diversity and fidelity of individual V(D)J coding joints from p53^{-/-} *scid* mice, TCR β transcripts from a 3-week-old p53^{-/-} *scid* thymus were cloned by RT-PCR amplification (Danska et al. 1994). Only 3 of 21 sequences analyzed were unique, and the structure of some TCR β coding joints revealed potential signatures of the *scid* V(D)J recombinase machinery (Fig. 3B and legend). Collectively, these phenotypic and molecular analyses suggest that p53 deficiency permits *scid* thymocytes to progress through multiple contingent steps characteristic of normal maturation: productive TCR β rearrangement, expression of CD4 and CD8 coreceptors, and onset of TCR α rearrangement and transcription. However, the limited diversity and unusual structural features of TCR transcripts suggested that p53 deficiency did not elicit WT V(D)J recombination in *scid* thymocytes.

Impact of p53 deficiency on B lineage development in scid mice

In agreement with a previous report (Hardy et al. 1989), only 5–10% of p53WT *scid* bone marrow cells expressed the B lineage marker B220, about one-third the frequency in normal mice, and all *scid* B220⁺ cells were large, CD43⁺, and iIg μ ⁻ (Fig. 4). In contrast, bone marrow from p53^{-/-} *scid* mice was enriched in B220⁺ cells as early as ten days of age, and the B220⁺ frequency rose to 20–65% by 5 weeks of age (Fig. 4B). Bone marrow cellularity increased significantly during this time period, indicating an expansion of absolute numbers of B220⁺ cells (Fig. 4B). Despite the increase in the frequency and number of B220⁺ cells in the double mutant mice, p53 deficiency did not appear to cause significant developmental progression of pro-B cell blasts in *scid* mice. Although we sometimes observed slightly decreased expression of CD43, this was not accompanied by expression of detectable intracellular Ig μ protein in B220⁺ cells (Fig. 4A). To examine Ig μ transcription, we performed RT-PCR of bone marrow from 5- to 10-day-old p53^{-/-} *scid* animals. C μ germ-line transcripts characteristic of the pro-B cell stage were present, but we were unable to detect full-length Ig μ mRNA in the p53^{-/-} *scid* samples using a 5' VH_{all} consensus primer paired with a 3' C μ primer (data not shown). Thus, in contrast to the promotion of T cell development, p53 deficiency promoted accumulation of B-cell precursors but did not appear to circumvent the block in B-cell maturation conferred by the *scid* mutation.

p53-deficiency results in rapid onset of lymphoma/leukemia in scid mice

At 7–12 weeks of age, all p53^{-/-} *scid* animals appeared moribund, and on necropsy, 16 of 16 animals were found to have variable degrees of thymic hyperplasia, lymphadenopathy, and splenomegaly, attributable to infiltration of large numbers of lymphoblastoid cells. Histological examination of infiltrated tissues revealed that normal architecture was replaced by densely packed lymphoblasts with abundant mitotic figures (data not shown). These findings are indicative of a significant lymphoma or leukemia burden. Tumors were observed in mice as young as 6 weeks of age, and the median age of onset was 8 weeks. In most animals, the lymphoma/leukemia was disseminated to multiple sites, including perithymic and inguinal lymph nodes, and spleen. Cell lines were readily derived from explanted thymic or lymph node tumor tissue of multiple animals.

Flow cytometric analyses of tumor phenotype demonstrated that both lymphoid lineages are highly prone to malignant transformation in p53-deficient *scid* mice. Fourteen of 16 tumors explanted from thymus and lymph nodes did not express T lineage markers (CD4, CD8, Thy-1; data not shown) but were comprised of uniformly large CD19⁺ B220⁺ CD43⁺ cells (Fig. 5A) that closely resembled normal pro-B cells (Hardy et al. 1991). In contrast, 2 of 16 animals had thymic tumors of an

Figure 3. T-cell receptor repertoire and coding joint structure in $p53^{-/-}$ *scid* thymocytes. (A) Thymus RNA was extracted from a BALB/c and four 3-week-old $p53^{-/-}$ *scid* mice and reverse transcribed into cDNA. (Top) RT-PCR amplification was performed using seven different Vβ-specific forward primers in concert with a Cβ reverse primer, and the products were resolved by electrophoresis, and evaluated by Southern blotting with a Cβ probe. Note that TCRβ mRNA is rarely detected in $p53^{+/+}$ *scid* mice using this technique (<1/30 animals) (Danska et al. 1994). (Bottom) cDNA from the same samples was RT-PCR amplified using a degenerate consensus Vα sense primer and a Cα antisense primer. The far right lane is a background control of BALB/c cDNA synthesized without reverse transcriptase. The PCR products were resolved by electrophoresis and evaluated by Southern blotting with a Cα probe. (B) Thymus cDNA from one 3-week-old $p53^{-/-}$ *scid* mouse was amplified with a Vβ-consensus primer in conjunction with a Cβ antisense primer. The PCR products were cloned, selected by hybridization with a Cβ probe, and sequenced by dideoxy chain termination using a Cβ antisense primer. The nucleotides for each germ-line TCR V gene segment (underlined) are shown above the $p53^{-/-}$ *scid* sequences. The lower sequence contained an in-frame sequence where the Vβ8.3 gene and an inverted repeat of the RSS heptamer were joined. Similar in-frame IgH signal-containing coding joints have been detected in *scid* bone marrow DNA (J. Pennycook and G. Wu, pers. comm.).

