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An Important Role of CD80/CD86-CTLA-4 Signaling during Photocarcinogenesis in Mice

Karin Loser,* Andrea Scherer,* Mathias B. W. Krummen,* Georg Varga,* Tetsuya Higuchi,* Thomas Schwarz,*§ Arlene H. Sharpe, † Stephan Grabbe, Jeffrey A. Bluestone, ‡ and Stefan Beissert1,2,*§

Although previous studies have shown that altered B7 costimulation plays a critical role in UV irradiation-induced regulation of immunity, the individual roles of the B7 receptors (CD28 and CTLA-4) or the B7 family members (CD80 and CD86) have not been explored. Thus, we investigated CTLA-4 signaling during photocarcinogenesis of chronically UV-B-exposed mice using an antagonistic anti-CTLA-4 Ab. Anti-CTLA-4-treated mice developed significantly fewer UV-induced tumors. Moreover, anti-CTLA-4 treatment induced long-lasting protective immunity because progressively growing UV tumors inoculated into anti-CTLA-4- and UV-treated mice that had not developed tumors were rejected. Next, we used mice deficient for CD80, CD86, or both in photocarcinogenesis studies to assess the relative contributions of these CTLA-4 ligands. Double-deficient mice showed significantly reduced UV-induced skin tumor development, whereas CD86−/− mice produced skin cancer earlier compared with CD80−/− and control mice. The growth of UV-induced tumors appears to be controlled by UV-induced suppressor T cells, because CD80−/−/ CD86−/− mice had strongly reduced numbers of UV-induced CD4+CD25+ suppressor T cells. In vitro, CTLA-4 blockade inhibited the suppressor activity of UV-induced CD4+CD25+ T cells, suggesting that reduced photocarcinogenesis might be due to decreased numbers of function or pressure of suppressor T cells. Together, these data indicate that blocking CD80/86-CTLA-4 signaling induced immune protection against the development of UV-induced skin tumors. Furthermore, CD86-mediated costimulation appears to play a more critical role in the protection against photocarcinogenesis than CD80.


Ultraviolet irradiation (UVR) constitutes the most important risk factor for the development of nonmelanoma skin cancer, such as basal and squamous cell carcinoma (1–3). These UV-induced tumors show a rapidly rising incidence not only in the United States, but also in Europe and especially in Australia. The two types of tumors are usually not fatal, but can cause significant disability. Although the use of broad-spectrum sunscreens has proven to have protective effects on the development of skin cancer, the rate of UV-induced skin malignancies is expected to rise significantly due to the cumulative nature of the factors inducing carcinogenesis in addition to more outdoor activities and the longer life expectancy of the population (4). Furthermore, decreasing stratospheric ozone levels have led in the past decades to increasing exposure to short wavelength UVB irradiation on the terrestrial surface (5).

The emergence of skin cancer appears to be controlled by the immune system. This hypothesis is strengthened by the observation of 30-fold increased numbers of cutaneous tumors within sun-exposed areas, such as the face or back of the hand, of therapeutically immunosuppressed, organ-transplanted patients (6, 7). In murine models of photocarcinogenesis, the growth of tumors seems to be regulated by T cell-mediated immune responses (8). Murine UV-induced skin tumors are often immunogenic and accordingly rejected upon transplantation into immunocompetent hosts (9, 10). These tumors grow only if the recipient animal is immunosuppressed by medication or chronic UV irradiation (11, 12). Also, UV-induced suppressor T cells have been detected in UV-treated mice, which apparently promote tumor growth by suppressing antitumor effector functions (13). Recent data suggest that UV-induced suppressor T cells belong to the CD4+CD25+ regulatory T cell lineage, but little is known about the factors that regulate UV-induced suppressor T cell homeostasis or function (14). This is in contrast to the much better characterized, naturally occurring CD4+CD25+ regulatory T cells. B7 costimulation plays an important role for these CD4+CD25+ T cells, because they express CTLA-4 and require CD28 ligation for their generation as well as their survival (15–18).

