Resistance of *CD1d*^{-/-} Mice to Ultraviolet-Induced Skin Cancer Is Associated with Increased Apoptosis

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Inhibition of p53-induced epidermal apoptosis, generation of p53 mutations, and suppressor T cells are the critical events responsible for the induction and development of UV-induced skin cancers. Recently, we demonstrated that *CD1d* knockout mice were resistant to UV-induced immunosuppression, prompting us to further address the role of *CD1d* in regulating UV carcinogenesis. We, therefore, investigated the response of wild-type (WT) and *CD1d*^{-/-} mice to UV carcinogenesis. We found that although 100% of WT mice developed skin tumors after 45 weeks of UV irradiation, only 60% of *CD1d*^{-/-} mice developed skin tumors. Surprisingly, keratinocytes and fibroblasts from *CD1d*^{-/-} mice were more sensitive to UV-induced apoptosis and persisted longer than cells derived from WT mice. In addition, epidermis and dermis taken from chronically UV-irradiated *CD1d*^{-/-} mice harbored significantly fewer p53 mutations than WT mice. Our findings identify an unexpected and novel function for *CD1d* as a critical molecule regulating UV carcinogenesis, by inhibiting apoptosis to prevent elimination of potentially malignant keratinocytes and fibroblasts. (*Am J Pathol* 2004, 165:879–887)

Skin cancer incidence is increasing at an astonishing rate. More than 1 million new cases of nonmelanoma skin cancer will be diagnosed in the United States this year.¹ Epidemiological, clinical, and biological studies indicated that solar ultraviolet (UV) radiation is the major etiological agent in skin cancer development.² Wavelengths in the UVB range (280 to 320 nm) of the solar spectrum are absorbed by the skin, producing erythema, burns, immunosuppression, mutations, and nonmelanoma skin cancer.

UV radiation targets the p53 tumor suppressor gene, and UV-induced mutations play a critical role in the induction of nonmelanoma skin cancer.^{3–6} The p53 protein serves as a guardian of the genome by aiding DNA repair

and/or causes elimination of cells with excessive DNA damage. Excessive UV exposure overwhelms DNA repair mechanisms, allowing the survival of mutations. Keratinocytes carrying p53 mutations acquire a growth advantage by virtue of their increased resistance to apoptosis, and so begin to undergo carcinogenesis.

CD1d is a novel antigen-presenting molecule encoded by nonmajor histocompatibility complex genes.⁷ CD1d presents glycolipid antigens to a unique subset of T cells, designated natural killer T (NKT) cells.^{8–10} NKT cell function and development is restricted by CD1d.^{11,12} NKT cells play a crucial role in UV-induced immune suppression because they suppress tumor immunity and allow for the progressive growth of highly antigenic UV-induced skin cancers.¹³ In addition, *CD1d*^{-/-} mice, which are deficient in NKT cells, are resistant to the immunosuppressive effects of UV radiation.¹³ Because immune suppression is a risk factor for UV-induced carcinogenesis,¹⁴ we hypothesized that *CD1d*^{-/-} mice may also be resistant to UV carcinogenesis. To test this hypothesis we compared the response of *CD1d*^{-/-} and wild-type (WT) mice to acute and chronic UV irradiation. Our results indicate that *CD1d*^{-/-} mice were more resistant to UV skin carcinogenesis than WT mice and that increased cell death and elimination of potentially malignant keratinocytes and fibroblasts accounted for this resistance.

Materials and Methods

Mice

Inbred *CD1d*^{-/-} and *CD1d*^{+/+} mice backcrossed onto the C57BL/6 background were obtained from Dr. Luc Van Kaer (Vanderbilt University School of Medicine, Vanderbilt, TN). The genetic identity of the *CD1d*^{-/-} and *CD1d*^{+/+} mice was confirmed by reciprocal skin grafting experiments. The animals were maintained in facilities approved by Association for Assessment and Accreditation of Laboratory Care, in accordance with current reg-

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ulations and standards of the National Institutes of Health. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee.