immature T-cell phenotype, as they expressed both CD4 and CD8, as well as intracellular TCRβ protein, and were negative for B-lineage markers (Fig. 5A; data not shown). In the example shown, both T- and B-lineage

cells were detectable in the peripheral lymph nodes. Adoptive transfer of thymocytes from 3-week-old $p53^{-/-}$ *scid* mice into $p53^{WT}$ *scid* recipients resulted in disseminated pro-B cell lymphoma within 6 weeks (data

Figure 4. Analysis of B-cell development in p53^{-/-} scid mice. (A) Bone marrow cell suspensions were obtained at the ages indicated and analyzed by flow cytometry for expression of B220 and intracellular Igμ protein. Shaded histograms in all plots represent staining with isotype-matched control antibodies. The percentage of B220⁺ cells in each bone marrow sample is indicated (clear histogram, far left). Both CD43 expression and FSC are displayed as single parameter histograms gated on B220⁺ cells. (B) (top) Bone marrow cellularity in individual p53^{-/-} scid mice (dark bars) at the indicated ages. Age-matched p53WT scid animals (light bars) are shown for comparison. (Bottom) Bone marrow cells from p53^{-/-} scid mice were obtained at 10 days, 3 weeks, and 5 weeks of age and stained for B220 expression as described in A. The bone marrow cellularity of p53WT scid age-matched controls are shown (light bars).

not shown), demonstrating that pre-malignant pro-B cells can be detected in the thymii of very young mice.

The Igμ locus was examined in pro-B lymphomas using a J_H4 probe, which was chosen because it is the most 3' J_H gene segment and is retained on Igμ alleles after normal V(D)J rearrangements. These p53^{-/-} scid tumors and lymphoma cell lines each contained apparently clonal Igμ rearrangements (Fig. 5B, top), but the TCRβ locus was in germ-line configuration (Fig. 5B, bottom), reinforcing the phenotypic evidence of their B-lineage derivation. Igμ protein was not detected, however, in any of the pro-B cell tumors (data not shown), suggesting that these rearrangements were incomplete (DJ only) or nonfunctional. Collectively, these data demonstrate that ablation of p53 activity in immature scid lymphocyte

precursors universally results in extremely rapid progression to a highly invasive lymphoma or leukemia.

Effects of p53 deficiency on cell cycle status, genomic instability, and survival of scid lymphocyte progenitors

The dramatic effects of p53 deficiency on lymphocyte development and neoplastic transformation in scid mice could be attributable to altered cell cycle regulation or to loss of an apoptotic pathway. CD25⁺ DN thymocytes and pro-B cells from WT mice have been demonstrated to undergo a G₁ arrest coincident with TCRβ or Igμ rearrangement, respectively (Pearse et al. 1989; Godfrey et al. 1994) suggesting that V(D)J recombination, like other

forms of DSB repair, is tightly coordinated with the cell cycle (Lin and Desiderio 1995). Because we did not detect p53 up-regulation in purified CD25⁺ DN WT thymocytes (see Fig. 1A), we reasoned that the G₁ arrest at this maturational stage in WT mice may be p53-independent. To examine this issue, we compared the cell cycle status of CD25⁺ DN thymocytes purified from WT B6 and p53^{-/-} (WT at the *scid* locus) mice, using propidium iodide staining and flow cytometric analysis (Table 1). These data showed that ≥90% of both WT and p53-deficient CD25⁺ DN thymocytes were arrested in the G₁ phase of the cell cycle. Similarly, CD25⁺ DN thymocytes from RAG-2^{-/-} and *scid* mice consist primarily of cells in G₀/G₁ (Table 1). Loss of p53 did not decrease the proportion of *scid* CD25⁺ DN thymocytes (Table 1) or bone marrow pro-B cells (Table 2) in G₀/G₁. Thus, the G₁ arrest accompanying V(D)J recombination in WT and *scid* lymphocyte precursors occurs independently of p53 accumulation.

