Prevention of UV radiation–induced immunosuppression by IL-12 is dependent on DNA repair

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The immunostimulatory cytokine IL-12 is able to antagonize immunosuppression induced by solar/ultraviolet (UV) radiation via yet unknown mechanisms. IL-12 was recently found to induce deoxyribonucleic acid (DNA) repair. UV-induced DNA damage is an important molecular trigger for UV-mediated immunosuppression. Thus, we initiated studies into immune restoration by IL-12 to discern whether its effects are linked to DNA repair. IL-12 prevented both UV-induced suppression of the induction of contact hypersensitivity and the depletion of Langerhans cells, the primary APC of the skin, in wild-type but not in DNA repair–deficient mice. IL-12 did not prevent the development of UV-induced regulatory T cells in DNA repair–deficient mice. In contrast, IL-12 was able to break established UV-induced tolerance and inhibited the activity of regulatory T cells independent of DNA repair. These data identify a new mechanism by which IL-12 can restore immune responses and also demonstrate a link between DNA repair and the prevention of UV-induced immunosuppression by IL-12.

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Ultraviolet radiation, in particular the mid-wave range (UVB, 290–320 nm), represents one of the most significant environmental factors affecting humans. Its hazardous effects on health include exacerbation of infectious diseases, skin cancer, and skin aging (1–3). These effects are partially mediated by the immunosuppressive properties of UV which are best demonstrated by the inhibition of cellular immune reactions, such as contact hypersensitivity (CHS) (4, 5). UV impairs sensitization to contact allergens applied directly to the UV-irradiated skin area (4, 5). In addition, hapten-specific tolerance develops, which is due to the induction of suppressor/regulatory T cells (T reg cells; references 6, 7).

The cytokine IL-12 is one of the major players involved in orchestrating both innate and acquired immune responses (8). It is critical for the development of T helper 1 responses. In addition, IL-12 is able to prevent UV-induced immunosuppression. Injection of IL-12 before hapten application onto UV-exposed skin prevents the development of immunosuppression (9–11). Even more importantly, IL-12 was discovered to break established immunotolerance and to antagonize the suppressive activity of T reg cells. However, the mechanisms by which IL-12 prevents UV-induced immunosuppression remain largely unclear.

UV-induced DNA damage, in particular cyclobutane pyrimidine dimers (CPDs), has been recognized to be an important molecular trigger for UV-mediated immunosuppression (12). Reduction of CPDs via application of DNA repair enzymes prevents UV-induced immunosuppression (13, 14). It has been recently observed that IL-12, in addition to its immunomodulatory activities, exhibits the capacity to remove UV-induced DNA damage (15). This effect was not present in *Xpa* KO ($Xpa^{-/-}$) mice (16) which, due to a mutation in the *Xpa* gene, lack functional nucleotide excision repair (NER), the endogenous DNA repair system removing UV-induced DNA lesions, indicating that IL-12 may reduce CPDs via induction of NER. Therefore, we were interested in whether the prevention of UV-induced immunosuppression by IL-12 may be due to its capacity to induce DNA repair. We postulated that if this should be the case IL-12 would not be able to prevent UV-induced immunosuppression in DNA repair–deficient mice.

The Journal of Experimental Medicine

RESULTS AND DISCUSSION

IL-12 prevents UV-induced suppression of the induction of CHS in WT but not in *Xpa*$^{-/-}$ mice

C57BL/6 and *Xpa*$^{-/-}$ mice were exposed to UV and sensitized after the last exposure by topical application of 2,4,-dinitrofluorobenzene (DNFB) onto the UV-exposed skin. Application of DNFB to UV-exposed skin failed to induce sensitization both in wild-type (WT) and *Xpa*$^{-/-}$ mice (Fig. 1). Injection of IL-12 3 h before DNFB application restored sensitization in UV-exposed WT mice as demonstrated by a vigorous CHS response upon ear challenge (Fig. 1 A). In contrast, UV-exposed *Xpa*$^{-/-}$ mice remained unresponsive to DNFB despite the application of IL-12 (Fig. 1 B), indicating that the preventive effect of IL-12 on UV-induced immunosuppression may depend on functional NER.