Radiation Source and Irradiation of Mice

A 1000 W xenon UV solar simulator equipped with a Schott WG-320 atmospheric attenuation filter (1 mm thick), a visible/infrared band pass blocking filter (Schott UG-11; 1 mm thick), and a dichroic mirror to further reduce visible and infrared energy (Oriol Corp., Stratford, CT) was used to provide solar-simulated UV radiation (UVA + UVB). The UV dose and spectral output of the light source were measured as previously described.¹⁵ For the acute UV experiments, groups of mice were irradiated with a single dose of 5 kJ/m² UVB (290 to 320 nm). Skin was excised 6 to 168 hours after UV irradiation. For the chronic UV experiments, the mice were irradiated with 5 kJ/m² of UVB radiation, three times a week for up to 30 weeks. For the carcinogenesis experiments, groups of 20 mice were irradiated with 10 kJ/m² of UVB radiation, three times a week until skin tumors developed. Sham-irradiated WT and *CD1d*^{-/-} mice were used as negative controls.

Isolation of Skin

Shaved dorsal skin (~2 × 4 cm) was excised from each mouse and cut into two pieces. One piece was immediately fixed in 4% formaldehyde for paraffin-embedded sectioning. The other piece was floated dermis-side down in buffered 0.5 mol/L ethylenediaminetetraacetic acid, pH 7.4, for 1 hour at 37°C. The skin was separated into epidermis and dermis, and DNA was extracted from each by phenol-chloroform method. In the carcinogenesis experiments, the mice were sacrificed when the skin tumors reached ~10 mm in diameter. The tumors were cut into two pieces, one of which was used for paraffin-embedded sectioning and the other for DNA extraction.

Immunohistochemical Analysis

Immunohistochemistry was performed as described.^{15,16} Briefly, 5-μm sections were deparaffinized, treated with target retrieval solution (DAKO, Carpinteria, CA), washed with phosphate-buffered saline (PBS), and incubated in 0.3% H₂O₂ to block endogenous peroxidase activity. After washing, the sections were preincubated for 10 minutes in 10% normal goat serum and then incubated overnight at 4°C with rabbit polyclonal anti-mouse p53 antibody (CM5; NovoCastra, Newcastle on Tyne, UK), rabbit polyclonal anti-mouse MDM2 antibody (H-221; Santa Cruz Biotechnology, Santa Cruz, CA), or rabbit polyclonal anti-mouse p19^{ARF} antibody (NB200-106; Novus Biologicals, Littleton, CO). After three washes with PBS plus 0.5% Tween, the sections were incubated for 15 minutes with biotin-conjugated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA). After three washes with PBS plus 0.5% Tween, the slides were treated with diaminobenzidine (Vectastain Elite ABC kit,

Vector Laboratories) as recommended by the manufacturer. Counterstaining was performed with hematoxylin. As a negative control, tissue sections were stained with the secondary antibody only.

Terminal Deoxyribonucleotidyl Transferase (TdT)-Mediated dUTP Nick-End Labeling (TUNEL) Assay

TUNEL assays were performed by using a commercial kit according to the manufacture's protocol (Promega Corp., Madison, WI) as described previously.^{15,16}

Allele-Specific Polymerase Chain Reaction (AS-PCR)

Genomic DNA was analyzed by AS-PCR for CC to TT tandem mutations at codons 154 to 155 and 175 to 176 and C to T transitions at codons 270 and 275 of the *p53* gene, as previously described.^{16,17} The mutant-specific forward primers used were: 5'-TTGTGGGTCAGCGC-CACTT-3' for mutations at codons 154 to 155, and 5'-TCGTGAGACGCTGCCCCATT-3' for mutations at codons 175 to 176. The reverse primer used for amplification of codons 154/155 and 175/176 was 5'-GCCT-GCGTACCTCTCTTTGC-3'. C to T hotspot mutations at codons 270 and 275, were detected using the forward mutant-specific primers 5'-GGACGGGACAGCTTTGAG-GTTT-3' and 5'-GTGTTTGTGCCTGCCT-3', respectively. The reverse primer used to detect both mutations was 5'-GCCTGCGTACCTCTCTTTGC-3'. The reaction mixture containing DNA (200 ng) was amplified by PCR in a 25-μl solution containing 10 mmol/L Tris-HCl (pH 8.3); 50 mmol/L KCl; 1.5 mmol/L MgCl₂; 0.001% gelatin; 75 μmol/L each of dATP, dGTP, dCTP, and dTTP; 2.5 μCi of [³²P]dCTP; upstream and downstream primers (200 nmol/L each); and 1.5 U of AmpliTaq (PE Xpress) for 25 cycles. An aliquot of the PCR product was separated on 2.0% agarose gels.

