p53 is required for both radiation-induced differentiation and rescue of *V(D)J* rearrangement in *scid* mouse thymocytes

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The murine *scid* mutation affects both *V(D)J* recombination and DNA repair. This mutation has been mapped to the gene encoding the catalytic subunit of the DNA-dependent protein kinase (DNA-PK), which is activated by DNA damage in normal cells. In *scid* mice, antigen receptor gene rearrangements are initiated normally, but impaired joining of coding ends prevents assembly of functional receptor genes, resulting in arrest of B- and T-cell development. Others have shown that exposure of *scid* mice to genotoxic agents such as γ -irradiation rescues rearrangement at the T-cell receptor (TCR) β locus and promotes thymocyte development. Here we demonstrate that irradiation rescues rearrangements at multiple TCR loci, suggesting a general effect on the recombination mechanism. Furthermore, our data show that p53 is required for irradiation-mediated rescue of both thymocyte development and *V(D)J* recombination. We also find that thymocyte proliferation and differentiation in the absence of DNA damage do not require p53 and are not sufficient to rescue *V(D)J* recombination. These results suggest that exposure to ionizing radiation facilitates a partial bypass of the *scid* defect, perhaps by inducing p53-dependent DNA damage response pathways.

[**Key Words:** p53; *scid*; ionizing radiation; *V(D)J* recombination; thymocyte differentiation; DNA repair]

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The mechanisms of *V(D)J* recombination and DNA repair are intimately connected (Pergola et al. 1993; Taccioli et al. 1993, 1994; Blunt et al. 1995; Roth et al. 1995). This relationship was first suggested by the observation that the mouse *scid* mutation impairs both *V(D)J* recombination and double-strand break repair (Fulop and Phillips 1990; Biedermann et al. 1991; Hendrickson et al. 1991). The discovery that irradiation of *scid* mice promotes both thymocyte development and rescue of rearrangements at the T-cell receptor β (TCR β) locus (Danks et al. 1994; Murphy et al. 1994) provides a valuable model system for investigating the connections among DNA repair, *V(D)J* recombination, and T-cell development. Here we investigate the mechanisms of irradiation-mediated rescue of TCR rearrangement and thymocyte differentiation in *scid* mice.

Our understanding of the intricate process of thymocyte development has advanced considerably in the past few years (for review, see Anderson and Perlmutter 1995; Levelt and Eichmann 1995). A simplified view of early intrathymic $\alpha\beta$ thymocyte development can be summarized as follows: immature precursor cells that express little or no CD4 or CD8, termed double-negative (DN)

cells, migrate to the thymus. TCR β rearrangement initiates in DN cells (Godfrey et al. 1993), and differentiation to the subsequent developmental stage normally proceeds when a functional TCR β chain has been expressed. Signaling of this event is thought to be mediated by the association of the TCR β chain with members of the CD3 complex and with the surrogate TCR α chain, pT α (Groettrup et al. 1993; Saint-Ruf et al. 1994; Fehling et al. 1995). Signaling through this complex results in rapid proliferation and differentiation to the CD4⁺ CD8⁺ double-positive (DP) stage (Godfrey et al. 1993, 1994), where most TCR α rearrangements are thought to occur (Groettrup and von Boehmer 1993; Wilson et al. 1994). Productive rearrangement of a TCR α allele allows replacement of the surrogate TCR α chain and surface expression of the mature $\alpha\beta$ TCR/CD3 complex, which is required for further maturation to single-positive (SP) CD4⁺ or CD8⁺ thymocytes.

V(D)J recombination plays a critical role in normal thymocyte development, as expression of functional TCR chains is required for both the DN to DP and the DP to SP developmental transitions. *V(D)J* recombination is initiated by the introduction of double-strand breaks (DSB) at specific sequences, termed recombination signal sequences, that are situated adjacent to the V, D, and J coding elements (Roth et al. 1992b). This site-

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specific cleavage, mediated by the RAG-1 and RAG-2 proteins (McBlane et al. 1995; van Gent et al. 1995), produces two kinds of broken DNA intermediates: coding ends, which are covalently sealed in the form of hairpins (Roth et al. 1992a; McBlane et al. 1995; Ramsden and Gellert 1995; van Gent et al. 1995; Zhu and Roth 1995) and signal ends, which are blunt (Roth et al. 1993; Schlissel et al. 1993; Zhu and Roth 1995). Joining of coding ends assembles the *V*, *D*, or *J* gene segments, producing coding joints. Signal ends are joined to form the reciprocal junction, termed a signal joint.

Mice homozygous for the *scid* mutation are able to join signal ends, but they fail to generate significant numbers of functional immunoglobulin and TCR gene rearrangements (Schuler et al. 1986, 1990) because of a severe defect in coding joint formation (Lieber et al. 1988; Malynn et al. 1988). In thymocytes of these mice, recombination is initiated normally but unresolved hairpin coding ends accumulate (Roth et al. 1992a; Zhu and Roth 1995), indicating that the *scid* factor is required for the joining of coding ends. Scid cells are also hypersensitive to ionizing radiation (Fulop and Phillips 1990; Biedermann et al. 1991; Hendrickson et al. 1991) and are deficient in the repair of chromosomal DSB (Biedermann et al. 1991; Hendrickson et al. 1991). The *scid* mutation has been mapped to the gene encoding the catalytic subunit of the DNA-dependent protein kinase (DNA-PK) (Blunt et al. 1995; Hartley et al. 1995; Kirchgessner et al. 1995; Peterson et al. 1995), and scid cells do not express detectable DNA-PK activity (Blunt et al. 1995; Boubnov and Weaver 1995). DNA-PK is activated by binding DNA lesions, including DSB (Gottlieb and Jackson 1993; Morozov et al. 1994) and is capable of phosphorylating a variety of substrates in vitro, including a number of transcription factors and the tumor suppressor protein p53 (Lees-Miller et al. 1992; Gottlieb and Jackson 1994). The precise roles of DNA-PK in *V(D)J* recombination and DSB repair remain elusive, although one obvious scenario involves phosphorylation of downstream target molecules in response to DNA lesions (Gottlieb and Jackson 1994). It has been suggested that DNA-PK may regulate the accessibility of hairpin coding ends to the hairpin opening machinery (Blunt et al. 1995; Roth et al. 1995; Zhu and Roth 1995).