UVR is able to suppress the APC function of dendritic cells, such as epidermal Langerhans cells, to induce protective antitumor immune responses (19). Langerhans cells (LC) are potent APC that reside within the epidermis and form a network of immune sentinels. They are able to take up tumor Ag and induce as well as elicit antitumor immune responses (20). UVR can inhibit LC APC function both directly via UV-induced LC cytotoxicity and indirectly via the release of suppressive mediators from neighboring keratinocytes. For the induction of Ag-specific immune responses by APC, interaction of B7 family molecules is of great importance.
The two initially discovered B7 family molecules, CD80 and CD86, are expressed on APC and bind to their coreceptors, CD28 and CTLA-4, on T cells. It was previously suggested that CD80/CD86-mediated costimulation supports the development of effective cell-mediated immunity (21). Data supporting this view include studies in which treatment of mice with CTLA-4Ig, a soluble form of CTLA-4 that effectively blocks CD80/CD86 engagement with CD28, suppressed antitumor immunity, transplant rejection, and autoimmune responses (22, 23). However, a growing body of evidence suggests a more complicated role of CD80/CD86-mediated costimulation during immune responses (24–26). There are several reports showing that CD28 costimulation induced T cell activation, whereas ligation of CTLA-4 down-regulated T cell function (23, 26, 27). Thus, there are documented differences in response outcomes due to the differential effects of CD28 and CTLA-4 ligation on immunity.

In previous studies we showed that functional blockade of CD80/CD86 costimulation, mediated by transgenic overexpression of CTLA-4Ig, significantly inhibited the development of UV-induced skin tumors (28). However, because CTLA-4Ig binding to both CD80 and CD86 could result in either CD28 or CTLA-4 blockade during carcinogenesis, the relative contribution of each receptor could not be differentiated in this model. Moreover, the recent observations that CD28 and CTLA-4 play integral roles in regulatory T cell function made interpretation of the previous work complex. Thus, in this study we focused on the relative role of CTLA-4-mediated signaling during UV-induced skin tumor development. We have chosen to investigate the CTLA-4 pathway during carcinogenesis, because ligation of CTLA-4 has been associated with impaired T cell stimulation (29, 30). Unfortunately, CTLA-4-deficient mice die prematurely due to a lymphoproliferative disorder. Thus, we used an antagonistic anti-CTLA-4 Ab that inhibits CD80/CD86-CTLA-4 binding (31, 32). The present data show that blocking CTLA-4 signaling leads to reduced development of skin tumors upon irradiation and induction of protective immunity. This anticanerinic effect correlated well with a diminished number or function of UV-induced CD4<sup>+</sup>CD25<sup>+</sup> suppressor T cells. Hence, interference with CD80/CD86-CTLA-4-mediated costimulation protects against UV-induced tumor development, possibly by counteracting UV-induced suppressor T cells.

Materials and Methods

**Mice**

CD80<sup>−/−</sup> and CD86<sup>−/−</sup> mice on a Sv129 background were generated as previously described (33). CD80<sup>−/−</sup> and CD86<sup>−/−</sup> mice were bred nine times onto the BALB/c (H-2<sup>d</sup>) background. CD80<sup>−/−</sup>/CD86<sup>−/−</sup> double-deficient mice were generated by breeding CD80<sup>−/−</sup> to CD86<sup>−/−</sup> mutants. Sv129 and BALB/c controls were purchased from Harlan-Winkelmann. Mice were housed under specific pathogen-free conditions and used according to institutional guidelines (G50/99). UV-B irradiation experiments were performed as described in detail elsewhere (33). CD80<sup>H11002</sup>/H11002 mice were generated by breeding CD80<sup>H11002</sup>/H11002 deficient mice were generated by breeding CD80<sup>H11002</sup>/H11002 and CD86<sup>H11002</sup>/H11002<sup>H11002</sup> double-deficient mice were irradiated (1 kJ/cm<sup>2</sup>) twice a week for 4 wk. One week after the last UV exposure, the spleens of the animals were removed. Single-cell suspensions were prepared, and cells were enriched for T cells using a nylon wool column. Afterward, CD4<sup>+</sup> T cells were purified using an Ab mixture and MACS technology as previously described (34). Purified T cells (>95%) were stained with the according fluorochrome-conjugated Abs (anti-CD3, clone 145-2C11; anti-CD4, clone RM4-5; anti-CD25, clone PC61; all purchased from BD Pharmingen) and examined for the expression of surface molecules using flow cytometry (FACSCalibur; BD Pharmingen).