Although p53 deficiency did not promote abnormal cell cycle progression of *scid* lymphocyte precursors, we noted that pro-B cells and thymocytes harvested from the double mutant animals during the premalignant phase displayed a significant degree of aneuploidy. Figure 6A shows that pro-B cells purified from p53^{-/-} *scid* animals have ≤20% greater G₀/G₁ DNA content than those isolated from p53^{+/+} *scid* animals. This was best demonstrated when the two purified populations were mixed together and then stained for DNA content (Fig. 6A, bottom). Hyperdiploidy was evident in purified pro-B cells from 75% of double mutant animals sacrificed at 5–6 weeks of age, but was not detected in bone marrow myeloid cells from the same animals (data not shown). We also examined primary tumor cell populations for evidence of aneuploidy. DNA content histograms showed bimodal G₀/G₁ peaks in bone marrow cells and thymocytes from a 10-week-old moribund p53^{-/-} *scid* mouse, but not from an age-matched BALB/c mouse (Fig. 6B). The B220 versus DNA content dot plot demonstrates that the B220⁺ cells are hyperdiploid in the double mu-

tant, but not in BALB/c animals. However, in contrast to the premalignant aneuploid populations, the malignant pro-B cells were actively cycling (50% in S/G₂/M). Similarly, we observed active cycling of hyperdiploid cells in CD4/CD8 DP thymic lymphoma (data not shown). These data suggest that the absence of p53 promotes genomic instability in *scid* lymphocyte progenitors.

The accumulation of noncycling, hyperdiploid lymphoid progenitors in p53-deficient *scid* mice also suggests that p53 may limit the survival of cells harboring broken V(D)J molecules. To assess the role of p53 in the survival of *scid* cells with DSB, we compared the relative survival of thymocytes from RAG-2^{-/-}, p53WT*scid*, p53^{+/+} *scid*, and p53^{-/-} *scid* mice after 1 or 2 days of culture. Multiple genetic studies have shown this assay to correlate well with survival of lymphocyte progenitors in vivo (Strasser et al. 1994b; Motoyama et al. 1995; Field et al. 1996). The surviving fraction (number of viable cells recovered/number of viable cells seeded) of p53WT *scid* and p53^{+/+} *scid* thymocytes was <0.2 (Fig. 7A). In contrast, the survival of RAG-2^{-/-} or p53^{-/-} *scid* thymocytes was respectively two- to fourfold greater or three- to sevenfold greater than *scid* thymocytes. p53-deficiency also enhanced dramatically the ability of *scid* lymphoma cell lines to survive DNA damage induced exogenously by low-dose γ-irradiation (Fig. 7B). As expected, the lymphoma cell lines derived from p53WT *scid* mice were extremely radiosensitive and few cells survived for 24 hr after treatment with 200 and 500 cGy. Lymphoma lines adapted from p53^{-/-} *scid* mice, however, were 4–14 times more radioresistant. Irradiated spleen cells from p53-deficient *scid* mice also showed substantially greater survival than those from p53WT *scid* mice, demonstrating that this effect was not limited to transformed cells, or to the lymphoid lineage (Fig. 7C). In accord with previous studies on p53-deficient cells (Strasser et al. 1994a), p53-deficient *scid* cells also displayed some p53-independent, radiation-induced cell death. Collectively, these observations support the notion that loss of the p53-dependent DNA damage checkpoint in *scid* lymphocytes confers protection from cell death associated with both RSS-dependent and radi-

Table 1. Effect of p53 deficiency on cell cycle status of CD25⁺ DN wild-type and *scid* thymocytes

Genotype	%G ₀ /G ₁	N ^a
WT	90 ± 2	2
p53 ^{-/-}	91 ± 2	2
RAG-2 ^{-/-}	94 ± 2	3
<i>scid</i>	95 ± 1	3
p53 ^{+/+} <i>scid</i>	96 ± 2	5
p53 ^{-/-} <i>scid</i>	94 ± 2	4

CD25⁺ DN thymocytes were purified from 6–8-week-old WT (B6) and p53^{-/-} mice, fixed, and stained with propidium iodide. Unfractionated thymocytes from the other genotypes were stained with FITC-anti-CD25 and fixed prior to staining with propidium iodide. Cells were analyzed by flow cytometry to determine the cell cycle distribution of the CD25⁺ subset. All mice were analyzed at 3–6 weeks after birth.

^aNumber of individual mice analyzed.

Table 2. Effect of p53 deficiency on cell cycle status of *scid* pro-B cells

Genotype	%G ₀ /G ₁	N ^a
<i>scid</i>	86 ± 3	2
p53 ^{+/+} <i>scid</i>	87 ± 5	4
p53 ^{-/-} <i>scid</i>	89 ± 2	4

1 × 10⁵ to 2 × 10⁵ CD19⁺ B220⁺ pro-B cells from individual mice (4–6 weeks old) of each genotype were purified by cell sorting, fixed, stained with propidium iodide, and analyzed by flow cytometry for DNA content. The double mutant mice used for this experiment appeared healthy and had only moderate accumulations of pro-B cells (33 ± 4%), and no lymphadenopathy, thymic hyperplasia, or splenomegaly.

^aNumber of individual mice analyzed.

Figure 5. (See facing page for legend.)

ation-induced DSB, directly promoting genomic instability of these recombinationally active progenitor cells.

