Exposure to Ultraviolet Radiation Causes Dendritic Cells/Macrophages to Secrete Immune-Suppressive IL-12p40 Homodimers[1]

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UV-induced immune suppression is a risk factor for sunlight-induced skin cancer. Exposure to UV radiation has been shown to suppress the rejection of highly antigenic UV-induced skin cancers, suppresses delayed and contact hypersensitivity, and depress the ability of dendritic cells to present Ag to T cells. One consequence of UV exposure is altered activation of T cell subsets. APCs from UV-irradiated mice fail to present Ag to Th1 T cells; however, Ag presentation to Th2 T cells is normal. While this has been known for some time, the mechanism behind the preferential suppression of Th1 cell activation has yet to be explained. We tested the hypothesis that this selective impairment of APC function results from altered cytokine production. We found that dendritic cells/macrophages (DC/Mϕ) from UV-irradiated mice failed to secrete biologically active IL-12 following in vitro stimulation with LPS. Instead, DC/Mϕ isolated from the lymphoid organs of UV-irradiated mice secreted IL-12p40 homodimer, a natural antagonist of biologically active IL-12. Furthermore, when culture supernatants from UV-derived DC/Mϕ were added to IL-12-activated T cells, IFN-γ secretion was totally suppressed, indicating that the IL-12p40 homodimer found in the supernatant fluid was biologically active. We suggest that by suppressing DC/Mϕ IL-12p70 secretion while promoting IL-12p40 homodimer secretion, UV exposure preferentially suppress the activation of Th1 cells, thereby suppressing Th-1 cell-driven inflammatory immune reactions. *The Journal of Immunology,* **2000, 165: 3162–3167.**

T he UV radiation found in sunlight has a number of adverse effects on the health and well-being of humans. It is the primary cause of nonmelanoma skin cancer (1) and has been implicated in the induction of malignant melanoma (2). Skin cancer is the most prevalent form of human neoplasia. It is estimated that in the United States alone, one million new cases of skin cancer were diagnosed in 1999. Exposure to UV radiation is also immune suppressive, and the immunosuppressive effects of UV have been identified as a major risk factor for skin cancer induction (3, 4). Because of the link between the carcinogenicity of UV radiation and its immunosuppressive effects, it is critically important to understand the mechanisms underlying the induction of immune suppression.

Exposure to UV radiation suppresses delayed (DTH)[3] and contact (CHS) hypersensitivity reactions to Ags or haptens applied at distant nonirradiated sites. Because UV radiation has a limited ability to penetrate the skin and directly irradiate distant lymphoid organs, its effects on distant immune elements are indirect. Cytokines released by UV-irradiated epidermal cells appear to be involved. Previous studies from this laboratory demonstrated that IL-10 is produced by UV-irradiated keratinocytes and plays an essential role in suppressing DTH (5). One consequence of UV-induced IL-10 secretion appears to be differential activation of Th cell subsets. This was illustrated by Brown et al. (6). In this study UV exposure suppressed the DTH response to *Borrelia burgdorferi*, the causative agent of Lyme's disease. In addition, UV exposure suppressed the production of Ab subclasses that are helped by Th1 cells (IgG2a and -2b) and caused a slight, but significant, increased in IgG1, an Ab whose production is helped by Th2 cells. The suppression of both DTH and IgG2a and IgG2b production was reversed when the UV-irradiated mice were injected with monoclonal anti-IL-10.

Total body UV exposure also results in a systemic impairment of APC function (7, 8). Here again cytokines released in response to UV exposure play an essential role in the systemic alteration of APC function. Cytokine secretion by Th1 clones was significantly suppressed when APC from UV-irradiated mice were used to present Ag to Th1 cells. In contrast, these same APC very effectively presented Ag to Th2 clones. Injecting UV-irradiated mice with anti-IL-10 reversed the APC defect (9), indicating the role of UV-induced IL-10 in the APC defect.

Direct UV irradiation of epidermal APCs, the Langerhans cells, causes a similar effect. Simon et al. found that normal Langerhans cells presented Ags equally well to both Th1 and Th2 clones, but UV-irradiated Langerhans cells lost their ability to present Ag to Th1 cells (10). In addition, not only were the Th1 cells incapable of proliferating in response to Ag presentation by UV-irradiated Langerhans cells, but these cells were rendered tolerant to subsequent Ag stimulation with normal Ag-bearing Langerhans cells (11). Some have suggested that the mechanism may involve UV-induced keratinocyte-derived IL-10. Enk et al. found that treating Langerhans cells with IL-10 mimicked the UV-induced effects. IL-10-treated cells presented Ag to Th2 cells and failed to present

Ag to Th1 cells, and Ag presentation by IL-10-treated Langerhans cells induced T cell tolerance (12).