**Analysis of UV-induced CD4<sup>+</sup>CD25<sup>+</sup> suppressor T cells**

BALB/c wild-type, CD80<sup>−/−</sup>/CD86<sup>−/−</sup>, and CD80<sup>−/−</sup>/CD86<sup>−/−</sup> double-deficient mice were irradiated (1 kJ/cm<sup>2</sup>) twice a week for 4 wk. One week after the last UV exposure, the spleens of the animals were removed. Single-cell suspensions were prepared, and cells were enriched for T cells using a nylon wool column. Afterward, CD4<sup>+</sup> T cells were purified using an Ab mixture and MACS technology as previously described (34). Purified T cells (>95%) were stained with the according fluorochrome-conjugated Abs (anti-CD3, clone 145-2C11; anti-CD4, clone RM4-5; anti-CD25, clone PC61; all purchased from BD Pharmingen) and examined for the expression of surface molecules using flow cytometry (FACSCalibur; BD Pharmingen).

**Analysis of UV-induced CD4<sup>+</sup>CD25<sup>+</sup> suppressor T cells**

To study the effects of anti-CTLA-4 Ab treatment and UV-B irradiation on the number and function of CD4<sup>+</sup>CD25<sup>+</sup> T cells, groups of BALB/c mice (n = 5) were irradiated on their shaved backs for 4 wk as described and subsequently treated i.p. with 50 μg of anti-CTLA-4 Ab in 100 μl of PBS after each UV exposure. After the last treatment, all mice were killed, and the numbers of splenic and lymph node CD4<sup>+</sup>CD25<sup>+</sup> T cells were analyzed using multicolor flow cytometry (FACSCalibur; BD Pharmingen).

**Growth of UV-induced regressor tumors in the presence of UV-suppressor T cells**

Groups of eight mice (naive BALB/c or CD80<sup>H11002</sup>/CD86<sup>H11002</sup> on the BALB/c background) were injected with 3 × 10<sup>6</sup> tumor cells isolated from a cutaneous UV-induced BALB/c tumor on day 0. On days 10, 14, 18, and 22, mice were injected i.p. with 50 μg of anti-CTLA-4 Ab in 100 μl of PBS after each UV exposure. After the last treatment, all mice were killed, and the numbers of splenic and lymph node CD4<sup>+</sup>CD25<sup>+</sup> T cells were analyzed using multicolor flow cytometry (FACSCalibur; BD Pharmingen).

**Generation and culture of bone marrow-derived dendritic cells**

Dendritic cells (DC) were generated as described by Inaba et al. (35) with some modifications. In brief, bone marrow cells were collected from murine tibias and femurs, suspended by vigorous pipetting, passed through a nylon mesh to remove debris, resuspended in complete medium (CM) and cultured in tissue dishes for 2 h. Nonadherent cells were collected, and aliquots of 1 × 10<sup>5</sup> cells were placed in 24-well plates (BD Pharmingen) containing 1 ml of CM medium supplemented with 150 U/ml GM-CSF (R&D Systems) and 75 U/ml IL-4 (BD Pharmingen). After 2 days of incubation (37°C, 5% CO<sub>2</sub>), 600 μl of medium was removed, and the same volume of fresh CM, containing 150 U/ml GM-CSF and 75 U/ml IL-4, was added. On days 5 and 7 of culture, nonadherent cells were transferred into six-well plates containing CM supplemented with cytokines (3 × 10<sup>5</sup> cells/3 ml/well). After a total of 8 days of incubation, most of the nonadherent cells in culture had acquired typical dendritic morphology. These cells were harvested and used as a source of DC in subsequent experiments.
Proliferation assays and MLRs

Proliferation of T cells was assessed by either [3H]thymidine incorporation or CFSA dilution. CD4+CD25- T cells (1 × 10⁵) were cultured in triplicates in 96-well, round-bottom plates in a final volume of 200 µl, and anti-CTLA-4 Ab-treated CD4+CD25+ T cells (1 × 10⁵) were added to each well. After a 5-day culture period using 1 µCi/ml anti-CD3 (clone 2C11) and 1 µg/ml anti-CD28 (clone 37.51) for stimulation, the cells were incubated with [3H]thymidine (1 µCi/well; Amersham Biosciences) for an additional 16 h. T cells were harvested onto filter membranes, and incorporation of thymidine was determined by liquid scintillation counting. For additional in vitro studies, purified CD4+CD25- and CD4+CD25+ T cells were incubated with 1 µM CFSE for 10 min at room temperature, and 2 × 10⁶ cells were cultured in the presence of anti-CTLA-4 Ab for 5 days at 37°C and 5% CO₂. Subsequently, FACS analyses for proliferation (progressive halving of the CFSE label) were performed.

