# UV irradiation augments lymphoid malignancies in mice with one functional copy of wild-type *p53*

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**Epidemiological studies have suggested an association between exposure to solar UV radiation and the incidence of lymphoid malignancies, which has increased substantially worldwide during the last two decades. Findings from animal studies have raised the question of whether UV radiation might influence the development of lymphoid malignancies by means of its immunosuppressive effect. In this study, we examined the effect of UV irradiation on the development of lymphoid malignancies in mice with no or only one functional copy of *p53*. Mice that lack both copies of *p53* spontaneously develop high frequency of lymphoid malignancies in the thymus and spleen. *p53* heterozygous mice with only one copy of the wild-type allele also develop lymphoid malignancies, but with a much lower frequency and a long latent period. In our study using mice of the C57BL/6 background, only one of the unirradiated mice lacking one copy of *p53* (*p53*$^{+/-}$) spontaneously developed a lymphoid tumor (6%), whereas 88% of UV-irradiated *p53*$^{+/-}$ mice developed lymphoid tumors in the spleen or liver. None of the control or UV-irradiated *p53* wild-type mice developed lymphoid tumors during the 60-week observation period. Both UV-irradiated and unirradiated mice lacking both copies of *p53* (*p53*$^{-/-}$) rapidly developed thymic lymphomas and/or lymphoid tumors in spleen or liver. All of the lymphoid tumors tested were of T cell type. The immune responses of the mice to contact sensitization were identical and were suppressed to the same extent by UV irradiation regardless of the genotype. These results indicate that differences in immune reactivity do not account for the different effects of UV radiation on lymphoid malignancies and, in addition, that p53 is not required for generation of T cell-mediated immunity. Interestingly, whereas *p53* mutations or loss of heterozygosity did not account for the accelerated development of lymphoid tumors in UV-irradiated *p53*$^{+/-}$ mice, deletions in the *p16*$^{INK4a}$ gene were quite common. These data provide the experimental evidence that UV irradiation induces lymphoid neoplasms in genetically susceptible mice and support the hypothesis that extensive sunlight exposure contributes to the induction of lymphoma in humans.**

tumor suppressor gene | immune suppression

The incidence of non-Hodgkin's lymphoma (NHL) worldwide has increased substantially during the last two decades (1–3). This surge cannot be totally explained by the improvement in diagnosis and recording. Recently, epidemiological studies have suggested an association between the UV radiation in sunlight and the incidence of NHL (3–7). The rise in the incidence of NHL parallels the global increase in melanoma incidence, and patients with NHL are at higher risk of developing melanomas (4, 5). Studies from several groups have demonstrated an increased incidence of nonmelanoma skin cancers in persons with a history of NHL or chronic lymphocytic leukemia, and of NHL or chronic lymphocytic leukemia in persons with a history of skin cancers (5–7). UV radiation, especially wavelengths in the UV-B (280–320 nm) region, can induce systemic immune suppression (8), and its immunosuppressive effect contributes to the development of nonmelanoma skin cancers (9). These findings from animal studies have raised the question of whether UV radiation might influence the development of lymphoid malignancies by means of its immunosuppressive effect. To date, few animal studies have explored the possible association between UV radiation and the development of lymphoid malignancies. One study found no effect of chronic UV irradiation on the incidence of leukemia in AKR strain mice, which develop a high incidence of leukemia early in life (10). A second study indicated that chronically UV-irradiated A strain mice developed leukemia at a very low frequency after a very long latent period (11). The development of *p53* knockout mice lacking one or both functional copies of *p53* has provided a useful tool for investigating the role of p53 in the development of a variety of cancers, including those of lymphoid origin. Mice that lack both copies of *p53* (*p53*$^{-/-}$ mice) spontaneously develop lymphoid malignancies in the thymus and spleen with a very high frequency; mice that have only one copy of the wild-type allele (*p53*$^{+/-}$ mice) also spontaneously develop lymphoid malignancies, but at a much lower frequency than do *p53*$^{-/-}$ mice (12). The genetic background of the mice affects the incidence, rate of development, and types of spontaneous tumors arising in *p53*$^{-/-}$ and *p53*$^{+/-}$ mice. For example, mice with the 129/Sv background developed spontaneous tumors earlier than mice with mixed background of C57BL/6 and 129/Sv. Nearly 50% of the 129/Sv *p53*$^{-/-}$ male mice developed malignant teratomas, which are rarely seen in mice with the mixed C57BL/6 and 129/Sv background (13). Exposure to ionizing radiation increases the incidence of lymphoid malignancies in both *p53*$^{+/-}$ and *p53*$^{-/-}$ mice of the mixed NIH and 129/Sv background, and this increased susceptibility in irradiated *p53*$^{+/-}$ mice is associated with loss of the functional copy of *p53* from the tumors (14). We recently used congenic C57BL/6 strain *p53*$^{-/-}$, *p53*$^{+/-}$, and *p53*$^{+/+}$ (two functional copies of *p53*) mice to show that p53 plays a protective role against skin cancer induced by UV radiation (15). During these studies, we also monitored the development of internal malignancies, all of which were of lymphoid origin in this strain. We now report the results of these studies in which we (*i*) compared the rate of tumor development and the histology of lymphoid tumors that arose in UV-irradiated and unirradiated *p53*$^{+/-}$ and *p53*$^{-/-}$ mice, (*ii*) analyzed the status of the residual copy of the *p53* allele, and (*iii*) investigated the role of p53 in T cell-mediated immune responses and UV-induced immune suppression. In addition, we analyzed lymphoid malignancies arising in UV-irradiated *p53*$^{+/-}$ as well as in unirradiated and UV-irradiated *p53*$^{-/-}$ mice for deletions in *p16*$^{INK4a}$ and *p19*$^{ARF}$ genes.

