Brief Definitive Report

p53 Prevents Maturation to the $CD4^+CD8^+$ Stage of Thymocyte Differentiation in the Absence of T Cell Receptor Rearrangement

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Summary

Rearrangement of the immunoglobulin (Ig) and T cell receptor (TCR) gene loci allows for the generation of B and T lymphocytes with antigen-specific receptors. Complete rearrangement and expression of the TCR-β chain enables immature thymocytes to differentiate from the $CD4^-CD8^-$ to the $CD4^+CD8^+$ stage. Mice in which rearrangement is impaired, such as severe combined immunodeficient (SCID) mice or recombinase activating gene–deficient ($RAG^{-/-}$) mice, lack mature B and T lymphocytes. Thymocytes from these mice are arrested at the $CD4^-CD8^-$ stage of T cell development. We previously observed that thymocytes from RAG-$2^{-/-}$ mice exposed to γ radiation differentiate from $CD4^-CD8^-$ into $CD4^+CD8^+$ without TCR-β chain rearrangement. We now report that irradiated RAG-$2^{-/-}$ thymocytes undergo direct somatic mutations at the p53 gene locus, and that p53 inactivation is associated with maturation of RAG-$2^{-/-}$ thymocytes to the $CD4^+CD8^+$ stage. Generation of RAG-$2^{-/-}$ and $p53^{-/-}$ double-deficient mice revealed that, in the absence of TCR-β chain rearrangement, loss of p53 function is sufficient for $CD4^-CD8^-$ thymocytes to differentiate into the $CD4^+CD8^+$ stage of T cell development. Our data provide evidence for a novel p53-mediated checkpoint in early thymocyte development that regulates the transition of $CD4^-CD8^-$ into $CD4^+CD8^+$ thymocytes.

Adaptive immunity requires the establishment of a large repertoire of lymphocytes, each bearing a unique antigen-specific receptor. Successful rearrangement of TCR gene loci is necessary for proper thymocyte development and for the generation of a mature T cell repertoire. Mice with recombination deficiencies, such as SCID and $RAG^{-/-}$, display a differentiation arrest early in T cell development and lack receptor-bearing T cells in the periphery (1–3). In these mice, thymocyte development does not proceed further than the early immature $CD4^-CD8^-$ (double-negative, DN) stage. Recent evidence supports the notion that signals derived from a pre–Tα chain and a fully rearranged TCR-β chain at the DN stage drive thymocyte differentiation to the next point in development, the $CD4^+CD8^+$ (double-positive, DP) stage (4–6). This TCR-β chain–mediated differentiation event has been termed "β selection" (6–8). Once thymocytes reach the DP stage, TCR-α chain rearrangement occurs, allowing for the TCR–MHC interactions that result in the negative or positive selection of developing thymocytes (9, 10).

We recently reported that DN thymocytes from RAG-$2^{-/-}$ mice differentiate into DP thymocytes after exposure to γ radiation, and that this induced differentiation occurs in the absence of TCR-β rearrangement (11). Because of their inability to recombine their TCR gene loci, DN thymocytes from RAG-$2^{-/-}$ mice have a limited developmental potential and a shortened life span (4, 7). This limited life span may reflect a dominant programmed cell death (apoptosis) pathway that is normally suppressed by TCR-β signals. We hypothesized that thymocytes from irradiated RAG-$2^{-/-}$ mice acquire a novel phenotype that permits them to proceed with thymocyte development by superseding the required TCR-β differentiation signals. DP thymocytes induced by radiation may have failed to respond to the appropriate death signals that DN cells normally receive in the absence of TCR-β selection. To address this possibility, we investigated genes involved in the apoptosis pathway that may be responsible for inducing death of DN thymocytes that fail to rearrange TCR-β gene loci.