MLRs were assessed by [3H]thymidine incorporation. T cells (1 × 10⁵/ml) were cultured in triplicates in 96-well, round-bottom plates in a final volume of 200 µl, and bone marrow-derived DCs isolated from BALB/c, CD80-/-, CD86-/-, or CD80-/-/CD86-/- double-deficient mice were added at the indicated ratios. MLRs were performed for 3 days, 1 µCi/well [3H]thymidine was added for the last 16 h of the experiment, and thymidine incorporation was measured by liquid scintillation counting.

Statistical analysis

The method of Kaplan and Meier was used to describe the probability of tumor development in the carcinogenesis study. This is a life table analysis and also takes into account animals that die before developing a tumor. Statistical differences for the development of tumors between the two strains of mice were determined using a log-rank test by Peto et al. (36). The differences in tumor latency periods were analyzed by 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 (20). To avoid unnecessary pain to the experimental animals, mice were killed upon transfer (UV progressor tumors) or killed after the tumor volume exceeded 1000 mm³. To avoid unnecessary pain to the experimental animals, mice were killed upon transfer (UV progressor tumors) or killed after the tumor volume exceeded 1000 mm³. 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 (version 1.3.2, Cricket Software; version 4.0, GraphPad PRISM) on a Macintosh G3 computer, and the significance of differences between the means of the slopes for the groups of interest was tested using two-tailed Student’s t test for unpaired data.

Results

Reduced development of UV-induced skin tumors after blockade of CD80/CD86-CTLA-4 signaling

In previous experiments using CTLA-4-4g transgenic mice we were able to show that in vivo disruption of CD80/CD86-CD28/CTLA-4 pathways induced a significant reduction in the probability of developing UV-induced skin tumors (28). Because anti-CTLA-4 Ab treatment induced tumor rejection in mice that had been injected with tumor cell lines, we were interested in investigating whether blocking CTLA-4 signaling plays a role during the development of autochthonous tumors in mice (37). Therefore, a photocarcinogenesis experiment was performed, and inhibition of CD80/CD86-CTLA-4 interactions was achieved by treatment with anti-CTLA-4 Ab after each UV exposure. Mice in the control groups were either UV-exposed only or UV-exposed and treated with an isotype-matched control Ab. All mice of both control groups developed UV-induced skin tumors after ~230 days (Fig. 1). Anti-CTLA-4 Ab treatment induced a significant reduction in the development of UV-induced tumors, suggesting that blockade of the CD80/CD86-CTLA-4 interaction protected mice from the carcinogenic effects of UVR. Even after an observation period of >1 year, <50% of the anti-CTLA-4-treated mice had developed a tumor.

Tumor specimens were obtained and subjected to histopathological analysis. Most UV-induced skin tumors were located on the ears and backs of the mice (Table I) (10, 28). The majority of the primary skin tumors that had developed in mice of both control groups were poorly differentiated squamous cell carcinomas that grew rapidly in vivo (data not shown). In contrast, more moderately differentiated squamous cell carcinomas developed in anti-CTLA-4 Ab-treated mice. Taken together, blocking CD80/CD86-CTLA-4 signaling not only reduced the probability of tumor development, but the few tumors that were seen were of a less malignant phenotype and accordingly grew less progressively in vivo.

Anti-CTLA-4 Ab treatment induced long-lasting, protective, anti-tumor immunity

The observation that the majority of UV-irradiated and anti-CTLA-4 Ab-treated mice failed to develop skin tumors even after an observation period of ~400 days suggested that blocking CD80/CD86-CTLA-4 signaling induced protective anti-tumor immunity. Previous studies have shown that the majority of UV-induced skin tumors are highly immunogenic and therefore rejected upon transfer into immunocompetent recipients (UV regressor tumors) (9). Thus, as expected, when cell lines derived from the skin tumors of both control groups of the photocarcinogenesis experiment were injected s.c. into naive recipients, most transferred cells were rejected (data not shown). However, we were able to identify two progressively growing tumor lines upon transfer (UV progressor tumors; data not shown). These two UV progressor tumors were used to investigate the antitumor immune status of the survivors of the photocarcinogenesis experiment, i.e., those mice that had been UV irradiated and anti-CTLA-4 treated, but did not develop any tumors (Fig. 1). The surviving animals were divided into two groups, and each group was s.c. injected with one of the two UV progressor tumor lines. Naive mice were used as the control group. As shown in Fig. 2, the naive recipients were unable to reject the tumor challenge; the inoculated two UV tumor cell lines grew progressively. Interestingly, UV-irradiated and anti-CTLA-4 Ab-treated mice, which had not developed a skin tumor, rapidly rejected the two UV tumors. These findings suggest that UV- and anti-CTLA-4 Ab-cotreated mice retained an extremely high immune reactivity.