Primary Keratinocyte and Fibroblast Culture

Primary keratinocyte and fibroblast cultures were prepared according to Hager and colleagues.¹⁸ Briefly, newborn skin (2 to 3 days after birth) was excised and floated, dermis down, on 0.25% trypsin at 4°C for 18 hours. The epidermis was separated from the dermis, minced with scissors, suspended in Eagle's minimal essential medium (MEM) with Earle's balanced salt solution without calcium (EMEM) (Cambrex, NJ), and stirred for 30 minutes at 4°C. The suspension was filtered through sterile gauze, centrifuged, and the cells were resuspended in 10 ml of EMEM. Keratinocytes were plated on BD BioCoat collagen IV 60-mm culture dishes (BD Biosciences, San Jose, CA) in EMEM plus 0.06 mmol/L CaCl₂ and 10% chelexed fetal bovine serum. Dermal fibroblasts were plated in Dulbecco's modified Eagle's medium with 10% fetal bovine serum.

UV Irradiation of Keratinocyte and Fibroblast Cultures

Keratinocytes or fibroblasts (1×10^5 cells per dish) were plated on 6-cm dishes and incubated overnight. The cells were washed with PBS and exposed to 30 J/m² (UVB component) from a Kodacel-filtered FS40 sunlamp (National Biological Corp., Twinsburg, OH). After a 24- to 72-hour incubation in EMEM, the cells were washed twice with ice-cold PBS, trypsinized, and collected. The cells were resuspended in 5 ml of ice-cold 70% ethanol, incubated at 4°C overnight, centrifuged, and resuspended in 500 μ l of PBS containing RNase (100 U) and PI (25 μ g). After incubation for 15 minutes at 37°C, the apoptotic cells (sub-G1 DNA content) were detected by flow cytometry.

Statistical Analysis

Data are presented as the means $\pm$ SEM. The data were analyzed by using an unpaired Student's *t*-test (StatView 4.0; SAS Institute, Cary, NC). Probabilities less than 0.05 were considered significant.

Results

CD1d^{-/-} Mice Are More Resistant to UV Skin Carcinogenesis than WT Mice

Because UV-induced immune suppression is a major risk factor for sunlight-induced carcinogenesis, and because CD1d^{-/-} mice were resistant to UV-induced immune suppression,¹³ we hypothesized that CD1d^{-/-} mice may also be resistant to UV skin carcinogenesis. To test this hypothesis, WT and CD1d^{-/-} mice ($n = 20$) were irradiated with solar-simulated UV radiation three times a week and tumor development was measured. The first skin tumor developed in a WT mouse 28 weeks after irradiation. Fifty percent of the WT mice developed skin tumors by week 36, and all WT mice had skin tumors at week 45 (Figure 1). In contrast, the first skin tumor in a CD1d^{-/-} mouse developed after 33 weeks of chronic UV irradiation, and 50% of CD1d^{-/-} mice developed skin tumors at week 42. At week 45, when all of the WT mice had skin tumors, only 60% (12 of 20) of CD1d^{-/-} mice had skin tumors. Continued irradiation until week 55 produced skin tumors in only 70% (14 of 20) of CD1d^{-/-} mice. In all, 25 skin tumors developed in 20 WT mice (average, 1.25 tumors per mouse), and 21 skin tumors in 14 CD1d^{-/-} mice (average, 1.50 tumors per mouse). Analysis of UV-induced skin tumors from WT and CD1d^{-/-} mice revealed the presence of p53 mutations in all of the expected hotspots. In addition, the mutation frequency did not differ significantly between WT and CD1d^{-/-} mice (Table 1).

