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IL-10 Controls Ultraviolet-Induced Carcinogenesis in Mice

Karin Loser,2* Jenny Apelt,2* Maik Voskort,* Mariette Mohaupt, ‡ Sandra Balkow,* Thomas Schwarz,*‡ Stephan Grabbe,*§ and Stefan Beissert3*

UV radiation-induced immunosuppression contributes significantly to the development of UV-induced skin cancer by inhibiting protective immune responses. IL-10 has been shown to be a key mediator of UV-induced immunosuppression. To investigate the role of IL-10 during photocarcinogenesis, groups of IL-10+/+, IL-10−/−, and IL-10−/+ mice were chronically irradiated with UV. IL-10+/+ and IL-10−/+ mice developed skin cancer to similar extents, whereas IL-10−/− mice were protected against the induction of skin malignancies by UV. Because UV is able to induce regulatory T cells, which play a role in the suppression of protective immunity, UV-induced regulatory T cell function was analyzed. Splenic regulatory T cells from UV-irradiated IL-10−/− mice were unable to confer immunosuppression upon transfer into naïve recipients. UV-induced CD4+CD25+ T cells from IL-10−/− mice showed impaired suppressor function when cocultured with conventional CD4+CD25+ T cells. CD4+CD25+ T cells from IL-10−/− mice produced increased amounts of IFN-γ and enhanced numbers of CD4+TIM-3+ T cells were detectable within UV-induced tumors in IL-10−/− mice, suggesting strong Th1-driven immunity. Mice treated with CD8+ T cells from UV-irradiated IL-10−/− mice rejected a UV tumor challenge significantly faster, and augmented numbers of granzyme A+ cells were detected within injected UV tumors in IL-10−/− animals, suggesting marked antitumoral CTL responses. Together, these findings indicate that IL-10 is critically involved in antitumoral immunity during photocarcinogenesis. Moreover, these results point out the crucial role of Th1 responses and UV-induced regulatory T cell function in the protection against UV-induced tumor development. The Journal of Immunology, 2007, 179: 365–371.

The Journal of Immunology

The UV spectrum of sunlight, in particular the mid-wave range (290–320 nm, UVB) represents the most important risk factor for the development of nonmelanoma skin malignancies, including basal cell and squamous cell carcinomas (1–3). Both of these UV-induced skin cancers show a rapidly increasing incidence worldwide (4–6). During the development of skin tumors the immune system appears to play an important role. Evidence to support this hypothesis is provided from organ transplant patients who are therapeutically on long-term immunosuppressive medication (7–9). These patients present with up to 30-fold increased numbers of skin malignancies, primarily on sun-exposed areas such as the head, upper lip, and back of the hands. In murine experimental models of photocarcinogenesis, tumor development seems to be regulated by T cells (10). There is increasing evidence that UV-induced regulatory T cells play an important role during photocarcinogenesis, because they are able to inhibit antitumoral effector functions (11, 12). Although UV-induced regulatory T cells were detected as far back as two decades ago—in those days they were called UV-induced suppressor T cells—the exact phenotype and mode of action remained unclear for a long time. In the past few years several subtypes of UV-induced regulatory T cells have been described in a variety of experimental models; most of them are CD4+ and coexpress CD25 plus CTLA-4 (13, 14). Because CD4+ UV-induced regulatory T cells produce IL-10 upon stimulation, it was suggested that IL-10 mediates suppressor function (13, 15). Recent investigations show that CD4+ UV-induced regulatory T cells are involved in the inhibition of effector cells during the development of UV-induced immunotolerance or skin tumors (16, 17).

In addition to inducing regulatory T cells, UV radiation is able to inhibit the Ag-presenting function of dendritic cells such as epidermal Langerhans cells (18). Langerhans cells can present tumor Ags, thereby mediating both the induction and elicitation of protective immunity (19). UV radiation is able to inhibit the Ag-presenting function directly via UV-induced cytotoxicity as well as indirectly via the release of immunosuppressive cytokines such as IL-10 (20). From several reports it was concluded that UV-induced expression of IL-10 contributes to the development of photocarcinogenesis by suppressing protective cellular immune responses. This view was supported by findings showing that invasively growing basal cell carcinomas secreted IL-10 (21). The expression of IL-10 has also been reported in human melanoma cells, and IL-10 production of melanomas correlates with a poorer prognosis (22–24). However, the injection of IL-10-overexpressing tumor cells into mice did not result in enhanced tumor growth kinetics but rather in tumor rejection (25). Furthermore, transgenic mice that overexpressed viral IL-10 under the control of a skin-specific keratin-14 promoter, resulting in increased IL-10 serum concentrations, developed significantly reduced skin tumors upon chronic UV irradiation (26). These unexpected findings were explained by the fact that the activation of NK cell function may contribute to impaired photocarcinogenesis in viral IL-10 transgenic mice. According to these discrepancies, the exact role of UV-mediated
IL-10 expression for the development of UV-induced skin tumors still remains to be determined.