IL-12 breaks established UV-induced tolerance in WT and *Xpa*$^{-/-}$ mice

Application of haptens to UV-exposed skin does not only inhibit the induction of CHS but also induces hapten-specific tolerance (4). Accordingly, WT and *Xpa*$^{-/-}$ mice which had had the hapten applied to UV-exposed skin did not respond with an ear swelling response upon resensitization with DNFB, indicating that both WT and *Xpa*$^{-/-}$ mice may have been tolerized to DNFB (Fig. 2). IL-12 is the only cytokine known that has the ability to break established tolerance (9–11). Sensitization responses after DNFB challenge were fully restored after injection of IL-12 3 h before resensitization of both tolerized WT and *Xpa*$^{-/-}$ mice, indicating that UV-induced tolerance was broken by IL-12 in both strains. Thus, in contrast with the prevention of UV-induced immunosuppression, breaking of UV-induced tolerance by IL-12 appears to be independent of functional NER.

Effect of IL-12 on the adoptive transfer of suppression in WT and *Xpa*$^{-/-}$ mice

UV-induced tolerance can be adoptively transferred into naive recipients via injection of T cells (6, 7). This indicates that application of haptens onto UV-exposed skin induces hapten-specific T reg cells (17). IL-12 can prevent the generation of UV-induced T reg cells but can also counteract the suppressive activity of these cells (10). To determine whether these two activities of IL-12 are linked to DNA repair, adoptive transfer studies were performed. LN cells were obtained from mice of both strains which had previously been tolerized by the application of DNFB to UV-exposed skin. Injection of these cells into naive syngeneic mice rendered recipients unresponsive to DNFB (Fig. 3, A and B, group 3). Transfer of cells obtained

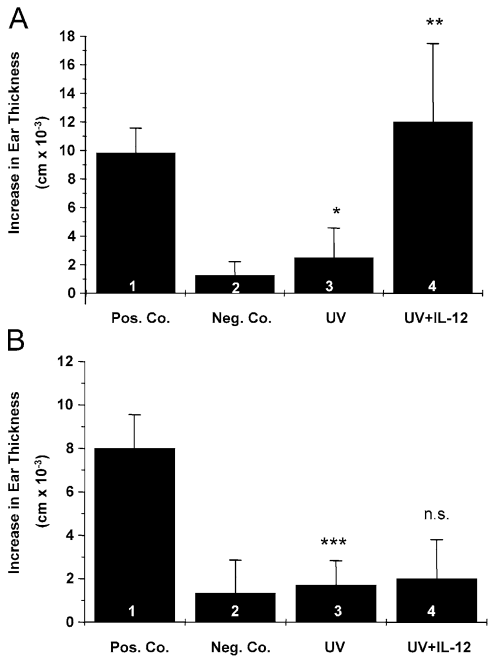

Figure 1. IL-12 prevents UV-induced suppression of the induction of CHS in WT but not in *Xpa*$^{-/-}$ mice. C57BL/6 (A) or *Xpa*$^{-/-}$ mice (B) were treated daily with UV (1,000 and 400 J/m^2, respectively) on 4 d on the back and sensitized 24 h after the last exposure through UV-exposed skin (groups 3 and 4). 5 d later, mice were challenged on the left ear and ear swelling was measured 24 h later. Group 4 received 1,000 ng of IL-12 3 h before sensitization. Positive control mice were sensitized and challenged (group 1), negative control animals were only challenged (group 2). Ear swelling is expressed as the difference (cm $\times$ 10^{-3}, mean $\pm$ SD) between the thickness of the challenged and that of the vehicle-treated ear. *P $<$ 0.00001 UV vs. positive control; **P $<$ 0.005 UV vs. UV+IL-12; ***P $<$ 0.00001 UV vs. positive control. n.s., UV vs. UV-IL-12.