Figure 1. Induction of skin tumors in chronically UV-irradiated WT and CD1d^{-/-} mice. The mice were irradiated with 10 kJ/m² (UVB component) three times per week. A mouse was scored as having a tumor when a skin lesion reached ~2 to 3 mm in diameter and persisted or increased in diameter for the duration of the experiment.

Persistence of Apoptotic Keratinocytes in UV-Irradiated CD1d^{-/-} Mouse Skin

Because UV-exposure induces apoptosis, we determined whether CD1d^{-/-} mice showed altered susceptibility to apoptosis by measuring TUNEL-positive cells in UV-irradiated mouse skin. The numbers of TUNEL-positive keratinocytes peaked at 24 hours in both WT and CD1d^{-/-} mice (Figure 2a). By 72 hours, TUNEL-positive cells decreased rapidly in the skin of WT mice but persisted in the CD1d^{-/-} mice. There were significantly more TUNEL-positive keratinocytes in CD1d^{-/-} mouse skin than in WT mouse skin (48.8 ± 4.9 versus 9.3 ± 2.2 , per 100 cells; $P < 0.001$) at 72 hours and at 96 hours (28.3 ± 3.6 and 4.8 ± 1.0 , $P < 0.001$) (Figure 2b). TUNEL-positive cells were also present in the dermis of both WT and CD1d^{-/-} mice and the pattern of expression at each time point correlated epidermal expression (data not shown).

Keratinocytes and Fibroblasts from CD1d^{-/-} Mice Are More Sensitive to UV-Induced Apoptosis in Vitro

The data presented above indicate slower removal of apoptotic keratinocytes and fibroblasts from the skin of CD1d^{-/-} mice. We wanted to determine whether this is an intrinsic property of CD1d-deficient keratinocytes or if

Table 1. Number of *p53* Mutations in Six WT and Six *CD1d*^{-/-} Mice after Chronic UV Exposure

UV treatment	Tissue	Mice	Codons 154 to 155	Codons 175 to 176	Codon 270	Codon 275	Total no. of mutations	P value
4 weeks	Epidermis	WT	0/6	0/6	1/6	1/6	2	—
		<i>CD1d</i> ^{-/-}	0/6	0/6	0/6	0/6	0	
	Dermis	WT	0/6	0/6	0/6	0/6	0	—
		<i>CD1d</i> ^{-/-}	0/6	0/6	0/6	0/6	0	
12 weeks	Epidermis	WT	4/6	4/6	3/6	5/6	16	<0.001
		<i>CD1d</i> ^{-/-}	2/6	1/6	1/6	1/6	5	
	Dermis	WT	1/6	2/6	0/6	1/6	4	<0.05
		<i>CD1d</i> ^{-/-}	0/6	0/6	0/6	0/6	0	
30 to 35 weeks	Tumors	WT	6/6	5/6	5/6	5/6	21	—
		<i>CD1d</i> ^{-/-}	4/6	4/6	5/6	5/6	18	

it reflects an alteration in systemic immune function in the NKT cell-deficient mice. Keratinocyte and fibroblast cultures, isolated from neonatal WT and *CD1d*^{-/-} mice, were exposed to 30 J/m² UV radiation *in vitro*, and apoptosis measured by flow cytometry (Figure 3). More apoptotic cells were found in irradiated keratinocytes from *CD1d*^{-/-} mice than from WT mice (8.4% versus 5.4% 24 hours after UV irradiation). The difference was even more pronounced at the later time points (28.0% versus 8.0% at 48 hours, *P* < 0.001 and 33.9% versus 11.8% at 72 hours, *P* < 0.001). Primary fibroblasts from *CD1d*^{-/-} mice were also more sensitive to UV-induced apoptosis than fibroblasts from WT mice (7.5% versus 3.5% at 24 hours, 15.7% versus 4.2% at 48 hours, *P* < 0.01, and 24.8% versus 10.8%, at 72 hours, *P* < 0.01). The fact that we show that keratinocytes and fibroblasts are more sensitive *in vitro* would suggest that the persistence of apoptosis observed in *CD1d*^{-/-} mouse skin exposed to acute UV likely reflects increased skin apoptosis rather than delayed removal.