To analyze the role of IL-10 during autochthonous tumor development after chronic UV irradiation, we used IL-10−/− mice and show that IL-10 deficiency results in protection from photocarcinogenesis.

Materials and Methods

Mice

IL-10−/− (knockout) and IL-10+/− (heterozygous) mice on a C57BL/6 background were generated as previously described (27). IL-10−/− (wild type) C57BL/6 controls as well as nude/nu mice were purchased from Harlan-Winkelmann. Mice were housed under specific pathogen-free-conditions and used according to institutional guidelines.

UV irradiation, tumor induction, and histology

For UV irradiation of mice, a bank of four Philips UV-B TL40W/12 sunlamps was used that has an emission spectrum ranging from 280 to 350 nm with a peak at 306 nm. The mice were placed on a shelf 20 cm below the light bulbs and the cage order was systematically rotated before each treatment to compensate for uneven lamp output (16, 28, 29). The location and growth of each tumor exceeding 2 mm in diameter was recorded. Sections from all tumor biopsies were stained with H&E and documented using an Olympus BX61 microscope with Visitron software (Visitron Systems).

Transfer of UV-induced skin tumors

Skin tumors that had reached a size of 5–7 mm in diameter were biopsied and tumor specimens were put in tissue culture flasks (BD Falcon) at 37°C and 5% CO2 containing RPMI 1640 supplemented with 10% heat inactivated FCS (PAA Laboratories), 10 U/ml penicillin (PAA Laboratories), 100 mg/ml streptomycin (PAA Laboratories), 0.1 mM essential and non-essential amino acids (PAA Laboratories), 2 mM l-glutamine (PAA Laboratories), 1 mM sodium pyruvate (PAA Laboratories), and 0.01 M HEPES buffer (PAA Laboratories). Tumor cells were allowed to grow to subconfluence. Subsequently, naive recipients (nu/nu or C57BL/6) were s.c. injected with 1 × 10^6 or 5 × 10^6 viable tumor cells, respectively, and tumor growth was documented over time.

Contact hypersensitivity (CHS)

Mice were sensitized by painting 100 μl of 2,4-dinitrofluorobenzene (DNFB; Sigma-Aldrich) solution (0.5% in acetone/olive oil, 4:1) on the shaved back as described (30). Each group consisted of at least five mice. Each experiment was performed at least three times.

UV irradiation

For induction of tolerance, mice were exposed to UV daily on four consecutive days (1 kJ/m^2 per exposure) (13, 17). Twenty-four hours after the last UV exposure, DNFB was applied to the irradiated skin as described above or mice were sacrificed for cell preparation. For the induction of Th1 or CTL activity, mice were UV irradiated six times (5 kJ/m^2 per exposure) at intervals of 48 h. Forty-eight hours after the last UV exposure, tumor cells were inoculated s.c. or mice were sacrificed for cell preparation.

Adoptive cell transfers

Donor mice were either UV irradiated or left untreated. Subsequently, regional lymph nodes were removed, single cell suspensions were prepared, and CD4^+ CD25−, CD4^+ CD25+ , CD4−, or CD8− T cells were purified by MACS (Miltenyi Biotec) and injected i.v. into recipient mice. After 24 h the recipients were challenged by painting 12 μl of 0.3% DNFB on both sides of the left ear and ear swelling was evaluated or they were inoculated with UV tumor cells and tumor growth was documented.

