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IL-12 Prevents the Inhibitory Effects of *cis*-Urocanic Acid on Tumor Antigen Presentation by Langerhans Cells: Implications for Photocarcinogenesis[1]

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UV radiation induces skin cancer primarily by its DNA-damaging properties, but also by its capacity to suppress the immune system. The photoisomer of urocanic acid (UCA), *cis*-UCA, is an important mediator of UV-induced immunosuppression and is involved in the inhibition of tumor immunity. The immunomodulatory cytokine IL-12 is known to counteract many of the immunosuppressive effects of UV radiation, including UV-induced immune tolerance. In this study, we addressed whether IL-12 also reverts the immunosuppressive activities of *cis*-UCA. *Cis*-UCA inhibits the ability of Langerhans cells to present tumor Ags for primary and secondary tumor immune responses. IL-12 treatment completely prevented the suppression by *cis*-UCA. IL-12 also protected mice from *cis*-UCA-induced suppression of contact hypersensitivity responses. To study the effects of *cis*-UCA on Ag-processing and Ag-presenting function in vitro, Langerhans cells were treated with UCA isomers and incubated with OVA or OVA peptide$_{323-339}$ before exposure to OVA-specific transgenic T cells. *Cis*-, but not *trans*-UCA suppressed Ag presentation, which was completely reversed upon addition of IL-12. Since these findings suggest that *cis*-UCA may play an important role in photocarcinogenesis by inhibiting a tumor immune response, mice were chronically UVB irradiated to induce skin cancer. Whereas all mice in the control groups developed tumors, mice treated with a mAb with specificity for *cis*-UCA showed a significantly reduced tumor incidence. These data strongly indicate the importance of *cis*-UCA during photocarcinogenesis and support the concept of counteracting *cis*-UCA as an alternative strategy to prevent UV-induced skin cancer, possibly via the application of IL-12. *The Journal of Immunology,* 2001, 167: 6232–6238.

U ltraviolet-induced nonmelanoma skin cancer is the most frequently occurring malignancy in Caucasians in Europe and the United States. Although in general not fatal, the incidence of these tumors is increasing greatly with an estimated 1.3 million new cases in the United States last year alone (www.cancer.org/statistics). These constantly growing numbers contribute to the increasing medical costs and underline the significance and importance of prevention campaigns. UV irradiation (UVR),[3] in particular the middle wavelength range (UVB, 290–320 nm), has been clearly shown to be the primary cause of nonmelanoma skin cancer (1, 2). UVR induces cancer primarily by its DNA-damaging properties, but also by its capacity to suppress the immune system (reviewed in Refs. 3 and 4).

UV-induced immunosuppression can be both local and systemic, and the effects of UVR on immunocompetent cells within the skin can be direct as well as indirect. To induce biological effects, photons have to be absorbed by chromophores. Basically, two major cutaneous chromophores for UVB have been identified in the epidermis, DNA and urocanic acid (UCA). Upon absorption of photons, DNA forms cyclobutane pyrimidine dimers and (6-4)-photoproducts (5). UV-induced dimer formation in particular leads to the release of immunosuppressive cytokines from keratinocytes, such as IL-10 (6). Furthermore, in >90% of UV-induced human skin cancers, cyclobutane pyrimidine dimers can be localized within the cell cycle regulatory gene *p53*, which also acts as a tumor suppressor gene, suggesting a significant role of UV-induced DNA damage in photocarcinogenesis (7, 8).

UCA, a histidine derivative synthesized by keratinocytes, accumulates within the epidermis in significant amounts since catabolic enzymes are absent from that site. Two isoforms exist, *trans*- and *cis*-UCA. *Trans*-UCA, the major cutaneous isomer of UCA, isomerizes to *cis*-UCA upon exposure to UV. Increased amounts of *cis*-UCA can be detected for several weeks after UV exposure in the skin as well as more transiently in the blood (reviewed in Ref. 9). *Cis*-UCA has been shown to suppress cellular immune reactions, such as delayed-type hypersensitivity (DTH) responses to HSV (10). In addition, systemic application of *cis*-UCA prolonged allograft survival in mice (11). Topically applied *trans*-UCA in combination with chronic UVR treatment resulted in enhanced photocarcinogenesis in mice, indicating that *cis*-UCA may be involved in the generation of UV-induced skin cancer probably by causing immunosuppression (12). To this end, previous investigations by our group have shown that *cis*- but not *trans*-UCA inhibits the ability of Langerhans cells (LC), the primary APC in the skin, to present tumor Ag (TA), both for the induction and elicitation of antitumoral immune responses in mice (13). Since

LC are important in the generation of antitumoral immunity, these findings are suggestive for a role of UCA in the development of UV-induced skin cancer.

IL-12 is a heterodimeric cytokine that is produced by a variety of immunocompetent cells, such as dendritic cells, macrophages, B cells, and even keratinocytes (Refs. 14 and 15; reviewed in Ref. 16). Besides its activating effects on NK and cytotoxic T cells, IL-12 exerts costimulatory and regulatory effects on Th cells skewing immune responses toward the Th1 type. Accordingly, IL-12 is crucially involved in the induction of contact hypersensitivity (CHS) and DTH reactions (17, 18). In addition, IL-12 was shown to be the first cytokine able to antagonize UV-induced immunosuppression (19). Intraperitoneal injection of IL-12 into UV-irradiated mice enabled sensitization to take place, even when the hapten was applied onto UV-exposed skin, a procedure that usually fails to induce sensitization (20). Moreover, IL-12 is able to break established immunotolerance induced by UVR. UV-tolerized mice can be resensitized with the respective hapten, if IL-12 is administered before resensitization. The mechanisms by which IL-12 counteracts UV-induced immunosuppression are still not established.