One of the key regulatory genes involved in the induction of apoptosis is *p53* (12). Cells with mutant forms of *p53* are resistant to several inducers of apoptosis, such as DNA damage (12, 13). This is most evident in the preponderance of human tumors known to have a mutated form of the *p53* gene, which is thought to lead to a deregulated

neoplastic phenotype (12, 14). Furthermore, the *p53* gene contains several regions, known as "hot spots," that are highly susceptible to direct somatic mutations from exposure to radiation or DNA-damaging agents (15). In this report, we show that irradiated RAG-$2^{-/-}$ thymocytes acquire radiation-induced somatic mutations of the *p53* gene, which can enable DN thymocytes to advance to the DP stage in the absence of TCR-β survival/differentiation signals. We directly demonstrate the involvement of *p53* during DN-to-DP thymocytes differentiation by showing that DN thymocytes differentiate to the DP stage of T cell development in RAG-$2^{-/-}$/$p53^{-/-}$ double-deficient mice. Thus, in the absence of TCR-β chain rearrangement, loss of *p53* function is sufficient for the transition of $CD4^-CD8^-$ into $CD4^+CD8^+$ thymocytes.

Materials and Methods

Mice. RAG-$2^{-/-}$ mice were obtained from Dr. Fred Alt (Howard Hughes Medical Institute, Children's Hospital, Boston, MA) (2) and bred in our animal facility. $p53^{-/-}$ mice were purchased from Taconic Farms, Inc. (Germantown, NY). Double-deficient $p53^{-/-}$/RAG-$2^{-/-}$ mice were bred in our facility. Double-mutation homozygosity was determined by flow cytometry analysis for RAG-$2^{-/-}$ phenotype or by PCR analysis for $p53^{-/-}$ genotype. Adult $p53^{-/-}$/RAG-$2^{-/-}$ mice (10–12 wk) were used for the flow cytometry analysis shown in Fig. 3; before analysis, mice were checked for the absence of any overt tumors. All other mice were obtained from the National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, MD). Irradiation of mice was performed as previously described (16).

Flow Cytometry. Antibodies for flow cytometry were purchased from PharMingen (San Diego, CA). Staining of cells was performed as previously described (16).

RNA and PCR Analysis. Total RNA from thymocytes was prepared with RNAzol (Tel-Test Inc., Friendswood, TX) as indicated by the manufacturer. Reverse transcriptase (RT)-PCR analysis and cloning of *p53* were performed using standard techniques. Briefly, RT reactions were performed with isolated RNA from thymocytes of control or 2-wk postirradiation RAG-$2^{-/-}$ mice using random hexamer primers. The first-strand cDNA products were amplified by PCR using *pfu* DNA polymerase with *p53* gene–specific primers (5′ CCT GTC ATC TTT TGT CCC TTC TCA 3′, for the upper primer at position 424, and 5′ ATA AGA CAG CAA GGA GAG GGG GAG 3′, for the lower primer at position 1361). The PCR product was analyzed by gel electrophoresis or cloned using the T/A cloning system (Invitrogen, San Diego, CA). Plasmids containing the *p53* gene were sequenced with several gene-specific primers using the Sequenase 2.0 system (Amersham Corp., Arlington Heights, IL).

Immunoprecipitation and Western Blot Analysis. Thymocyte nuclear extracts were prepared as previously described (17). Nuclear extracts were immunoprecipitated with either Pab-246 or Pab-240 mAb (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for the wild-type or mutant forms of *p53*, respectively. SDS-PAGE was performed on immunoprecipitated material and electroblotted onto nitrocellulose membranes. Immunoblots were hybridized using Pab-240 mAb, which recognizes both wild-type and mutant forms of denatured/membrane-bound *p53*. Polyclonal anti–mouse-κ/λ-HRP–labeled Ig (Southern Biotechnology Associates, Birmingham, AL) was used to detect the hybridizing antibody. The ECL system (Amersham Corp.) was used to detect the secondary antibody.