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Abbreviations: CHS, contact hypersensitivity; DTH, delayed-type hypersensitivity; NHL, non-Hodgkin's lymphoma; T4N5, T4 endonuclease V; DNFB, 1-fluoro-2,4-dinitrofluorobenzene.

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## Materials and Methods

**Mice.** Specific pathogen-free *p53* knockout mice (TSG-p53), derived from those originally produced by Donehower *et al.* (12), were obtained from GenPharm (Mountain View, CA). Specific pathogen-free C57BL/6 mice were obtained from the Frederick Cancer Research Facility Animal Production Area. The TSG-p53 mice were then backcrossed onto the C57BL/6 background for 12 generations to produce a congenic line. Genetic homogenicity was confirmed by isoenzyme analysis and second-set skin graft. All mice were housed in a pathogen-free barrier facility accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care, in accordance with current regulations and standards of the United States Department of Agriculture, Department of Health and Human Services, and National Institutes of Health. All procedures were approved by the Institutional Animal Care and Use Committee.

**UV Irradiation.** UV irradiation was provided by a bank of six FS40 sunlamps (National Biological, Twinsburg, OH), which emit a continuous broad spectrum of light ranging from 250 to 400 nm, with a peak emission of 313 nm. Approximately 65% of the radiation emitted was within the UV-B range; the incident dose rate averaged 3.75 W/m$^2$ UV-B over the course of the experiment. Age- and sex-matched cohorts of mice of the $p53^{-/-}$, $p53^{+/-}$, and $p53^{+/+}$ genotypes (littermates from 12th backcross generation) were irradiated with 5 kJ/m$^2$ UV-B radiation three times per week for 30 weeks, beginning at 12 weeks of age; all irradiated mice had their dorsal fur shaved weekly. Age- and sex-matched mice of the same genotypes that were not exposed to UV irradiation were used as controls. Mice were individually numbered and observed three times per week for health status.

**Immunohistochemical Analysis of T and B Cell Markers.** Fixed cryosections were incubated with primary and secondary antibodies, as recommended by the manufacturer. The primary antibodies used were rat anti-mouse CD3 (Serotec/Harlan Bioproducts for Science, Indianapolis, IN), biotin-conjugated rat anti-mouse CD45R/B220 (PharMingen), and rat anti-mouse sIgM (Pierce). After staining with appropriated secondary antibodies, the sections were counterstained with Gill no. 3 hematoxylin (Sigma) and examined under a bright-field microscope.

**p53 Immunohistochemistry.** Deparaffinized 5-$\mu$m sections were stained with a rabbit polyclonal anti-mouse p53 antibody and counterstained with a biotin-conjugated goat anti-rabbit antibody as described before (16).

***p53* Mutation.** Cell lysates from microdissected tumor nodules and foci by laser capture microdissection (Arcturus, Mountain View, CA) were amplified for *p53* exons 5–8 by PCR using primers in exon 5 (forward) and exon 8 (reverse) and sequenced, as described (15).

**Loss of Heterozygosity.** Cell lysates prepared from tumor tissues were amplified for 30 cycles using primer sets [5′-GTGTTTCATTAGTTCCCCACCTTGAC and 5′-ATGGGAGGCTGCCAGTCCTAACCC, which amplifies wild-type p53, and 5′-CCCTACTCTACAACTAAAACTGAA and 5′-AAGCTGGGGAAGAAACAGGCTAAC, which amplifies *p53* exon 8 (internal control) to indicate successful amplification] and conditions recommended by the manufacturer (GenPharm). DNAs from $p53^{+/+}$, $p53^{+/-}$, and $p53^{-/-}$ mouse skin were used as controls. The PCR products were run on 2% agarose gels.

**PCR Amplification of $p16^{INK4a}$ and $p19^{ARF}$.** Six microliters of cell lysates were amplified in a 25-$\mu$l reaction mixture for 30 cycles using primers for mouse *p16* exon 1 (5′-ATGGAGTCCGCTGCAGACAG-3′ and 5′-CTGAATCGGGGTACGACCGA-3′) and exon 2 (5′-GTGATGATGATGGGCAACGT-3′ and 5′-TGGGCGTGCTTGAGCTGAGG-3′), and for $p19^{ARF}$ exon 1$\beta$ (5′-TACAGCAGCGGGAGCATGGGT-3′ and 5′-CTGGTCCAGGATTCCGGT-3′). Aliquots of PCR products were run on 2% agarose gels.