Cell preparations and flow cytometry

Single-cell suspensions of regional lymph nodes were prepared as described (31). The expression of cell surface and intracellular markers was analyzed by multicolor flow cytometry on a FACSCanto cytometer (BD Biosciences) and cells were stained in PBS containing 1% FCS using the following Abs from eBioscience/NatuTec: FITC-conjugated anti-Foxp3 (clone FJK-16s); PE-conjugated anti-TIM-3 (clone RMT3-23); anti-perforin (clone d9); anti-CTLA-4 (clone UC10-4F10-11); and anti-IFN-γ (clone XMGI1.2); and allopurinol-conjugated anti-CD25 (clone PC61) and mouse polyclonal anti-T-bet. PerCP-conjugated anti-CD4 (clone RM4-5) and anti-CD8 (clone M53-6.7) were obtained from BD Biosciences. Intracellular staining of CTLA-4, perforin, and IFN-γ or allopurinol staining of Foxp3 and T-bet was performed using the Cytofix/Cytoperm kit (BD Biosciences) or the Foxp3 staining set (eBioscience/NatuTec).

Proliferation assays

CD4^+ CD25− and CD4^+ CD25+ cells from UV-irradiated or naive IL-10+/+ or IL-10−/− mice were sorted by MACS (Miltenyi Biotec). CD4^+ CD25− and CD4^+ CD25+ T cells (1 × 10^6/ml; alone or mixed at indicated ratios) were cultured in triplicate in 96-well-round-bottom plates and stimulated with 1 μg/ml anti-CD3 (clone 2c11) and 1 μg/ml anti-CD28 (clone 37.51; both eBioscience/NatuTec). Proliferation assays were cultured in a final volume of 200 μl, 1 μCi/well [3H]thymidine was added for the last 12 h of the experiment, and thymidine incorporation was measured by liquid scintillation counting.

Cytokine quantification

The cytokine activity in culture supernatants of CD4^+ CD25− or CD4^+ CD25+ T cells from IL-10+/+ and IL-10−/− mice was assayed using the mouse Th1/Th2 10plex kit (Bender MedSystems) according to the manufacturer’s instructions. T cells (2 × 10^6/ml) were incubated for 4 days with a combination of anti-CD3 and anti-CD28 Abs (1 μg/ml each Ab) at 37°C and 5% CO2 in 96-well-round-bottom plates in a volume of 200 μl of RPMI 1640 containing 10% FCS. Supernatants were collected and subjected to cytokine quantification using mouse Th1/Th2 10plex kits.

Immunofluorescence stainings

Immunofluorescence stainings were performed on cryostat sections of tumors (3–4 μm) fixed in acetone according to standard methods. Slides were incubated in the appropriate dilutions ofAbs (anti-CD4, anti-Foxp3, and anti-TIM-3; all purchased from eBioscience/NatuTec) or an isotype control (eBioscience/NatuTec). The anti-granzyme A (GrzA) Ab was kindly provided by Dr. M. M. Simon, Max-Planck-Institute for Immunobiology, Freiburg, Germany. Subsequently slides were incubated with Oregon Green- or Texas Red-labeled secondary Abs (Molecular Probes) and examined using a Leica confocal microscope.

Statistical analysis

The method of Kaplan and Meier was used to describe the probability of tumor development in the carcinogenesis study. Statistical differences for the development of tumors between the two strains of mice were determined using a log-rank test by Peto et al. (32). The differences in tumor latency periods were analyzed by using a Mann-Whitney U test. Tumor volumes were calculated as the product of the maximal tumor diameter in three perpendicular directions measured with a Vernier caliper (Mitutoyo). This method has previously been confirmed to correlate well with the tumor weight (19). Mice were sacrificed after the tumor volume exceeded 1,000 mm^3. To evaluate statistical differences between the mean tumor volume in the various experimental groups, the “best-fit” slope of the tumor growth in each animal was determined (Cricket software, version 1.3.2; GraphPad Prism, version 5.0) on a Macintosh G4 computer, and the significance of differences between the means of the slopes for the groups of interest were tested by the two-tailed Student t test for unpaired data. Data from the CHS experiments and proliferation assays were analyzed by Student’s t test and differences were considered significant at p < 0.05.

Results

Reduced development of UV-induced skin cancer in IL-10−/− mice

To determine the role of IL-10 for the generation of UV-induced cutaneous malignancies, groups of IL-10−/−, IL-10+/+ , and IL-10−/− mice (all H-2b) were chronically irradiated with UVB on their shaved backs and tumor development was documented over time. Upon irradiation, the vast majority of IL-10−/− and IL-10+/+ mice developed UV-induced skin tumors (Fig. 1). Tumor development occurred to a similar extent, latency period, and rate in both IL-10−/− and IL-10+/+ mice. Also, autochthonous tumor growth in vivo was similar both in IL-10−/− and IL-10+/+ mice.
recipient mice (28, 33). Accordingly, the vast majority of cell lines cancers produced by UV irradiation are highly immunogenic and development.