Discussion

V(D)J recombination activates a DNA damage checkpoint in scid lymphocytes

Numerous studies have demonstrated that imposition of DNA breaks by exogenous agents are sufficient to initiate a p53-mediated cell cycle arrest or apoptosis, but normal physiological conditions that activate p53 pathways have not been identified previously. Here we provide the first demonstration that this DNA damage checkpoint is activated physiologically by V(D)J recombination in *scid* mice. Examination of p53 protein levels in *scid* versus RAG-2-deficient *scid* thymocytes demonstrated that RAG-mediated introduction of DSB in *scid* thymocytes provides a necessary signal to cause p53 accumulation (Fig. 1A). Given that (1) TCR β , TCR γ , and TCR δ are all recombinationally active in this cell population, (2) that V to D joining is not usually attempted before successful D to J joining, and (3) rearrangement of both alleles can be attempted simultaneously, the number of broken V(D)J ends present in an individual CD25⁺ DN thymocyte is likely between 2 and 16. This implies that the DNA damage-sensing mechanism is highly sensitive, in accord with a recent study suggesting that a single DSB can activate the p53 checkpoint (Huang et al. 1996). We could not detect p53 accumulation, however, in recombinationally active WT lymphocyte precursors. We suggest that the differential accumulation of p53 in WT versus *scid* lymphocyte progenitors reflects the abnormal persistence of V(D)J coding ends caused by the *scid* mutation in DNA-PK (Roth et al. 1992a,b; Zhu and Roth 1995). Nonetheless, rare WT precursors in which V(D)J coding ends persist may also activate the p53-dependent checkpoint.

Several properties of DNA-PK suggest that it may play a direct role in activation of the p53 checkpoint. It belongs to a subfamily of PI-3 kinase-related genes implicated in yeast, *Drosophila*, and humans that regulate DNA damage checkpoints (Zakian 1995), and defects in

the human *ATM* family member cause abnormalities in DNA damage-induced p53 accumulation, *trans*-activation function, and cell cycle arrest (Kastan et al. 1992; Khanna and Lavin 1993; Lu and Lane 1993; Khanna et al. 1995). Intriguingly, the enzymatic activity of DNA-PK requires association with DNA breaks, and serine residues in the p53 amino-terminal *trans*-activation domain are targets of phosphorylation by human and rodent DNA-PK in vitro (Lees-Miller et al. 1992; Finnie et al. 1995). Although *scid* DNA-PK fails to phosphorylate this site in vitro (Blunt et al. 1995), the biological significance of phosphorylation at this site is unclear (Ko and Prives 1996). We show here that p53 protein is up-regulated in *scid* thymocytes in vivo as a consequence of attempted V(D)J recombination. Moreover, *scid* and WT thymocytes accumulated similar levels of p53 in response to ionizing radiation. We also observed radiation-induced p21^{waf-1/cip-1} mRNA expression in *scid* cells (data not shown), indicative of normal p53-mediated *trans*-activation. Thus, in contrast to mutations in *ATM*, the *scid* mutation does not compromise the DNA damage-induced accumulation and function of p53.

Effects of p53 deficiency on survival, V(D)J recombination, and developmental progression of scid lymphocyte precursors

Our results show that p53 deficiency enhances survival of T and B cell precursors in *scid* mice, as evidenced by prolonged in vitro survival of thymocytes, and by accumulation of noncycling pro-B cells in vivo. We observed both productive TCR β rearrangements and progression to the DP stage, in thymocytes, whereas productive Ig μ rearrangements and developmental progression of pro-B cells were not seen. Previously, we observed a similar lineage dichotomy in restoration of V(D)J recombination and development in newborn *scid* mice given low doses of ionizing radiation (Danska et al. 1994). Productive TCR δ rearrangements were also documented in the latter system (Bogue et al. 1996; Livak et al. 1996), which can facilitate development of DP thymocytes in the absence of TCR β (Mombaerts et al. 1992a; Kersh et al.

Figure 5. Phenotype and lineage of p53^{-/-} *scid* lymphoid tumors. (A) Phenotype of cells from the thymus, lymph nodes, and bone marrow of a normal BALB/c mouse and two moribund p53-deficient *scid* mice. These animals had moderate (10-week-old) or severe (11-week-old) thymic hyperplasia, lymphadenopathy, and the 10-week old had hypercellular bone marrow. Cells from each tissue were stained with the indicated antibodies and analyzed by multiparameter flow cytometry as described for Fig. 2. (B) Southern blot analysis of rearrangements at the Ig μ and TCR β loci in p53^{-/-} *scid* lymphoid tissues and primary pro-B cell tumors and cell lines. (Top) Southern blot of EcoRI-digested genomic DNA samples were evaluated with a radioabeled Ig J_H4 probe. Bone marrow cells were obtained from B6, *scid*, and p53^{-/-} mice, as well as 3-week-old p53^{-/-} *scid* and p53^{+/+} *scid* littermates. DNA was also extracted from two p53^{-/-} *scid* pro-B lymphoma cell lines (SC53-1 and SC53-2), and primary pro-B tumors (#1 and #2) from 8-week-old p53^{-/-} *scid* mice. DNA combined from two tumors (lane 7), and a single tumor (lane 8) are shown. Note that SC53-1 had lost both J_H4 alleles. The filter was stripped and rehybridized with a housekeeping gene (HPRT) to control for DNA loading (middle). In normal mouse bone marrow, distinct Ig μ rearrangements were undetectable by Southern blot analysis presumably because B lineage cells comprise only 20–30% of bone marrow and have highly diverse rearrangements. (Bottom) Southern blots of PvuII-digested genomic DNA samples were evaluated with a TCR C β probe. DNA samples from BALB/c thymus, p53^{-/-} thymus, and a p53WT *scid* immature T cell lymphoma cell line (LK8; Danska et al. 1994) were also evaluated for comparison to the pro-B cell tumors. In addition to the prominent germ-line band, the “smears” evident in the BALB/c and p53^{-/-} thymus samples are characteristic of polyclonal TCR rearrangements.