**Contact Hypersensitivity Reaction (CHS).** The dorsal and ventral fur of the mice was removed with electric clippers, the ears were covered with opaque tape, and the dorsal skin of the mice was exposed to various doses of UV irradiation in a single acute exposure. To measure the CHS response, the shaved ventral skin was sensitized 3 days after UV irradiation with either 50 $\mu$l of 0.3% solution of 1-fluoro-2,4-dinitrofluorobenzene (DNFB) (Sigma) in acetone or 100 $\mu$l of 3% oxazolone in 100% ethanol. Five days after sensitization, the ear thickness was measured, and the mice were challenged by applying 10 $\mu$l of 0.2% DNFB or 5 $\mu$l of 3% oxazolone solution to each ear. Ear thickness was measured again 18–24 h later and recorded. The specific ear swelling was calculated by subtracting the swelling found in negative control mice that were not immunized but were challenged, from the swelling seen in mice that were both immunized and challenged.

**Delayed-Type Hypersensitivity Reaction (DTH).** The dorsal fur of the mice was removed with electric clippers, and the mice were exposed to various doses of UV irradiation in a single acute exposure. The control mice were shaved but not exposed to UV. Three days later, the mice were immunized by injecting $10^7$ formalin-fixed *Candida albicans* or $2.5 \times 10^7$ allogeneic BALB/c spleen cells into each flank. Nine days later, each hind footpad was measured with an engineer's micrometer (Mitutoyo, Tokyo), the thickness was recorded, and the mice were challenged by injecting 50 $\mu$l of *Candida* antigen (ALerChek, Portland, ME) or $10^7$ BALB/c spleen cells into each hind footpad. Footpad thickness was again measured 18–24 h later and recorded. The specific footpad swelling was calculated by subtracting the swelling found in negative control mice that were not immunized but were challenged, from the swelling seen in mice that were both immunized and challenged.

**T4 Endonuclease V (T4N5) Liposomes.** T4N5 liposomes were prepared by encapsulating purified, recombinant T4N5 in liposomes composed of phosphatidylcholine/phosphatidylethanolamine/oleic acid/cholesteryl hemisuccinate (2:2:1:5 molar ratio) as described (17). Control preparations of liposomes contained heat-inactivated (65°C for 1 h) T4N5. The liposomes were diluted with minimum essential medium, supplemented with 1% FBS; the final concentration was 0.3 $\mu$g of T4N5/ml of liposome–hydrogel mixture.

## Results

**Lymphoid Malignancies in UV-Irradiated $p53^{-/-}$ and $p53^{+/-}$ Mice.** The rate of development of internal lymphoid malignancies in UV-irradiated $p53^{-/-}$ and $p53^{+/-}$ mice was determined (Fig. 1). The data are presented as the probability of tumor development versus the age of the animals (life table analysis), which permitted the inclusion of animals that died from other causes during the experiment.

For $p53^{-/-}$ mice, UV irradiation did not increase the incidence or shorten the latent period of lymphoid malignancies. The probability of 50% lymphoid tumor development occurred at 23.9 weeks of age in UV-irradiated $p53^{-/-}$ mice compared with 23.8 weeks for lymphoid tumors that arose in unirradiated mice. However, for $p53^{+/-}$ mice, UV irradiation substantially increased the incidence and shortened latency of lymphoid malignancies. The $p53^{+/-}$ mice that were irradiated developed internal tumors after a mean latency of 40 weeks of age, whereas

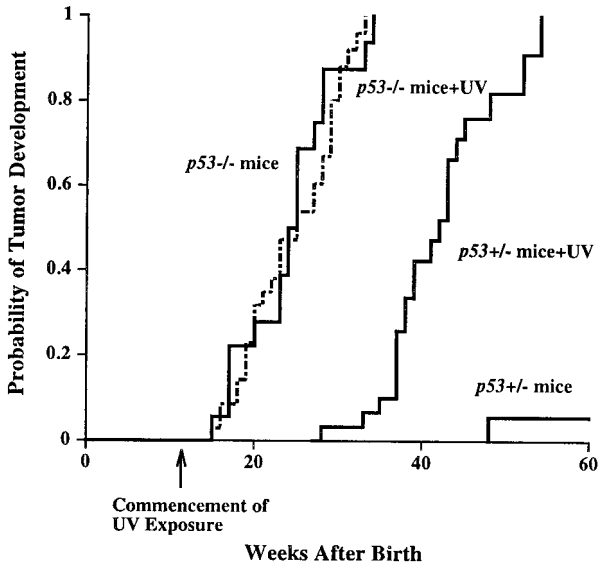


**Fig. 1.** Probability of development of lymphoid malignancies in $p53^{-/-}$ and $p53^{+/-}$ mice with and without UV irradiation. Shaved dorsal skin of congenic C57BL/6 $p53^{-/-}$ and $p53^{+/-}$ mice were exposed to 5 kJ/m$^2$ UV three times per week for 30 weeks. Age- and sex-matched $p53^{-/-}$ and $p53^{+/-}$ mice without UV irradiation were also included. UV irradiation started at 12 weeks of age.

only 1 of 18 unirradiated $p53^{+/-}$ mice developed a lymphoid tumor at 49 weeks of age, and the rest of the mice remained tumor free at 60 weeks of age.

