Prevention of Ultraviolet Radiation–Induced Immunosuppression by (−)-Epigallocatechin-3-Gallate in Mice Is Mediated through Interleukin 12–Dependent DNA Repair

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Abstract

Purpose: Solar UV radiation–induced immunosuppression is considered to be a risk factor for melanoma and nonmelanoma skin cancers. We previously have shown that topical application of (−)-epigallocatechin-3-gallate (EGCG) prevents UV-induced immunosuppression in mice. We studied whether prevention of UV-induced immunosuppression by EGCG is mediated through interleukin 12 (IL-12)–dependent DNA repair.

Experimental Design: IL-12 knockout (KO) mice on C3H/HeN background and DNA repair–deficient cells from xeroderma pigmentosum complementation group A (*XPA*) patients were used in this study. The effect of EGCG was determined on UV-induced suppression of contact hypersensitivity and UV-induced DNA damage in the form of cyclobutane pyrimidine dimers (CPD) in mice and *XPA*-deficient cells using immunohistochemistry and dot-blot analysis.

Results: Topical treatment with EGCG prevented UV-induced suppression of the contact hypersensitivity in wild-type (WT) mice but did not prevent it in IL-12 KO mice. Injection of anti-IL-12 monoclonal antibody to WT mice blocked the preventive effect of EGCG on UV-induced immunosuppression. EGCG reduced or repaired UV-induced DNA damage in skin faster in WT mice as shown by reduced number of $CPDs^+$ cells and reduced the migration of CPD^+ antigen-presenting cells from the skin to draining lymph nodes. In contrast, this effect of EGCG was not seen in IL-12 KO mice. Further, EGCG was able to repair UV-induced CPDs in *XPA*-proficient cells obtained from healthy person but did not repair in *XPA*-deficient cells, indicating that nucleotide excision repair mechanism is involved in DNA repair.

Conclusions: These data identify a new mechanism by which EGCG prevents UV-induced immunosuppression, and this may contribute to the chemopreventive activity of EGCG in prevention of photocarcinogenesis.

Polyphenols from green tea (*Camellia sinensis*) have been shown to have anticarcinogenic activity in various tumor models, with (−)-epigallocatechin-3-gallate (EGCG) being the constituent with the greatest chemopreventive activity (1, 2). Oral administration of green tea polyphenols (a mixture of several epicatechin derivatives) or topical application of EGCG inhibits solar UV radiation–induced skin carcinogenesis in mice in terms of tumor incidence, tumor multiplicity, and tumor size (3–5). We have found that treatment with green tea polyphenols or EGCG also prevents UV radiation–induced immunosuppression in mice (6, 7) and that this effect is associated with the induction of the immunoregulatory cytokine, interleukin 12 (IL-12; ref. 7).

The immunosuppressive effects of solar UV radiation, in particular the mid-wave range (UVB, 290-320 nm), are well established, having been shown most clearly by the effects of UV radiation on the inhibition of contact hypersensitivity, which is a prototypic T-cell–mediated immune response (8, 9). Many of the adverse effects of solar UV radiation on human health, including exacerbation of infectious diseases, premature aging of the skin, and induction of skin cancer, are mediated at least in part by this ability of UV radiation to induce immune suppression (10–12). As UV-induced immunosuppression is considered to be a risk factor for the induction of skin cancer (13, 14), prevention of UV-induced immunosuppression represents a potential strategy for the management of skin cancer. Thus, the mechanism by which IL-12 prevents UV-induced suppression of contact hypersensitivity is of considerable interest.

IL-12 is a 70-kDa heterodimer composed of two covalently linked p35 and p40 subunits, both of which are required for its biological activities (15). These activities are diverse including several immunomodulatory activities, particularly the development of T helper 1 responses (16–18), as well as antitumor

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activity (19). In addition to these direct immunomodulatory activities, IL-12 also is capable of removing or repairing UV-induced DNA damage (20). Notably, UV-induced DNA damage, particularly in terms of the formation of cyclobutane pyrimidine dimers (CPD), is an important molecular trigger for UV-induced immunosuppression (21) and reduction of the formation of CPDs through application of DNA repair enzymes can prevent UV-induced immunosuppression (22, 23). Recently, Schwarz et al. (24) reported that the prevention of UV radiation–induced immunosuppression by IL-12 is dependent on DNA repair and acts through the induction of nucleotide excision repair enzymes. Thus, EGCG may prevent UV-induced immunosuppression through enhancement of the levels of IL-12, which acts to facilitate DNA repair. To test this possibility, we explored the effects of EGCG treatment on UV-induced immunosuppression in IL-12-deficient mice.

Materials and Methods

Animals. C3H/HeN mice (6-7 weeks old) were purchased from Charles River Laboratory (Wilmington, MA). Male IL-12 (−/−) knockout (KO) mice on C57BL/6J genetic background were purchased from The Jackson Laboratory (Bar Harbor, ME). These IL-12 (−/−) mice carry a germ-line disruption of the gene in the p53 chain of IL-12 protein molecule and thus completely eliminate the synthesis of IL-12 protein. Male IL-12 (−/−) and female C3H/HeN mice were mated to obtain male and female IL-12 (+/−) mice. We then mated male IL-12 (−/−) mice with IL-12 (+/−) female or IL-12 (+/−) males with IL-12 (+/−) females and genotyped the progeny by using a new rapid method of backcrossing for the generation of IL-12 KO mice onto a C3H/HeN mice background. The Transgenic Animal Core Facility at the University of Alabama at Birmingham provided marker-assisted mouse genome scanning with genetic monitoring of mouse strains and the accelerated development of congenic mice (25).

Both strains of mice (IL-12 KO and C3H/HeN) were housed in the Animal Resource Facility of the University of Alabama at Birmingham under the following conditions: 12 hours dark:12 hours light cycle, 24 ± 2°C temperature, and 50 ± 10% relative humidity. They were fed a standard Purina Chow diet (Harlan Teklad, Madison, WI) and water *ad libitum*. The experimental animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Determination of IL-12 genotype. Chromosomal DNA extractions from the tail tissues of mice were done following a standard protocol (26). The PCR protocol suggested by The Jackson Laboratory was used with two pairs of primers in the same reaction. The DNA fragment (280 bp) from the bacterial neomycin resistance gene insert was amplified with 5′-CTTGGGTGGAGAGGCTATTC-3′ and 5′-AGGTGAGATGACAG-GAGATC-3′ whereas the DNA fragment (680 bp) from the *IL-12* gene was amplified with 5′-AGTGAACCTCACCTGTGACACG-3′ and 5′-TCTTTGCACCAGCCATGAGC-3′. The PCR product from the IL-12 (−/−) homozygote is 280 bp, the PCR product from the IL-12 (+/−) heterozygote is 680 and 280 bp, and the PCR product from the IL-12 (+/+) wild-type (WT) mouse (C3H/HeN) is 680 bp.