Figure 6. (See facing page for legend.)

1995). Thus, we suggest that productive TCR β or TCR δ rearrangements promoted DP development in p53^{-/-} *scid* mice, and that the absence of pro-B cell maturation in these animals reflects the failure to generate productive Ig μ rearrangements. It remains unclear whether the differential effects of p53 deficiency and irradiation on promoting successful TCR versus Ig rearrangements in *scid* pro-B cells reflect unique, Ig μ -specific constraints on V(D)J recombination, or owe to different properties of the thymus versus bone marrow microenvironments.

We observed oligoclonal, in-frame TCR β transcripts, suggesting expansion of a limited cohort of p53^{-/-} *scid* precursors with productive coding joints (Fig. 3). An interesting implication of these results is that *scid* DNA-PK has a higher capacity to mediate coding joint formation than has been appreciated previously. The frequency of coding joint formation in *scid* cells has been measured as 10- to 1000-fold below WT levels (Lieber et al. 1988; Malynn et al. 1988; Pennycook et al. 1993), and normal V(D)J rearrangements have been seen in rare lymphocytes in aged *scid* mice (Carroll and Bosma 1988; Blackwell et al. 1989; Carroll et al. 1989; Hendrickson et al. 1990; Kotloff et al. 1993; Young and Kearney 1995). Our results help to rationalize this "leakiness" by suggesting that the *scid* mutation causes an inefficiency in the V(D)J recombination process that limits cell survival. However, the limited diversity and "scid-like" TCR coding joints we observed suggest that *scid* DNA-PK still functions relatively ineffectively to coordinate DSB repair when thymocyte survival is enhanced by p53 ablation.

Independent support for the notion that attempted V(D)J recombination is deleterious for *scid* lymphocyte precursors has been reported. Transgenic expression of Bcl-2, which can inhibit DNA damage/p53-mediated cell death (for review, see White 1996), improved survival and developmental progression of *scid* pro-B cells, but this study did not evaluate whether this was associated with productive Ig μ recombination (Strasser et al. 1994b). In another study, restoration of B-cell production by transgenic expression of Ig μ and Ig light chains in *scid* mice was dependent on the capacity of different transgenes to inhibit endogenous light chain rearrangement (Chang et al. 1995). We posit that p53-mediated apoptosis eliminated pre-B cells attempting light chain gene rearrangement, but this pathway was not invoked in pre-

cursors bearing a transgene that suppressed endogenous rearrangements. From this perspective, the profound immune deficiency conferred by the *scid* mutation does not result exclusively from failure to generate positive selection signals through pre-T and pre-B cell surface antigen receptors. Rather, *scid* lymphocyte survival is limited by V(D)J coding breaks that trigger p53-mediated apoptosis.

Effects of p53 deficiency vs. low-dose irradiation on T-cell development and V(D)J recombination in scid mice

Previously, we have shown that low-dose irradiation of *scid* newborns provokes a rapid, transient burst of diverse TCR β and TCR δ rearrangements coincident with DP thymocyte maturation and expansion (Danska et al. 1994; Livak et al. 1996). However, multiple features distinguish the irradiated *scid* and p53-deficient *scid* models. Irradiation of p53WT *scid* mice provokes rapid generation of thymocytes with normal, polyclonal TCR β and TCR δ , but not TCR α , coding joints, suggesting a transient induction of repair activities that compensate for the *scid* mutation (Danska et al. 1994; Livak et al. 1996). In contrast, V(D)J coding joints in p53-deficient *scid* mice are oligoclonal, include nonstandard coding joints, and accumulate slowly over a 5- to 8-week postnatal period, consistent with enhanced precursor survival revealing the leakiness of the *scid* mutation. These differences in efficiency, kinetics, and fidelity of coding joint formation suggest that mechanisms operative in promoting radiation-induced maturation and survival of *scid* thymocytes are likely to be distinct from those operative in p53-deficient *scid* mice.