Although it is clear from the studies mentioned above that UV-induced cytokines, particularly IL-10, are involved in modulating APC function, the exact mechanism by which APC isolated from UV-irradiated mice distinguish between Th1 and Th2 cells is not known. Here we test the hypothesis that total body UV exposure suppresses the activation of Th1 cells by suppressing dendritic cell/macrophage (DC/Mϕ) IL-12 production. We concentrated on IL-12 for two reasons. First, it is the major cytokine involved in activating Th1 cells (13), but it is not involved in activating Th2 cells (14). Second, injecting rIL-12 into UV-irradiated mice overcomes UV-induced immune suppression (15, 16) and blocks UV-induced tolerance induction (17). Our findings indicate that total body UV exposure not only significantly suppresses the secretion of biologically active IL-12 byDC/Mϕ, but, in addition, increases DC/Mϕ secretion of IL-12p40 homodimer, a natural antagonist of biologically active IL-12.

Materials and Methods

Animals

Specific pathogen-free female C3H/HeNCr (MTV⁻) mice were obtained from the National Cancer Institute Frederick Cancer Research Facility Animal Production Area (Frederick, MD). The animals were maintained in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International, in accordance with current regulations and standards of the U.S. Department of Agriculture, the Department of Health and Human Services, and the National Institutes of Health. All animal procedures were reviewed and approved by the institutional animal care and use committee. Within each experiment all mice were age and sex matched. The mice were 8–10 wk old at the start of each experiment.

Radiation sources

A bank of six FS-40 sunlamps (National Biological, Twinsberg, OH) was used to irradiate the mice. These lamps emit a continuous spectrum from 270–390 nm; with peak emission at 313 nm; ~65% of the irradiation is within the UVB range (280–320 nm) of the solar spectrum. The irradiance of the six bulbs averaged 10 W/m², as measured by an IL-1700 research radiometer (International Light, Newburyport, MA).

DC enrichment

Adult mice were irradiated with 15 kJ/m² UV (280–320 nm) radiation on their shaved dorsal skin. Three days later each mouse was sensitized by applying 400 μl of a 0.5% solution of FITC diluted in acetone dibutylphthalate (1/1, v/v) to the unirradiated abdominal skin. The inguinal, axilary, and brachial lymph nodes draining the site of sensitization were harvested 18 h later. Single-cell suspensions were prepared in PBS supplemented with 5% FCS and 2 mM EDTA, and the cells were incubated with the anti-FITC-coated microbeads (20 μl/10⁷ cells; Miltenyi Biotec, Gladbach, Germany) at 4° for 15 min. The cell suspension was washed and added to a stainless steel mesh column that was held next to a high gradient magnet. The unbound cells were removed by washing three times with medium. Removing the column from the magnet and forcing 1 ml of medium through the column yielded the hapten-bearing FITC⁺ cells. The cells were washed in PBS, resuspended in complete RPMI 1640 medium, and counted (18). The predominant DC nature of the resulting cell population was determined by direct microscopic examination and was confirmed by flow cytometry. By morphology (Fig. 1) this procedure results in a population of cells enriched for DC/Mϕ (65–75% DC, the rest Mϕ). Control populations of DC were obtained from the draining lymph nodes of normal nonirradiated mice that were handled in an identical fashion.