UV irradiation of mice. The shaved backs of the mice were UVB irradiated as described earlier (3, 5) using a band of four FS20 UVB lamps (Daavlin, UVA/UVB Research Irradiation Unit, Bryan, OH) equipped with an electronic controller to regulate UV dosage. The UV lamps emit UVB (280-320 nm; ≈80% of total energy) and UVA (320-375 nm; ≈20% of total energy) with UVC emission being insignificant. The Daavlin UV radiation unit consists of a fixture mounted on fixed legs. Mounted within the exposure unit are four UVB lamps and the exposure is controlled using Daavlin Flex Control Integrating Dosimeters. In this system, UVB dose can be entered in millijoules per square centimeter. This unit enables us to enter dose in millijoules and variations in energy output are automatically compensated so the desired UV dose can be delivered. Mice were kept under the UV lamps at a fixed distance of 24 cm. Monitoring indicated that the majority of the resulting wavelengths of UV radiation were in the UVB (290-320 nm) range with a peak emission at 314 (5).

UV-induced contact hypersensitivity response/protocol. The shaved backs of the mice were exposed to UV radiation (100 mJ/cm^2) for 4 consecutive days. Twenty-four hours after the last UV exposure, the mice were sensitized by painting 25 μL of 0.5% 2,4-dinitrofluorobenzene in acetone/olive oil (4:1, v/v) on the UV-irradiated skin site. The contact hypersensitivity response was elicited 5 days later by challenging the both surfaces of the ears of each mouse with 20 μL of 0.2% 2,4-dinitrofluorobenzene in acetone/olive oil (4:1, v/v). The ear swelling was measured 24 hours after the challenge using an engineer's micrometer (Mitutoyo, Tokyo, Japan) and was compared with the ear thickness just before the challenge as previously detailed (7). Mice that received the same dose of 2,4-dinitrofluorobenzene but were not UV irradiated served as a positive control whereas the nonirradiated mice which received only ear challenge without sensitization with 2,4-dinitrofluorobenzene served as a negative control. To determine the chemopreventive effect of EGCG against UV-induced immunosuppression, EGCG was applied topically (1 mg/cm^2 skin area/mouse/100 μL acetone) 30 minutes before each exposure to UV radiation in a separate group of mice. During UV exposure of the mice, the ears of mice were protected from the UV irradiation by opaque black tape, which was removed after exposure. The mice that were not exposed to UV radiation were also shaved to maintain the identical regimen. The percent suppression of contact hypersensitivity was determined as previously described (27). Each group consisted of five mice and each experiment was done at least twice.

Immunohistochemical detection of CPDs. To detect UV-induced CPD$^+$ cells in skin or draining lymph nodes, immunostaining of CPD was done using a procedure previously described (28). Briefly, frozen skin or lymph node sections (5 μm thick) were thawed and kept in 70 mmol/L NaOH in 70% ethanol for 2 minutes to denature nuclear DNA, followed by neutralization for 1 minute in 100 mmol/L Tris-HCl (pH 7.5) in 70% ethanol. Slides were washed with PBS and then incubated for 30 minutes with 10% goat serum in PBS. Sections were then incubated with thymine dimer–specific monoclonal antibody (Kamiya Biomedical Company, Seattle, WA) or an isotype control antibody [immunoglobulin G1 (IgG1)]. Bound anti-CPD antibody was detected by incubation with biotinylated goat anti-mouse IgG1 followed by peroxidase-labeled streptavidin. Sections were then incubated with diaminobenzidine plus peroxidase substrate for 5 minutes. The sections were rinsed with distilled water and counterstained with H&E.

Southwestern dot-blot analysis. Genomic DNA from the skin was isolated following the standard procedures. Genomic DNA (500 ng) was transferred to a positively charged nitrocellulose membrane by vacuum dot-blotting (Bio-Dot Apparatus, Bio-Rad, Hercules, CA) and fixed by baking the membrane for 30 minutes at 80°C. After blocking the nonspecific binding sites in blocking buffer (5% nonfat dry milk, 1% Tween 20 in 20 mmol/L TBS, pH 7.6), the membrane was then incubated with the antibody specific to CPDs for 1 hour at room temperature. After washing in washing buffer, the membrane was again incubated with horseradish peroxidase–conjugated secondary antibody. The CPD dimers were detected by chemiluminescence using an enhanced chemiluminescence detection system. The experiments were repeated thrice.

In vivo ***treatment of anti-IL-12 antibody or recombinant IL-12.*** To verify the role of EGCG-induced IL-12 on EGCG-induced prevention from UVB-induced suppression of contact hypersensitivity response in mice, anti-IL-12 antibody was diluted in sterile endotoxin-free saline and injected i.p. The mice received two doses (500 ng each) 24 and 3 hours before 2,4-dinitrofluorobenzene sensitization. Control mice were injected i.p. with equal volumes of rat IgG1 (isotype control of anti-IL-12) in saline. Recombinant murine IL-12 (1,000 ng/100 μL PBS) or an equal volume of PBS was injected s.c. 3 hours before UV irradiation.

Cells, culture conditions, and UV irradiation. Xeroderma pigmentosum (XP) complementation group A (*XPA*)–deficient and *XPA*-proficient human fibroblasts were obtained from the Coriell Institute for Medical Research (Camden, NJ). These *XPA*-deficient cells originally were obtained from patients suffering from XP and the *XPA*-proficient cells from healthy human donors. Cells were cultured in DMEM with Earle's salts supplemented with 2 mmol/L L-glutamine, 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), and maintained in an incubator at 37°C in a humidified atmosphere of 5% CO_2. The cells were UV irradiated using the same UV source as employed for irradiation of the mice as described above. The cells were exposed to the UV radiation through PBS, and on UV irradiation, cells were reincubated with EGCG for indicated time periods.