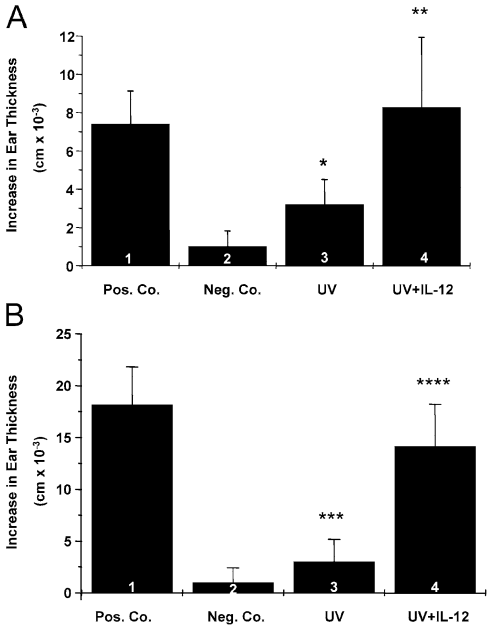

Figure 2. IL-12 breaks established UV-induced tolerance in WT and *Xpa*$^{-/-}$ mice. C57BL/6 (A) or *Xpa*$^{-/-}$ mice (B) were treated daily with UV (1,000 and 400 J/m^2, respectively) on 4 d on the back and sensitized 24 h after the last exposure through UV-exposed skin. 14 d after the first sensitization, mice were resensitized with DNFB applied onto the abdomen (groups 3 and 4). 5 d later, mice were challenged on the right ear and ear swelling was measured 24 h later. Group 4 received 1,000 ng of IL-12 3 h before resensitization. Positive control mice were sensitized and challenged (group 1), negative control animals were only challenged (group 2). *P $<$ 0.05 UV vs. positive control; **P $<$ 0.05 UV vs. UV+IL-12; ***P $<$ 0.0005 UV vs. positive control; ****P $<$ 0.0005 UV vs. UV+IL-12.

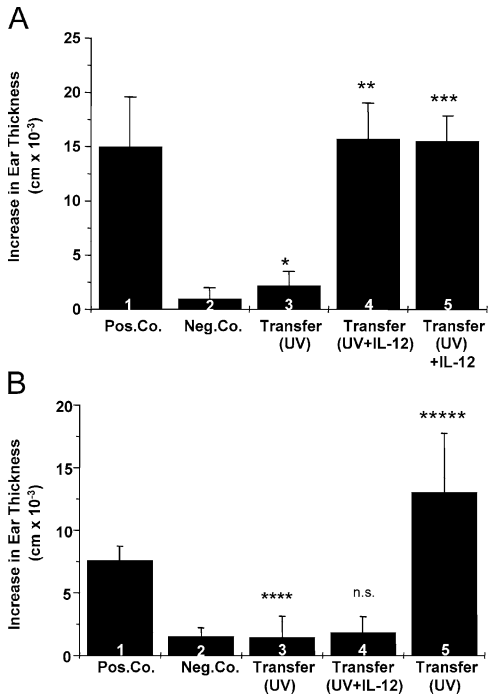

Figure 3. IL-12 inhibits transfer of suppression by UV-induced T reg cells. Naive C57BL/6 (A) or *Xpa*$^{-/-}$ mice (B) were injected i.v. with LN cells obtained from syngeneic donors which were tolerized against DNFB by application of DNFB onto UV-exposed skin. 24 h after injection mice were sensitized against DNFB and 5 d later DNFB challenge was performed on the left ear (group 3). Mice in group 5 received 3 h before and 24 h after cell transfer 1,000 ng IL-12 i.p. Mice in group 4 received cells obtained from UV-exposed donors, which were treated with IL-12 before sensitization through UV-exposed skin, and were sensitized 24 h after cell transfer. *$P < 0.001$ UV vs. positive control; **$P < 0.00001$ UV vs. (UV+IL-12); ***$P < 0.0001$ UV vs. (UV)+IL-12; ****$P < 0.0005$ UV vs. positive control; *****$P < 0.00005$ UV vs. (UV)+IL-12; n.s., UV vs. (UV+IL-12).

from donors that had also been injected with IL-12 did not cause suppression in WT mice (Fig. 3 A, group 4), whereas transfer of cells obtained from identically treated *Xpa*$^{-/-}$ donors still caused suppression in *Xpa*$^{-/-}$ recipients (Fig. 3 B, group 4). This indicates that IL-12 prevented the induction of UV-induced T reg cells in WT but not in *Xpa*$^{-/-}$ mice.

IL-12 has also been shown to suppress the activity of UV-induced T reg cells as demonstrated by the transfer experiments in which transferred T reg cells cannot elicit suppression if the recipients have been treated with IL-12 (10, 11). Therefore, T reg cells of both strains were injected into the respective naive recipients that received IL-12 i.p. 3 h before and 24 h after transfer. Recipients were sensitized with DNFB 24 h later. In both strains suppression after transfer of T reg cells was inhibited by IL-12 (Fig. 3, A and B, group 5), indicating that IL-12 can antagonize the suppressor activity of T reg cells in WT as well as *Xpa*$^{-/-}$ mice.