The number and types of internal tumors that developed in *p53* null mice were evaluated (Table 1). All $p53^{-/-}$ mice developed internal tumors regardless of UV irradiation. There was no difference in the histologic types of tumors that arose in UV-irradiated and unirradiated mice. The majority of the internal tumors were thymic lymphomas, lymphoid malignancies arising in the spleen and/or liver, or combinations of the two. Lymphomas arising in gut-associated lymphoid tissues occurred at low frequency in both UV-irradiated and unirradiated $p53^{-/-}$ mice. Eighty-eight percent of the $p53^{+/-}$ mice developed lymphoid malignancies after UV irradiation. All of those malignancies were lymphoid tumors of the spleen and/or liver. The only spontaneous tumor that arose in the unirradiated $p53^{+/-}$ mice was a splenic lymphoid tumor in one animal.

Immunohistochemical analysis of T and B cell markers (CD3 for T cells and B220 and sIgM for B cells) in some of the internal tumors induced in the UV-irradiated *p53*-deficient mice showed that all of the tested tumors were of T cell origin. The thymic lymphomas were strongly positive for anti-CD3 and negative for anti-B220 and sIgM. The lymphomas in the gut were strongly positive for anti-CD3 and, except for two focal areas, negative for anti-B220. Lymphoid malignancies in the spleen and liver showed the same pattern of staining. About 20% of the lymphoid malignancies in UV-irradiated $p53^{-/-}$ and $p53^{+/-}$ mice were tested, including four thymic lymphomas, two lymphomas in the gut, three lymphoid malignancies in the spleen, and two lymphoid malignancies in the liver (data not shown).

To investigate whether the increased incidence of lymphoid malignancies after UV irradiation in $p53^{+/-}$ mice was associated with loss of the wild-type *p53* allele, immunohistochemical analysis using anti-p53 antibody was performed. The results indicated that 82% (27 of 33) of the tumors from UV-irradiated $p53^{+/-}$ mice expressed p53 protein, and 48% (16 of 33) expressed a high level of p53 protein (data not shown). In contrast, neither tissues from $p53^{-/-}$ mice nor tissues from unirradiated $p53^{+/-}$ mice expressed p53 protein. However, spleen and liver tissues from UV-irradiated, nontumor-bearing $p53^{+/-}$ mice expressed low level of p53 (data not shown). These data suggested that the remaining wild-type *p53* was not lost in the majority of leukemias that developed in the UV-irradiated $p53^{+/-}$ mice.

***p53* Genotyping of Lymphoid Tumors from UV-Irradiated $p53^{+/-}$ Mice.** To further confirm the presence of wild-type p53 in lymphoid malignancies, cell lysates from microdissected tumor tissues from UV-irradiated $p53^{+/-}$ mice were analyzed for loss of heterozygosity in a multiplex PCR reaction using primer sets that amplify wild-type *p53* exon 5 and exon 8 (internal control). Gel electrophoresis data revealed that DNAs from 7 of 10 lymphoid tumors in liver and spleen, respectively, gave rise to 320-bp bands corresponding to the expected wild-type *p53* band (data not shown). This 320-bp band was also present in control $p53^{+/+}$ and $p53^{+/-}$ mouse skin DNA but was absent in $p53^{-/-}$ mouse skin DNA. These results indicate that most lymphoid tumors from UV-irradiated $p53^{+/-}$ mice retained the wild-type allele.

***p53* Mutation in Lymphoid Tumors Arising in UV-Irradiated $p53^{+/-}$ Mice.** Twenty lymphoid tumors (10 each from spleen and liver) were analyzed for *p53* mutations in exons 5–8. The results indicated that one lymphoid tumor each from spleen and liver had a C→T base substitution in codon 149, resulting in a sequence change from CCA→CTA, with a predicted Pro→Leu amino acid change. One splenic lymphoid tumor had a C→T mutation in codon 200, resulting in a sequence change from CCC→TCC, and a predicted amino acid change from Pro to Ser. No other mutations were detected. Thus, only 15% of the tumors tested had mutations in *p53*. All three mutations occurred at dipyrimidine sites, but the mutated codons were different from those found in skin cancers induced by UV in $p53^{+/-}$ and $p53^{+/+}$ mice (15).