Thymocytes of scid mice are arrested at the DN stage (Shores et al. 1990) because of the *scid* defect in coding joint formation that precludes the formation of functional TCR rearrangements. This early block in thymocyte development can be partially relieved by treatment with agents that cause DSB, including γ -irradiation (Danska et al. 1994; Murphy et al. 1994). Within 1–2 weeks after treatment, roughly normal proportions of DP thymocytes are observed, and the cellularity of the thymus is increased. DP cells persist in the thymus for months; however, further developmental progression either does not occur or is very rare, as distinct SP populations are not observed (Danska et al. 1994; Murphy et al. 1994). Polyclonal TCR β rearrangements are also detected within 1–2 weeks after the irradiation of newborn scid mice; furthermore, sequence analysis revealed that

90% of the TCR β transcripts are in-frame and appear normal (Danska et al. 1994), lacking the structural anomalies associated with aberrant rearrangements isolated from scid mice (Kienker et al. 1991; Schuler et al. 1991). These data indicate that DNA-damaging agents may induce “bypass” DNA repair processes that can alleviate, at least partially, the block in coding joint formation conferred by the *scid* mutation.

Because p53 is involved in early responses to DNA damage, we considered the possibility that p53 might play a critical role in irradiation-induced rescue of *V(D)J* rearrangement and development in scid thymocytes. p53 binds to a variety of DNA lesions (Bakalkin et al. 1994; Lee et al. 1995) and accumulates rapidly in response to genotoxic insults such as γ -irradiation (Lu and Lane 1993; Nelson and Kastan 1994). p53 in turn stimulates transcription of a variety of genes responsible for cell cycle arrest or apoptosis (Clarke et al. 1993; Lowe et al. 1993; Kastan et al. 1995). Interestingly, the transcriptional activation domain of p53 contains DNA-PK consensus phosphorylation sites and is phosphorylated in vitro by DNA-PK (Lees-Miller et al. 1992). The proposed link between DNA-PK and p53 is further supported by the observation that a mutation in one of the phosphorylation consensus sites impairs p53-dependent inhibition of cell cycle progression (Fiscella et al. 1993). However, the physiological relevance of p53 phosphorylation by DNA-PK has not been determined.

We have used scid and p53-deficient scid mice to demonstrate the following. (1) p53 is required for irradiation-induced rescue of both rearrangement at multiple TCR loci and proliferation and differentiation to the DP stage in scid thymocytes. (2) p53 is not required for anti-CD3 ϵ induction of proliferation and differentiation in scid thymocytes, underscoring the specific role of p53 in the irradiation response. (3) Whereas treatment with antibodies to CD3 ϵ causes significant proliferation and differentiation of scid thymocytes, rescue of TCR δ rearrangements is not observed, indicating that rescue of rearrangement and differentiation can be dissociated. (4) p53-Dependent responses are not impaired in scid thymocytes, indicating that DNA-PK activity is not required for activation of p53.

Results

Absence of p53 prevents irradiation-induced rescue of thymocyte development and V(D)J recombination

How does irradiation induce differentiation and the rescue of TCR gene rearrangement in scid mice? One obvious possibility is that the signal for both events is provided by irradiation-induced DSB. This hypothesis is supported by the observation that treatment of newborn scid mice with bleomycin, which induces DSB, also promotes the appearance of DP thymocytes (Danska et al. 1994). Because p53 is thought to play a key role in orchestrating the early cellular response to DNA damage (Kastan et al. 1995), we asked whether the irradiation-induced rescue of either thymocyte development or

V(D)J recombination in scid mice is affected by the lack of functional p53.

Scid mice were crossed to p53-deficient mice (Donehower et al. 1992), generating animals that were homozygous for the *scid* defect and homozygous or heterozygous for the mutated p53 allele. Newborn mice were treated with 100 centigrays (cGy) of γ -irradiation within the first 24 hr after birth, and thymocytes were harvested and analyzed 11–16 days after irradiation. Figure 1a shows representative CD4/CD8 profiles of thymocyte preparations from individual unirradiated or irradiated scid p53 +/– and scid p53 –/– littermates. Irradiation of scid p53 +/– mice promotes increased thymic cellularity, with DP cells reaching wild-type proportions. These results are consistent with previous reports (Danska et al. 1994; Murphy et al. 1994). However, after irradiation of scid p53–null mice, no significant increase in thymic cellularity or in numbers of DP thymocytes was observed in comparison to unirradiated scid p53–null mice, as shown in the bottom set of profiles in Figure 1a. Data from a number of independent experiments are summarized in Figure 1b. These results demonstrate that p53 is required for irradiation-induced rescue of thymocyte development in scid mice.