dysplastic keratinocytes or pathologic mitosis. Taken together, for IL-10

n/H11005/H11569 (data not shown). In contrast, none of the IL-10

n/H11002

Impaired development of UV-induced skin tumors in IL-

FIGURE 1. Impaired development of UV-induced skin tumors in IL-10+/– mice. The rates of UV-induced skin cancer in wild-type (IL-10+/+; n = 20), heterozygous (IL-10+/-; n = 10), and knockout (IL-10–/–; n = 16) mice are shown. Mice were irradiated with 2.5 kJ/m2 UV for 4 wk, with 5 kJ/m2 UV for another 4 wk, and subsequently with 10 kJ/m2 UV for 6 mo and shaved weekly. This experiment was performed once. *, p < 0.00001 for IL-10+/- vs IL-10–/–.

data not shown). In contrast, none of the IL-10–/– mice developed a skin tumor, not even after an observation period of 1 yr. These findings strongly indicate that the expression of IL-10 is required for UV-induced skin tumor development in mice.

Analysis of skin tumors produced by UV irradiation

Tumor specimens were obtained and subjected to histopathological analysis. Most of the UV-induced skin tumors that developed in IL-10+/+ and IL-10–/– mice were located on the ears and backs (Table I). The majority of the primary skin tumors induced by UV irradiation in mice of both groups were poorly differentiated squamous cell carcinomas that grew rapidly in vivo (data not shown).

In contrast, histopathological analysis of chronically UV-irradiated skin demonstrated that not even skin cancer in situ had developed in IL-10–/– mice. There were no signs of intraepidermal dysplastic keratinocytes or pathologic mitosis. Taken together, IL-10 deficiency resulted in no detectable UV-induced skin tumor development.

Previous studies have shown that the majority of murine skin cancers produced by UV irradiation are highly immunogenic and are therefore rejected upon injection into immunocompetent naive recipient mice (28, 33). Accordingly, the vast majority of cell lines

derived from the skin tumors of both IL-10+/+ and IL-10+/- groups from the photocarcinogenesis experiment were rejected upon injection into naive immunocompetent recipients as indicated in Fig. 2A. To compare the growth kinetics of UV tumors from IL-10+/+ and IL-10+/- mice upon transplantation, tumor specimens were s.c. injected into immunodeficient nu/nu mice. The data depicted in Fig. 2B show that the tumor cell lines from the IL-10+/+ and IL-10+/- mice grew with similar kinetics in recipient animals, indicating a similar malignant phenotype of the UV-induced skin tumors from IL-10+/+ and IL-10+/- mice.

Impaired UV-induced immunotolerance in IL-10–/– mice

Because previous work has shown that the inhibitory effects of UV-induced regulatory T cells play a role in immunotolerance (13, 17), we were interested to analyze whether IL-10 is required for the in vivo function of UV-induced regulatory T cells (34, 35). Therefore, IL-10+/+ and IL-10–/– mice were DNPB-sensitized via UV-irradiated skin. Subsequently, splenic T cells were prepared from the different groups of animals and transferred into naive recipients. After cell transfer, all recipients were DNFB sensitized and ear challenged to DNFB to elicit CHS responses (Fig. 3). The transfer of splenic T cells from UV-tolerized IL-10+/- donor mice suppressed CHS responses in recipients, indicating that UV-induced regulatory T cells had been induced. Interestingly, the transfer of splenic T cells from UV-tolerized IL-10–/– mice failed to transfer suppression, because recipient animals were still able to mount significant ear swelling responses (Fig. 3). Together, these results suggest that IL-10 is a critical mediator of UV-induced tolerance. The question arising is whether UV-induced regulatory T cells are not induced in IL-10–/– mice or whether IL-10 is important for their suppressor function.