Since *cis*-UCA plays an important role in UV-induced immunosuppression, we were interested in investigating whether IL-12 also antagonizes the immunoinhibitory effects of *cis*-UCA. Ex vivo treatment of epidermal LC with IL-12 completely prevented *cis*-UCA-induced suppression of the induction as well as the elicitation of protective tumor immunity. Accordingly, IL-12 prevented *cis*-UCA-induced inhibition of Ag presentation by LC, indicating that IL-12 can antagonize the immunosuppression induced by *cis*-UCA. To assess the in vivo relevance of *cis*-UCA-induced immunosuppression for photocarcinogenesis, chronically UV-exposed mice were treated with a mAb with specificity for *cis*-UCA. Ab treatment significantly reduced the probability of tumor development. Together, these data indicate an important role of *cis*-UCA in the development of UV-induced skin cancer and point to a therapeutic alternative in the prevention of photocarcinogenesis by inhibiting the effects of *cis*-UCA, possibly by application of IL-12.

Materials and Methods
Mice

Six- to 8-wk-old female A/J (H-2$^{a/d}$) and BALB/c (H-2^d) mice were obtained from Harlan-Winkelmann (Borchen, Germany). OVA-TCR-specific transgenic (DO11) mice (BALB/c; H-2^d) originally generated by Murphy et al. (21) were kindly provided by Dr. T. Blankenstein (Max-Delbrück Center for Molecular Medicine, Berlin, Germany).

Tumors

The S1509a methylcholanthrene-induced spindle cell tumor cell line, orginally derived from A/J mice, was kindly provided by Dr. M. I. Green (University of Pennsylvania, Philadelphia, PA). S1509a cells were maintained in tissue culture at 37°C and 5% CO$_2$ in RPMI 1640 supplemented with 10% heat-inactivated FCS (PAA, Linz, Austria), 100 U/ml penicillin (PAA), 100 μg/ml streptomycin (PAA), 0.1 mM essential and nonessential amino acids (Biochrom, Berlin, Germany), 2 mM L-glutamine (PAA), 1 mM sodium pyruvate (Biochrom), and 0.01 M HEPES buffer (Life Technologies, Parsley, U.K.) (complete medium). S1509a cells usually grow progressively in naive syngeneic recipients and have been demonstrated to induce a variety of immunological responses (22).

Reagents

Murine rGM-CSF was a kind gift from Immunex (Seattle, WA). Murine rIL-12 (p70 heterodimer) was kindly provided by Dr. S. Wolf (Genetics Institute, Boston, MA). Anti-Thy-1.2 mAb (clone 53-2.1) was obtained from BD PharMingen (San Diego, CA) and used at 1:2000. Low-toxicity rabbit complement was obtained from Harlan Sera-Lab (Loughborough, U.K.) and used at 1:40 in PBS. Enzymes used during preparation and dissociation of epidermal cells (EC) were dispase (Boehringer Mannheim,

Mannheim, Germany), DNase (Sigma-Aldrich, St. Louis, MO), and trypsin (Life Technologies).

Trans-UCA was purchased from Sigma-Aldrich. *Cis*-UCA was synthesized from *trans*-UCA in three steps by photoisomerization of the *trans*-methyl ester and saponification to the acid (23). The chemical and isomeric purity of *cis*-UCA was 99.5%, as tested by HPLC.

A mAb with specificity *cis*-UCA was generated in BALB/c mice, as described by Norval and colleagues (24, 25), and used as previously optimized. Mice were injected i.p. with 300 μl of 1/500 dilution of the *cis*-UCA Ab ascitic fluid (equivalent to 0.1 μg of IgG1) in PBS. For controls, mice were injected i.p. with the same amount of an irrelevant isotype-matched IgG1 Ab (Sigma-Aldrich) diluted in 300 μl of PBS.

Soluble tumor-associated Ags (TA) were prepared from freeze-thaw lysates of S1509a tumor cells, as described elsewhere (22, 26). Briefly, S1509a cells (10^7/ml in complete medium) were disrupted by three freeze-thaw cycles and centrifuged at 600 × g for 20 min. The supernatant was collected and spun again at 13,000 × g for 1 h. The remaining supernatant was used as a source of soluble TA.

Preparation of epidermal cells and immunization protocol

EC were prepared using a standard protocol, as described elsewhere (22, 26). Briefly, truncal skins of shaved and chemically depilated (Pilca med Crème; Asid Bonz, Böblingen, Germany) mice were removed and depleted of s.c. panniculus carnosus. The skins were floated dermis side down on 0.5 U/ml dispase and 0.4% trypsin in Ca^{2+}/Mg^{2+}-free PBS for 40 min at 37°C; epidermal sheets were then collected and dissociated by gentle stirring for 20 min. The resulting EC were filtered through nylon gauze (Nitex; Tetco, Elmsford, NY) and washed. Thy-1$^+$-bearing cells were deleted by incubation in anti-Thy-1.2$^+$ mAb for 30 min on ice, followed by washing and subsequent incubation in low-toxicity rabbit complement for 30 min at 37°C. Dead cells were removed by treatment with 0.05% trypsin and 80 μg/ml DNase in Ca^{2+}/Mg^{2+}-free PBS for 5 min at room temperature. Typically, the EC population contained between 5 and 15% I-A$^+$ cells. Viability and percentage of I-A$^+$ cells were assessed by flow cytometry analysis immediately before injection into mice, and differences between groups within experiments were negligible.