Decreased latency period of UV-induced skin cancer development by disrupting the CD86-CTLA-4/CD28 pathway

We next wanted to differentiate the individual functional role of CD80- or CD86-mediated costimulation during photocarcinogenesis. To address this question, mice deficient for CD80, CD86, or...
both molecules were used for a UV carcinogenesis study. Groups of CD80−/−, CD86−/−, and CD80−/−/CD86−/− double-deficient mice as well as naïve controls were chronically UV-irradiated on their shaved backs to induce skin tumor development as described above. After ~1 year (day 383), all mice from the control group had developed skin tumors (Fig. 3). The latency in tumor development between the control groups shown in Fig. 1 and in this experiment is due to the fact that nonpigmented BALB/c were used in the previous experiments, whereas in the present experiment, pigmented SV129 mice had to be used because the B7 mutants were only available in that background at that time. CD80−/− mice developed UV-induced skin tumors at a frequency comparable to that of wild-type mice. In contrast, CD80−/−/CD86−/− double-deficient animals showed a significantly reduced rate of UV-induced tumor development. This finding is in accordance with our previous report on the reduced carcinogenesis rate in transgenic mice that overexpressed CTLA-4Ig, resulting in a functional inhibition of CD80/CD86-mediated costimulation (28).

Surprisingly, CD86−/− mice showed a significantly shorter latency period to develop UV-induced skin tumors compared with wild-type mice (Fig. 3). Together, these data suggest a differential relevance of CD80- vs CD86-mediated costimulation during carcinogenesis. It appears that the CD86-CD28/CTLA-4 interaction is prominently involved in generating tumor immunity compared with CD80-CD28/CTLA-4 signaling.

### Reduced numbers of UV-induced suppressor T cells in CD80−/−/CD86−/− double-deficient mice

The development of murine UV-induced skin tumors appears to be controlled at least in part by UV-induced suppressor T cells (13). There is currently increasing evidence that UV-induced suppressor T cells belong to the CD4+CD25+ regulatory T cell lineage (14). Additionally, it was shown that the peripheral homeostasis of naturally occurring CD4+CD25+ regulatory T cells is modulated by molecules of the B7 family (17, 38). Therefore, we investigated whether the differences in UV tumor development among B7 mutant mice correlated with the presence of UV-induced CD4+CD25+ suppressor T cells. Splenic and lymph node T cells were prepared from naïve and UV-irradiated wild-type as well as CD80−/−, CD86−/−, and CD80−/−/CD86−/− double-deficient mice, and CD4+CD25+ suppressor T cells were quantitated by multicolor flow cytometry. The data shown in Fig. 4 indicate that normal numbers of regulatory T cells were present in wild-type mice, and that this number increased upon UV irradiation. Similar numbers of CD4+CD25+ T cells were detectable in CD80−/− and CD86−/− mice, which again increased after UV exposure. As previously shown for natural CD4+CD25+ regulatory T cells, the UV-induced CD4+CD25+ T cells from CD80−/− and CD86−/− mice suppressed the proliferation of CD4+CD25+ T cells in response to a polyclonal mitogenic stimuli (data not shown). In fact, the suppression was equivalent to that in B7-sufficient wild-type animals. However, CD80−/−/CD86−/− double-deficient mice revealed strongly reduced numbers of CD4+CD25+ regulatory T cells, and no increase was detectable after UV irradiation. This reduced number of CD4+CD25+ T cells in CD80/CD86 double-deficient mice correlated with the reduced skin cancer development produced by UVR. Therefore, the number of UV-induced CD4+CD25+ T cells correlated with the probability of UV-induced skin tumor development.