Reduced suppressor function of UV-induced CD4+CD25+ T cells from IL-10–/– mice

There is accumulating evidence that UV-induced regulatory T cells belong to the CD4+CD25+ T cell subset (14, 16, 17). In mice, lineage development of naturally occurring CD4+CD25+ T

Table I. Number, site, and histological type of skin tumor produced by chronic UV irradiation

<table>
<thead>
<tr>
<th>Tumor Bearers</th>
<th>Site</th>
<th>No. of Tumors</th>
<th>Histological Differentiation</th>
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<tr>
<td></td>
<td></td>
<td>Poor</td>
<td>Moderate</td>
</tr>
<tr>
<td>IL-10+/+</td>
<td>16/20</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>Ear</td>
<td>Back</td>
<td>8</td>
<td>5</td>
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<tr>
<td>Foot</td>
<td>Eye</td>
<td>1</td>
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</tr>
<tr>
<td>IL-10+/-</td>
<td>8/10</td>
<td>4</td>
<td>2</td>
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<td>Ear</td>
<td>Back</td>
<td>4</td>
<td>3</td>
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<tr>
<td>Foot</td>
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<tr>
<td>IL-10–/–</td>
<td>0/16</td>
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* Wild type; n = 20.
Heterozygous; n = 10.
Knockout; n = 16.

FIGURE 2. Growth kinetics of UV-induced skin tumors from IL-10+/+ and IL-10+/- animals in nu/nu and C57BL/6 mice. A, Groups of naive C57BL/6 mice (n = 5) were injected s.c. with 5 x 106 tumor cells isolated from UV-tumors of IL-10+/+ or IL-10–/– mice. Afterward, tumor growth was documented over time by two independent investigators. B, UV-induced skin tumors were biopsied from IL-10+/- and IL-10–/– mice and subsequently inoculated into nu/nu mice.
cells is controlled by the transcription factor Foxp3 (36). Because our data indicate that the transfer of splenic T cells from UV-tolerized IL-10−/− mice failed to induce tolerance in naive recipients. Naïve recipient mice were injected i.v. with 1 × 10⁶ regulatory T cells obtained from donors that were tolerantized against DNPB by an application of DNPB onto UV-exposed skin (4 × 1000 J/m²). Twenty-four hours after injection, mice (n = 8 mice per group) were sensitized against DNPB and 5 days later a challenge with DNPB was performed on the left ear. Positive (pos.) control mice were sensitized and challenged without transfer, negative (neg.) control mice were ear challenged only. Ear swelling was measured 24 h after challenge. Ear swelling response is expressed as the difference between the thickness (cm ± 10⁻²) of the challenged left ear compared with the thickness of the vehicle-treated right ear. *, p < 0.0001 for positive vs negative control; positive vs recipients injected with UV-induced (UV-ind.) regulatory T cells from UV-tolerized IL-10−/− mice.

Analysis of proliferation and cytokine production of CD4⁺CD25⁺ or CD4⁺CD25− T cells from IL-10−/− mice

Because T cell-derived mediators are critical for the stimulation of anti-tumoral immune responses, lymphocytes from IL-10−/− and IL-10−/− mice have been phenotypically characterized. IL-10−/− and IL-10−/− mice have similar numbers of peripheral CD4⁺ or CD8⁺ T cells (data not shown). Upon TCR stimulation with mitogenic Abs (anti-CD3/anti-CD28), splenic and lymph node CD4⁺ CD25− T cells from IL-10−/− mice showed a reduced proliferative response compared with IL-10−/− CD4⁺ CD25⁺ T cells (Fig. 5A). Subsequently, supernatants from stimulated CD4⁺ CD25− and CD4⁺ CD25⁺ lymphocytes were assayed for the presence of cytokines. The data shown in Fig. 5B indicate that stimulated CD4⁺ CD25− T cells from IL-10−/− mice produced significantly more IFN-γ and less IL-4 compared with stimulated CD4⁺ CD25⁺ T cells from naive IL-10−/− mice.

**FIGURE 3.** Transfer of T cells from UV-tolerized IL-10−/− mice failed to induce tolerance in naïve recipients. Naïve recipient mice were injected i.v. with 1 × 10⁶ regulatory T cells obtained from donors that were tolerantized against DNPB by an application of DNPB onto UV-exposed skin (4 × 1000 J/m²). Twenty-four hours after injection, mice (n = 8 mice per group) were sensitized against DNPB and 5 days later a challenge with DNPB was performed on the left ear. Positive (pos.) control mice were sensitized and challenged without transfer, negative (neg.) control mice were ear challenged only. Ear swelling was measured 24 h after challenge. Ear swelling response is expressed as the difference between the thickness (cm ± 10⁻²) of the challenged left ear compared with the thickness of the vehicle-treated right ear. *, p < 0.0001 for positive vs negative control; positive vs recipients injected with UV-induced (UV-ind.) regulatory T cells from UV-tolerized IL-10−/− mice.