A recent study concluded that p53 is required for radiation-induced T-cell development in *scid* mice (Bogue et al. 1996). This conclusion is surprising, given that low-dose radiation further elevates p53 levels in *scid* thymocytes (Fig. 1), and that p53 activates an apoptotic pathway in thymocytes (Clarke et al. 1993; Lowe et al. 1993), and it provides an apparent paradox with our demonstration that p53 deficiency promotes survival and V(D)J recombination in *scid* thymocytes. However, several variables not considered in the Bogue study may impact the conclusion that p53 plays an obligatory role in radiation-induced development of *scid* thymocytes.

Figure 6. Presence of hyperdiploid premalignant and malignant pro-B cells in p53-deficient *scid* mice. (A) Bone marrow-derived CD19⁺ B220⁺ pro-B cells were purified by cell sorting from 6-week-old p53^{+/+}, p53^{+/-}, and p53^{-/-} *scid* mice. The latter appeared healthy and tumor free (no thymic hyperplasia or lymphadenopathy) at the time of sacrifice. Cells were then fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry. The G₀/G₁ DNA content of pro-B cells from p53^{+/+} *scid* and p53^{+/-} *scid* mice was identical, therefore only the former cells are shown. To ensure that the apparent hyperdiploid shift of the p53^{-/-} *scid* cells was not attributable to minor differences in dye or cell concentration, approximately equal numbers of pro-B cells from p53^{+/+} *scid* and p53^{-/-} *scid* were mixed before propidium iodide staining. The bimodal G₀/G₁ peak of the mixture confirms the unequal DNA content of the two populations. (B) Bone marrow cells and thymocytes from the indicated mice were stained for surface expression of B220 as described for Fig. 3, followed by fixation and propidium iodide staining for DNA content. The B220 versus DNA content dot plot demonstrates a hyperdiploid shift of the B220⁺ cells from both tissues of double mutant mice. Numbers on each plot refer to the percentage of B220⁺ cells in G₀ or G₁/S/G₂ or M phases. Note that in normal thymocytes, a significant B220⁺ population is not detectable (ND), and that the majority of B220⁺ bone marrow cells in normal mice are in G₀/G₁.

Figure 7. Effect of p53-deficiency on in vitro survival of *scid* thymocytes and radiosensitivity of *scid* lymphoma cell lines and spleen cells. (A) The surviving fraction (number recovered/number seeded) of thymocytes from RAG-2^{-/-}, *scid*, or p53^{+/-} *scid* and p53^{-/-} *scid* littermates (3 weeks old) was determined after 1 (dark bars) or 2 (light bars) days of culture. (B) Cell lines established from independent *scid* (LK3 and LK6.2) and p53^{-/-} *scid* lymphomas (SC53-1, SC53-2, and SC53-TL3) were cultured without γ -irradiation (control) or after irradiation with 200 cGy (dark bars) or 500 cGy (light bars). The surviving fraction (number viable irradiated/number viable control) of cells was determined the next day. (C) Spleen cells from 3-week-old *scid* and p53^{-/-} *scid* mice were depleted of red blood cells, and the fraction of cells surviving 24 hr after irradiation was determined as described in B.

First, similar to the data presented here, Bogue and colleagues showed that nontreated p53^{-/-} *scid* mice have an elevated frequency of DP thymocytes compared with age-matched p53^{+/-} *scid* animals. Thus, a radiation-dependent effect on DP thymocyte expansion would have to be detectable above the background of “spontaneous” DP thymocyte development in the double mutant animals. Second, this study examined a single 2-week time point, which may have obscured detection of a similar developmental process that occurs with altered kinetics in irradiated p53^{+/-} versus p53^{-/-} *scid* mice. Finally, we show here that loss of DP thymocytes in nontreated p53^{-/-} *scid* mice occurs coincident with immigration of growth dysregulated pro-B cells as early as 3 weeks of age. Others have shown that irradiation of p53-deficient

(but otherwise WT) mice greatly accelerates lymphoma onset (Kemp et al. 1994). Thus, the kinetics of lymphomagenesis in irradiated p53-deficient *scid* is predicted to be extremely rapid, potentially confounding distinctions between effects on “normal” DP thymocyte maturation and the consequences of rapid lymphomagenesis. Thus, the role of p53 in radiation-induced rescue of TCR rearrangement and lymphocyte maturation in *scid* mice may not be readily resolved in these double mutant mice.

The oncogenic potential of V(D)J recombination

On a p53WT background, *scid* mice develop thymic lymphoma with low incidence (15%) and long latency, but susceptibility to other tumors is not significantly elevated over that seen in BALB/c mice (Custer et al. 1985). p53-deficient mice with WT DNA-PK function also develop primarily thymic lymphoma (50% incidence at 16–20 weeks) (Donehower et al. 1992; Harvey et al. 1994, 1995; Jacks et al. 1994). Thus, lymphoma is the predominant tumor caused by two different genetic lesions that affect DNA repair and damage detection in all tissues. Here we show that loss of p53 synergizes with the *scid* mutation to promote extremely rapid development of thymic lymphoma or lymphocytic leukemia by 6–12 weeks after birth.