Another interesting feature of the data shown in Figure 1 is the presence of DP thymocytes in unirradiated scid p53 –/– mice. This irradiation-independent DP population increases with age from 8% of total thymocytes at day 9 to 45%–50% by 6 weeks of age (M. Bogue, C. Zhu, L. Donehower, and D. Roth, unpubl.). A similar phenomenon has also been observed in mice deficient for both RAG-1 and p53 (Mombaerts et al. 1995). Taken together, these results suggest that in the absence of p53, limited developmental progression to the DP stage can occur in rearrangement-deficient mice. We are currently investigating this phenomenon in more detail.

We have shown that p53 is required for irradiation-induced rescue of thymocyte proliferation and differentiation (Fig. 1). We next asked whether the lack of p53 also prevents irradiation-induced rescue of *V(D)J* recombination in scid mice. As shown in Figure 2 there is no detectable rearrangement at the TCR β locus in scid p53 –/– thymocytes after irradiation (lane 3) as compared with irradiated scid p53 +/– mice (lane 4). (The non-germ-line fragments visible in unirradiated scid thymus DNA result from DSB at D β 1; see legend to Fig. 2 for details.) These data indicate that p53 is required for the irradiation-induced rescue of TCR β rearrangements in scid thymocytes.

Previous studies examined TCR α and TCR β rearrangement and found consistent rescue only of TCR β rearrangements in irradiated scid mice (Danska et al. 1994; Murphy et al. 1994), suggesting that the irradiation effect might be locus specific. We therefore examined other loci for irradiation-induced rearrangements. Figure 3 shows a highly diverse pattern of rearrangement at the TCR δ locus in thymocytes from irradiated scid p53 +/– mice (lane 5; cf. with the pattern obtained from unirradiated scid mice shown in lane 2). Irradiation also rescues rearrangement at the TCR γ locus, as shown below.

Figure 1. p53 is required for irradiation-induced rescue of thymocyte development in scid mice. (a) CD4-PE/CD8-FITC profiles are shown from thymocytes of individual unirradiated or irradiated wild-type (WT) (day 13), scid p53 +/–, and scid p53 –/– mice. The number in the second quadrant is the percentage of DP cells, and the number below the histogram denotes the cellularity of the thymus ($\times 10^6$). (b) Accumulated data from CD4/CD8 profiles and thymic cellularity are shown from several individual unirradiated and irradiated scid p53 +/– and scid p53 –/– mice, ranging from day 11 to day 16. The bar graph shows percent thymocytes (with standard deviation) that are DN (solid) or DP (open). Numbers below the bars indicate the average thymic cellularity (with standard deviation). (n) Number of mice tested.

As we have shown for the TCR β locus, p53 is also required for irradiation-mediated rescue of TCR δ rearrangements, as rearrangement is not observed in irradiated scid p53–null mice (lane 4). These data indicate that irradiation promotes rearrangement at multiple TCR loci by a p53-dependent mechanism.

Figure 2. p53 is required for irradiation-induced rescue of rearrangement at TCR β in scid mice. (Top) TCR β Southern blot analysis of unirradiated or irradiated scid p53 $^{+/-}$ and scid p53 $^{-/-}$ thymocyte DNAs digested with *Eco*RI. For comparison, unirradiated wild-type (WT) thymus and testis DNAs are also shown. (The same wild-type DNA samples were used for both blots; the slight difference in the appearance of the patterns is attributable to different electrophoresis conditions.) The TCR β map (bottom) shows the location of the probe (heavy line spanning D β 1) in relation to D β 1 and J β 1 coding gene segments (open boxes) and their recombination signal sequences (solid triangles). This probe primarily detects germ-line and D to J rearrangements, but also detects D β 1 coding and signal ends in scid thymocyte DNA (C. Zhu, M. Bogue, F. McBlane, L. Donehower, and D. Roth, in prep.). The diagram illustrates the 8.8-kb TCR β germ-line (GL) fragment and two non-germ-line 5.2-kb fragments representing D β 1 hairpin coding ends (loop structure; CE) and D β 1 signal ends (SE). These DSB at D β 1 (D), germ-line (GL) fragment, and the positions of DNA size markers (1-kb ladder, GIBCO-BRL) are indicated on the Southern blot.

Time course of irradiation-induced development and rearrangement at multiple TCR loci

As shown above, irradiation of newborn scid mice triggers several events, including thymocyte proliferation, differentiation, and rescue of rearrangement at multiple TCR loci. To define the nature of the irradiation response more carefully, we examined the time course of these events in scid mice. Profiles of cell surface staining for CD4 and CD8 on thymocytes from individual mice are shown in Figure 4. A distinct DP population is first evident at day 8. By day 11, the DP population is predominant at ~90% of total thymocytes, persisting until at least day 25. The appearance of DP thymocytes is

accompanied by an increase in thymic cellularity (up to 15-fold), although total cell numbers remain substantially lower than wild-type animals.