**FIGURE 4.** Reduced suppressor function of UV-induced CD4⁺ CD25− T cells from IL-10−/− mice. Groups of naïve IL-10−/− and IL-10−/− mice were irradiated with UV and CD4⁺ CD25− T cells were subsequently prepared from lymph nodes and spleen. A, Peripheral CD4⁺ CD25− FoxP3⁺ T cells were quantitated before and after UV irradiation using multicolor flow cytometry (cells were gated for CD4). B, Equal numbers (2 × 10⁶) of UV-induced CD4⁺ CD25− T cells from UV-irradiated IL-10−/− or IL-10−/− mice were added to CD4⁺ CD25− T cells from naïve IL-10−/− mice and stimulated with anti-CD3/anti-CD28. T cell proliferation was quantitated by [³H]thymidine incorporation. Mean values of [³H] uptake ± SD are shown from one of three independent experiments. *, p < 0.01 for CD4⁺ CD25− vs CD4⁺ CD25⁺ plus UV-induced (UV-ind.) CD4⁺ CD25⁺ T cells from IL-10−/− mice.

**FIGURE 5.** Increased proliferation and IFN-γ production by CD4⁺ CD25− T cells from IL-10−/− mice. A, CD4⁺ CD25− and CD4⁺ CD25⁺ T cells from lymph nodes of IL-10−/− and IL-10−/− mice were separated by MACS and proliferation assays were performed by stimulating 2 × 10⁶ cells with anti-CD3 plus anti-CD28. T cell proliferation was quantitated by [³H]thymidine incorporation and mean values of [³H] uptake ± SD are shown from one of three independent experiments. *, p < 0.01 for CD4⁺ CD25− T cells from IL-10−/− vs IL-10−/− mice. B, For cytokine production, CD4⁺ CD25− and CD4⁺ CD25⁺ T cells (2 × 10⁵) were stimulated with anti-CD3 and anti-CD28, and IL-4 as well as IFN-γ levels were analyzed using the mouse Th1/Th2 10plex kit. Data show one of three different experiments.
cells from IL-10−/− mice. Together, these findings support the concept that IL-10 plays an essential role in down-regulating Th1-driven immune responses. We speculate that this enhanced proliferative response plus cytokine expression contributes to the reduced photocarcinogenesis observed in IL-10−/− mice.

**Enhanced antitumoral immunity of CD4+ and CD8+ T cells from IL-10−/− mice**

Because UV-induced CD4+CD25+ regulatory T cells can inhibit protective antitumoral immunity, we were interested to scrutinize whether UV-induced CD4+CD25+ T cells from IL-10−/− mice would regulate tumor rejection. To address this question, immunocompetent recipient mice were treated three times with CD4+CD25+ or CD4+CD25− T cells from IL-10−/− or IL-10+/+ mice and s.c. challenged with viable UV-induced regressor tumor cells. Mice that were injected with CD4+CD25+ T cells from IL-10−/− or IL-10+/+ mice rejected the tumor challenge, similar to animals that received CD4+CD25− T cells from IL-10−/− mice (Fig. 6A). However, treatment with CD4+CD25+ T cells from IL-10−/− mice significantly inhibited tumor rejection, suggesting that IL-10 plays an important role in the suppressor function of CD4+CD25+ T cells during the down-regulation of antitumoral immune responses. This hypothesis is supported by the detection of reduced numbers of CD4+Foxp3+ T cells in tumor tissue of mice that had been treated with CD4+CD25+ T cells from IL-10−/− mice (Fig. 6B).

To determine CTL responses and the role of IL-10-deficiency during UV irradiation, groups of mice were treated with two injections of CD8+ T cells from UV-irradiated IL-10−/− or IL-10+/+ mice. Subsequently, CD8+ T cells were phenotypically characterized in tumor-draining lymph nodes. Interestingly, mice that were treated with CD8+ T cells from UV-irradiated IL-10−/− mice rejected the tumor challenge significantly faster compared with controls, suggesting strong antitumoral CD8-mediated effector functions (Fig. 7A). Moreover, in the draining lymph nodes of IL-10−/− mice that had been challenged with UV-tumor cells and were UV irradiated, increased expression of T cell activation and cytotoxic markers was detected in CD8+ T cells (Fig. 7B).