EC were then incubated in *trans*- or *cis*-UCA (100 μg/ml) or complete medium and 50 U/ml GM-CSF for 16 h of culture at 37°C. Other groups of EC were cultured in 200 ng/ml IL-12 3 h before addition of 50 U/ml GM-CSF and *cis*-UCA or in GM-CSF, *cis*-UCA, and IL-12 together. Another group of EC was incubated in GM-CSF and *cis*-UCA for 16 h and subsequently exposed to IL-12 for 3 h. These concentrations were chosen according to previously published data (13, 20, 27). Control cells were cultured in 50 U/ml GM-CSF alone for 16 h. After depletion of dead cells by incubation for 3 min at room temperature in 0.05% trypsin and 80 mg/ml DNase, EC were washed three times and incubated in a suspension containing TA from 1 × 10^7/ml S1509a tumor cells or in complete medium alone for 2 h at 37°C. After TA pulsing, the EC were washed extensively to remove soluble TA. Each naive recipient was then injected s.c. on the lower back with 2 × 10^5 EC. This immunization was repeated three times at weekly intervals. One week after the last immunization, mice were challenged with 2 × 10^6 live S1509a tumor cells s.c. on the lower lateral abdomen, and tumor growth was assessed every 48 h by measurement with a Vernier caliper.

Earlier studies showed that this immunization protocol generates tumor immunity in immunized mice, leading to immunological rejection of the tumor over a period of 7–14 days. The specificity of tumor immunity in this system was previously demonstrated by showing that immunization with tumor cell lysates from an unrelated tumor line (UV-5496-1) did not lead to immunity against S1509a (22).

Elicitation of S1509a tumor immunity and measurement of DTH

Mice ($n = 5$) were immunized against S1509a by three injections of 0.5–1 × 10^6 dead S1509a cells (killed by repetitive freeze thawing) s.c. at 5- to 7-day intervals. Generation of protective immunity in these mice was confirmed by rejection of a subsequent tumor challenge and induction of DTH against this tumor (data not shown).

EC from naive donor mice were generated and Thy-1$^+$ cells deleted, as described above. EC were then pulsed with S1509a TA for 2 h and washed extensively, and 5 × 10^5 cells then injected into a hind footpad of tumor-immune mice. Some groups of EC were treated with *cis*- or *trans*-UCA (100 μg/ml) for 3 h before TA pulsing. Other groups were treated with *cis*-UCA and various concentrations of IL-12 (200, 100, or 10 ng/ml) for 3 h. Specific footpad swelling was measured as the mean difference between the footpad thickness of the injected vs the uninjected side 24 h after injection.

Contact hypersensitivity (CHS)

CHS experiments were performed as described previously (28). Briefly, mice (groups $n = 5$) were sensitized by painting 25 μl of 0.5% 2,4-dinitrofluorobenzene or 50 μl 2% oxazolone (both from Sigma-Aldrich), respectively, in acetone:corn oil (4:1) on the shaved back. For elicitation of CHS responses, 10 μl of 0.3% dinitrofluorobenzene, or 0.5% oxazolone, respectively, was painted on both sides of each ear 5 days after immunization. CHS was determined by the degree of ear swelling of the hapten-exposed ear compared with the ear thickness before challenge and measured with a micrometer (Mitutoyo, Tokyo, Japan) 24 h after challenge. Mice that were ear challenged without prior sensitization served as negative controls.

Cis- or *trans*-UCA were diluted in sterile endotoxin-free saline, and treatment (200 μg/200 μl) was performed i.p. 1 h before immunization. IL-12 was diluted in endotoxin-free saline, and treatment (100 ng/100 μl) was given 1 h before application of *cis*-UCA. This dose of IL-12 has been shown to effectively block UV-induced immunosuppression (19, 20). Heat-inactivated IL-12 (95°C for 30 min) was used as a negative control (data not shown).

OVA Ag presentation and Ag-processing assay (DO11 assay)

OVA is taken up by APCs, processed, and presented to T cells. On the other hand, the small OVA peptide$_{323-339}$ is only presented, but not processed by APCs. To investigate the effects of UCA on Ag processing or Ag presentation, EC were prepared and enriched for LC content, as described. These LC were then incubated in medium, medium plus *cis*- or *trans*-UCA (100 μg/ml) in the presence of either OVA (5 mg/ml) or OVA peptide$_{323-339}$ (5 μg/ml) for 24 h. OVA-TCR-specific T cells were prepared from the spleens of OVA-TCR transgenic (DO11) mice. Sixty to 70% of these transgenic T cells express a specific TCR that recognizes the processed small variant of OVA, the OVA peptide$_{323-339}$. The differently treated LC suspensions were incubated in serial dilutions with OVA-specific transgenic (DO11) T cells for 5 days. Subsequently, [^{3}H]thymidine incorporation was used as a measure of T cell stimulation.