***p16$^{INK4a}$* Deletions in Lymphoid Tumors Arising in UV-Irradiated $p53^{+/-}$ Mice.** The murine *INK4a-ARF* locus encodes two alternative reading frame proteins (p16$^{INK4a}$ and p19$^{ARF}$) known to function as tumor suppressors via the retinoblastoma or the p53 pathway (18, 19). Inactivation of *INK4a-ARF* can lead to dysregulation of these two pathways (18, 19). To determine whether genetic

**Table 1. Lymphoid malignancies in UV-irradiated and unirradiated $p53^{-/-}$ and $p53^{+/-}$ mice**

| | | | | Number of mice with | | | |
|---|---|---|---|---|---|---|---|
| Genotype | Treatment | Number of mice | Number with tumor | Lymphoma in thymus (%) | Leukemia in spleen/liver (%) | Lymphomas + leukemia (%) | Lymphoma in gut (%) |
| $p53^{-/-}$ | UV | 31 | 31 | 8 (26) | 12 (38) | 8 (26) | 3 (10) |
| $p53^{-/-}$ | None | 18 | 18 | 2 (11) | 11 (61)* | 5 (28)* | 1 (6)† |
| $p53^{+/-}$ | UV | 24 | 21 | 0 (0) | 21 (100)‡ | 0 (0) | 0 (0) |
| $p53^{+/-}$ | None | 18 | 1 | 0 (0) | 1 (100)‡ | 0 (0) | 0 (0) |

*Each contained one hemangiosarcoma.
†The mouse also contained leukemia in the spleen and liver.
‡$P = <0.01$ versus unirradiated $p53^{+/-}$ mice.

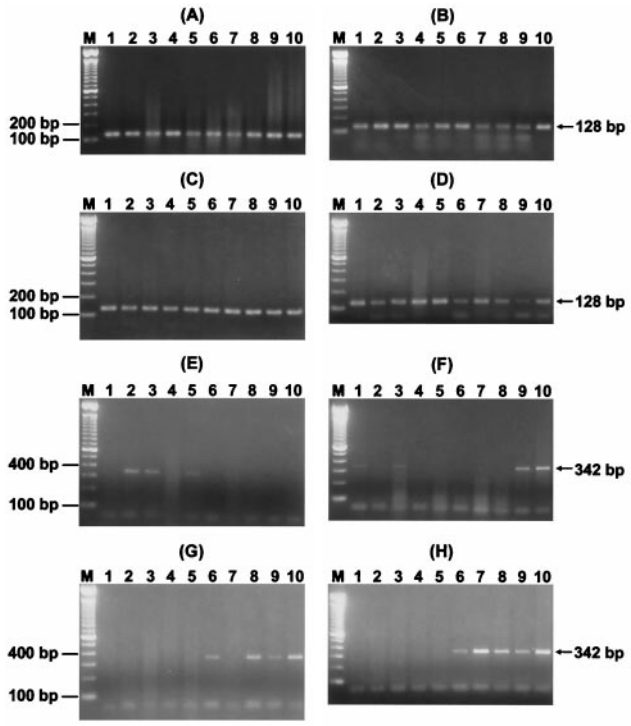


**Fig. 2.** *p16*$^{INK4a}$ deletions in lymphoid tumors arising in UV-irradiated *p53*$^{+/-}$ and *p53*$^{-/-}$ mice. DNAs were amplified for exons 1 and 2 by PCR, as described in *Materials and Methods*. (*A–D*) Exon 1; (*E–H*) exon 2. (*A* and *E*) Ten lymphoid tumors in spleen from UV-irradiated *p53*$^{+/-}$ mice. (*B* and *F*) Ten lymphoid tumors in liver from UV-irradiated *p53*$^{+/-}$ mice. (*C* and *G*) Ten thymic lymphomas from UV-irradiated *p53*$^{-/-}$ mice. (*D* and *H*) Ten thymic lymphomas from unirradiated *p53*$^{-/-}$ mice. Lanes marked M represent molecular marker. Arrows on the right indicate expected band size for each exon.

alterations in *p16*$^{INK4a}$ contributed to the accelerated development of lymphoid malignancies in UV-irradiated *p53*$^{+/-}$ mice, cell lysates from microdissected tumor tissues were analyzed by PCR using primer sets that amplify *p16* exons 1 and 2. Gel electrophoresis data shown in Fig. 2 *A* and *B* reveal that all 20 tumors (10 lymphoid tumors in liver and in spleen) arising in UV-irradiated *p53*$^{+/-}$ mice gave rise to 128-bp bands corresponding to the expected size of *p16* exon 1. Similarly, all 20 thymic lymphomas arising in *p53*$^{-/-}$ mice (10 each from unirradiated and UV-irradiated mice) also amplified for *p16* exon 1 (Fig. 2 *C* and *D*). In contrast, *p16* exon 2 was deleted in 13 of 20 lymphoid tumors from UV-irradiated *p53*$^{+/-}$ mice [Fig. 2 *E* (lanes 1, 4, 6–10) and *F* (lanes 2, 4–8)] and in 11 of 20 thymic lymphomas from unirradiated and UV-irradiated *p53*$^{-/-}$ mice [Fig. 2 *G* (lanes 1–5 and 7) and *H* (lanes 1–5)]. Thus, about 65% of lymphoid tumors from UV-irradiated *p53*$^{+/-}$ mice and about 55% of tumors from unirradiated and UV-irradiated *p53*$^{-/-}$ mice had deletions in *p16* exon 2 (Table 2). However, exon 1$\beta$ corresponding to *p19*$^{ARF}$ was unaffected in most spontaneous and UV-induced lymphomas (data not shown).