IL-12 prevents UV-induced depletion of Langerhans cells (LC)

A key event in the suppression of CHS by UV is the depletion of LC, the crucial APC in the epidermis (4, 18). Initially, disappearance of LC from the epidermis after UV was attributed to induction of apoptosis. However, recent evidence indicates that UV-induced LC depletion is mainly caused by the emigration of LC from the skin to the draining LN (19). Accordingly, CPD-containing APC were found in the draining LN of UV-exposed mice (20). These APC were identified to be of epidermal origin and exhibited impaired Ag presenting function. Removal of CPDs by DNA repair enzymes restored the immunostimulatory capacity (21). This suggests that UV-induced DNA damage is the major trigger for the emigration of LC from the epidermis and that Ag presentation by UV-damaged LC in the LN induces T reg cells, which ultimately are responsible for mediating immunotolerance. Therefore, we next studied whether IL-12 prevents UV-induced LC depletion and whether this may be linked to DNA repair. Ears of WT and *Xpa*$^{-/-}$ mice were exposed to UV. One group of mice was injected with IL-12 3 h before sensitization. Ear sheets were prepared 48 h later and the number of LC evaluated. UV caused pronounced depletion of LC in WT and *Xpa*$^{-/-}$ mice (Fig. 4). Upon injection of IL-12, the vast majority of LC was preserved in WT mice despite UV exposure of the skin. In contrast, IL-12 was not able to prevent LC depletion in *Xpa*$^{-/-}$ mice.

IL-12 reduces the number of APC with DNA damage in the LN

There is evidence that UV-induced DNA damage is the major molecular trigger for the emigration of LC to the draining LN. It also impairs their capacity to present Ag, which in turn results in the lack of sensitization and the induction of tolerance (21). Bearing in mind that IL-12 has the capacity to induce NER, we then commenced studies to determine

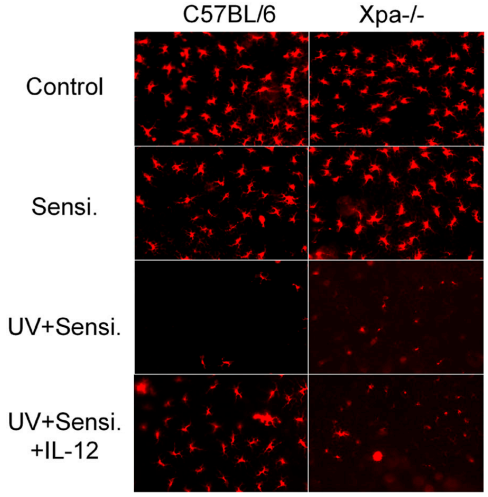

Figure 4. IL-12 prevents UV-induced emigration of LC in WT but not in *Xpa*$^{-/-}$ mice. Ears of C57BL/6 and *Xpa*$^{-/-}$ mice were exposed to 1,000 J/m^2 and to 400 J/m^2, respectively. 24 h later 0.5% DNFB was applied (UV+Sensi.). One group of animals received 1,000 ng IL-12 i.p. 3 h before application of DNFB (UV+Sensi.+IL-12). An additional group was only sensitized (Sensi.). Unirradiated animals served as negative controls (Control). 48 h after hapten application, ears were cut and sheet preparations performed. Sheets were stained with an anti-I-A/I-E Ab, followed by an anti-rat IgG coupled with Texas red and subjected to fluorescence microscopy.