Precipitous lymphoma onset may indicate that broken V(D)J molecules in p53-deficient *scid* lymphocyte progenitors frequently participate in aberrant rejoining events initiating an oncogenic process. In support of this notion, we show that loss of p53 in the context of the *scid* mutation permits the rapid accumulation of aneuploid pro-B cells, but not myeloid cells, in very young mice. Thus, recombinationally active lymphocyte progenitors appear to be highly dependent on p53-mediated checkpoints to maintain genomic integrity. Several classes of aberrant V(D)J joining events have been observed in human lymphocytic leukemias and lymphomas. These include translocation of antigen receptor regulatory elements to proto-oncogenes (for review, see Rabbitts 1994), and recombination mediated through “cryptic” RSS (for review, see Lewis 1994). These abnormal joining events highlight the opportunities for RAG-1/2-mediated generation of DSB at inappropriate sites in immature lymphocytes, underscoring the importance of the p53 DNA damage checkpoint during lymphocyte development.

Materials and methods

Mice

All *scid* and RAG-2^{-/-} animals were maintained under SPF conditions, and WT strains (BALB/c, B6) were maintained under conventional conditions at the Hospital for Sick Children. C.B-17.*scid* mice were maintained as homozygotes. TCR β -transgenic mice heterozygous for the *scid* mutation were obtained from E. Shores and A. Singer (Shores et al. 1993) and bred to C.B-17 *scid* mice. TCR β ⁺ *scid*/*scid* mice were detected by PCR amplification of tail DNA using the V β 8incl/C β uni primer pair (Danska et al. 1994). p53^{-/-} mice (Donehower et al. 1992) were bred to ICR.*scid* homozygotes (*scid*), and F₁ offspring were intercrossed to derive p53^{-/-} and p53^{+/-} *scid* animals. Homozy-

gous *scid* mice were identified by flow cytometric analysis of peripheral blood cells stained with monoclonal anti-Ig μ and anti-Thy 1.2 antibodies. WT and targeted (KO) p53 alleles were identified by PCR of tail DNA with two primer pairs; p53WT: sense 5'-GTC CGC GCC ATG GCC ATC TA-3', antisense 5'-ATG GGA GGC TGC CAG TCC TAA CCC-3', yields a 137-bp fragment and, p53KO: sense 5'-GTG GGA GGG ACA AAA GTT CGA GGC C-3', antisense 5'-TTT ACG GAG CCC TGG CGC TCG ATG T-3', yields a 170-bp fragment. PCR conditions were 30 cycles of 30 sec at 94°C, 60 sec at 55°C, and 40 sec at 72°C. The mice used in this study were the progeny of p53^{+/-} *scid* parents, or p53^{+/-} *scid* females and p53^{-/-} *scid* males. p53 genotypes were determined retrospectively. RAG-2^{-/-} *scid* double mutants were generated by backcrossing (RAG-2^{-/-} $\times$ CB-17.*scid*) F₁ progeny to CB-17.*scid*. The RAG-2^{-/-} line has been described previously (Shinkai et al. 1992). Peripheral blood from backcross progeny was screened for the presence of T- and B-cells by flow cytometry to identify *scid* homozygotes. WT and KO RAG-2 alleles were then identified in *scid* homozygotes by PCR amplification of tail DNA using a primer trio of RAG 2-3 (5'-GCC TGC TTA TTG TCT CCT GGT ATG-3'), Neo-3 (5'-CCA ACG CTA TGT CCT GAT AGC GGT-3'), and RAG 2-1 (5'-TTA ATT CAA CCA GGC TTC TCA CTT-3'). Thermal cycling conditions for this trio were 30 cycles of 30 sec at 94°C, 1.5 min at 60°C, and 2 min at 72°C. Under these conditions, RAG 2-3 and Neo-3 amplify a 937-bp KO fragment, and RAG 2-3 and RAG 2-1 amplify a 973-bp WT fragment that were resolved by 2% agarose gel electrophoresis. RAG-2^{-/-} *scid* mice were maintained as double homozygotes.

Flow cytometry

Staining of thymocyte, bone marrow, and lymph node cell suspensions or lymphoma cell lines for surface or intracellular expression of various lineage and developmental markers was performed as previously described (Guidos et al. 1990; Danska et al. 1994) using a FACScan flow cytometer with Lysis II software (Becton Dickinson & Co., Mountain View, CA). The following monoclonal antibodies were affinity purified from hybridoma culture supernatants and conjugated to various fluorochromes using standard techniques, or were purchased from Pharmingen (San Diego, CA): TCR β (H57), CD4 (YTS 191.1), CD8 (YTS 169.4), B220 (6B2 or 14.8), CD43 (anti-S7), Ig μ (R40-97), CD19 (1D3), and Thy-1.2 (53-2.1). Purification of DN thymocyte subsets was performed by magnetic bead depletion of CD4, CD8, CD3-expressing thymocytes, followed by staining with anti-CD25 and and fluorescence-activated cell sorting. Both DN populations were reanalyzed and found to be >98% pure (data not shown). Purification of bone marrow pro-B cells was done by sorting cells staining positive with fluorescein isothiocyanate (FITC)-anti-B220 and biotin-anti-CD19/avidin-phycoerythrin. Reanalysis of these populations showed them to be >98% pure. Cell cycle analysis of both CD25⁺ DN thymocytes, and B220⁺CD19⁺ pro-B cells was done by staining ethanol-fixed cells with propidium iodide as previously described (Groves et al. 1995).