Immunostaining of CPDs in cultured cells. UV-induced CPD^+ cells in the cultured cells were detected using a protocol as described previously with some modification (28). Briefly, at indicated time period after UV irradiation (15 mJ/cm^2), cells were trypsinized and centrifuged. Cell pellets were resuspended in PBS buffer and processed for cytospin preparation ($\approx 1 \times 10^5$ cells per slide). Cells were washed in PBS and fixed with 45% ethanol for 5 minutes followed by 70% ethanol at −20 °C for 10 minutes. Cells were subsequently permeabilized with 0.3% Triton X-100 for 30 minutes. DNA denaturation was done by treating the cells with 0.5 N HCl and 0.05% pepsin at 37°C for 30 minutes. Slides were then incubated with the thymine dimer–specific (CPD) monoclonal antibody for 1 hour at room temperature, and after washing, the bound anti-CPD antibody was detected by incubation with biotinylated goat anti-mouse IgG1, followed by peroxidase-labeled streptavidin. Cells were then incubated with diaminobenzidine plus peroxidase substrate for 5 minutes. After washing with distilled water, the cells were counterstained with methyl green. CPD^+ cells were counted at five to six different places and data were represented as mean percentage of CPD^+ cells ± SD. Experiments were repeated at least twice.

Statistical analysis. In the contact hypersensitivity experiments, the statistical significance of differences in the ear swelling responses among different treatment groups was analyzed using the two-tailed Student's *t* test for unpaired samples. $P < 0.05$ was considered significant.

Results

EGCG prevents UV-induced suppression of contact hypersensitivity response in WT but not in IL-12 KO mice. Previously, we have shown that EGCG prevents UV-induced suppression of contact hypersensitivity in C3H/HeN (WT) mice and that the ability of EGCG to prevent immunosuppression was associated with the induction of IL-12 (7). To determine whether the prevention of UV-induced immunosuppression by EGCG is mediated by IL-12, we conducted experiments using IL-12 KO mice with a C3H/HeN background and their WT (C3H/HeN) counterparts. Application of 2,4-dinitrofluorobenzene to UV-exposed skin did not induce sensitization in either WT or IL-12 KO mice (Fig. 1A-C, *fourth column*), but did induce sensitization in the positive control group which were not exposed to UV radiation (*second column*). Treatment with EGCG prevented UV-induced suppression of contact hypersensitivity in WT mice as shown by significant enhancement of the contact hypersensitivity response (ear swelling) on ear challenge (Fig. 1A, *fifth column*). In contrast, UV-exposed IL-12 KO mice remained unresponsive to 2,4-dinitrofluorobenzene despite the application of EGCG on the mouse skin (Fig. 1C, *fifth column*), indicating that the immunopreventive effect of EGCG on UV-induced suppression of contact hypersensitivity may require IL-12 or is mediated through IL-12. It should be noted that

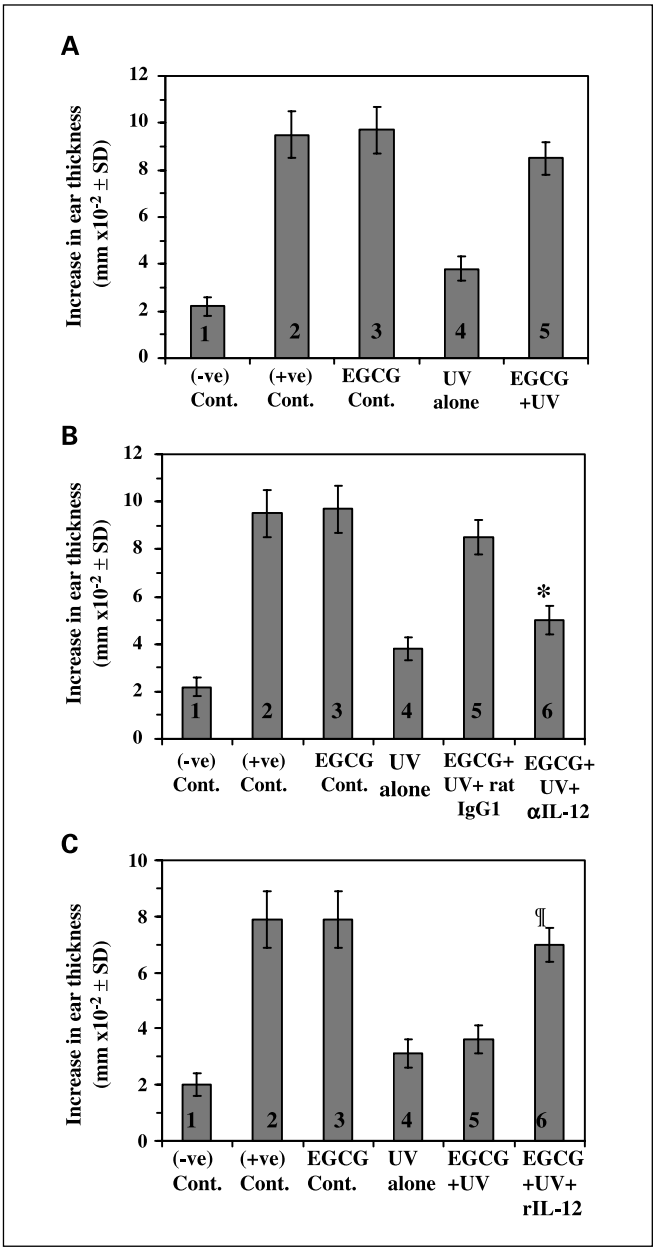

Fig. 1. EGCG prevents UV-induced suppression of the contact hypersensitivity response in WT (C3H/HeN) but not in IL-12 KO mice. The shaved backs of C3H/HeN (*A* and *B*) or IL-12 KO (*C*) mice were exposed to UV radiation (100 mJ/cm^2) for 4 consecutive days. Twenty-four hours after the last exposure, the mice were sensitized with 2,4-dinitrofluorobenzene (*DNFB*) through UV-exposed skin (groups/column 3-6). Five days later, the mice were challenged by painting 2,4-dinitrofluorobenzene on the ear and ear swelling was measured 24 hours later as detailed in Materials and Methods. *B,* WT mice (group 6) received two doses of anti-IL-12 antibody i.p. (500 ng each/100 μL saline) 18 and 3 hours before 2,4-dinitrofluorobenzene sensitization. Control mice (group 5) were injected i.p. with equal volumes of rat IgG1 (isotype control of anti-IL-12) in saline. *C,* IL-12 KO mice (group 6) received 1,000 ng of IL-12 i.p. 3 hours before sensitization. Columns, mean change in ear thickness expressed in millimeter (mm × 10^{-2}; $n = 5$ per group); bars, SD. The experiment was repeated once. *, $P < 0.001$, significant difference versus EGCG + UV + rat IgG1; ¶, $P < 0.005$, significant sensitization versus EGCG + UV.

treatment with EGCG alone does not affect the 2,4-dinitrofluorobenzene-induced sensitization (Fig. 1A-C, *third column*).