Results and Discussion

Thymocytes from recombination-deficient (RAG-$2^{-/-}$) mice are blocked at the DN stage of T cell development (Fig. 1). Thymocytes from RAG-$2^{-/-}$ mice exposed to sublethal doses of γ radiation show the appearance of DP cells 2 wk after treatment (Fig. 1). Surprisingly, DN thymocytes reach the DP stage in the absence of TCR gene rearrangement (11, 18). This finding provides evidence for a DN-to-DP differentiation event independent of rearranged TCR-β chain and pre-Tα signals. Thus, thymocytes from irradiated RAG-$2^{-/-}$ mice must overcome the requirement for survival/differentiation signals normally provided by TCR-β and pre–Tα chains (4–6). We hypothesized that γ radiation may be affecting genes that normally control cell death or survival events during thymocyte development. Therefore, we investigated the potential role of *p53* in the generation of DP cells in irradiated RAG-$2^{-/-}$ mice. We chose *p53* as a candidate gene because of its involvement in controlling cell cycle and apoptosis after exposure to DNA-damaging agents (12–14). Furthermore, *p53* has been shown to readily undergo somatic mutation in cells after exposure to radiation (15).

We isolated mRNA from normal RAG-$2^{-/-}$ thymocytes or from RAG-$2^{-/-}$ thymocytes 2 wk after irradiation (IR-RADRAG; 8.5 Gy). After RT with random hexamer primers, PCR with gene-specific primers was used to amplify mRNA transcripts from the *p53* gene. The RT-PCR product was generated using *Pfu* polymerase to avoid PCR-mediated errors in the DNA sequence. The RT-PCR product was cloned, and plasmids containing *p53* from several different colonies were sequenced. The *p53* sequence derived from normal RAG thymocytes was identical to the previously reported sequence (12). By contrast, several *p53* gene sequences derived from irradiated RAG thymocytes revealed various nucleotide changes that would result in an altered p53 protein (Table 1). Thus, thymocytes that developed 2 wk after radiation exposure may express mutated *p53* genes. However, not all of the *p53* cDNAs derived from irradiated thymocytes showed genetic alterations. This may be due to the use of total thymus rather than isolated DP thymocytes to prepare the mRNA. Nonetheless, mutated cDNAs of *p53* were obtained in 42% of the colonies we analyzed from irradiated thymuses but in none of the thymuses from nonirradiated RAG-$2^{-/-}$ mice (Table 1). These findings demonstrated that treatment with γ radiation can directly mutate the *p53* gene.

Our analysis of p53 protein expression revealed the presence of mutant forms of *p53* in thymocytes from irradiated RAG-$2^{-/-}$ but not from control RAG-$2^{-/-}$ mice. Fig. 2 shows a Western blot analysis of immunoprecipitated *p53* from irradiated or control RAG-$2^{-/-}$ thymocyte nuclear extracts using wild-type or mutant form–specific mAbs. As a positive control for the immunoprecipitation and Western blot, we used nuclear extracts from A431 cells (Santa

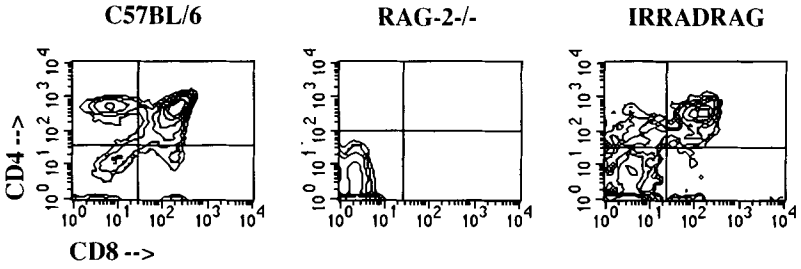

Figure 1. Two-parameter flow cytometry analysis of thymocytes from C57Bl/6, RAG-$2^{-/-}$ mice, and RAG-$2^{-/-}$ mice 2 wk after sublethal (750 cGy) γ irradiation (IRRADRAG). Thymocyte single-cell suspensions were stained with CD8-FITC versus CD4-PE and analyzed by flow cytometry. Propidium iodide was used to exclude dead cells from the analysis. Data shown are representative of >10 independent FACS® analyses.