### Blocking anti-CTLA-4 signaling inhibits the suppressive function of UV-induced CD4+CD25+ suppressor T cells

The data obtained to date suggest that interfering with CD80/CD86-mediated costimulation mitigates UV-induced tumor development, possibly by counteracting CD4+CD25+ regulatory T cell homeostasis. Because UV-induced regulatory T cells express CTLA-4, and anti-CTLA-4 Ab treatment significantly reduced tumor incidence after UVR, we investigated whether anti-CTLA-4 Ab treatment would influence the number or function of UV-induced CD4+CD25+ regulatory T cells. To this end, groups of mice were UV-irradiated and treated with anti-CTLA-4 Ab for 4 wk. Subsequently, lymph nodes and spleens were prepared and analyzed for the presence of UV-induced CD4+CD25+ suppressor T cells. UV-exposed and anti-CTLA-4 Ab-treated mice had similarly increased numbers of CD4+CD25+ T cells compared with isotype-matched, control Ab-treated mice (data not shown). Next, we addressed whether blocking CTLA-4 signaling would affect the inhibitory function of UV-induced suppressor T cells. Therefore, CD4+CD25+ T cells were prepared from UV-irradiated mice and cocultured with CD4+CD25− in the presence of various concentrations of anti-CTLA-4 Ab. Interestingly, anti-CTLA-4 Ab treatment abrogated the suppressive function of UV-induced suppressor T cells, as evidenced by the vigorous proliferation of CD4+CD25− T cells upon stimulation with anti-CD3 and anti-CD28 (Fig. 5A). In another experiment we addressed whether the anti-CTLA-4 Ab affected CD4+CD25− effector T cells rather than UV-induced CD4+CD25+ suppressor T cells, because CTLA-4 also becomes up-regulated during effector T cell stimulation. To address this, purified CD4+CD25− or CD4+CD25+ T cells were labeled with CFSE and stimulated via anti-CD3/anti-CD28 in the presence or the absence of anti-CTLA-4 Ab. Subsequently, T cell proliferation was evaluated by measuring CFSE dilution in the labeled T cells. Addition of anti-CTLA-4 Ab to CD4+CD25+ T cells inhibited the proliferation dose-dependently to a greater extent than the proliferation of CD4+CD25− T cells (Fig. 5B). Although at a higher concentration (10 μg/ml), anti-CTLA-4 Ab was also able to influence CD4+CD25− T cell proliferation. These findings suggest that the reduced photocarcinogenesis rate induced
by anti-CTLA-4 Ab treatment might be due to inhibition of UV-induced suppressor T cell function and growth by blocking CTLA-4 signaling.

To directly address this, groups of naive mice (H-2d) were s.c. inoculated with a UV-induced regressor tumor (H-2d). One group of animals was injected with UV-induced CD4+CD25+ T cells from UV-irradiated donor mice, and another group was treated with UV-induced CD4+CD25+ T cells plus anti-CTLA-4 Ab. Subsequently, tumor growth was measured in all groups of mice. The results presented in Fig. 6 show that naive mice are able to reject the tumor challenge. In contrast, mice that were injected with UV-induced CD4+CD25+ T cells failed to reject the tumor; the inoculated tumor grew progressively. Interestingly, the injected tumor did not grow in mice that were treated with UV-induced CD4+CD25+ T cells plus anti-CTLA-4 Ab, suggesting that indeed anti-CTLA blockades interfere with the activity of UV-induced CD4+CD25+ T cells to inhibit antitumoral immune responses.

**Discussion**

UV-induced carcinogenesis is an ideal model to study tumor development under control of the immune system. UV-induced tumors in mice are predominantly immunogenic and therefore regress upon transplantation into naive recipients. The growth of such tumors can only be observed in therapeutically immunosuppressed or UV-treated mice. UV can inhibit cellular immune responses via different mechanisms (40, 41). UV-induced systemic immunosuppression correlates with a general shift of immunity from a Th1 to a Th2 response. This shift is supposed to be mediated by the UV-induced release of cytokines, such as IL-4 and IL-10 (42, 43). In support of this concept, we showed in previous experiments that functional blockade of CD80/CD86-mediated co-stimulation impaired the development of UV-induced skin cancer, possibly by counteracting UV-induced immunosuppression and the shift toward a Th2 response after UVR (28). In the present study we have investigated and identified the CTLA-4 pathway as being critically involved in the development of skin cancer produced by chronic UVR (Fig. 1). Moreover, our findings provide evidence that CTLA-4 signaling is involved in antitumor memory responses (Fig. 2). The failure to reject syngeneic UV-induced tumors has been attributed mostly to T cells; in particular, UV-induced suppressor T cells, which inhibit antitumor effector functions within the host (13). These UV-induced suppressor T cells have also been shown to participate in other consequences of UVR, such as UV-induced immunosuppression and immunotolerance (44, 45).