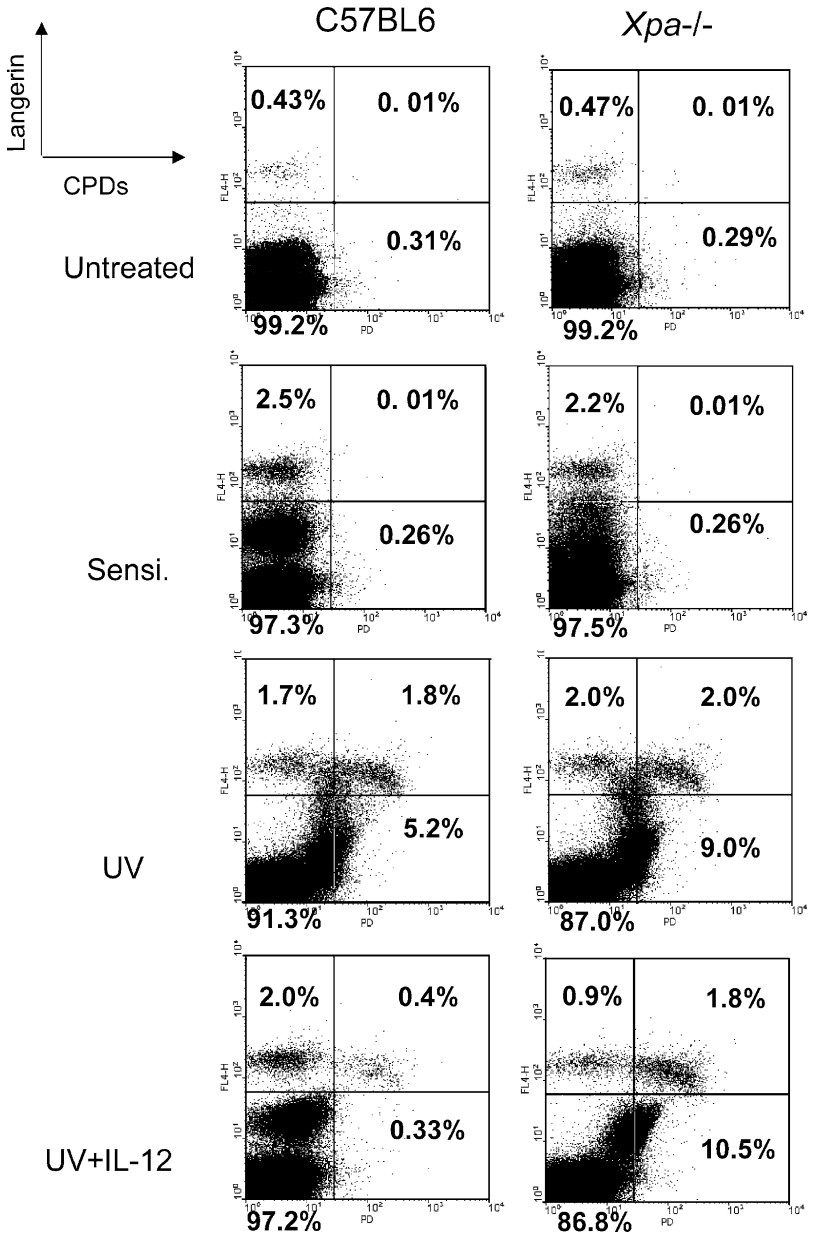

Figure 5. IL-12 reduces the number of CPD-positive LC in LN draining UV-exposed skin in WT but not in *Xpa*$^{-/-}$ mice. C57BL/6 or *Xpa*$^{-/-}$ mice were treated daily with UV on 4 d on the shaved back and sensitized 24 h after the last exposure through UV-exposed skin (UV). One group received 1,000 ng of IL-12 3 h before sensitization (UV+IL-12). As controls LN of untreated and DNFB-sensitized (Sensi.) mice were used. 48 h after sensitization CD11c-positive cells were obtained from draining LN by magnetobead separation. Cells were double stained for Langerin and CPDs and subjected to FACS analysis.

whether IL-12 can reduce the number of CPD-positive cells in the draining LN. DNFB was painted onto UV-exposed skin of both WT and *Xpa*$^{-/-}$ mice, whereby one group was injected with IL-12 3 h before application of the hapten. 48 h later, CD11c-positive cells were obtained from the draining LN by magnetobead separation. These cells were then stained with an Ab directed against the LC specific marker Langerin (22) and another directed against CPDs and subsequently subjected to FACS analysis. In LN from untreated mice almost no Langerin-positive cells were detected (Fig. 5). Sensitization caused an increase in the number of Langerin-expressing cells. When mice were treated with UV and sensitized, the majority of Langerin-positive cells also stained positive for CPDs in both WT and *Xpa*$^{-/-}$ mice. The number of double-positive cells was significantly reduced in WT mice that had been treated with IL-12. In contrast, injection of IL-12 did not reduce the number of CPD-carrying LC in the LN of *Xpa*$^{-/-}$ mice. This indicates that the reduction of CPDs in LC by IL-12 is critically dependent on functional NER. Together, the data suggest that the ability of IL-12 to prevent UV-induced immunosuppression may be due to its capacity to remove UV-induced DNA damage via induction of NER.