Expression of *p53* Protein in UV-Irradiated WT and *CD1d*^{-/-} Mouse Skin

Because the *p53* protein plays an important regulatory role in UV-induced apoptosis, we determined whether the alteration of apoptosis observed in *CD1d*^{-/-} cells was

because of altered *p53* expression. In both WT and *CD1d*^{-/-} mice, intense nuclear immunostaining was observed 24 hours after UV irradiation (Figure 4a). Although the number of *p53*-positive keratinocytes had decreased dramatically at 72 hours after UV irradiation in WT mouse skin, the *p53*-positive cells were still present in *CD1d*^{-/-} mouse skin. To determine whether the persistent expression of *p53* protein was because of altered expression of MDM2 protein, which binds and degrades *p53*, we analyzed MDM2 expression in UV-irradiated WT and *CD1d*^{-/-} mice. Figure 4b shows intense MDM2 protein expression in the nuclei and weak cytoplasmic expression 24 hours after UV exposure in WT mouse skin. In contrast, MDM2 was not expressed after UV exposure of *CD1d*^{-/-} mice. These results suggest that persistent expression of *p53* in *CD1d*^{-/-} mouse skin is because of absence of MDM2. *p19*^{ARF} protein, which could affect MDM2 expression, was expressed at similar levels in both WT and *CD1d*^{-/-} mouse skin at 24 hours after UV exposure (data not shown).

Persistence of TUNEL-Positive Cells in Chronically UV-Irradiated *CD1d*^{-/-} Mouse Skin

Although acute UV irradiation induces apoptosis, after chronic exposure to UV radiation, mice develop resis-

Figure 2. Response of WT and *CD1d*^{-/-} mouse skin to acute (5 kJ/m²) UV irradiation. **a:** At 72 hours after UV irradiation, more TUNEL-positive keratinocytes were present in *CD1d*^{-/-} mice than in WT mice. DNA was counterstained with PI to identify the nuclei (**left**). **b:** Quantitation of TUNEL-positive cells 12 to 120 hours after acute UV exposure. Four mice were sacrificed at each time point, and four microscopic fields in each mouse were used to calculate average number of TUNEL-positive keratinocytes. ***, *P* < 0.001.

Figure 3. Representative cell-cycle analysis of UV-irradiated (30 J/m² of UVB) primary keratinocytes (**a**) and fibroblasts (**b**) from 2- to 3-day-old WT and *CD1d*^{-/-} mice. Forty-eight hours after UVB irradiation, there were significantly more apoptotic keratinocytes in *CD1d*^{-/-} mice (28.0%) than in WT mice (8.0%, $P < 0.001$), and significantly more apoptotic fibroblasts in *CD1d*^{-/-} mice (15.7%) than in WT mice (4.2%, $P < 0.01$). The horizontal lines correspond to the apoptotic cell fractions.

tance to apoptosis.¹⁶ To determine whether CD1d deficiency alters the apoptotic response in chronic UV-irradiated mice, mice were irradiated with UV, three times per week for 30 weeks, and TUNEL-positive cells were counted at various time points. Figure 5 shows that early

Figure 5. Increased keratinocyte apoptosis in chronically UV-irradiated *CD1d*^{-/-} mice compared with WT mice. The mice were exposed to UV radiation three times per week for 1 to 12 weeks and TUNEL-positive cells in the skin were measured. The results are averages of four different microscopic fields from two mice at each time point. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

Figure 4. p53 and MDM2 expression in UV-irradiated WT and *CD1d*^{-/-} mice. **a:** p53 protein expression was measured in both WT and *CD1d*^{-/-} skin 24 and 72 hours after UV irradiation. **b:** MDM2 protein expression was measured both in WT and *CD1d*^{-/-} skin 24 and 72 hours after UV irradiation.

during the course of UV irradiation (1 to 2 weeks), *CD1d*^{-/-} mouse skin contained significantly more TUNEL-positive keratinocytes than WT mice did. After 1 week of chronic UV exposure, there were 7.5 ± 1.1 TUNEL-positive keratinocytes per 100 cells in *CD1d*^{-/-} mouse skin but only 3.3 ± 0.7 per 100 in WT mice ($P < 0.001$). Significant differences were also observed after 2 (6.7 ± 1.4 versus 3.8 ± 0.7 , $P < 0.01$) and 3 weeks (4.8 ± 0.7 versus 3.3 ± 1.1 , $P < 0.05$) of chronic UV exposure. However, there were no significant differences in the numbers of TUNEL-positive cells between *CD1d*^{-/-} and WT mice after 4 weeks of chronic UV irradiation. These results indicate that *CD1d*^{-/-} keratinocytes are more sensitive to UV-induced apoptosis and are eliminated from the epidermis of UV-irradiated mice.