Cruz Biotechnology), which express a mutant form of *p53* (19) (data not shown). Consistent with our DNA sequence analysis, the immunoblot shows that thymocytes from irradiated RAG-$2^{-/-}$ mice express mutant and wild-type *p53*. Thus, our analysis provides direct evidence that exposure to radiation can cause the emergence of a cell population in which the *p53* gene is mutated and an altered p53 protein is expressed. Therefore, we hypothesized that mutant *p53* may allow DN thymocytes from RAG-$2^{-/-}$ mice to escape apoptosis and progress to the DP stage.

To test the participation of *p53* in the prevention of DP thymocyte differentiation in RAG-$2^{-/-}$ mice, we bred RAG-$2^{-/-}$ mice with *p53*-deficient mice (*p53*$^{-/-}$). Although T cell development appears to occur normally in *p53*$^{-/-}$ mice (20), thymocytes from these mice are resistant to certain forms of induced programmed cell death (20). Analysis of thymocytes from *p53*$^{-/-}$/RAG-$2^{-/-}$ (*p53*/RAG) mice demonstrated the presence of $CD4^+CD8^+$ thymocytes (Fig. 3), whereas thymic cellularity did not increase by >50% from that observed in RAG-$2^{-/-}$ mice (1–3 × 10^6 cells/thymus). Further analysis showed that the emergence of DP cells is not simply an aberrant expression of CD4 and CD8, but rather a progression to the next differentiation stage (7). Fig. 3 shows that, in several aspects of their developmental status, the new population of DP thymocytes in *p53*/RAG mice displayed a phenotype normally associated with the $CD4^+CD8^+$ stage of development, including loss of IL-2R expression, decreased MHC class I expression, and smaller cell size (Fig. 3, *a–d*) (11). We failed to detect TCR-β chain rearrangement or cell surface expression of CD3 on DP thymocytes from *p53*/RAG mice (Fig. 3 *e* and data not shown). Therefore, lack of *p53* function is sufficient to allow for the differentiation of $CD4^-CD8^-$ into $CD4^+CD8^+$ thymocytes in RAG-$2^{-/-}$ mice in the absence of TCR rearrangement. Although DP thymocytes develop in *p53*/RAG mice, the lack of TCR-α/β rearrangement and expression precludes positive selection on self-MHC from occurring and prevents further thymocyte differentiation. Thus, positive selection of $CD4^+CD8^-$ and $CD4^-$ $CD8^+$ thymocytes is apparently controlled by a *p53*-independent pathway.

DP thymocyte differentiation normally requires the expression of a rearranged TCR-β chain and a monomorphic pre-Tα chain (4–8). Developing thymocytes that fail to properly rearrange their TCR-β loci undergo programmed cell death (7). This phenotype is most apparent in SCID and RAG$^{-/-}$ mice. The mechanism by which DN thymocytes without TCR are prevented from further maturation is not understood. We propose that *p53* is involved in the regulatory pathway that directs TCR^- DN thymocytes to undergo apoptosis. In the absence of *p53* control, TCR^- DN thymocytes are free to enter the next stage of thymocyte differentiation. While in the presence of functional *p53*, DN thymocytes require differentiation/survival signals provided by the TCR-β chain. There is strong evidence to suggest that the signals delivered by the TCR-β chain to DN cells are mediated by the protein tyrosine kinase $p56^{lck}$ (21–24). This is evident from reports that show reduced numbers of DP cells present in *lck*$^{-/-}$ mice (21, 22) and from findings that show the appearance of DP cells in transgenic RAG$^{-/-}$ mice expressing a constitutively active form

Table 1. *p53 Mutations in RAG-$2^{-/-}$ Thymocytes after Sublethal γ Radiation*

Clone	Nucleotide change	Codon location	Amino acid change
Clone 2	CAG→TAG	164	gln→stop
Clone 10	ATC→GTC	157	ile→val
Clone 6	TAC→TTC	160	tyr→phe
Clone 6	ATG→ATT	165	met→ile
Clone 6	CGC→TGC	175	arg→cys
Clone 5	TCT→CCT	124	ser→pro
Clone D2	AAC→AGC	237	ser→asn

Mutation frequency in *p53* cDNAs from irradiated RAG-$2^{-/-}$ thymocytes is 42% (5/12), and from thymocytes of control RAG-$2^{-/-}$ mice is 0% (6/6).