The timing of the appearance of rearrangements at TCR δ is illustrated in Figure 5a. Rescue of TCR δ rearrangement is not apparent prior to day 9, at which time diverse rearrangements are detected. By day 10, consid-

Figure 3. p53 is required for irradiation-induced rescue of rearrangement at TCR δ in scid mice. (Top) TCR δ Southern blot analysis of irradiated scid p53 $^{+/-}$ and scid p53 $^{-/-}$ thymocyte DNA digested with *Eco*RI. For comparison, unirradiated scid, wild-type (WT) thymus and testis DNAs are also shown. Unirradiated scid p53 $^{-/-}$ (not shown) is similar in pattern to unirradiated scid (see Fig. 7b, lane 5, for unirradiated scid p53 $^{-/-}$). The TCR δ map (bottom) shows the location of the probe (heavy line 3' of J δ 1) in relation to D δ 2 and J δ 1 coding gene segments (open boxes) and their recombination signal sequences (solid triangles). This probe detects germ-line (GL) and D to D, D to J, V to J, and V to D/J rearrangements (Chien et al. 1987a,b; Carroll and Bosma 1991; Roth et al. 1992b). This probe also detects D and J coding ends (CE) in thymocyte DNA from scid mice (Roth et al. 1992a), as illustrated in the map. Non-germ-line fragments of 4.0 and 4.9 kb that have been identified previously as hairpin coding ends (Roth et al. 1992a) are prominent in DNA from unirradiated scid thymocytes. Previous work has established that these coding ends accumulate in scid, but not wild-type, thymocytes (Roth et al. 1992a), presumably because they are resolved quickly into coding joints in wild-type cells (Ramsden and Gellert 1995). Another non-germ-line fragment of 6.6 kb, which is derived from D δ 2 to J δ 1 rearrangement (R), is seen in unirradiated scid thymocytes (Carroll and Bosma 1991).

Figure 4. Time course of irradiation-induced proliferation and differentiation. CB.17 *scid/scid* (*scid*) mice were irradiated and harvested at various times. CD4–CyChrome/CD8–PE thymic profiles are shown for individual irradiated *scid* mice from days 3–25. Unirradiator wild-type (WT) and *scid* are shown for comparison. The age of the mouse is indicated above each profile; the number in the second quadrant corresponds to the percentage of DP cells, and the number below the profile indicates the thymic cellularity ($\times 10^6$).

erable rearrangement is evident, resulting in substantially diminished intensity of the germ-line fragment. This suggests that TCR δ rearrangements are present in a large fraction of thymocytes from irradiated *scid* mice at day 10.

Because TCR β rearrangements normally play a role in early thymocyte development, we considered the possi-

bility that rearrangement at this locus might precede the appearance of DP thymocytes in irradiated *scid* mice. Reprobing the time course blot shown in Figure 5a with a DB1-specific probe revealed that the timing of TCR β rearrangement after irradiation parallels that of TCR δ (data not shown). We also assayed for rescued rearrangements at TCR β and TCR γ using the polymerase chain reaction (PCR) to amplify rearranged products. Thymocyte DNA samples from irradiated and unirradiator *scid* mice were amplified using primers specific for V β 8–J β 2.6 and V γ 2–J γ 1 rearrangements. As shown in Figure 5b, whereas little or no PCR products could be detected from DNA prepared from unirradiator thymocytes, both of these rearrangements were detected easily by V-region-specific probes on day 10 after irradiation. PCR products derived from both TCR β and TCR γ rearrangements are the same size on acrylamide gels as products generated by amplification of rearrangements from wild-type mice. Sequence analysis (not shown) of several clones from V β 8–J β 2.6 PCR revealed coding joints that are indistinguishable from wild-type thymocytes. These data indicate that the irradiation-induced rescue of recombination produces grossly normal coding joints at these loci, in agreement with previous nucleotide sequence analysis of TCR β rearrangements (Danska et al. 1994). In summary, DP thymocytes and rearrangements at TCR δ , TCR β , and TCR γ all become apparent at about the same time after irradiation.

Irradiation activates recombination at TCR α

The data reported here show that irradiation of newborn *scid* mice rescues rearrangement at three of the four TCR loci. However, previous studies did not detect significant TCR α rearrangement or cell surface expression in irradiated *scid* mice (Danska et al. 1994; Murphy et al. 1994). We wondered whether the differential effects of irradiation might be dependent on the recombinational activity of the locus at the time of irradiation. Because rescue of rearrangements presumably involves bypass of the *scid* block to joining hairpin coding ends, one would expect only actively rearranging loci to be available for rescue.

Figure 5. Irradiation rescues rearrangement at multiple TCR loci in *scid* mice. (a) TCR δ Southern blot analysis of *Eco*RI-restricted DNA from thymocytes of irradiated *scid* mice from days 5, 7, 9, and 10. Thymocyte DNA from unirradiator wild-type (WT) and adult newborn *scid* are shown for comparison (see Fig. 3 for map and probe details). Germ-line (GL), D δ 2 to J δ 1 rearrangement (R), and D δ 2 (D), and J δ 1 (J) coding ends are indicated. (b) PCR analysis of V β 8–J β 2.6 and V γ 2–J γ 1 rearrangements in unirradiator and irradiated day 10 *scid* thymocyte DNA (100 ng). Amplification of wild-type (WT) thymocyte DNA (10 ng) is shown for size comparison. The negative control contained all reagents used for amplification except DNA. Markers (radiolabeled 1-kb ladder) are shown in the left-most lane; pertinent sizes are indicated.