Conclusion

IL-12 not only prevents UV-induced immunosuppression but also breaks established tolerance. The present findings support the concept that IL-12 mediates these two effects via different mechanisms. Prevention of UV-induced immunosuppression by IL-12 appears to be based on the induction of NER and its capacity to remove UV-induced DNA damage. In contrast, inhibition of UV-induced T reg cell activity by IL-12 seems to be independent of this effect because only prevention of immunosuppression but not breaking of tolerance by IL-12 was impaired in $Xpa^{-/-}$ mice. The *XPA* gene is an essential component of the NER. Therefore, $Xpa^{-/-}$ mice are severely deficient in NER (16). Because UV-induced DNA damage is known to be the major molecular trigger for the induction of apoptosis (23) $Xpa^{-/-}$ mice have a higher number of apoptotic keratinocytes than WT mice (24) and a higher risk of developing UV-induced skin cancer upon chronic UV exposure (16) due to the impaired capacity to remove UV-induced DNA damage. $Xpa^{-/-}$ mice are also more susceptible to UV-induced immunosuppression as lower UV doses are required to achieve the same level of immunosuppression as in WT mice (25). This also supports the crucial role of UV-induced DNA damage as a molecular mediator of the impairment of the immune system by UV. Because IL-12 had little effect on the emigration of LC or the induction of CHS by UV in $Xpa^{-/-}$ mice, nor did it lead to a reduction of the number of CPD-positive cells in the draining LN we conclude that IL-12 exerts these effects via NER.

$Xpa^{-/-}$ mice are quite similar to WT mice with the exception of the impaired capacity to remove DNA damage. However, one has to consider the possibility that $Xpa^{-/-}$ mice respond differently to IL-12 than WT mice. This appears unlikely because IL-12 was fully capable of breaking established tolerance and inhibiting the suppressive capacity of T reg cells in both $Xpa^{-/-}$ and WT mice. In this context, it should be mentioned that DNA damage appears to be important for the initial development of T reg cells but their suppressive activity should then be independent of DNA damage because T reg cells are never exposed to UV radiation in vivo.

The mechanisms leading to the induction of NER by IL-12 are still not clear. Although it was shown that IL-12 induces certain components of the NER at the RNA level (11), these data have not yet been confirmed at the protein level. It is also not yet clear whether IL-12 affects global genome or transcription-coupled repair. Because the latter is associated with UV-induced LC depletion and local immunosuppression (25) one may indirectly conclude that IL-12 should affect at least transcription-coupled repair. Furthermore, we have not analyzed by which signaling mechanisms IL-12 induces DNA repair. Usage of STAT-$4^{-/-}$ cells will help to answer the question whether STAT-4, the major protein in IL-12 signal transduction (26), is involved.

The observation that IL-12 prevents UV-induced apoptosis of keratinocytes (15) implied that keratinocytes express the IL-12 receptor which is composed of two subunits, designated β1 and β2 (27). PCR and FACS analysis revealed that murine epidermal cells and the transformed keratinocyte cell line PAM-212 express both chains (unpublished data). Hence, the question arises whether the prevention of UV-induced immunosuppression by IL-12 is mediated via keratinocytes, LC or both. BM chimeras with LC of $Xpa^{-/-}$ origin appear as an ideal tool to address this question. The failure of IL-12 to prevent UV-induced immunosuppression in these chimeras should indicate that LC are the primary target cells for IL-12. However, Merad et al. recently reported that in lethally irradiated mice that had received BM transplants, LC of host origin remained for at least 18 mo, whereas DC in other organs were almost completely replaced by donor cells within 2 mo (28). Only upon high dose UV exposure LC disappeared and were replaced by circulating LC precursors. However, high dose UV causes systemic immunosuppression. Thus, it would be extremely difficult to dissect which immunosuppressive effect is caused by the initial UV dose applied to deplete the host LC and by the next dose applied to affect the donor LC. We are currently trying to identify UV doses which deplete the donor LC but do not cause systemic immunosuppression.