p53 Expression in Chronically UV-Irradiated WT and *CD1d*^{-/-} Mouse Skin

p53-positive clusters of cells, which are thought to undergo further clonal expansion into skin cancer, are present in UV-damaged skin.¹⁹⁻²¹ Because *CD1d*^{-/-} mouse skin exhibited increased apoptosis after chronic UV irradiation, we hypothesized that continued elimination of UV-damaged keratinocytes should decrease the number of p53-positive clusters in *CD1d*^{-/-} mice. To test this hypothesis, we counted p53-positive clusters in WT and *CD1d*^{-/-} mouse skin after chronic UV irradiation. After 1 to 6 weeks of chronic UV irradiation, p53-positive keratinocytes were scattered mainly in the basal layer of the epidermis in both WT and *CD1d*^{-/-} mice

(data not shown). After 12 and 30 weeks of chronic UV irradiation, clusters of atypical keratinocytes with relatively large and irregular nuclei were present in WT mouse skin (Figure 6a). Such clusters were also present in *CD1d*^{-/-} mouse epidermis but were of lesser frequency (Figure 6b). The average number of p53-positive clusters at week 12 was significantly higher in the epidermis of WT mice than in *CD1d*^{-/-} mouse epidermis. At week 30, the number of p53-positive clusters increased in *CD1d*^{-/-} mice, but only marginally in WT mice. Atypical p53-positive fibroblast clusters were also present in the dermis of WT mice UV-irradiated for 30 weeks (Figure 6c). At this time point, only one *CD1d*^{-/-} mouse had a cluster of atypical cells in the dermis. After 35 to 38 weeks of irradiation, the numbers of dermal p53-positive clusters in WT and *CD1d*^{-/-} were not statistically different (Figure 6c).

CD1d^{-/-} Mice Are More Resistant to UV-Induced p53 Mutation

Although fewer p53-positive clusters in the skin of chronically UV-irradiated *CD1d*^{-/-} mice may account for the fewer skin cancers in *CD1d*^{-/-} mice, a more direct test of that hypothesis is to measure the frequency of UV-induced p53 signature mutations.^{16,17} Therefore, we analyzed the genomic DNA from the epidermis and dermis of UV-irradiated WT and *CD1d*^{-/-} mice by mutant AS-PCR.^{9,19,20} The representative gel of AS-PCR (Figure 7) indicates that three of six epidermal samples from WT mice exposed to UV radiation for 12 weeks but only one of six epidermal samples from *CD1d*^{-/-} mice had codon 270 mutations. Similarly, two of six dermal samples from WT mice had p53 mutations at codons 175 to 176, but none of six dermal samples from *CD1d*^{-/-} mice did. The p53 mutation data for all of the hotspot codons in mice exposed to UV radiation are shown in Table 1. After 4 weeks of irradiation, two p53 mutations were found in the epidermis of the six WT mice but none in the dermis. After 12 weeks of irradiation, 16 mutations were found in the epidermis of the six WT mice. In contrast, none of six *CD1d*^{-/-} mice irradiated for 4 weeks had mutations in the epidermis or dermis. Moreover, the total number of p53 mutations in the epidermis of *CD1d*^{-/-} mice UV irradiated for 12 weeks was significantly lower (five mutations per six mice, $P < 0.001$) than that detected in the epidermis of WT mice (16 mutations per six mice). The number of p53 mutations in the dermis of *CD1d*^{-/-} mice exposed to UV for 12 weeks was also significantly different (0 mutations per six mice, $P < 0.05$) than the number in the dermis of WT mice (four mutations per six mice).