Figure 6. Recombination at TCR α is initiated in thymocytes of irradiated scid mice. (a) TCR α Southern blot analysis of *Eco*RI-digested thymocyte DNA from unirradiated scid and wild-type (WT) mice and irradiated scid mice. Germ-line (GL) and J α 50 (J50) and J α 49 (J49) coding ends are indicated. To compare loading of these lanes, these blots were stripped and reprobed with RAG-1, as shown in the bottom panel. (b) TCR α Southern blot analysis of *Eco*RI-digested and *Sph*I-digested thymocyte DNA. Unirradiated and irradiated scid thymocyte DNA samples are shown. Germ-line (GL) and J α 50 (J50) and J α 49 (J49) coding ends are indicated. Testis DNA was also digested separately with these enzymes. Lane 1 is loaded with aliquots of both digests. **Note:** Although sequence data for the TCR α locus (Koop et al. 1992; Wilson et al. 1992) does not indicate an *Sph*I site in the vicinity of the upstream *Eco*RI site shown in this map, we discovered an additional *Sph*I site by restriction mapping ~40 bp downstream of the indicated *Eco*RI site. (c) Map of the TCR J α region showing the coding elements (open boxes) and their recombination signal sequences (solid triangles) that reside on this *Eco*RI fragment. Although this proximal region of J α has been shown to be recombinationally active in normal mice (Livak et al. 1995), the large size of the TCR α locus precludes the ability to directly visualize novel fragments resulting from rearrangements involving more than a small fraction of the numerous coding elements. We therefore chose a probe (heavy line between J α 49 and J α 48) that has been used (Livak et al. 1995) to assess rearrangements by measuring a decrease in germ-line hybridization intensity, which occurs as a consequence of deletional rearrangement. Expected sizes for germ-line (GL) and J α 50 (J50) and J α 49 (J49) coding ends are indicated for *Eco*RI and *Sph*I digests.

The TCR α locus is presumed to be recombinationally inactive in scid thymocytes. Germ-line transcripts, which are an indicator of recombinational accessibility, are conspicuously absent from the TCR α locus, whereas TCR β , TCR δ , and TCR γ transcripts are detected easily in scid thymocytes (Schuler et al. 1988).

We examined the status of the TCR α locus in unirradiated and irradiated scid mice by Southern blotting. Since detection of rearrangements at this locus is somewhat problematic because of its large size (see Fig. 6 legend), we first established the normal rearrangement pattern using wild-type thymocytes that should be active for TCR α recombination. Thymocyte DNA from wild-type mice shows a notable decrease in germ-line intensity along with the appearance of several non-germ-line fragments (Fig. 6a, lane 2). Both of these phenomena provide evidence for TCR α rearrangement (Livak et al. 1995). [To ensure that lanes were loaded equally, we re-probed the blot with a probe that hybridizes to a gene (RAG-1) that does not undergo rearrangement (Fig. 6a, bottom)] The situation in unirradiated scid thymocyte DNA is quite different, as there is only a single, intense germ-line fragment (Figure 6a, lane 1). Thus, as suggested by the germ-line transcription data (Schuler et al. 1988),

we do not detect TCR α rearrangement in unirradiated scid mice.

In contrast, in thymocytes of irradiated scid mice, several non-germ-line fragments appear that are not observed in wild-type or unirradiated animals (Fig. 6a,b, lanes 3). The sizes of these novel *Eco*RI fragments suggest that they might be derived from J α coding ends, as illustrated in the map shown in Figure 6c. To map these putative coding ends with higher resolution, DNA samples were digested with *Sph*I. This digest generates novel fragments whose sizes are consistent with the presence of coding ends at J α 50 and J α 49, as shown in Figure 6c. This interpretation is further supported by ligation-mediated PCR analysis (Zhu and Roth 1995), which confirmed the presence of J α 50 and J α 49 hairpin coding ends in thymocyte DNA preparations from irradiated but not from unirradiated scid mice (C. Zhu, M. Bogue, F. McBlane, L. Donehower, and D. Roth, in prep.).

These results indicate that initiation of TCR α rearrangement (as measured by the formation of coding ends) is induced by irradiation, perhaps as a consequence of differentiation to the DP stage. This hypothesis is consistent with the observation that TCR α germ-line transcription can be induced in RAG-deficient mice follow-

ing a treatment that induces developmental progression to the DP stage (Levelt et al. 1995). Although our data show that irradiation promotes the appearance of hairpin coding ends at TCR α gene segments, we have not detected completed TCR α rearrangements by Southern blotting, and others have failed to detect significant levels of TCR α rearrangement in irradiated scid mice using more sensitive PCR-based techniques (Danska et al. 1994). These results imply that irradiation-induced effects on rearrangement in scid mice may be transient and may not persist for the several days that elapse between irradiation and the appearance of DP thymocytes.

Rescue of thymocyte development can be dissociated from rescue of TCR gene rearrangement

Because rescue of *V(D)J* rearrangement and thymocyte development both require p53 and occur with similar timing after irradiation, we wondered whether these two processes might be interdependent. An initial assessment of our results suggests that rescue of TCR rearrangement might be responsible for the rescue of development, as a substantial fraction of thymocytes—most of which are DP cells—have rearrangements at one or more TCR loci (see diverse rearrangements and diminished germ-line bands in irradiated scid samples in Fig. 2, lane 4, Fig. 3, lane 5, and Fig. 5, days 9 and 10). However, this simplistic view is clouded by the fact that irradiation also promotes the appearance of DP thymocytes in RAG-deficient mice (Zúñiga-Pflücker et al. 1994; Guidos et al. 1995). Therefore, rescue of thymocyte development can clearly occur in the absence of TCR rearrangements.