UV irradiation, tumor induction, and histology

Within the solar spectrum, the UVB range (290–320 nm) is responsible for carcinogenesis and immunosuppression. Therefore, a bank of four Philips UV-B TL40W/12 sunlamps (Philips, Hamburg, Germany) with an emission spectrum from 280 to 350 nm with a peak at 306 nm was used for irradiation. These lamps deliver an average dose of 8 W/m^2 as measured with an IL-1700 UV detector and a SED 24 (3124) filter (both from International Light, Newburyport, MA). The mice (BALB/c) were placed on a shelf 20 cm below the light bulbs for irradiation. The cage order was systematically rotated before each treatment to compensate for uneven lamp output along the shelf, as described before (29–31). The mice, 20 for each group (10 males and 10 females per cage), were shaved with electric clippers on the entire dorsum once per week. Beginning at 10 wk of age, mice were irradiated three times per week with 2.5 kJ/m^2 for 4 wk, 5 kJ/m^2 for 4 wk, and then 10 kJ/m^2 for 4 mo. Before each UV treatment, one group of mice was injected i.p. with anti-*cis*-UCA Ab (0.1 μg/300 μl), and another group received an irrelevant IgG Ab (0.1 μg/300 μl). Afterward, all mice were observed twice weekly for tumor development for an additional 6 mo. The location and growth of each tumor exceeding 2 mm in diameter were recorded. Excision biopsies from all tumors were fixed in paraformaldehyde and embedded in parablast. Sections were stained with H&E and documented by a video computer-assisted digital image-processing technique (DISKUS version 3.99 for Windows 95; C. H. Hilgers, Königswinter, Germany).

Data generation and statistical evaluation

Tumor volumes were calculated as the product of the maximal tumor diameter in three perpendicular directions, measured with a Vernier caliper. This method has previously been confirmed to correlate well with the tumor weight (22, 26). To avoid unnecessary pain to the experimental animals, mice were sacrificed after the tumor volume exceeded 1000 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 using Cricket Graph software (version 1.5.1; Computer Associates International, Islandia, NY) on a Macintosh computer and the significance of differences between the means of the slopes for the groups of interest was tested by the two-tailed Student's *t* test for unpaired data. The significance of differences between the mean values obtained for CHS and DTH experiments was assessed by the two-tailed Student's *t* test for unpaired data.

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 differentially treated groups of mice were determined using a log rank test by Peto et al. (32). The differences in tumor-latent periods were analyzed by the Mann-Whitney *U* test.

Results

IL-12 prevents cis-*UCA-induced inhibition of TA presentation by LC*

Cis- but not *trans*-UCA has been shown to inhibit the function of LC to present TA for the induction and elicitation of antitumoral immunity (13). To investigate whether IL-12 can block the immunosuppressive effects of *cis*-UCA on LC Ag-presenting function, epidermal cells were prepared and enriched for LC. Groups of LC were treated with either *cis*- or *trans*-UCA in the presence of GM-CSF for 16 h. IL-12 was added either along with *cis*-UCA, or 3 h before and 3 h after *cis*-UCA, respectively. Subsequently, all groups were washed and pulsed with TA. Appropriate controls (no IL-12, no *cis*-UCA, or no TA) were included. LC were then washed extensively to remove all unbound TA and injected s.c. into naive mice. These immunizations were repeated three times at weekly intervals. One week after the last immunization, viable S1509a tumor cells were injected s.c. at a site different from that of immunization, and tumor size was scored over time. As demonstrated previously (13, 22), GM-CSF-treated and TA-pulsed epidermal cells induced protective tumor immunity, which was suppressed upon exposure of LC with *cis*-UCA (Fig. 1). Irrespective of whether given together, before, or after *cis*-UCA, IL-12 treatment completely prevented tumor growth. This indicates that IL-12 prevents *cis*-UCA-induced inhibition of the induction of antitumoral immune responses.

In the next experiments, we investigated whether IL-12 affects *cis*-UCA-induced inhibition of the elicitation of an antitumoral immune response. Therefore, mice were immunized against the S1509a tumor by s.c. injection of freeze-thawed cell lysates three times at 5-day intervals, which renders animals immune against a subsequent challenge with viable tumor cells (22). Groups of these
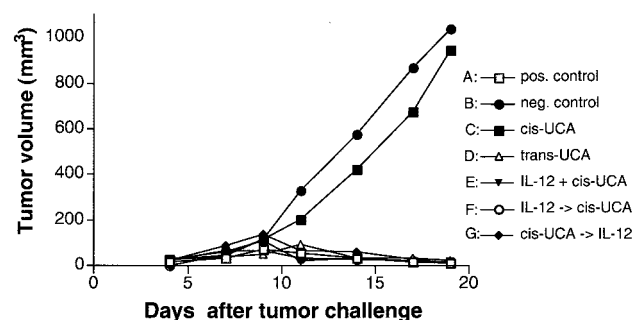

FIGURE 1. IL-12 prevents *cis*-UCA-induced suppression of TA presentation by LC to induce tumor immunity. LC were incubated in either *cis*-UCA (*C*) or *trans*-UCA (*D*) (100 μg/ml), 50 U/ml GM-CSF, or in medium alone plus GM-CSF for 16 h (*A* and *B*). Other groups of LC were treated with GM-CSF plus *cis*-UCA along with 200 ng/ml IL-12 (*E*); IL-12 3 h before culture in GM-CSF plus *cis*-UCA (*F*); or IL-12 3 h after 16-h exposure to GM-CSF plus *cis*-UCA (*G*). Subsequently, all treated groups of LC were pulsed with TA, except the negative control group (*B*). These differentially treated LC were injected at a concentration of 2–3 × 10^5 s.c. into naive groups of mice ($n = 5$) at weekly intervals for a total of three immunizations. All mice were challenged with viable S1509a cells s.c., and tumor growth was scored over time. The graph shows the mean tumor volume in these differentially immunized mice from one experiment of three. The $p < 0.001$ for *A* vs *B*, *C*; NS for *A* vs *D–G*.