Discussion

Here, we provide evidence that skin tumor incidence is decreased in *CD1d*^{-/-} mice. Mice that do not express the CD1d protein eliminated UV-damaged, potentially malignant keratinocytes and fibroblasts by prolonging p53 protein expression and, at the same time, down-

Figure 6. Induction of p53-positive, abnormal cell clusters in chronically UV-irradiated WT and *CD1d*^{-/-} mouse skin. **a:** Clusters of p53-positive atypical keratinocytes (**top**) and fibroblasts (**bottom**) in a representative WT mouse after 30 weeks of chronic UV irradiation. **b** and **c:** Quantitation of p53-positive atypical clusters in the epidermis (**b**) and dermis (**c**) of mice exposed to chronic UV irradiation. ***, $P < 0.001$.

regulating MDM2, a negative regulator of p53. Repair of UV-induced DNA damage and removal of cells containing DNA damage decrease the probability of tumor development.^{4,20-22} Although there was no difference in the repair of UV-induced pyrimidine dimers in *CD1d*^{-/-} and WT mouse skin (data not shown), more UV-damaged keratinocytes and fibroblasts were removed from

Figure 7. Representative results of AS-PCR detecting *p53* mutations. Genomic DNA from epidermal samples of six WT mice and six *CD1d*^{-/-} mice after 12 weeks of UV irradiation were analyzed for codon 270 (**top**), and genomic DNA from dermal samples of the same mice were analyzed for codons 175 to 176 (**bottom**). Amplification of mutant sequences of the sizes expected for codon 270 (134 bp) and codons 175 to 176 (55 bp) are indicated by **arrows**. M, molecular marker (ϕ X174/*Hae*III); P, tumor DNA known to contain mutation at the specified codons; N, negative control (genomic DNA from normal WT mouse skin).

CD1d^{-/-} than WT mouse skin. Even after chronic UV irradiation for 3 weeks, many more apoptotic cells were present in *CD1d*^{-/-} mouse skin than in WT mouse skin. We previously showed that chronic UV irradiation results in dysregulation of apoptosis, which leads to hyperproliferation of keratinocytes.¹⁶ Hyperproliferative keratinocytes can accumulate *p53* mutations and begin to undergo carcinogenesis. According to the theory of field cancerization,²³⁻²⁶ numerous mutations accrue throughout a UV-irradiated field, and some of the initiated cells undergo clonal expansion and progress to form skin tumors. These observations are consistent with our finding that the skin of WT mice exposed to chronic UV radiation had more *p53*-positive clusters than *CD1d*^{-/-} mice did. These atypical *p53*-positive clusters are considered to be the seeds of skin cancers that arise after chronic UV irradiation.¹⁹⁻²¹ In fact, the frequency of *p53*-positive atypical foci correlates well with the appearance of UV-induced skin cancers.²⁰

It is well known that human and mouse UV-induced skin cancers harbor unique mutations in the *p53* gene that are not commonly found in other cancers. These UV signature mutations are tandem CC to TT or C to T transitions occurring at dipyrimidine sequences.³⁻⁵ Mutations in the *p53* gene give rise to *p53*-positive clusters^{4,19-21} and keratinocytes containing *p53* mutations are more susceptible to the tumor-promoting effects of UV radiation. Previously, we noted that UV-induced *p53* mutations arise early, well before the development of skin cancers. Further, inhibition of *p53* mutations protects mice against skin cancer development.^{16,17,27} These observations are quite consistent with our finding that *p53* mutations were present in the chronically UV-irradiated, noncancerous skin of both WT and *CD1d*^{-/-} mice. That skin from *CD1d*^{-/-} mice exposed to chronic UV irradiation had significantly fewer *p53* mutations as well as fewer *p53*-positive atypical clusters than WT mice could explain the delay in the onset of skin tumors in *CD1d*^{-/-} mice. However, the tumors from both WT and *CD1d*^{-/-} mice harbored *p53* mutations in all of the hotspots, anal-

ogous to those detected in UV-irradiated skins. The presence of multiple mutations in the same tumor is consistent with our earlier observation that suggest field cancerization.⁵ Field cancerization is postulated to result from prolonged exposure to carcinogens.²⁸⁻³⁰ Numerous mutations accrue throughout the field or area sharing the carcinogen exposure(s), and some of the initiated cells progress to multiple primary tumors. The presence of multiple *p53* mutations observed in UV-induced mouse skin cancers suggests that clones harboring an initial mutation on one allele were targets for a second mutational event on the other allele or that these mutations may have arisen independently, perhaps in different clonal subpopulations during tumor development. This possibility is supported by the finding that primary tumor masses often consist of clones of cells that have different biological properties such as karyotype, surface receptors, growth rate, and metastatic ability.³¹