Another way in which rescue of development might be linked to rescue of rearrangements is the possibility that rearrangements are somehow promoted by irradiation-induced proliferation and differentiation. The underlying mechanism of such an effect could be related to the well-documented “leakiness” of the *scid* defect. A substantial fraction of older scid mice are leaky, and TCR rearrangements are readily detected (Bosma et al. 1988; Petrini et al. 1990). Even in newborn scid mice some rearrangements occur normally, most notably *D δ 2– δ 1* coding joints (Carroll and Bosma 1991), which can be detected by Southern blotting (for example, see Figure 3, lane 2). We therefore considered the possibility that the irradiation-induced developmental progression might somehow enhance this intrinsic leakiness, perhaps through proliferation and amplification of cells containing rare rearrangements.

To address this possibility, we sought a means to induce proliferation/developmental progression without introducing DNA damage. Development to the DP stage is promoted in thymocytes of scid or RAG-deficient mice or fetal thymic organ culture by treatment with antibodies to CD3 ϵ (Levelt et al. 1993; Jacobs et al. 1994; Shinkai and Alt 1994). This treatment cross-links surface CD3 molecules, presumably mimicking the signal that is normally made through the pre-TCR/CD3 complex. Anti-CD3 ϵ treatment of scid p53 +/– mice re-

sulted in proliferation and appearance of DP thymocytes, as shown in Figure 7a. Thymic cellularity was increased to a level comparable to that induced by irradiation treatment. Similar results were obtained with anti-CD3 ϵ treated scid p53 –/– mice (Fig. 7a), indicating that p53 is not required for rescue of thymocyte development in response to anti-CD3 ϵ treatment. This observation supports the hypothesis that p53 is specifically involved in the response to irradiation, as differentiation and proliferation triggered by anti-CD3 ϵ treatment can proceed efficiently in the absence of p53.

To assess the effects of anti-CD3 ϵ treatment on *V(D)J* recombination, we examined the rearrangement status of the TCR δ locus. TCR δ was chosen because it exhibits the most striking pattern of rearrangements in response to irradiation (e.g., see Fig. 3, lane 5). Although treatment with anti-CD3 ϵ promotes the appearance of DP thymocytes and increased cellularity as efficiently as irradiation, no rescue of TCR δ rearrangements was observed in scid p53 +/– mice (Fig. 7b, left panel). As expected,

Figure 7. Rescue of thymocyte development can be dissociated from rescue of TCR gene rearrangement. (a) CD4–CyChrome (y-axis)/CD8–PE (x-axis) profiles are shown for anti-CD3 ϵ -treated day 12 scid p53 +/– and scid p53 –/– mice. The percentage of DP cells is 77% and 84% for scid p53 +/– and scid p53 –/–, respectively. Numbers below the histograms indicate thymus cellularity ($\times 10^6$). (b) TCR δ Southern blot analysis of *Eco*RI-restricted DNA from thymocytes of untreated, irradiated, and anti-CD3 ϵ -treated (day 12) scid p53 +/– and scid p53 –/– mice. Because the anti-CD3 ϵ -treated scid p53 +/– sample (lane 3) is underloaded, we overloaded a second *Eco*RI digest, (lane 4), which shows no significant rearrangement.

anti-CD3 ϵ treated scid p53-null mice failed to exhibit rescue of rearrangements (Fig. 7b, right). These data suggest that relief of the developmental block in the absence of irradiation is not sufficient to promote rescue of rearrangements. This argues against the possibility that rearrangement is merely a consequence of thymocyte proliferation or differentiation. Instead, this result strongly suggests that DNA damage has a specific effect on the rearrangement process. One simple explanation would be that irradiation-induced DNA damage initiates DNA repair pathways that are somehow able to promote joining of stalled recombination intermediates, as discussed below.

Discussion

The experiments presented here demonstrate that γ -irradiation is capable of promoting rearrangement and thymocyte development in scid mice and that these events are dependent on the presence of functional p53. We have shown that exposure of newborn scid mice to ionizing radiation rescues rearrangement at the TCR β , TCR γ , and TCR δ loci, initiates TCR α rearrangement, and rescues thymocyte development to the DP stage. Our data also demonstrate that rescue of thymocyte development can be uncoupled from rescue of *V(D)J* recombination in scid mice treated with anti-CD3 ϵ antibodies (Table 1). Therefore, thymocyte proliferation and differentiation in the absence of DNA damage are not sufficient to relieve the block to coding joint formation conferred by the *scid* mutation. These results suggest that irradiation may specifically affect the processing of stalled *V(D)J* recombination intermediates that accumulate in scid thymocytes, perhaps by inducing DNA damage response pathways. Possible mechanisms of irradiation-induced rescue of thymocyte development and *V(D)J* recombination are discussed below.

Although it has long been recognized that mammalian cells are capable of responding to many different types of DNA lesions, surveillance proteins capable of sensing DNA damage have been identified only recently. Candidates include DNA-PK, ATM, p53, and poly(ADP-ribose) polymerase (for review, see Enoch and Norbury 1995; Jackson and Jeggo 1995; Lindahl et al. 1995). Irradiation-induced DNA lesions are presumably detected by one of these proteins or by some other unidentified sensing mechanism, resulting in a direct or indirect activation of p53. In vitro studies have shown that DNA-PK is capable of phosphorylating the *trans*-activation domain of p53, suggesting that DNA-PK is upstream of

p53. That this is physiologically relevant has not been established. Our observation of p53-dependent responses to irradiation in scid mice indicates that DNA-PK activity is not absolutely required for DNA damage response pathways involving p53. p53 is also not essential for normal *V(D)J* recombination, as there is no obvious deficiency in T- or B-cell development in p53-null mice (Donehower et al. 1992) nor is there discernible impairment in rearrangement (even after irradiation) or in processing of DSB recombination intermediates in these animals (C. Zhu, M. Bogue, F. McBlane, L. Donehower, and D. Roth, in prep.). Although these data suggest that there is no absolute requirement for p53 in DNA-PK-mediated end-processing events, the possibility remains that p53 plays a redundant role that is revealed in the absence of DNA-PK activity.