The present findings are in agreement with previous reports on enhanced antitumor effects of CTLA-4 blockade in mice with inoculated tumor lines (37, 46). Also, in a tumor immunotherapy model, treatment of anti-CTLA-4 Ab in combination with GM-CSF tumor cell vaccination induced strong antitumor immunity against B16 melanoma cells (47). Together, these results suggest that interfering with CTLA-4 signaling strengthens antitumor immunity (48). In principle, two scenarios can be envisioned of how anti-CTLA-4 Ab treatment leads to increased tumor immunity: first, by enhancing effector cell responses, and second, by inhibiting (UV-induced) immunosuppression. Direct in vitro activation of CD4+ T cells or CTL by anti-CTLA-4 stimulation has not been observed in our model system (Fig. 5; data not shown). In contrast, our data provide evidence that anti-CTLA-4 inhibits the functional response of tumor cells by inhibiting the generation of tumor-specific antigens and their presentation.

**FIGURE 3.** Differential roles of CD80- or CD86-mediated costimulation during the development of UV-induced skin tumors. Shown are the rates of UV-induced skin tumor development in CD80−/−, CD86−/−, CD80−/−CD86−/− double-deficient, and wild-type control mice (all on an Sv129 background). Mice were initially treated with 2.5 kJ/m2 UVR for 4 wk, then with 5 kJ/m2 UVB for 4 wk, and then with 10 kJ/m2 UVR for 4 mo. The dorsal hair was shaved weekly. This experiment was performed once. n = 20 for the different groups. *, p < 0.001, wild-type vs CD86−/− mice; **, p < 0.05, wild-type vs CD80−/−CD86−/− double-deficient mice.
suppressor activity of UV-induced CD4<sup>+</sup>CD25<sup>+</sup> suppressor T cells in CD80<sup>−/−</sup>/CD86<sup>−/−</sup> double-deficient mice. Groups of CD80<sup>−/−</sup>, CD86<sup>−/−</sup>, CD80<sup>−/−</sup>/CD86<sup>−/−</sup>, and wild-type mice were irradiated with UVB. Subsequently, T cells were isolated from lymph nodes and spleen and analyzed by flow cytometry. The numbers of CD4<sup>+</sup>CD25<sup>+</sup> T cells slightly increased in wild-type, CD80<sup>−/−</sup>, and CD86<sup>−/−</sup> mice upon UV exposure. CD80<sup>−/−</sup>/CD86<sup>−/−</sup> double-deficient mice showed strongly reduced numbers of CD4<sup>+</sup>CD25<sup>+</sup> T cells, which did not increase upon UVR.

These results extend previous findings that UV-induced suppressor T cells participate in the control of UV-induced carcinogenesis most likely by inhibiting antitumor immune responses (49). It was shown that significantly more skin tumors developed in transplanted UV-irradiated skin when the recipient mice were treated with bulk splenic T cells from chronically UV-exposed syngeneic mice (49). Additionally, UV-induced suppressor T cells appear to detect UV-induced tumors as a class, because inoculated non-UV-produced tumor cells in mice that were coinjected with UV-induced suppressor T cells were rejected. Although several attempts have been made to characterize UV-induced suppressor T cells, they have remained largely ill defined (44). In one investigation it was elegantly shown that NK T cells (CD3<sup>+</sup>DX5<sup>+</sup>) from UV-irradiated donor mice can function upon transfer into naive recipients as suppressor T cells to down-regulate antitumoral immunity against injected skin tumors (50). However, recently, more evidence has been provided that CD4<sup>+</sup>CD25<sup>+</sup> T cells are able to mediate several immunoregulatory effects induced by UVR (14). In our experiments we demonstrate that the number or function of these UV-induced CD4<sup>+</sup>CD25<sup>+</sup> suppressor T cells correlated well with the probability of skin tumor development after chronic UVR. Hence, significantly fewer tumors developed in CD80<sup>−/−</sup>/CD86<sup>−/−</sup> double-deficient mice, which had almost no detectable (UV-induced) CD4<sup>+</sup>CD25<sup>+</sup> suppressor T cells. In contrast to CD3<sup>+</sup>DX5<sup>+</sup> T cells, which produce high amounts of IL-4, UV-induced CD4<sup>+</sup>CD25<sup>+</sup> T cells show IL-4 production below detectable levels (data not shown). It is currently unclear whether the suppressor activity of UV-induced CD4<sup>+</sup>CD25<sup>+</sup> T cells is mediated via the release of immunosuppressive cytokines or via a cell contact-dependent mechanism. The results presented are in keeping with our previously reported reduced carcinogenesis rate in keratin 14-CTLA-4Ig transgenic mice with a functional blockade in CD80/CD86-mediated costimulation (28). These transgenic mice also have strongly reduced numbers of UV-induced CD4<sup>+</sup>CD25<sup>+</sup> suppressor T cells (data not shown).