To confirm that prevention of UV-induced suppression of contact hypersensitivity by EGCG requires IL-12, we also treated WT mice with anti-IL-12 monoclonal antibody i.p. 24

and 3 hours before sensitization. In EGCG-treated mice, the i.p. injection of anti-IL-12 antibody significantly reversed the preventive effect of EGCG on UV-induced suppression of contact hypersensitivity (Fig. 1B, *sixth column*; $P < 0.001$) whereas treatment with an IgG1 isotype control antibody did not (Fig. 1B, *fifth column*). Moreover, the restoration of IL-12 in IL-12 KO mice by injection of IL-12 3 hours before 2,4-dinitrofluorobenzene application resulted in an enhanced sensitization reaction in these mice after treatment with EGCG and UV irradiation (Fig. 1C, *sixth column*) as compared with the reaction in mice treated with either UV alone (*fourth column*) or EGCG + UV (*fifth column*). Taken together, these studies provide strong evidence that prevention of UV-induced suppression of contact hypersensitivity by EGCG is mediated, at least in part, through IL-12.

EGCG removes UV-induced DNA damage more rapidly in WT mice than in IL-12 KO mice. To determine whether IL-12 contributes to the ability of EGCG to prevent UV-induced immunosuppression by inducing DNA repair, we evaluated the formation of CPDs in the UV-exposed skin. The shaved backs of IL-12 KO and WT mice were exposed to UVB (60 mJ/cm^2) with and without the treatment with EGCG. Mice were sacrificed either immediately (≈30 minutes) or 24 hours later; samples of the skin were obtained and the presence of CPDs was determined by immunohistochemistry using an antibody directed against CPDs. In skin samples obtained immediately after UV exposure, no differences in the staining pattern of CPDs were observed between IL-12 KO and WT mice whether or not they were treated with EGCG (Fig. 2A). This observation also eliminated the speculation that EGCG might have significant filtering effect on UV radiation (Fig. 2A and B). In contrast, in samples obtained 24 hours after UVB exposure, the numbers of CPD$^+$ cells were significantly lower in the EGCG-treated WT mice than in the WT mice that were not treated with EGCG but were exposed to UVB ($P < 0.001$). As anticipated, the UVB-induced DNA damage in the IL-12 KO mice that had been treated with EGCG did not differ from that in the IL-12 KO mice that had not been treated with EGCG (Fig. 2). It was noted that the skin samples obtained 24 hours after UVB exposure from WT and IL-12 KO mice that were not treated with EGCG showed a reduction in the number of CPD$^+$ cells, indicating that some endogenous defense mechanism independent of IL-12 may be involved in repair of UV-damaged DNA. However, this spontaneous DNA repair being greater in WT mice was greater than IL-12 KO mice (Fig. 2A and B). This suggests that endogenous mechanisms, including both IL-12-dependent and IL-12-independent mechanisms, may contribute to the repair of UV-damaged DNA and that the difference in DNA repair in between WT and IL-12 KO may be due to the absence of IL-12 in IL-12 KO mice. The skin samples obtained from the groups

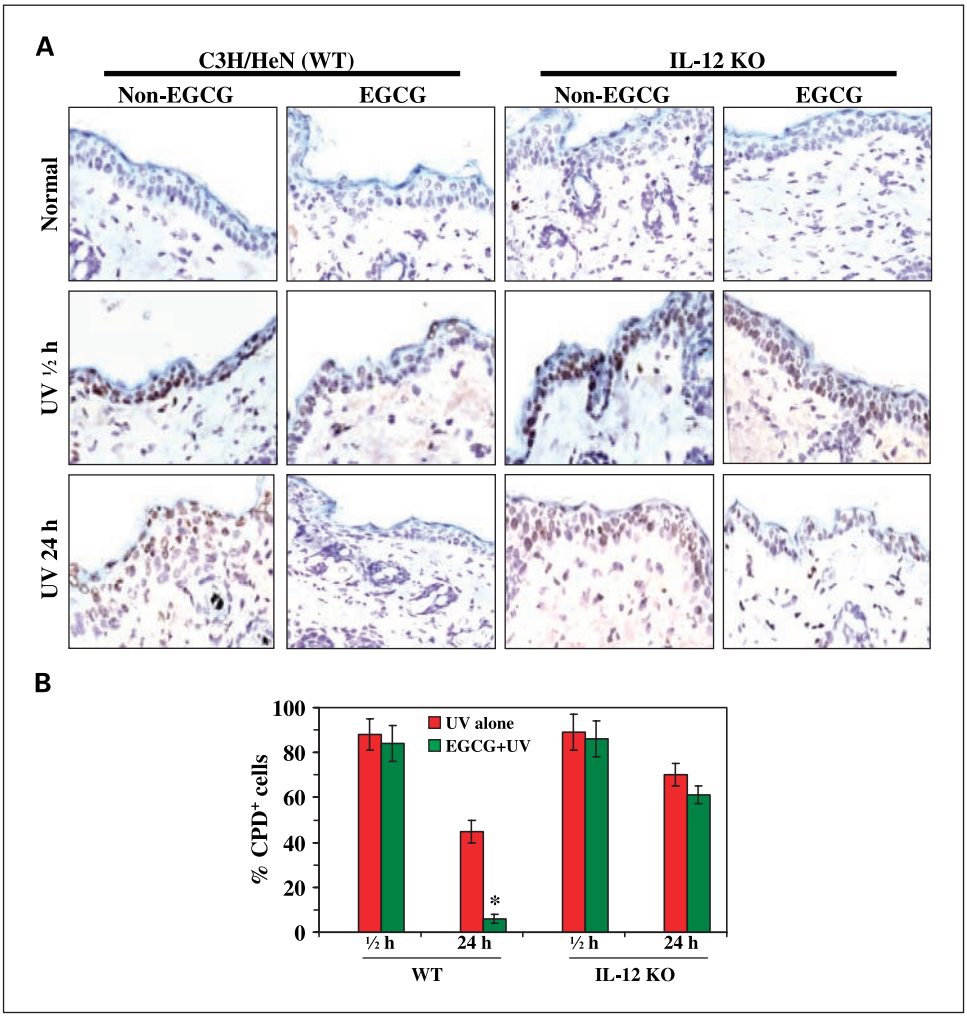

Fig. 2. EGCG repairs or removes UV-induced CPDs more rapidly in WT mice than in IL-12 KO mice. *A,* C3H/HeN or IL-12 KO mice were treated topically with EGCG then exposed to UV (60 mJ/cm^2) 30 minutes later as described in Fig. 1. Mice were sacrificed at 30 minutes (immediate) or 24 hours later and skin samples were collected and frozen in optimum cutting temperature medium. Frozen sections (5 μm thick) were subjected to immunoperoxidase staining to detect CPD$^+$ cells. CPD$^+$ cells are shown in dark brown. CPD$^+$ cells were not detected in non-UVB exposed skin. Magnification, ×40. *B,* the numbers of CPD$^+$ cells were counted in five to six different areas of the sections under microscope. Columns, mean percentage of CPD$^+$ cells in epidermis ($n = 5$); bars, SD. *, $P < 0.001$, versus UV alone.

of mice that were not exposed to UV (normal skin), including those that were or were not treated with EGCG, were devoid of any CPD^+ cells.