**Table 2. Frequency of *p16*$^{INK4a}$ deletions in lymphoid tumors from *p53*$^{+/-}$ and *p53*$^{-/-}$ mice**

| Genotype | Treatment | No. of tumors with deletion/no. of tumors analyzed (%) | |
|---|---|---|---|
| | | Exon 1 | Exon 2 |
| *p53*$^{+/-}$ | UV | 0/20 (0) | 13 /20 (65) |
| *p53*$^{-/-}$ | UV | 0/10 (0) | 6 /10 (60) |
| *p53*$^{-/-}$ | No UV | 0/10 (0) | 5 /10 (50) |

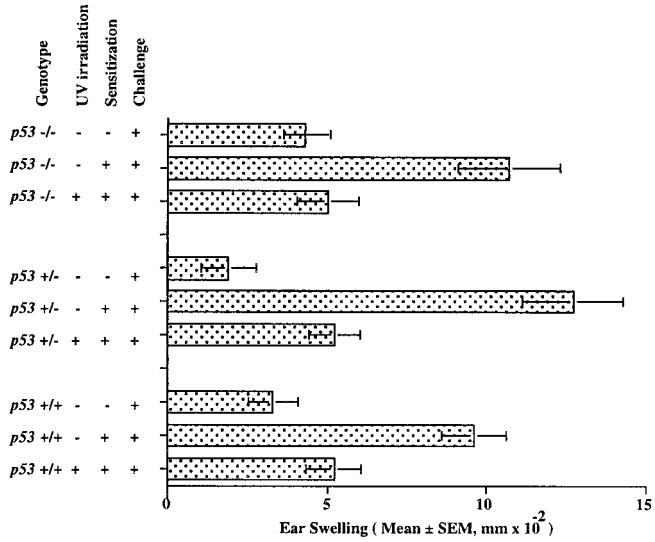


**Fig. 3.** Effect of UV irradiation on CHS response to DNFB in *p53*$^{-/-}$, *p53*$^{+/-}$, and *p53*$^{+/+}$ mice. Groups of five shaved congenic C57BL/6 *p53*$^{-/-}$, *p53*$^{+/-}$, and *p53*$^{+/+}$ mice were exposed to 10 kJ/m$^2$ UV radiation on dorsal skin. Three days later, they were sensitized on the shaved ventral skin with 50 $\mu$l of 0.3% DNFB. Five days after sensitization, the ear thickness was measured, and the mice were challenged by applying 10 $\mu$l of 0.2% DNFB. Ear thickness was measured again 18–24 h later. * $P < 0.05$ versus positive control.

**Immune Status of Congenic *p53*$^{-/-}$, *p53*$^{+/-}$, and *p53*$^{+/-}$ Mice.** *p53*$^{+/-}$ mice showed increased susceptibility to development of lymphoid malignancies after UV irradiation, whereas *p53*$^{-/-}$ mice did not. To determine whether this could be explained by differences in the immune status of UV-irradiated *p53*$^{+/-}$ and *p53*$^{-/-}$ mice, we compared their ability to mount a T cell-mediated immune response and the extent to which their immune responses were suppressed by UV irradiation. To induce systemic immune suppression with UV irradiation, mice were first UV-irradiated and then immunized 3–5 days later at an unirradiated site with either *C. albicans* or alloantigen to measure DTH, or with a contact sensitizer (oxazolone or DNFB) to measure CHS. A representative experiment showing the effect of UV irradiation in *p53*$^{-/-}$, *p53*$^{+/-}$, and *p53*$^{+/+}$ mice is shown in Fig. 3. The immune response to DNFB was identical in mice of all of the three genotypes, and the immune response was suppressed to the same extent in *p53*$^{-/-}$, *p53*$^{+/-}$, and *p53*$^{+/+}$ mice. The same results were obtained regardless of the UV doses used (2, 5, or 10 kJ/m$^2$) or the antigens used (DNFB, oxazolone, *C. albicans*, or alloantigen) (data not shown). In addition, there was no difference in the ability of UV irradiation to suppress the induction of CHS to DNFB applied locally to UV-irradiated skin of mice of the three genotypes (data not shown).

Previous studies showed that DNA damage induced by UV irradiation in wild-type mice initiated suppression of the DTH response to *C. albicans* (20). Because p53 plays an important role in repairing DNA damage (21), it is possible that UV radiation suppressed immune responses in *p53*$^{-/-}$ mice by another mechanism. To investigate whether DNA damage was responsible for immune suppression by UV irradiation in mice lacking functional *p53*, we used liposomes containing T4N5 to repair DNA damage and assessed the effect on immune suppression. T4N5 is an endonuclease that specifically repairs cyclobutyl pyrimidine dimers, the main photoproduct induced by UV radiation. In both *p53*$^{-/-}$ and *p53*$^{+/-}$ mice, the DTH response was reduced by more

than 90% after UV irradiation, compared with that in untreated mice ($P < 0.001$, Student's $t$ test). Application of T4N5-containing liposomes to UV-irradiated mice abrogated the suppressive activity. Treating UV-irradiated mice with heat-inactivated T4N5 in liposomes had no effect. The reversal of UV-induced immune suppression by T4N5-containing liposomes was identical in all three genotypes and indicated that DNA damage was the triggering event for this form of immune suppression in mice of all three genotypes (data not shown).