animals were challenged 5 days after the last immunization by footpad injection of TA-pulsed LC. Twenty-four hours later, footpad swelling was assessed as a measure of DTH responses to TA. Three hours before TA pulsing, LC were treated with *cis*-UCA or *cis*-UCA plus various concentrations of IL-12 (200, 100, 10 ng/ml). Injection of TA-exposed LC into immunized mice elicited a significant footpad-swelling response, which was much less pronounced upon injection of LC that had been exposed to *cis*-UCA (Fig. 2). Incubation of LC with IL-12 at concentrations of 100 and 200 ng/ml prevented *cis*-UCA-induced suppression of DTH responses against TA (Fig. 2), whereas heat-inactivated IL-12 had no effect (data not shown). At a concentration of 10 ng/ml, IL-12 failed to suppress the inhibitory effects of *cis*-UCA in this assay system. Together, these data suggest that IL-12 is able to prevent the inhibitory effects of *cis*-UCA on the induction as well as elicitation of tumor immunity by LC.

IL-12 treatment protects mice from cis-*UCA-induced suppression of CHS responses*

To study whether IL-12 protects from *cis*-UCA-induced suppression only in the tumor immunity model or whether this applies also for other types of immune responses, the CHS model was used. Mice were immunized against the hapten oxazolone by epicutaneous painting. One group was treated i.p. with *cis*-UCA 1 h before immunization; another group received IL-12 i.p. 1 h before *cis*-UCA application. Upon challenge with oxazolone on the ear 24 h later, mice that were only immunized mounted a significant ear-swelling response (positive control), whereas mice that were only challenged without prior immunization failed to do so (neg-
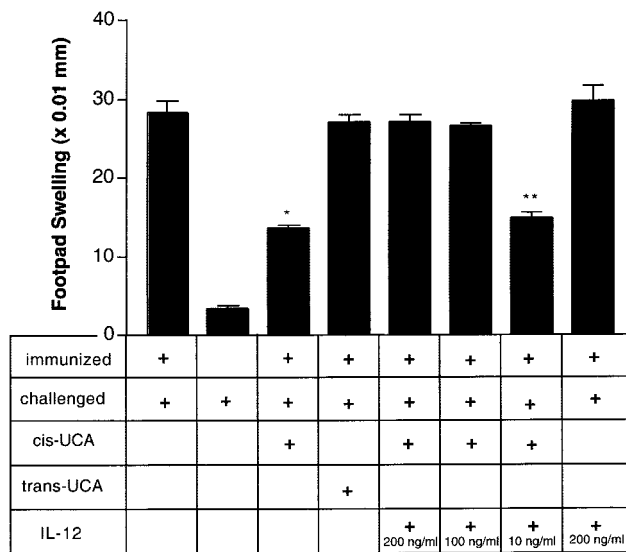
ative control). The data in Fig. 3 show that *cis*-UCA injection significantly suppressed hapten-specific immune responses. Interestingly, IL-12 treatment was able to protect mice from the immunosuppressive effects of *cis*-UCA, since mice that were injected with IL-12 before *cis*-UCA application and immunization mounted a significant ear-swelling response after hapten challenge indistinguishable from the positive control. Injection of heat-inactivated IL-12 before *cis*-UCA did not restore the CHS response (data not shown). These findings suggest that IL-12 prevents the inhibitory effects of *cis*-UCA on hapten-specific immune responses in vivo.

Cis-*UCA suppresses Ag-specific T cell activation by LC*

To date little is known of how *cis*-UCA exerts its inhibitory effects on APC. Our own investigations as well as others revealed no significant effects of *cis*-UCA on the expression of I-A or costimulatory molecules on LC or monocytes (13, 33). To determine whether *cis*-UCA affects Ag processing and/or Ag presentation by LC, the OVA-DO11 assay was used. To study Ag processing in this assay, LC are pulsed with the Ag OVA, which is intracellularly processed and presented to transgenic OVA-DO11 T cells that express a TCR specific for the OVA peptide$_{323-339}$ (Fig. 4A). To evaluate Ag presentation independent of processing, LC are exposed to the OVA peptide$_{323-339}$ that is presented to transgenic OVA-DO11 T cells without the necessity of being processed (Fig. 4B). Subsequently, T cell proliferation can be quantitated as a measure of stimulation. Three hours before exposure to OVA or peptide, LC were exposed to *cis*-UCA, *trans*-UCA, or left untreated. *Cis*- but not *trans*-UCA significantly suppressed the Ag-processing function of OVA-pulsed LC (Fig. 4A). Ag presentation by peptide-pulsed LC was significantly suppressed by *cis*- but not *trans*-UCA to the same degree (Fig. 4B), implying that *cis*-UCA may exert its inhibitory effects primarily via impairment of Ag presentation. *Cis*-UCA-induced inhibition of both Ag processing and Ag presentation was completely lost when LC were preincubated with IL-12 (Fig. 4).
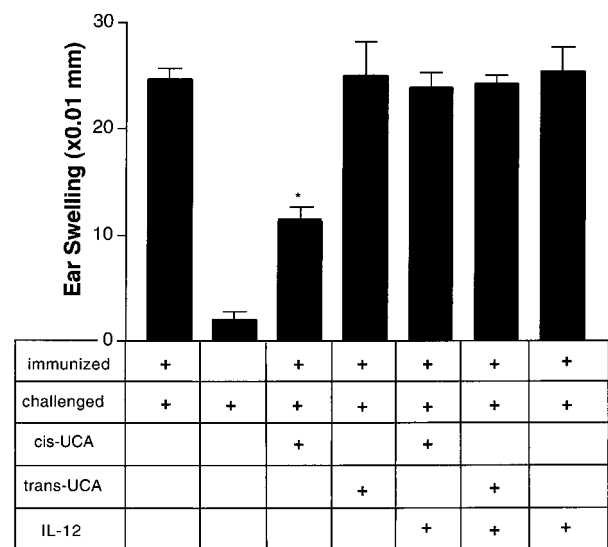
immunized	+		+	+	+	+	+	+
challenged	+	+	+	+	+	+	+	+
cis-UCA			+		+	+	+	
trans-UCA				+				
IL-12					+ 200 ng/ml	+ 100 ng/ml	+ 10 ng/ml	+ 200 ng/ml