Our results indicate that a p53-dependent DNA damage response pathway promotes *V(D)J* rearrangement in irradiated scid mice. Although the mechanisms responsible for this phenomenon remain undefined, several possibilities will be discussed in the context of our current knowledge about the *scid V(D)J* recombination defect. Because hairpin coding ends, which are normal intermediates in *V(D)J* recombination (McBlane et al. 1995; Ramsden and Gellert 1995; van Gent et al. 1995), accumulate in thymocytes of scid mice (Roth et al. 1992a; Zhu and Roth 1995), functional DNA-PK may be required for hairpin opening. Perhaps the kinase activity of DNA-PK is required to activate or recruit hairpin opening nucleases or to regulate the accessibility of hairpin coding ends to the hairpin opening machinery (Blunt et al. 1995; Roth et al. 1995; Zhu and Roth 1995). DNA damage could provide signals that might somehow restore DNA-PK activity or bypass the requirement for DNA-PK in scid mice. For example, irradiation might induce DNA repair activities capable of facilitating the opening and joining of hairpin coding ends.

An alternative possibility is that irradiation of scid mice rescues rearrangement by inducing cell cycle arrest. In this case, perhaps activation of a p53-dependent DNA damage checkpoint substitutes for a checkpoint function normally performed by DNA-PK. It is currently not known whether the presence of hairpin coding ends activates a cell cycle checkpoint in scid thymocytes. The joining of these ends, which is thought to occur quite rapidly in normal cells, may require an extended period of time in scid thymocytes (Roth et al. 1992a; Ramsden and Gellert 1995; Zhu and Roth 1995). Experiments in normal diploid human fibroblasts have shown that low doses of irradiation induce a prolonged p53-dependent

Table 1. Summary of results from irradiation and anti-CD3 ϵ treatments

	scid p53 + / -		scid p53 - / -	
	rearrangement	development	rearrangement	development
Untreated	-	-	-	-
Irradiated	+	+	-	-
Anti-CD3 ϵ	-	+	-	+

G₁ arrest (Di Leonardo et al. 1994). If this response also occurs in irradiated DN thymocytes, the arrest could provide time for “dead-end” recombination complexes containing sequestered hairpins to fall apart, allowing hairpin opening and joining.

Irradiation can promote thymocyte development from the DN to DP stage in scid mice by several potential mechanisms, as illustrated in Figure 8. One possibility is that a functional TCR chain, produced by successful joining of coding ends, could be directly involved in signaling developmental progression. On the other hand, irradiation-mediated rescue of development can also proceed in a TCR-independent manner, as shown by induction of DP cells in irradiated RAG-deficient mice, which do not undergo TCR rearrangement (Guidos et al. 1995). The mechanism responsible for this effect is unclear, although it is conceivable that the DNA damage response and thymocyte activation cascades are interconnected at some point, so that DNA damage mimics signaling through the CD3 complex (Fig. 8). Thus, in scid mice both rearrangement-dependent and -independent mechanisms might be responsible for irradiation-induced development of DP thymocytes. However, it is likely that functional rearrangements confer a selective growth advantage, as ~90% of TCR β rearrangements arising in irradiated scid mice are in-frame (Danska et al. 1994).

Regardless of the nature of the signal that initiates proliferation, the developmental program in this transition not only involves the expression of CD4 and CD8 but also initiates events leading to accessibility of the TCR α locus (Levelt et al. 1995) and to expression of a second wave of *V(D)J* recombinase activity (Wilson et al. 1994). Although we have shown that hairpin coding ends at the TCR α locus are induced by irradiation of scid mice, significant levels of completed TCR α rearrange-

ments have not been observed (Danska et al. 1994). These observations suggest DP thymocytes that proliferate in response to irradiation still express the *scid* defect and are not phenotypic revertants (Petrini et al. 1990). These data also indicate that the effect of irradiation on rescue of rearrangement is transient, affecting loci that are recombinationally active at the time of irradiation, and does not persist for the several days that elapse before the TCR α locus becomes accessible in rescued DP thymocytes.

Several aspects of the irradiation-mediated rescue of *V(D)J* recombination remain particularly puzzling. Whereas one might expect rearrangements to occur soon after irradiation, they are not detected until day 8–9 after irradiation. Rearrangements are detectable at about the same time as the thymus increases in cellularity and DP cells appear. Perhaps rearrangements occur in a relatively small fraction of thymocytes, and amplification by proliferation is required to allow their detection. The time delay in detection of irradiation-induced rearrangements in scid mice is similar to the time required for thymic regeneration in normal mice following irradiation (Kadish and Basch 1975).

Another puzzling facet of these results is the reported absence of an irradiation effect on rearrangement in B-cell precursors of scid mice (Danska et al. 1994; Murphy et al. 1994). The differential effects of irradiation in B cells versus T cells may be related to the different cellular requirements for proliferation and differentiation. For example, Bcl-2 levels are low in pre-B cells (Merino et al. 1994), which is the stage at which rearrangement occurs; in contrast, Bcl-2 levels are quite high in DN thymocytes (Veis et al. 1993). Because pre-B cells are low in Bcl-2, they may undergo irradiation-induced apoptosis, which would preclude rearrangement and proliferation.