UV-induced DNA damage is certainly one of the major molecular mediators of photoimmunosuppression. Our findings indicate that this regulation does not appear to be unidirectional because a cytokine, like IL-12, can in turn control DNA repair and thereby counteract UV-induced immunosuppression. Based on the present data it may be concluded that the immunoreconstitutive activities of IL-12 might be in part due to its activity to induce DNA repair. Because induction of DNA damage by UV is a crucial event in photocarcinogenesis, this crosstalk may represent a new defense mechanism of the host against UV-induced immunosuppression and carcinogenesis. In addition, these data identify a new mechanism by which IL-12 restores an immune response and demonstrate for the first time, a link between DNA repair and the prevention of UV-induced immunosuppression by IL-12. Because UV-induced DNA damage and immunosuppression play an important role in photocarcinogenesis, it is tempting to speculate about a therapeutic and preventive potential of IL-12 for UV-induced cancer, one of the most frequent malignancies today.

MATERIALS AND METHODS

Animals. C57BL/6 mice were purchased from Harlan-Winkelmann. $Xpa^{-/-}$ mice were generated at the RIVM (16). Animal care was provided by expert personnel in compliance with the relevant laws and institutional guidelines. rIL-12 was diluted in sterile endotoxin-free saline and 1,000 ng were injected i.p. per mouse.

CHS. Mice were sensitized by painting 50 μl of DNFB (Sigma-Aldrich) solution (0.5% in acetone/olive oil, 4:1) on the shaved back on day 0. On day 5, 20 μl 0.3% DNFB were applied to the left ear. Ear swelling was quantified with a spring-loaded micrometer 24 h later. CHS was determined as the amount of swelling of the hapten-challenged ear compared with the thickness of the vehicle-treated ear and expressed in cm $\times 10^{-3}$ (mean ± SD). Resensitization was performed on abdominal skin 14 d after the first sensitization. Second challenge was performed on the right ear 5 d

after second sensitization. Each group consisted of at least seven mice. Each experiment was performed at least two times.

UV irradiation. The shaved back was exposed to UV from TL12 fluorescent lamps (Philips) which emit most of their energy within the UVB range. Mice were exposed to UV daily for four consecutive days. WT mice received 1,000 J/m^2 per exposure. Because *Xpa*$^{-/-}$ mice are more UV-susceptible (25) they received only 400 J/m^2 per exposure to achieve the same level of immunosuppression.

Adoptive transfer of immune response. Donor mice were treated as indicated, spleens and regional LN were removed and single-cell suspensions prepared. 200 μl (2.5×10^8/ml) were injected i.v. into each recipient mouse, which were sensitized 24 h later against DNFB. After 5 d, mice were challenged on the left ear and ear swelling was evaluated 24 h later. Injection of these cells into naive mice suppresses induction of CHS in a hapten-specific fashion, indicating the presence of T reg cells within this population (7). For the sake of simplicity in the present manuscript the term UV-induced T reg cells was used for the LN cells obtained from UV-tolerized mice, although we are aware of the fact that we did not inject pure T reg cells but rather bulk cells containing T reg cells.

Immunofluorescence stainings. Ears were mechanically split into dorsal and ventral sides, incubated in 2 mM EDTA, washed with PBS, and fixed in acetone. Sheets were stained overnight with an anti–I-A/I-E Ab (BD Biosciences), then incubated with a Texas red–coupled secondary anti–rat Ab (IgG) and examined using a microscope (model BX61; Olympus).

FACS analysis. Cells obtained from spleens and LN were enriched for CD11c-positive cells using magnetobead separation (autoMACS; Miltenyi Biotec). Cells were washed with PBS and incubated for 5 min at 4°C with 0.8% paraformaldehyde. After washing, cells were incubated for 5 min on ice with 0.3% saponin. Cells were incubated at RT for 30 min with an Ab directed against Langerin (Hybridoma 929F3; Schering-Plough) (22) coupled with anti–rat Oregon green and an Ab directed against CPDs (Kamiya Biomedical Company) coupled with anti–mouse FITC Ab. Samples were analyzed by flow cytometry (FACS Calibur; BD Biosciences).

Statistical analysis. Data were analyzed by Student's *t* test and differences were considered significant at $P < 0.05$.

The authors are grateful to Sem Saeland for providing the 929F3 Ab, Annette Mehling for editorial help, and Thomas Brzoska for help in preparing the figures.

This work was supported by grants from the German Research Foundation (SFB 293, B9 to T. Schwarz, BE1580/7-1 to S. Beissert), from the Federal Ministry of Environmental Protection (St.Sch_4373 to T. Schwarz), from the European Community (QLK4-CT-2001-00115 to T. Schwarz), and the CERIES Research Award to T. Schwarz.

The authors have no conflicting financial interests.

Submitted: 18 June 2004
Accepted: 3 December 2004

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