FIGURE 2. IL-12 blocks *cis*-UCA-induced suppression of DTH responses elicited by TA-pulsed epidermal cells. Mice were immunized against S1509a by s.c. injections of 1×10^6 dead S1509a cells (freeze-thawed S1509a lysates) three times at weekly intervals. One week after the last immunization, mice were challenged for a DTH response by injection of 5×10^5 epidermal cells treated as below, and 24-h footpad swelling was assessed as a measure of DTH responses. Epidermal cells were incubated in either *cis*- or *trans*-UCA (100 μg/ml) or *cis*-UCA plus IL-12 at various concentrations for 3 h. Epidermal cells were then washed and pulsed with TA for 2 h. Epidermal cells incubated in complete medium and either pulsed with TA or not served as controls. Data represent one experiment of three. *, $p = 0.005$ for immunized and challenged (positive control) vs *cis*-UCA. **, $p = 0.006$ for positive control vs *cis*-UCA plus IL-12 (10 ng/ml); NS for positive control vs *cis*-UCA plus IL-12 (200 ng/ml), or *cis*-UCA plus IL-12 (100 ng/ml), or IL-12 (200 ng/ml) only.

immunized	+		+	+	+	+	+
challenged	+	+	+	+	+	+	+
cis-UCA			+		+		
trans-UCA				+		+	
IL-12					+	+	+

FIGURE 3. IL-12 prevents *cis*-UCA-induced suppression of CHS responses. Groups of mice ($n = 5$) were sensitized against oxazolone. One group was treated i.p. 1 h before sensitization with *cis*- or *trans*-UCA (200 μg/200 μl) in PBS. Other groups were treated i.p. 1 h before UCA isomer administration with IL-12 (200 ng/200 μl) or with IL-12 only. Data represent one experiment of three. *, $p = 0.007$ for immunized and challenged (positive control) vs *cis*-UCA treated; NS for positive control vs *trans*-UCA, IL-12 before *cis*-UCA, IL-12 before *trans*-UCA, and IL-12.
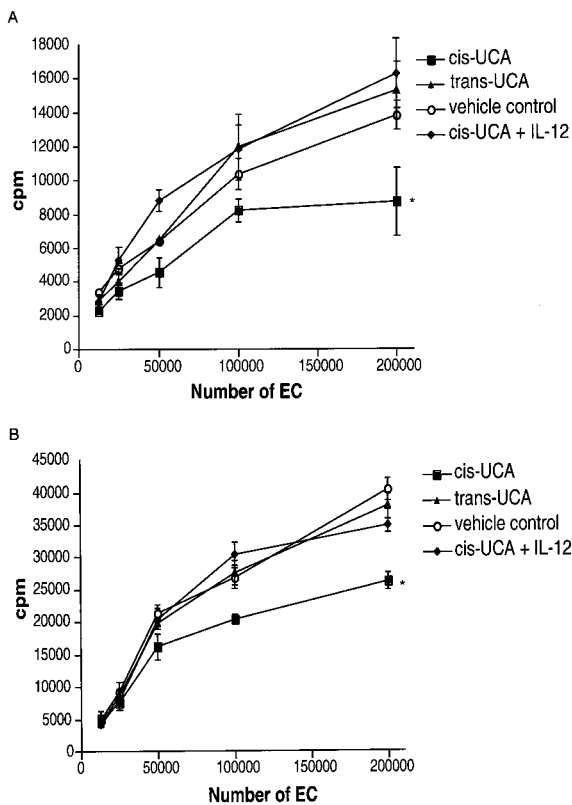
FIGURE 4. *Cis-* but not *trans*-UCA inhibits Ag-presenting function of LC. Epidermal cell suspensions enriched for LC were treated with *cis-* or *trans*-UCA and incubated with either OVA or OVA peptide$_{323-339}$ and used to stimulate OVA-specific (DO11) transgenic T cells (200,000). Other groups of LC were coincubated with *cis*-UCA (100 μg/ml) and IL-12 (200 ng/ml) before addition of transgenic OVA-DO11 T cells. *A*, The data show the effects of *cis*-UCA on the processing of OVA by LC and the stimulation of OVA-specific (DO11) transgenic T cells. The data from one representative experiment of four are depicted. *, *p* value <0.01 for medium-control plus OVA vs *cis*-UCA plus OVA; NS for medium-control plus OVA vs *trans*-UCA plus OVA, *cis*-UCA plus IL-12 plus OVA, and IL-12 only (data not shown). *B*, The data show the effects of *cis*-UCA on the presentation of OVA peptide$_{323-339}$ by LC and the stimulation of OVA-specific (DO11) transgenic T cells. The data from one representative experiment of four are depicted. *, *p* < 0.01 for medium-control plus OVA peptide vs *cis*-UCA plus OVA peptide; NS for medium-control plus OVA peptide vs *trans*-UCA plus OVA peptide, *cis*-UCA plus IL-12 plus OVA peptide.