In vivo ***treatment of EGCG-treated mice with IL-12 removes or repairs UV-induced DNA damage in IL-12 KO mice.*** To further confirm the central role of IL-12 in the EGCG-mediated repair of UV-induced DNA damage, the shaved backs of EGCG-treated IL-12 KO mice were exposed to UV (60 mJ/cm^2) with one group of mice receiving IL-12 s.c. 3 hours before UV exposure. Mice were sacrificed 24 hours later, skin samples were obtained, and the presence of CPDs was assessed using immunohistochemical analysis. As shown in Fig. 3, in the UV-irradiated IL-12 KO mice, the number of CPD^+ cells in the EGCG-treated group ($62 \pm 8\%$) was similar to the number observed in the group that did not receive EGCG ($70 \pm 7\%$). In contrast, the number of CPD^+ cells in the skin samples of IL-12 KO mice that had received IL-12 before UV exposure was only $15 \pm 3\%$, indicating a significant lower level of DNA damage 24 hours after UV exposure in the presence of IL-12 ($P < 0.001$). These data further support the concept that EGCG promotes the removal or repair of damaged DNA in UVB-exposed skin through a mechanism that involves IL-12 activity.

EGCG reduces the number of CPD^+ cells in the draining lymph nodes of UV-exposed WT mice but not in IL-12 KO mice. It has been recognized that UV-induced DNA damage is an important molecular trigger for the migration of antigen-presenting cells (i.e., Langerhans cells in the epidermis) from the skin to the draining lymph nodes. DNA damage in antigen-presenting cells impairs their capacity to present antigen, which in turn results in a lack of sensitization (29). CPD-containing antigen-presenting cells have been found in the draining lymph nodes of UV-exposed mice (30). These antigen-presenting cells were identified to be of epidermal origin and exhibited an impaired antigen presentation capacity. As we have found that EGCG induces IL-12 (7) and IL-12 has the capacity to induce DNA repair (20), we next determined whether EGCG acts to reduce the migration of CPD^+ cells from the skin to the draining lymph nodes. For this purpose, WT and IL-12 KO mice were treated with EGCG and UV irradiated. Mice were sacrificed 36 hours later, the draining lymph nodes were collected, and the presence of CPDs in the draining lymph nodes was estimated by immunohistochemical analysis. CPD^+ cells were not detectable in the draining lymph nodes of mice that were not UV irradiated, whether or not they were treated with EGCG (Fig. 4A). The significant numbers of CPD^+ cells in the draining lymph nodes were found in both UV-exposed WT and IL-12 KO mice, with the numbers of CPD^+ cells in the draining lymph nodes of the UV-exposed IL-12 KO mice being ~4-fold higher ($P < 0.001$) than in the draining lymph nodes of their WT counterparts. The lower percentage of CPD^+ cell in the draining lymph nodes of UV-exposed WT mice compared with IL-12 KO mice was not unexpected and may be attributable to the presence of endogenous IL-12 in the WT mice at levels that are capable of partial removal of the damaged DNA in the migrating cells. Treatment with EGCG resulted in a 71% reduction in the numbers of CPD^+ cells in the draining lymph nodes of UV-exposed WT mice compared with UV-exposed WT mice that did not receive EGCG (Fig. 4B; $P < 0.001$). In contrast, there was no significant difference in the number of CPD^+ cells in the draining lymph nodes of EGCG-treated and non-EGCG-treated UV-exposed IL-12 KO mice. This observation indicates that the reduction in the numbers of CPD^+ cells in the draining lymph nodes of WT mice after EGCG treatment may be due to EGCG-induced, IL-12-mediated repair of CPD^+ cells.