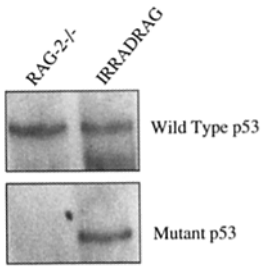

Figure 2. Immunoprecipitation and Western blot analysis for wild-type and mutant forms of *p53*. Nuclear extracts from control RAG-$2^{-/-}$ thymocytes and IRRADRAG thymocytes (2 wk after exposure to 750 cGy) were immunoprecipitated with specific mAbs for the wild-type or mutant forms of *p53* (Pab-246 or Pab-240 mAb, respectively) Immunoblots of SDS-PAGE–immunoprecipitated material were performed with Pab-240 mAb.

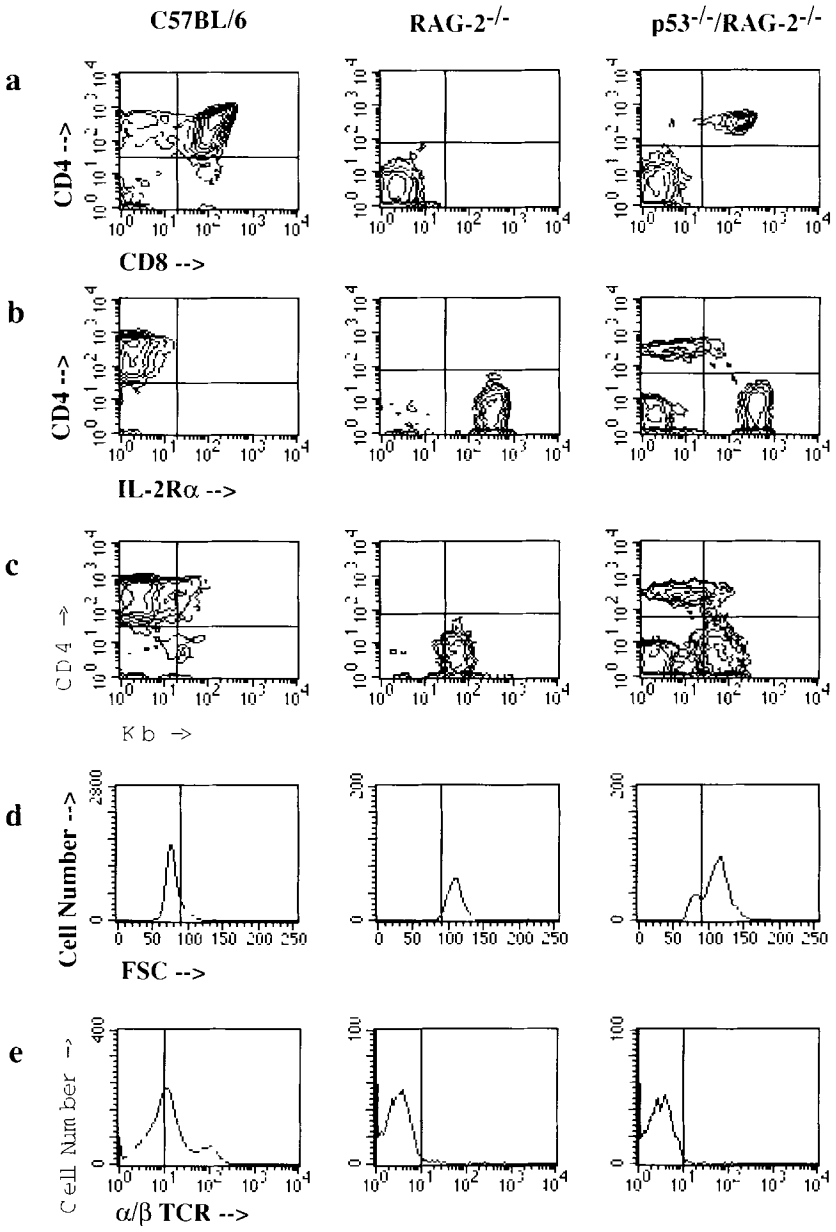

Figure 3. Two-parameter flow cytometry analysis of thymocytes from C57Bl/6, RAG-2$^{-/-}$ mice, or from double-deficient RAG-2$^{-/-}$ × *p53*$^{-/-}$ mice (>8 wk old). Thymocyte single-cell suspensions were stained with (*a*) CD8-FITC versus CD4-PE, (*b*) IL-2Rα–FITC versus CD4-PE, (*c*) MHC-K^{b}–FITC versus CD4-PE, (*d*) forward side scatter (*FSC*), and (*e*) TCR-α/β-FITC for FACS® analysis, as indicated.

of *lck* (23). Furthermore, the ability to generate DP cells in RAG$^{-/-}$ mice treated with anti-CD3ε mAb was recently shown to involve *lck*-mediated signals (24).

TCR-β chain *lck*-derived signals in DN cells may influence the survival potential of DN cells by activating the *bcl-2* gene and/or by preventing *p53*-mediated apoptosis. Involvement of the *bcl-2* survival-promoting oncogene at the DN-to-DP transition stage was shown by the ability to generate DP cells in *bcl-2*–transgenic RAG-1$^{-/-}$ mice (25). Thus in the absence of TCR-β (*lck*) signals, *bcl-2* was sufficient to block apoptosis and allow DN cells to differentiate to the DP stage (25). Importantly, the *bcl-2* survival signals can be countered by the *bax* gene (26–28). In this context, it is interesting that *p53* has been shown to promote apoptosis by repressing *bcl-2* expression and by upregulating *bax* transcription (29, 30). Taken