That there is a relationship between apoptosis and skin cancer development is well established. *p53*^{-/-} mice are more resistant to UV-induced sunburn cell formation²² and are highly sensitive to developing UV-induced skin cancers.^{32,33} Similarly, the keratinocytes from *bax*^{-/-} mice are more resistant to UV-induced cell death than are WT mouse keratinocytes, and a significant increase in tumor incidence was observed in *bax*^{-/-} mice in response to chemical carcinogenesis.³⁴ In *Gadd45a* knockout mice, the number of apoptotic keratinocytes is dramatically reduced by UV radiation, and the knockout mice are more susceptible to UV-induced carcinogenesis.³⁵ Together these results imply that increased apoptosis of target cells reduces skin cancer development.

Tiano and colleagues³⁶ found that initiated keratinocytes from mice deficient in cyclooxygenase-1 and -2 undergo premature terminal differentiation, which reduces skin tumorigenesis. This mechanism does not appear to be a significant factor in the study reported here, because no differences in keratin 10 expression, a marker of terminal differentiation, or cellular proliferation, or keratinocyte transit speed were observed between *CD1d*^{-/-} and WT mice exposed to chronic UV irradiation for 12 or 30 weeks (data not shown).

Our finding that MDM2 protein was not induced by UV irradiation in *CD1d*^{-/-} mouse skin could explain why *p53* protein was expressed longer in UV-irradiated *CD1d*^{-/-} mice. MDM2 binds to *p53* and blocks its function primarily by promoting ubiquitination and proteasomal degradation.³⁷ The balance between *p53* and MDM2 dictates the state of activity of *p53* within a living cell.³⁵ One of the most important components that affects this autoregulatory feedback loop is the tumor suppressor protein p19^{ARF} (p14^{ARF} in humans).^{38,39} By binding MDM2 protein, p19^{ARF} inhibits E3 ligase activity of MDM2,⁴⁰ and sequesters it in the nucleus,^{41,42} thus preventing MDM2 binding to *p53* and disrupting the negative feedback inhibition of *p53*. In the study reported here, we found no differences in p19^{ARF} expression in UV-irradiated *CD1d*^{-/-} and WT mice (data not shown).

During UV carcinogenesis there appear to be at least two distinct stages at which the *CD1d* gene product plays a role. One involves suppression of tumor rejection

by NKT cells.¹³ NKT cells are CD1d restricted, and *CD1d*^{-/-} mice do not have functional NKT cells.¹³ Control of skin tumor rejection by NKT cells probably occurs late in the process of carcinogenesis, after the initiation of the skin cancers. The data presented here, however, suggest an early and unexpected role for *CD1d* in UV-induced skin carcinogenesis. CD1d protein has been reported to be expressed both in normal human and murine keratinocytes.^{43,44} That *CD1d*^{-/-} mice are tumor resistant may not be solely because of lack of NKT cells, because there was no significant difference in the degree or time course of infiltrative cell type or dermal inflammation between *CD1d*^{-/-} and WT mice after acute or chronic UV radiation, and primary keratinocytes and fibroblasts from *CD1d*^{-/-} mice were more sensitive to UV-induced apoptosis *in vitro* (ie, in the absence of NKT cells) than keratinocytes and fibroblasts from WT mice. Our data suggests that the resistance of *CD1d*^{-/-} mice to UV-induced skin carcinogenesis is because of increased cell death and elimination of potentially malignant keratinocytes and fibroblasts, in part because of a failure to up-regulate the induction of MDM2. How exactly CD1d, or the absence of CD1d, modulates the cell signaling pathways leading to MDM2 expression is unclear, but our findings suggest that this cell surface antigen-presenting cell molecule may have more diverse functions than presently recognized.

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