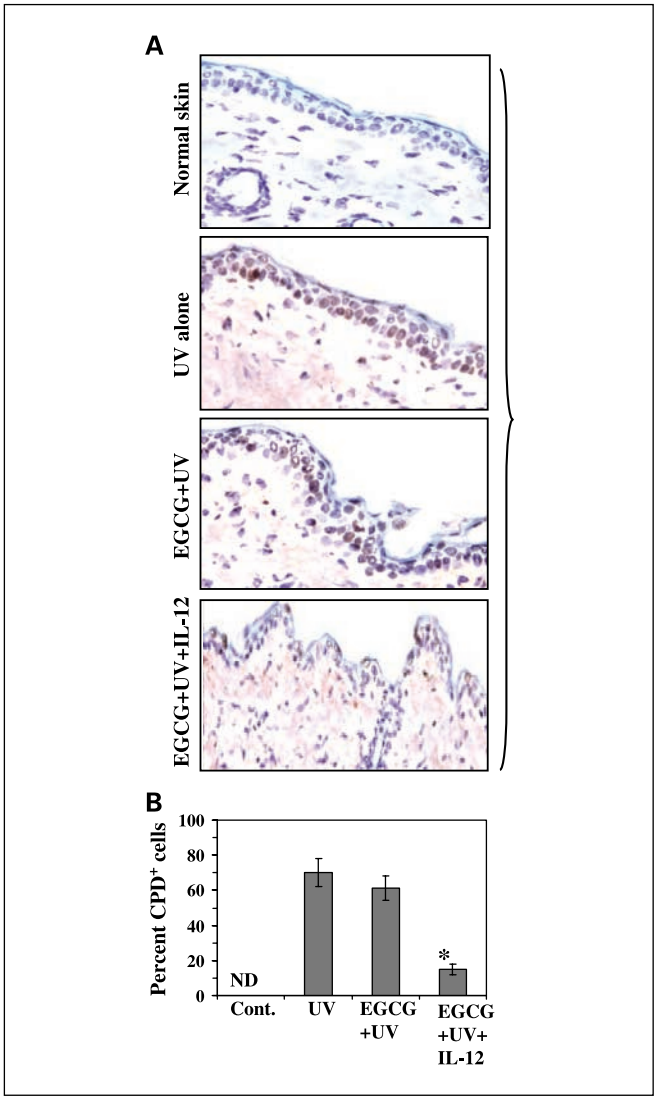

Fig. 3. *In vivo* treatment with IL-12 of IL-12 KO mice reduces or repairs UV-induced DNA damage in the form of CPDs. IL-12 KO mice were treated topically with EGCG, then exposed to UV (60 mJ/cm^2) 30 minutes later as described in Fig. 1. In the indicated group, mice were injected with 1,000 ng/mouse of IL-12 s.c. 3 hours before UV irradiation. Mice were sacrificed 24 hours after UV irradiation and skin samples were collected and frozen in optimum cutting temperature medium. *A,* frozen skin sections were subjected to immunoperoxidase staining to detect CPDs as detailed in Materials and Methods. Magnification, ×40. *B,* the numbers of CPD^+ cells were counted in five to six different areas of the sections. Columns, mean percentage of CPD^+ cells in the epidermis ($n = 5$); bars, SD. ND, not detectable. *, $P < 0.001$, versus UV alone and/or EGCG + UV.

Most of the CPD^+ cells, both in WT and IL-12 KO mice, were localized in an area extending from the subcapsular sinus to the paracortical region of the lymph nodes, including the interfollicular areas (Fig. 4A). The CPD^+ cells were counted in these areas and reported in terms of percentage of total cells in those areas. As the interfollicular areas are the sites of T-cell localization, the presence of CPD^+ cells in these areas may adversely affect the induction of the sensitization response. These data suggest that the ability of EGCG to prevent

UV-induced immunosuppression in mice may be due to its capacity to repair UV-induced DNA damage in epidermal antigen-presenting cells.

EGCG induces repair of UV-induced damage of DNA in XPA-proficient cells but does not reduce UVB-induced DNA damage in XPA-deficient cells. It has been shown that application of DNA repair enzymes that reduce the numbers of CPD^+ cells prevents UV-induced immunosuppression (22, 23). Moreover, IL-12 has been shown to repair UV-induced DNA damage in healthy cells but not in cells from patients with XPA (20), which are devoid of the nucleotide excision repair enzymes that are necessary for the repair of UV-induced DNA damage in mammalian cells. Therefore, we further examined whether the IL-12-mediated repair of UV-induced DNA damage induced by EGCG treatment requires nucleotide excision repair enzyme activity. For this purpose, we used nucleotide excision repair–deficient fibroblasts from *XPA* patients and repair-proficient fibroblasts from healthy persons. *XPA*-deficient and *XPA*-proficient human fibroblasts were exposed to UV radiation in the presence or absence of EGCG. Induction of CPDs by UV radiation is an immediate event and, thus, is detectable shortly after UV exposure. When the cells were analyzed for CPDs immediately after UV exposure, no differences were observed in the cells treated with or without EGCG in terms of the number of CPD^+ cells (data not shown). This finding implies that EGCG does not prevent immediate induction of CPDs, thereby further excluding a UV radiation filtering effect. As expected, CPDs were absent in non-UV-irradiated cells whether or not they were treated with EGCG. However, 24 hours after UV irradiation, the number of CPD^+ cells was significantly reduced (62%; $P < 0.001$) in *XPA*-proficient cells [Fig. 5A (*left column*) and B], suggesting that EGCG might accelerate the repair of UV-induced CPDs through an nucleotide excision repair mechanism. Because nucleotide excision repair is the major mechanism by which CPDs are removed in mammalian cells, the effect of EGCG on UVB-induced DNA damage of nucleotide excision repair–deficient cells was also determined. The *XPA* gene is an essential component of the nucleotide excision repair; thus, cells with a mutated *XPA* gene completely lack a functional nucleotide excision repair. As shown in Fig. 5A (*right column*) and B, treatment with EGCG did not significantly remove or repair the UV-induced DNA damage in *XPA*-deficient cells. This observation indicated that EGCG-induced DNA repair is mediated through the functional nucleotide excision repair mechanism.

We also examined the chemopreventive effect of EGCG on UV-induced CPDs in *XPA*-proficient and *XPA*-deficient cells using Southwestern dot-blot analysis. As clearly indicated in Fig. 5C, EGCG treatment of *XPA*-proficient cells for 24 hours resulted in remarkable repair or reduction of UV-induced CPDs. However, this chemopreventive effect of EGCG was not evident in the *XPA*-deficient cells 24 hours after UV irradiation. This may be due to absence of nucleotide excision repair enzymes in these cells. The cells, whether *XPA*-deficient or *XPA*-proficient and either treated with EGCG or not treated with EGCG, did not show the presence of CPD^+ cells as reflected from the absence of dot-blot. Thus, the data from dot-blot analysis further confirm our observation that EGCG-induced DNA repair requires functional nucleotide excision repair.