Cell lines and survival assays

p53^{-/-} *scid* cell lines were derived by explanting tumors from lymph nodes and thymy into Iscove's medium supplemented with 10% fetal bovine serum, 10 mM HEPES (pH 7.0), 50 μ M 2-mercaptoethanol, 2 mM glutamine, and 1% penicillin/streptomycin using standard techniques. Derivation of lymphoma cell lines from p53WT *scid* mice was described previously (Danska et al. 1994). These cell lines display radiation-induced p53 protein expression, p21^{waf-1/cip-1} mRNA expression, and apop-

tosis, and thus appear to have normal p53 function (data not shown). For determination of sensitivity to ionizing radiation, 2 $\times$ 10⁶ cells were irradiated with a ¹³⁷Cs source, and cultured overnight in 4 ml of RPMI supplemented as described above. The number of viable cells remaining was determined by counting trypan blue excluding cells 24 hr later. For thymocyte survival assays, 1–2 $\times$ 10⁶ cells in 2 ml of supplemented RPMI were seeded into 24-well plates and cultured for 1 or 2 days. The number of surviving cells was determined by counting with trypan blue.

Molecular analysis of antigen receptors

Reverse-transcriptase coupled PCR amplification was performed as previously described (Danska et al. 1994). Seven TCR V β -specific primers were used in separate reactions with a TCR C β antisense primer (Danska et al. 1994; Galley and Danska 1995). RT-PCR to examine TCR α was done with a consensus V α /C α primer pair as previously described (Danska et al. 1990). RT-PCR products were cloned into the pTA vector (Invitrogen Corp., San Diego, CA), screened by hybridization with a C β probe, and sequenced as previously described (Galley and Danska 1995). For genomic Southern blot analyses, DNA was prepared from the thymus, bone marrow, or kidney of WT or p53^{-/-} *scid* mice at the indicated ages and from cell lines. DNA samples were digested with *Eco*RI or *Pvu*II, and electrophoresed on 0.8% agarose gels. Southern blot filters were hybridized to IgJ μ 4 (Atkinson et al. 1991), HPRT, or TCRC β probes. RT-PCR analysis of Ig μ transcripts was performed with a pair of C μ primers (forward, 5'-CTA CGG AGG CAA AAA CAA AGA-3' and reverse, 5'-TAG CCA CAC CCT TAG CAC TGA-3'), and with a degenerate VH forward primer (VHall, see Guidos et al. 1995) paired with the reverse C μ primer. PCR products were resolved by electrophoresis, transferred to Southern blots and hybridized to a radiolabeled C μ cDNA probe. PhosphorImages were obtained with a Molecular Dynamics imaging system.

Western blot analysis

Monoclonal antibody Pab 242 (ATCC, Rockville, MD) was used to detect p53 expression in nuclear extracts. Briefly, cells were swelled on ice in 10 mM HEPES (pH 7.9), 10 mM KCl, 1 mM DTT, and protease inhibitors, lysed by addition of NP-40 to 0.6% and microcentrifuged for 30 min at 4°C. Nuclear proteins were eluted from the pellet in 20 mM HEPES (pH 7.9), 0.4 M KCl, 1 mM EDTA, 1 mM DTT, and protease inhibitors by agitation at 4°C. The supernatant was recovered after microcentrifugation, and adjusted to 120 mM KCl by addition of 10 mM HEPES (pH 7.9). Protein concentrations were measured (Bio-Rad protein assay) and 50–100 μ g of each sample was subjected to SDS-PAGE (reducing conditions), Western blotting, and enhanced chemiluminescence detection (ECL, Amersham) using standard methods.

Acknowledgments

We wish to thank D. Holland for expert technical assistance, Dr. G. Wu for sharing unpublished results, Dr. E. Shores for TCR β -transgenic *scid* mice, Drs. S. Lewis and J. Dick and for their interest in the work and insightful comments on the manuscript, and the members of our laboratories for advice and encouragement. M.T.F.H. thanks T. Cruz, M. Chang, S. Phelan, and R. Hoyt for assistance with breeding p53-deficient mice. This work was supported by a Hospital for Sick Children Research Institute (RESTRACOM) studentship to C.J.W., and grants to J.S.D. and C.J.G. from the Medical Research Council,

the Leukemia Research Fund, and the National Cancer Institute of Canada (NCIC) with funds from the Canadian Cancer Society. J.S.D. is a Research Scientist of the Canadian Cancer Society NCIC.

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Genes Dev. 1996, **10**:

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