Neutralization of endogenous cis-*UCA by anti-*cis-*UCA Ab treatment reduces photocarcinogenesis*

The data to date suggest that *cis*-UCA blocks Ag presentation by LC, which can be reversed by IL-12. Accordingly, IL-12 prevents *cis*-UCA-induced inhibition of CHS and tumor immunity. The inhibitory effect of *cis*-UCA on tumor Ag presentation by LC as well as the elevated concentrations of *cis*-UCA within the skin after UV treatment (13, 34) suggest that *cis*-UCA may play a role in the development of UV-induced skin cancer. Accordingly, topical application of UCA, followed by chronic UVB exposure resulted in augmented skin tumor development (12). These data clearly indicated that *cis*-UCA can promote photocarcinogenesis. However, due to the external application of UCA, amounts greater than those found naturally were present, and therefore this study does not give an answer as to whether endogenously produced *cis*-UCA plays a role in photocarcinogenesis. To address this issue, photocarcinogenesis experiments were performed in which mice were injected with an anti-*cis*-UCA Ab during chronic irradiation. Groups of

mice were UVB irradiated three times per week on their shaved backs for 6 mo. Immediately before the UV exposure, one group of animals was treated i.p. with an anti-*cis*-UCA Ab (0.1 μg). Another group of mice received equivalent amounts of an isotype-matched irrelevant IgG Ab. The Ab treatment was discontinued after termination of the irradiation period. One group of mice was only UVB irradiated as a positive control. During the course of the experiment, skin tumor development and tumor growth were documented over a period of 400 days in total. The data in Fig. 5 indicate that in the two control groups (untreated or IgG treated), all mice had developed UV-induced skin tumors after ~6 mo (≈182 days). However, the development of UV-induced cutaneous malignancies in the anti-*cis*-UCA Ab-treated group of mice was significantly reduced. At 200 days, only ~50% of mice in the anti-*cis*-UCA Ab-treated group developed tumors compared with almost 100% of the mice for the other two groups. Together, these findings suggest an important role for endogenous *cis*-UCA during photocarcinogenesis.

Discussion

Induction of skin cancer by UVR is mostly due to the powerful DNA-damaging properties of UVR. In addition, there is clear evidence that suppression of the immune system by UVR also contributes to photocarcinogenesis, since the probability of developing skin cancer can be increased by suppressing the immune system (35). Accordingly, renal transplant patients have a significantly increased risk of developing skin cancer, and this risk increases with the cumulative solar UV load (36). UV-induced immunosuppression is a highly complex process in which a variety of pathways is involved (reviewed in Refs. 4 and 27). For example, UV impairs APC function (37), it induces the release of immunosuppressive cytokines like IL-10 (38), and it drives T lymphocytes into apoptotic cell death (39). Many of the immunosuppressive effects are much more weakly pronounced when UV-induced DNA lesions are reduced, e.g., by acceleration of DNA repair (40, 41). Therefore, UV-induced DNA damage has been recognized as the major, but not the only mediator of the immunosuppressive effects of UVR.
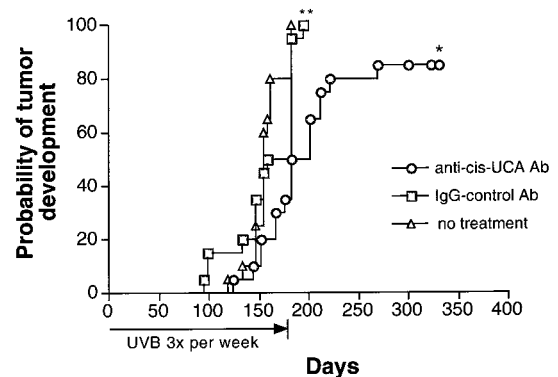

FIGURE 5. Reduced photocarcinogenesis in mice after anti-*cis*-UCA Ab treatment. Mice were initially irradiated with 2.5 kJ/m^2 UVB for 4 wk, then with 5 kJ/m^2 UVB for 4 wk, and then with 10 kJ/m^2 UVB for 4 mo. Their dorsal hair was shaved weekly. One group (*n* = 20) received a neutralizing anti-*cis*-UCA Ab (0.1 μg/300 μl) i.p. before each irradiation. Control groups were either left untreated after irradiation (*n* = 20) or injected with equivalent amounts of an IgG control Ab (*n* = 10). This experiment was performed once. *, *p* < 0.02 for untreated control vs anti-*cis*-UCA Ab treatment; **, *p* < 0.02 for IgG control Ab vs anti-*cis*-UCA Ab treatment; NS for untreated control vs IgG control Ab treatment.