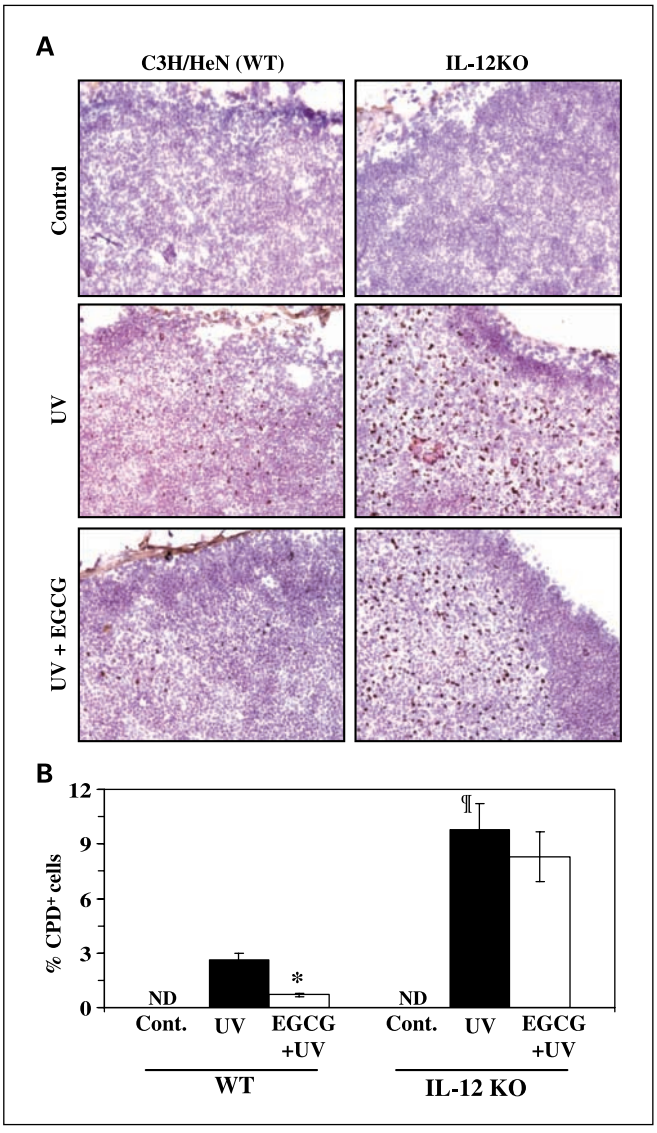

Fig. 4. EGCG reduces the migration of CPD^+ antigen-presenting cells from the skin to draining lymph nodes of UV-exposed WT mice but does not affect the migration in IL-12 KO mice. *A,* C3H/HeN or IL-12 KO mice were treated topically with EGCG, then exposed to UV (60 mJ/cm^2) 30 minutes later. Mice were sacrificed 36 hours later and the draining lymph nodes harvested and frozen in optimum cutting temperature medium. Draining lymph nodes obtained from non-UV-exposed mice were used as controls. CPD^+ cells were detected by immunoperoxidase staining of frozen sections as described in Materials and Methods. CPD^+ cells were not detectable in draining lymph nodes obtained from non-UV-exposed control mice. Five mice were used per group and the experiment was repeated once. Magnification, ×20. *B,* the numbers of CPD^+ cells were counted in five to six different areas of the sections. Columns, mean percentage of CPD^+ cells in the draining lymph nodes ($n = 5$); bars, SD. ND, not detectable in non-UV-exposed mice. *, $P < 0.001$, significant difference versus UV alone; ¶, $P < 0.001$, significantly higher versus WT mice.

Discussion

Exposure of skin to UV radiation initiates a variety of harmful effects on human health, including squamous and basal cell carcinoma and melanoma, as well as premature aging of the skin and susceptibility to infection (10–12). These skin disorders have been associated with the immunosuppressive effect of UV radiation. We have shown that green tea polyphenols, and in particular EGCG, which is the major and most chemopreventive constituent, have the ability to prevent UV-induced immunosuppression (7). However, the mechanism

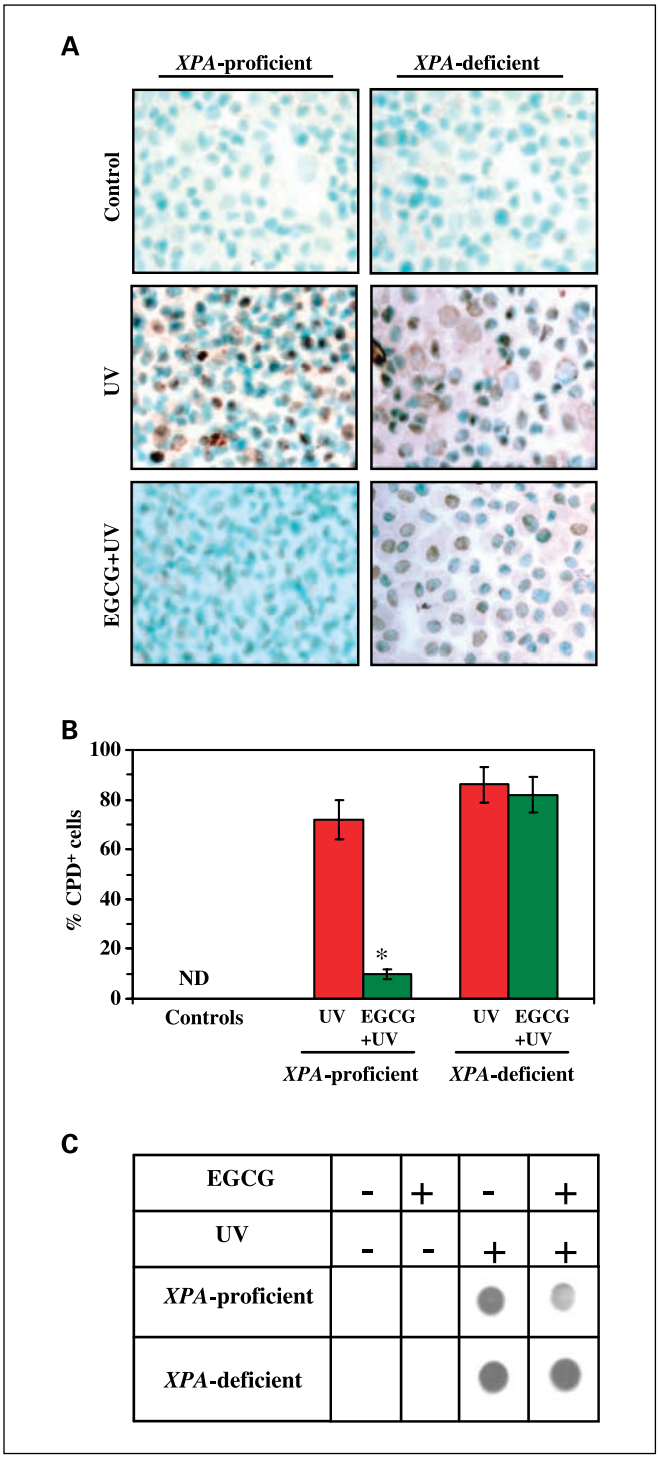

Fig. 5. EGCG reduces UV-induced DNA damage in *XPA*-proficient cells but does not reduce UV-induced DNA damage in *XPA*-deficient cells. *A, XPA*-proficient cells from a healthy person and *XPA*-deficient cells from a patient suffering from XP with a deficiency in *XPA* were exposed to UV (15 mJ/cm^2) in the presence or absence of EGCG (20 μg/mL) and cells were harvested 24 hours after UV irradiation, cytospun, and subjected to immunocytostaining to detect CPD$^+$ cells as detailed in Materials and Methods. CPD$^+$ cells are dark brown. Magnification, ×40. *B,* the numbers of CPD$^+$ cells were counted in five to six areas of the slide. Columns, mean percentage of CPD$^+$ cells from three independent experiments; bars, SD. *, $P < 0.001$, significant decrease versus non-EGCG (UV alone) treatment. *C,* genomic DNA was extracted from cells treated as described above and subjected to Southwestern dot-blot analysis using an antibody against CPD. The cells which were not exposed to UV did not show the presence of CPDs in the dot-blot analysis. Experiments were repeated thrice with identical results.