**Discussion**

Investigating the development of UV-induced skin tumors is an ideal experimental model for studying carcinogenesis under control of the immune system. It is well established that UV-induced murine skin tumors are highly immunogenic and are therefore rejected upon transfer into immunocompetent recipients. Such tumors only grow in therapeutically immunosuppressed or UV-treated mice (28, 33, 37). It was suggested that tumor development and growth are controlled by UV-induced regulatory T cells and/or by skewing immunity toward Th2-type responses (12, 38, 39).
This concept was supported by findings showing increased IL-10 as well as IL-4 production after UV irradiation.

The presented results indicate that IL-10−/− mice are resistant to photocarcinogenesis. Because other effects of UV on the skin such as epidermal hyperplasia, erythema, sunburn cell formation, and changes in Langerhans cell or dendritic epidermal T cell numbers were unaffected by IL-10 deficiency (data not shown), we surmise that the documented effects on UV-induced tumor development are due to the immunomodulation of IL-10. To this end, IL-10 has been shown to be a key mediator of UV-induced systemic immunosuppression (40–42). Irradiation of mice with UVB resulted in enhanced IL-10 serum concentrations and inhibition of the sensitization phase toward s.c. injected Ags even when the Ags were injected at a distant nonirradiated site (40, 43). This UV-induced systemic immunosuppression can be transferred to naïve recipients by injecting either serum from UV-treated mice or supernatants from UV-exposed keratinocyte cultures. In line with these results, the treatment of UV-irradiated mice with a neutralizing anti-IL-10 Ab or the addition of anti-IL-10 Abs to the serum resulted in strongly reduced UV-induced tumor development (16). In addition, animals with an impaired suppressor function of UV-induced CD4+CD25+ regulatory T cells induced by treatment with anti-CTLA-4 Abs also demonstrated strongly reduced photocarcinogenesis. Our data indicate that IL-10 is dispensable for the development of UV-induced CD4+CD25+Foxp3+ T cells. Importantly, our results suggest that IL-10 is an essential factor for the suppressor function of UV-induced regulatory T cells because UV-irradiated CD4+CD25+ T cells from IL-10−/− mice show a significantly reduced ability to inhibit the proliferation of conventional CD4+CD25− T cells. This impaired suppressor activity detected in IL-10−/− mice allows for the enhanced stimulation of protective antitumoral immunity against incipient skin malignancies. Together, these results extend our understanding of the molecular mechanisms of how UV-induced regulatory T cells regulate cutaneous tumor growth and antitumoral immunity.

Other reports have argued that, in addition to CD4+CD25+ T cells, CD3−CD4−DX5−NKT cells play a role in inhibiting antitumoral immunity against UV tumors. Upon UV irradiation of mice these CD3−CD4−DX5−NKT cells were able to secrete significant concentrations of IL-4 (44). Treatment of mice with CD3−CD4−DX5−NKT cells from UV-irradiated syngeneic donors resulted in the growth of a previously inoculated UV-induced regressor tumor that was otherwise rapidly rejected in control mice. Whether CD3−CD4−DX5− NKT cells regulate immunity during ongoing tumor development after chronic UV exposure remains to be scrutinized. An analysis of NKT cells revealed increased numbers of splenic CD3−NK1.1−IFN-γ+ NKT cells in IL-10−/− compared with IL-10+/+ mice (data not shown). Whether this enhanced number of activated NKT cells in IL-10−/− mice contributes to antitumoral immunity is currently under investigation.

The functional analysis of CD4+CD25+ T cells from IL-10−/− mice demonstrated increased secretion of IFN-γ. This is most likely due to the impaired Th1/Th2 balance in IL-10−/− mice in favor of Th1-mediated immunity, a hypothesis that is supported by the detection of increased numbers of CD4+Tim-3+ T cells within the UV tumor tissue of IL-10−/− mice. In line with this concept, previous photocarcinogenesis experiments have indicated that mice with impaired UV-induced Th2 responses were protected against UV-induced skin tumor generation (16, 45). Furthermore, IFN-γ has been shown in other experimental tumor models to induce protective antitumoral immunity (46, 47). Our findings additionally indicate a strong CD8-mediated antitumoral CTL response induced by IL-10 deficiency (Fig. 7, A–C). Taken together, the reduced tumor development and the increased Th1 and CTL immunity in IL-10−/− mice indicate the importance of IL-10 for the inhibition of antitumoral immune responses during photocarcinogenesis and allow for the development of new strategies for the immunotherapy of skin cancer such as topical application of IL-10 inhibitors.

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Disclosures

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