We also tested the ability of $p53^{-/-}$ mice to reject antigenic tumors by challenging them with transplants of regressor skin tumors from UV-irradiated $p53^{+/+}$ mice. The ability of the $p53^{-/-}$ mice to reject two different regressor tumors was indistinguishable from that of wild-type mice in four separate experiments (data not shown).

## Discussion

Our data clearly demonstrate the extreme sensitivity of $p53^{+/-}$ mice to the development of lymphoid malignancies following exposure to UV irradiation and indicate a causal relationship between UV irradiation and the development of lymphoid malignancies in genetically susceptible hosts. These results also support the idea that p53 plays a role in preventing the development of lymphoid malignancies in these mice. All of the lymphoid tumors examined in our study were of T cell origin, even though p53 is thought to play a prominent role in the development of B cells (22, 23). The reason for this is not clear, nor do we have an explanation for the absence of thymic lymphomas in the $p53^{+/-}$ mice, which were clearly the predominant tumors in the $p53^{-/-}$ mice. It is possible that chronic inflammation, resulting from UV irradiation of the skin, provided a stimulus for malignant transformation of peripheral T cells without altering immature cells present in the thymus. Thus, in $p53^{-/-}$ mice the thymus may be the most vulnerable tissue because of the extensive proliferation of this tissue in young animals. In contrast, in $p53^{+/-}$ mice thymocytes may be protected from malignancy because of the remaining copy of *p53*, and additional events affecting peripheral T cells may be required for the development of lymphoid malignancies in these animals.

Unlike the investigators who studied $p53^{-/-}$ mice of mixed NIH and 129/Sv background exposed to ionizing radiation (14), we did not see an increase in the susceptibility of our $p53^{-/-}$ mice to the development of lymphoid malignancies. This could be due to the fact that the animals were exposed to UV irradiation beginning at 12 weeks of age, and this may have been too late to affect the development of malignancies that began to become clinically apparent at 15 weeks of age. Alternatively, the lymphoid malignancies in our mice may have developed at such a rapid rate that further acceleration by UV irradiation was not possible. Our experiments demonstrate that the immunological competence of the mice of the three genotypes and their susceptibility to UV-induced immune suppression were indistinguishable. These results argue against the hypothesis that intrinsic differences in immune reactivity between $p53^{-/-}$ and $p53^{+/-}$ mice accounted for the differential effect of UV irradiation on lymphoid tumor development in mice of these two genotypes. The latter experiments are also important because they demonstrate that p53 is not required for either the development of normal T cell-mediated DTH and CHS responses or the ability to reject transplanted regressor tumors. This is somewhat surprising in light of the early development of T cell lymphomas in the $p53^{-/-}$ mice, which suggests that control of T cell proliferation is abnormal in these mice.

In the previous studies of $p53^{+/-}$ mice treated with ionizing radiation, an extremely high percentage of the lymphoid tumors that developed lost their remaining wild-type allele of *p53* (14). This is in sharp contrast to our results with UV (non-ionizing) radiation, in which the majority of the resulting lymphoid tumors expressed p53 protein, indicating that the wild-type allele of *p53* was still present in these tumors. That loss of heterozygosity did not contribute to the pathogenesis of lymphoid malignancies in UV-irradiated $p53^{+/-}$ mice was confirmed by genotyping analysis. However, sequence analysis of the *p53* revealed mutations in 3 (15%) of the 20 tumors tested, suggesting that in most cases, normal, rather than mutant, p53 was being overexpressed. Our finding that all three mutations in *p53* that we detected were C→T mutations at dipyrimidine sites is intriguing because such mutations are characteristic of UV-induced *p53* mutations in skin cancers and have been termed UV signature mutations (24). It is conceivable that these mutations occurred in T cells during their residence in inflamed, UV-irradiated skin and represent true UV-induced leukemias. Alternatively, these tumors may resemble pyothorax-associated lymphomas (25). These NHL arising in the pleural cavity of tuberculosis patients have a high frequency of *p53* mutations at dipyrimidine sites, which are unlikely to result from direct exposure to UV radiation. The authors suggest that long-term chronic inflammation may generate a UV-mimetic mutagenic agent. The majority of the lymphoid tumors in $p53^{+/-}$ mice showed no detectable mutations in the functional allele of *p53*, however. Thus, these tumors are reminiscent of those described by Fero *et al.* (26) in $p27^{+/-}$ mice. Mice of the $p27^{+/-}$ genotype were predisposed to the development of tumors in multiple tissues when given ionizing radiation or a chemical carcinogen, and molecular analysis of the tumors from those mice showed that the remaining wild-type allele was neither mutated nor silenced. Fero *et al.* therefore concluded that *p27* represents a new class of tumor suppressor genes haplo-insufficient for tumor suppression, meaning that loss of both alleles is not required for tumor formation, and a mere reduction in the level of the gene product may predispose to malignancy (26). Similarly, we propose that loss of a single *p53* allele may predispose T cells to malignant transformation and that UV irradiation may induce genetic alterations in other genes and perhaps provide additional, indirect stimulus for development of malignant T cells in peripheral lymphoid tissues.