of action of EGCG has not been elucidated. Our present study shows that prevention of UV-induced immunosuppression by EGCG is mediated through the induction of IL-12. This observation was supported by our experiments conducted using IL-12 KO mice and their wild-type counterparts. Our data show that EGCG prevents UV-induced immunosuppression in WT mice but does not prevent it in IL-12 KO mice, indicating that the prevention of immunosuppression by EGCG is mediated through IL-12. The role of IL-12 in mediating the chemopreventive effect of EGCG against UV radiation was further supported when it was observed that anti-IL-12 injection in WT mice blocked the prevention of UV-induced immunosuppression in mice treated with EGCG. Further, the injection of recombinant IL-12 resulted in prevention of UV-induced immunosuppression in EGCG-treated IL-12 KO mice. The role of IL-12 in prevention of UV-induced immunosuppression has been shown (31). Injection of anti-IL-12 monoclonal antibody before allergen painting prevents sensitization *in vivo* whereas administration of IL-12 before hapten application onto UV-exposed skin prevents the development of UV-induced immunosuppression (31). Previously, we reported that treatment of mouse skin with EGCG stimulates IL-12 and that the EGCG acts synergistically with UV radiation in terms of this IL-12 stimulation (7). Our present observation that treatment with EGCG removes or repairs UV-induced DNA damage in the form of CPDs faster in WT mice than in IL-12 KO mice indicates that prevention of UV-induced immunosuppression by EGCG may be associated with its ability to stimulate DNA repair through induction of IL-12.

IL-12 has been shown to possess potent antitumor activity in a wide variety of murine tumor models (19, 32–34) and has the ability to repair UV-induced DNA damage (20). It has been documented that UV-induced DNA damage, predominantly in the form of CPDs, is an important molecular trigger for UV-induced immunosuppression and initiation of photocarcinogenesis (21, 22). Damaged DNA lesions can be repaired by the process of nucleotide excision repair, and thus the UV-induced immunosuppression can be reduced or reversed. It has been shown that UV-induced CPDs are significantly reduced by administration of IL-12 and that this process is mediated through the induction of DNA repair (20). Therefore, we were interested to determine whether EGCG-induced DNA repair is mediated through induction of IL-12. To show this mechanism, the IL-12 KO mice and their WT counterparts were used as a tool. Both immunohistochemical analysis and Southwestern dot-blot analysis indicated that the treatment with EGCG removes or repair UV-induced CPDs faster in WT mice than in IL-12 KO mice. However, when EGCG-treated IL-12 KO mice were injected s.c. with IL-12, the numbers of UV-induced CPDs were significantly reduced or repaired (Fig. 3).

There is evidence that UV-induced DNA damage is the molecular trigger for the migration of Langerhans cells (antigen-presenting cells in the epidermis) from the skin to the draining lymph nodes. The UV-induced DNA damage also impairs the antigen-presenting capacity of Langerhans cells, which results in a lack of sensitization and the induction of tolerance to contact sensitizers. We observed that treatment with EGCG on the UV-exposed mouse skin inhibited the migration of epidermal antigen-presenting cells to draining lymph nodes in WT mice but did not prevent it in IL-12 KO mice, indicating that treatment with EGCG might

be able to repair UV-induced CPD in the WT mice because of induction of IL-12. In contrast, this effect was not found in the IL-12 KO mice. As the UV-induced DNA damage in antigen-presenting cells in the epidermis of WT mice might be repaired by EGCG, the number of the CPD^+ cells in lymph nodes of WT mice was estimated and found to be lower than in IL-12 KO mice. We speculate that, as the migrating antigen-presenting cells in the epidermis were either not damaged or were repaired in WT mice, they were able to present antigen to T cells in the draining lymph nodes, resulting in induction of sensitization to 2,4-dinitrofluorobenzene after challenge. This was not the case in IL-12 KO mice, further supporting our hypothesis that prevention of UV-induced immunosuppression by EGCG may be mediated through IL-12 induction. Further, the numbers of CPD^+ cells were significantly higher in IL-12 KO mice in the subcapsular sinus to the paracortical region of the lymph nodes, including the interfollicular areas, which are the sites of T-cell localization. Thus, in the IL-12 KO mice, the damaged DNA may adversely affect the ability of the antigen-presenting cells to present antigen to T cells, thus abrogating sensitization after 2,4-dinitrofluorobenzene challenge.

Nucleotide excision repair is the main mechanism of repair in mammalian cells for the removal of UV radiation–induced DNA damage. Because EGCG induces IL-12 synergistically with UV radiation and IL-12 exhibits the capacity to remove UV-induced DNA damage (20), we further examined whether the removal or repair of UV-induced CPDs by EGCG is mediated via induction of nucleotide excision repair. For this purpose, we used nucleotide excision repair–deficient cells from *XPA* patients and nucleotide excision repair–proficient cells from healthy persons. Cells derived from patients suffering from XP either lack or have reduced DNA repair capacity due to genetic mutations in several components of the nucleotide excision repair. The *XPA* complementation type represents the most severe phenotype because the *XPA* gene is the most crucial component in the repair process and, thus, cells lacking the *XPA* gene are completely deficient in nucleotide excision repair (35, 36). Our immunocytochemical and dot-blot analysis indicated that EGCG was able to remove UV-induced CPDs in nucleotide excision repair–proficient cells but was not able to remove or repair in nucleotide excision repair–deficient cells. These observations indicate that repair of UV-induced DNA damage by EGCG may be mediated through the nucleotide excision repair mechanism. These findings have important implications for the chemopreventive mechanism of skin cancer protection by EGCG and identify a new mechanism by which EGCG prevents UV-induced immunosuppression. Based on the information obtained in this study, it can be suggested that the consumption of five to six cups (one cup = 150 mL) of green tea (1 g green tea leaves/150 mL) per day by humans may provide the same level of photoprotective effect in human system. However, the magnitude of photoprotective effect of green tea may differ person to person based on the differences in race and intensity and exposure time of UV radiation.

Taken together, our data indicate that the prevention of UV radiation–induced immunosuppression by EGCG is mediated through an IL-12-dependent DNA repair mechanism that requires nucleotide excision repair. As UV-induced DNA damage and immunosuppression play an important role in melanoma and nonmelanoma skin cancer, it is tempting to suggest that EGCG may be tested as a therapeutic agent for the prevention of skin cancers in humans.

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