Figure 8. Models for p53-mediated differentiation and TCR gene rearrangement in irradiated scid mice. Two signaling mechanisms—one is TCR dependent and the other TCR independent—can promote thymocyte development in irradiated scid mice (see text).

The absence of a functional p53 allele presumably results in reduced ability to eliminate those cells containing unrepaired DNA lesions. Although p53-deficient mice have no gross abnormality in the assembly of antigen receptor genes, these animals show a high incidence of thymic lymphoma (Donehower et al. 1992). It is noteworthy that the thymocyte-specific effects of irradiation on proliferation, developmental progression, and rescue of *V(D)J* recombination parallel the characteristics of irradiation-induced neoplasms. Both in scid and wild-type mice, γ -irradiation frequently induces neoplasms of T-cell, but not B-cell, lineage (Danska et al. 1994; Murphy et al. 1994; Sen-Majumdar et al. 1994). A role for *V(D)J* recombination in tumorigenesis is supported by the failure to observe irradiation-induced T-cell neoplasms in RAG-deficient animals (Guidos et al. 1995). Unraveling the connections between the cellular response to irradiation and the mechanism of both normal and abnormal *V(D)J* recombination should aid our understanding of irradiation-induced lymphomagenesis.

Materials and methods

Mice and treatment

CB.17 *scid/scid* (scid) (Bosma et al. 1983) and p53-deficient mice (Donehower et al. 1992) were maintained in microisolator cages in our animal facility at Baylor College of Medicine. Matings of these two strains provided offspring that were backcrossed to obtain mice that were scid p53^{+/−} and scid p53^{−/−}. Animals were typed as scid based on serum immunoglobulin levels, which were determined by ELISA. Mice were screened for wild-type and mutant alleles of p53 by Southern (Donehower et al. 1992) or PCR analysis (Timme and Thompson 1994). BALB/c and C57BL/6 strains were used as wild-type controls. The birth of a litter was recorded as day 1, and newborn animals were treated within 24 hr. γ -Irradiation (100 cGy) was accomplished by exposure to a ¹³⁷Cs source. Anti-CD3 (145-2C11; Pharmingen) was administered by intraperitoneal injection at a dose of 10 μ g/gram of body weight. Thymocytes were harvested at various times after treatment. Animals are reported by age, for example, irradiated day 5 means the treatment was administered on day 1 and the mouse was harvested 4 days later on day 5.

Cell preparation and flow cytometry

Thymuses were homogenized, and cells were washed and counted. Cells were stained with anti-CD4 (RM4-4) and anti-CD8 (53-6.7) that were either FITC, phycoerythrin, or Cy-Chrome conjugated (Pharmingen). Thymocytes were analyzed on a Coulter Profile I flow cytometer. Thymocytes were gated by forward and side scatter properties; two parameter histograms are shown (Figs 1, 4, and 7, log scale).

DNA preparation and analyses

DNA was prepared as described previously (Roth et al. 1992b). Briefly, thymic cell suspensions were subjected to lysis buffer containing SDS and proteinase K, phenol extracted, and ethanol precipitated. For Southern analysis, DNA from 11- to 16- day

thymocytes was used unless otherwise indicated. DNA (5–10 μ g) was digested with restriction enzymes, separated by agarose gel electrophoresis, and transferred to GeneScreen Plus nylon membranes as described previously (Roth et al. 1992b). Membranes were hybridized with the following random primed ³²P-labeled probes: the 400-bp TCR β locus probe includes and flanks D β 1 as shown in Figure 2; the TCR δ locus probe (Carroll and Bosma 1991) is a 2-kb *SacI* fragment between *J* δ 1 and *J* δ 2 as shown in Figure 3; the TCR α locus probe (probe 10; Livak et al. 1995), is derived from a 400-bp fragment between *J* α 49 and *J* α 48 gene segments, as shown in Figure 6; and the RAG-1 probe (Schatz et al. 1989) is a 3.2-kb *SalI*–*Bam*HI cDNA fragment.

For PCR analysis, products were amplified from DNA with the following primers: *V* β 8.1, 2, 3, 5'-GAGGAAAGGTGACAT-TGAGC (Chou et al. 1987); *J* β 2.6, 5'-GCCTGGTGCCGGGAC-CGAAGTA (Malissen et al. 1984); *V* γ 2, 5'-AAGGAATTCATC-GAAAGCTTTAGGAG (Garman et al. 1986); *J* γ 1, 5'-CCCTC-GAGCTTTGTTCTTCTGCAAATAC (Aguilar and Belmont 1991). One round of 30 cycles (94°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec) was performed with 2 mM MgCl₂ and 1 unit of *Taq* polymerase. Products were separated on a 6% polyacrylamide gel, blotted onto GeneScreen Plus nylon membrane, and hybridized to internal oligonucleotide probes that were ³²P end-labeled. (*V* β 8 probe, 5'-GGGCTGAGGCTGATCCATTA (Chou et al. 1987); *V* γ 2 probe, 5'-ACCATACACTGGTACCGGCA (Garman et al. 1986).

Radiolabeled products from Southern and PCR blotting were detected and analyzed using a Molecular Dynamics Phosphor-Imager.

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M A Bogue, C Zhu, E Aguilar-Cordova, et al.

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