Several studies have shown that the *INK4a-ARF* locus, which encodes two tumor suppressor proteins, p16INK4a and p19ARF (p14ARF in humans), are frequently deleted or mutated in mouse and human lymphoid malignancies (27–30). In addition, inactivation of *p16INK4a-ARF* by deletion, mutation, or hypermethylation has been reported in other human and mouse tumors, including skin tumors (18, 31–35). Our results also suggest an important role for *p16* in the development of spontaneous as well as UV-induced lymphoid malignancies in $p53^{-/-}$ and $p53^{+/-}$ mice. Whereas deletions in exon 1 were absent in lymphoid tumors from UV-irradiated $p53^{+/-}$ and unirradiated and UV-irradiated $p53^{-/-}$ mice, deletions in exon 2 were found in 65% of lymphoid tumors from UV-irradiated $p53^{+/-}$ mice. Interestingly, deletions in exon 2 were also found in 55% of thymic lymphomas from unirradiated and UV-irradiated $p53^{-/-}$ mice. Although the mechanisms involved in *p16* exon 2 deletion in lymphoid malignancies from unirradiated $p53^{-/-}$ mice and UV-irradiated $p53^{+/-}$ and $p53^{-/-}$ mice are unknown, we can speculate that the absence of one or both alleles of *p53* may cause genomic instability leading to further genetic alterations in other genes including *p16*. In addition, it is possible that some of the lymphoid tumors from $p53^{+/-}$ and $p53^{-/-}$ mice that did not have deletions in exons 1 or 2 may still have functional inactivation of p16 protein because of point mutations or promoter hypermethylation. However, we have not tested this possibility.

In addition to deletions in $p16^{INK4a}$ gene, other factors may indirectly contribute to the accelerated development of lymphoid malignancies in UV-irradiated $p53^{+/-}$ mice. First, UV irradiation causes systemic suppression of certain T cell-mediated immune responses, as we demonstrated in these experiments. This transient immune suppression may be sufficient to provide a growth advantage for incipient tumor cells that

would otherwise be eliminated or held in check by immunological mechanisms. Second, UV irradiation of the skin causes an increase in the production and secretion into the bloodstream of a variety of cytokines. One of them, IL-10, is a growth factor for certain subpopulations of lymphocytes (36). This is an attractive hypothesis because IL-10 has been shown to be a prognostic factor for poor survival of patients with NHL (37). Another cytokine, macrophage migration inhibitory factor, was recently shown to suppress the transcriptional activity of p53, thus functionally inactivating it (38). Either UV irradiation or the chronic inflammation induced by UV irradiation could upregulate migration inhibitory factor or other factors that affect p53 activity. The third possibility relates to the ability of chronic UV irradiation to induce suppressor T cells that specifically inhibit the rejection of UV-induced skin cancers (39). If the lymphoid tumors that arise in the $p53^{+/-}$ mice expressed the UV-associated common antigen found on UV-induced skin cancers, they would be able to survive and grow only in UV-irradiated mice, which are unable to reject tumor cells bearing this antigen. This raises the interesting and testable hypothesis that perhaps p53 plays a critical role in expression of the elusive UV-associated common antigen recognized by UV-induced suppressor T cells (40).

The biological impact of UV exposure is well known. In addition to skin cancer induction, UV exposure has a number of adverse effects on human health and well being. These include acute effects, such as sunburn, inflammation, edema, necrotic and/or apoptotic cell death, as well as long-term chronic effects such as premature aging of the skin, cataract formation, and immune suppression. The data reported here demonstrate that chronic UV exposure can also contribute to the development of lymphoid neoplasms. This observation is unique in at least three respects. First, all of the energy of UV radiation is absorbed in uppermost layers of the skin; none can directly penetrate to the underlying lymphoid tissue. Yet, UV exposure promotes the development of lymphoid tumors in these mice. This suggests that an alternative pathway must be involved in the development of lymphoid neoplasms, one that is clearly different from the well described mechanisms of skin cancer induction (i.e., induction of *p53* mutations). Determining how this ubiquitous environmental carcinogen induces lymphoid malignancy by a previously unrecognized route is important. Second, the induction of lymphoid malignancies by UV is more than an interesting laboratory phenomenon because epidemiological evidence shows a latitude gradient for human NHL. Our findings support the hypothesis that sunlight exposure contributes to the induction of lymphoma in humans. Third, we describe here a unique animal model for studying the induction of lymphoma by UV radiation. Although there are many differences between this model of lymphoid malignancy and NHL and chronic lymphocytic leukemia in humans, the mechanisms by which UV irradiation influences the development of lymphoid tumors may be similar in mice and humans. Our studies also provide evidence for the involvement of the $p16^{INK4a}$ gene and propose several new hypotheses to account for the previously unexplained association between solar UV exposure and NHL. This will allow us and others to design and test hypotheses to discern the mechanism by which UV exposure promotes the induction of lymphoma.

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