Recent investigations of the maintenance of peripheral, naturally occurring, regulatory T cell homeostasis suggest that low level expression of CD80/CD86 is required for CD4<sup>+</sup>CD25<sup>+</sup> T cell survival (17). CTLA-4, which binds with higher affinity to CD80/CD86 than CD28, is predominantly expressed on CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (15, 16). Our group has also previously identified CTLA-4 as a molecular marker of UV-induced suppressor T cells, which confer Ag-specific immunotolerance upon transfer (46). Taking the present data from UV-treated CD80<sup>−/−</sup>/CD86<sup>−/−</sup> double-deficient mice into account, CD80/86 signaling appears to be of importance for the peripheral maintenance and expansion of UV-induced CD4<sup>+</sup>CD25<sup>+</sup> suppressor T cells. Blocking CTLA-4 signaling by anti-CTLA-4 Ab treatment did not result in a reduction of UV-induced CD4<sup>+</sup>CD25<sup>+</sup> T cell frequencies (data not shown). These data indirectly support the concept that CD80/
CD86-CD28 ligation is needed for CD4+CD25+ T cell survival rather than CTLA-4 engagement (18). Blocking CTLA-4 signaling in vitro inhibited the proliferation of UV-induced suppressor T cells stimulated by mitogenic Ab, rather than the growth of effector T cells (Fig. 5B). Our results provide evidence that the reduced tumor development in anti-CTLA-4 Ab-treated mice might be a consequence of impaired UV-induced suppressor T cell function and support the concept that CD80/CD86-CTLA-4 signaling is required for UV-induced suppressor T cell function.

It is presently unknown whether UV-induced suppressor T cells impair antitumor immune responses via contact-dependent suppression of effector cells as do naturally occurring CD4+CD25+ T cells or whether the release of inhibitory factors, such as IL-10, is essential for suppressor function. For inhibition of contact hypersensitivity responses by UV-induced suppressor T cells, IL-10 production seems to be involved (14). Recently, the tryptophan-degrading enzyme, indoleamine 2,3-dioxygenase (IDO), has been identified to play a role in the regulation of T cell function (51). It was demonstrated that ligation of CD80/CD86 on human DCs by CD4+ T cells induced IDO activity, which allowed DCs to suppress the proliferation of CD4 and CD8 T cells (52). Whether IDO activity plays a role in UV-induced suppressor T cell function is currently under investigation.

The consequences of CD80- or CD86-mediated costimulation can be very different, although both receptors bind to the same coreceptors (23). In murine diabetes transplantation models, it was shown that anti-CD86 Ab treatment of mice that had been transplanted with allogeneic pancreatic islets significantly suppressed T cell proliferative responses and prolonged allograft survival (53). Whether IDO activity plays a role in UV-induced suppressor T cell function is currently under investigation.

Activation of CD86-mediated costimulation via administration of B7-2Ig fusion proteins significantly enhanced T cell and CTL responses (54). Similar results have been reported when tumor-bearing mice were treated with B7-2Ig fusion proteins (55). These findings are in keeping with our finding that CD86-/- DC were less efficient allostimulators compared with DC from CD80-/- or wild-type mice (Fig. 6). It is increasingly apparent that for the initiation of antitumoral immunity, presentation of tumor-derived Ags by professional APCs is required in the context of sufficient costimulation. We, therefore, speculate that the impaired T cell stimulatory capacity of CD86-/- DC contributes to the differences in UV-induced tumor development observed between CD80-/- and CD86-/- mice, because removal of CD86 costimulation led to an enhanced probability of developing skin cancer. These findings are in agreement with studies using anti-CD80 mAb or anti-CD86 mAb for the short term blockade of these pathways in UV-treated mice. It was shown that injections of anti-CD86 mAb played a more important role in regulating UV-induced immunosuppression and induction of suppressor T cells than application of anti-CD80 mAb (56). The photocarcinogenesis results (Fig. 3) indicate that CD86 signaling is more important than CD80-mediated costimulation for the orchestration of antitumor immunity in this model system. Furthermore, our carcinogenesis findings support the importance of costimulation, both positive and negative, in the regulation of protective antitumoral immunity and allow the development of new strategies for immunotherapy of skin malignancies.

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References