together with our findings, we suggest that the progression to the DP stage in the absence of TCR-β can be normally prevented by a *p53*-regulated programmed cell death pathway. TCR-β/pre–Tα chains most likely coordinate the signals that lead to the survival of DN cells to the DP stage by mediating *lck*-derived signals and regulating *bcl-2/bax* activation and *p53* inactivation in order to allow further thymic differentiation to proceed unhampered.

Although thymocyte development in RAG$^{-/-}$ mice is blocked at the DN stage, six different experimental manipulations in RAG$^{-/-}$ mice show that this impediment can be overcome: (*a*) transgenic expression of a rearranged TCR-β chain (4); (*b*) anti-CD3 mAb treatment (24); (*c*) transgenic expression of a constitutively active *lck* (23); (*d*) transgenic

overexpression of *bcl-2* (25); (*e*) sublethal γ radiation treatment (11, 18); and (*f*) inactivation of the *p53* gene (this report). In the first three settings, DP thymocyte differentiation results from *lck*-mediated signals, which allow DN thymocytes to differentiate and expand (up to a 100-fold increase in thymic cellularity) into the next stage. Thus, positive signals from *lck* drive differentiation and proliferation. In the last three settings, DP thymocyte differentiation is observed without a dramatic increase in thymic cellularity. In these situations, where the programmed cell death pathway is inhibited, differentiation without proliferation into DP stage is observed. Therefore, DN-to-DP thymocyte differentiation can occur in the absence of proliferation, and these processes are molecularly distinct. Thus, TCR-β selection may achieve two critical functions, differentiation and thymocyte expansion, in causing the emergence of DP thymocytes.

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