There is ample evidence that *cis*-UCA contributes to UV-induced immunosuppression (42), and may also be involved in photocarcinogenesis (12, 13). Accordingly, Reeve et al. (12) observed >10 years ago that topically applied UCA markedly increased the overt tumor yield and the degree of malignancy in hairless mice exposed chronically to UVR. Although this was the first in vivo demonstration that *cis*-UCA supports the development of UV-induced skin cancer, its implications concerning the role of endogenously produced *cis*-UCA were limited since UCA was applied topically, and thus, excess amounts of UCA were used. To answer whether endogenous *cis*-UCA is involved in the generation of UV-induced skin cancer, we used a *cis*-UCA Ab that reverses some of the effects of UV on the immune system (24) in the photocarcinogenesis model. Intraperitoneal injection of this Ab after each UV exposure caused a significant reduction in the development of skin tumors following an overall irradiation period of 6 mo. Thus, these findings demonstrate for the first time that endogenous *cis*-UCA does play a role in the development of UV-induced skin cancer in the murine model.

Despite the clear in vivo data presented in this study and in previous reports (11, 43–45), it still remains to be determined how *cis*-UCA impairs an immune response. Since incubation of LC with *cis*-UCA inhibits the ability of these cells to sensitize mice for CHS and DTH reactions against haptens and tumor Ags, respectively, *cis*-UCA may directly suppress the function of LC. These reports are in agreement with the finding that *cis*-UCA altered vimentin expression among LC, leading to the destruction of their cytoskeleton and reduced APC function (46). Additionally, *cis*-UCA was able to significantly suppress the mixed epidermal cell lymphocyte reaction, but murine bone marrow-derived dendritic cells exposed to *cis*-UCA demonstrated only feeble impairment of their allostimulatory capacity (47, 48). Although the epidermal cell suspensions we used were enriched for LC, we were not using purified LC. Therefore, an indirect effect mediated via contaminating keratinocytes cannot be excluded. On the other hand, previous studies have shown that incubation of keratinocytes with *cis*-UCA does not induce the release of immunosuppressive cytokines, including IL-10 or TNF-α, which ultimately may impair LC (13, 49). In addition, release of PGE_2 does not appear to play an important role, since the inhibitory effects of *cis*-UCA were still preserved in the presence of indomethacin (13). In addition, *cis*-UCA does not affect the expression of surface molecules, including MHC class II and costimulatory molecules (33).

To test whether *cis*-UCA interferes with Ag processing, we used the OVA-DO11 assay. For studying Ag processing, LC are pulsed with OVA, while the OVA peptide$_{323-339}$ does not need to be processed. If *cis*-UCA exclusively interferes with Ag processing, the stimulatory response upon OVA pulse should be inhibited, while the peptide response should be unaltered. However, *cis*-UCA inhibited both OVA- and peptide-induced proliferation to the same extent, which excludes the possibility that *cis*-UCA interferes only with Ag processing. Based on the fact that OVA- and peptide-driven proliferation were equally affected by *cis*-UCA, it is likely that *cis*-UCA affects primarily Ag presentation.

Since UV-induced immunosuppression may lead to the exacerbation of infectious diseases and also contributes to photocarcinogenesis (reviewed in Refs. 3, 4, and 50), strategies to counteract UV-induced immunosuppression are of practical importance. IL-12 has been recognized to exert such capacities (reviewed in Ref. 16). We and others observed that IL-12 is able to prevent UV-induced inhibition of the induction of CHS and DTH when injected i.p. between UV exposure and Ag application (19, 20). Even more importantly, IL-12 was found to be able to break established UV-mediated tolerance. Mice, which had been tolerized

in a hapten-specific way by application of haptens onto UV-exposed skin, were rendered again susceptible to the respective hapten when IL-12 was injected i.p. before resensitization (20). Although the detailed mechanisms by which IL-12 breaks tolerance are not known, there are indications that IL-12 might protect APCs from apoptotic cell death induced by hapten-specific regulatory T cells (19).

Since *cis*-UCA also contributes to UV-induced immunosuppression, we were interested to study whether IL-12 is also able to antagonize the immunosuppressive effects of *cis*-UCA. The present study provides several lines of evidence that this is indeed the case. Intraperitoneal injection of IL-12 prevented the suppression of the induction of CHS caused by i.p. administered *cis*-UCA. In addition, in vitro exposure of LC to IL-12 prevented *cis*-UCA-mediated inhibition of the induction as well as the elicitation of tumor immunity. Interestingly, IL-12 protected the TA-presenting function of LC when administered before, together with, and even after *cis*-UCA incubation. Likewise, *cis*-UCA-mediated inhibition of both OVA- and peptide-induced proliferation in the OVA-DO11 assay was completely prevented when LC were preincubated with IL-12.

Taken together, the present study further confirms the immunosuppressive properties of *cis*-UCA both in vitro and in vivo. In addition, it shows for the first time that IL-12 is able to reverse the inhibitory effects of *cis*-UCA on the induction of CHS and tumor immunity. Inhibition of tumor immunity by *cis*-UCA may play a critical role in UV-induced carcinogenesis, since treatment with *cis*-UCA Ab in vivo is associated with a decreased incidence of skin tumors in the photocarcinogenesis model. In conclusion, these data indicate an important role of *cis*-UCA in the development of UV-induced skin cancer and point to an additional strategy for the prevention of photocarcinogenesis by inhibiting the effects of *cis*-UCA, possibly by the application of IL-12.

Acknowledgments

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