Cytokine determinations

IL-12 secreted into the supernatant fluids of LPS-activated (10 ng/ml in complete RPMI 1640 medium for 72 h) DC/Mϕ was measured by use of sandwich ELISAs performed according to manufacturer's instructions as reported previously (19). For total IL-12, clone C15.6 was the capture Ab and biotin-labeled C17.8 was the detecting Ab (PharMingen, San Diego, CA). For IL-12p40, homodimer clone C17.15 was the capture Ab, and biotin-labeled C17.15 was the detecting Ab (Genzyme, Cambridge, MA). Recombinant IL-12p70 was purchased from PharMingen. Recombinant IL-12p40 homodimer was purchased from R&D Systems (Minneapolis, MN).
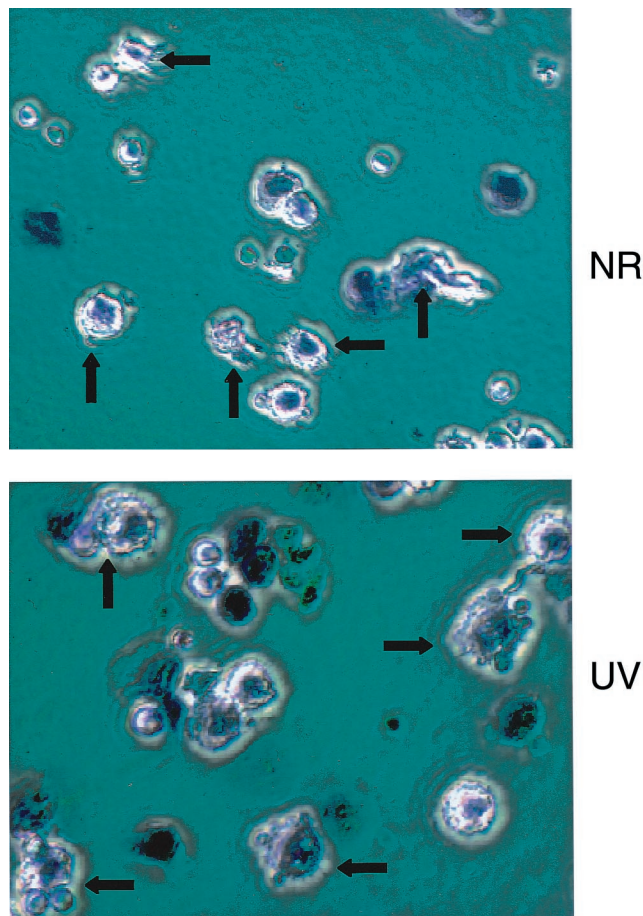

FIGURE 1. The morphological appearance of DC/Mϕ isolated from the draining lymph nodes of normal (*top panel*) or UV-irradiated mice (*bottom panel*) was examined using cytospin preparations stained with Wright's Giemsa stain. The arrows denote dendritic processes. By morphology, it was determined that this cell population is 65–75% DC and 25–35% macrophages.

For IFN-γ measurements, clone RA4-6A2 was used as the capture Ab, and biotin-labeled XMG1.2 was the detecting Ab. Both these Abs as well as rIFN-γ were purchased from PharMingen. Generally the limit of sensitivity of the IL-12 and IFN-γ ELISAs was between 5 and 25 pg/ml.

IL-12 bioassay

IL-12 biological activity was measured by inducing normal T cells to produce IFN-γ. Whole spleen cells (10⁶ cultured in 1 ml of complete RPMI 1640 in 24-well Costar dishes (Costar, Cambridge, MA)) were cultured in the presence or the absence of rIL-12 or supernatants from DC/Mϕ for 72 h at 37°. At the end of the incubation period the supernatants were harvested, and IFN-γ levels were measured by ELISA. Standard curves were constructed by adding rIL-12 p70 to the spleen cells cultures and measuring the amount of IFN-γ produced. The data are expressed as picograms of IL-12 per milliliter. The specificity of the reaction was determined by blocking IL-12-induced IFN-γ secretion with monoclonal anti-IL-12. In some experiments the ability of UV-induced IL-12p40 homodimer to block the activity of rIL-12 was measured. In these experiments rIL-12 (250 pg/ml) was added to normal spleen cells in the presence or the absence of supernatants from LPS-activated UV or normal DC/Mϕ. Control cultures received rIL-12p40 homodimer (10 μg/ml). Suppression of IL-12-induced IFN-γ production was used as an indication of IL-12p40 homodimer activity.

Flow cytometry

DC/Mϕ were isolated as described above. The cells were resuspended in PBS containing 2% goat serum and 0.5% BSA and were incubated on ice for 30 min with PE-conjugated Ab. The PE-labeled anti-CD3, anti-B220, anti-CD11b, anti-CD86, and anti-MHC class II (Ia^k) were purchased from

PharMingen. After washing, cell staining was analyzed by fluorescence-activated flow cytometry (FACS EPICS Profile, Coulter Electronics, Hialeah, FL).

Results

Characterization of the DC population isolated by magnetic bead enrichment

Mice were exposed to UV radiation and 3 days later were painted with FITC on a distant nonirradiated site as described previously (20). Eighteen hours later the lymph nodes draining the site of allergen sensitization were removed, and the Ag-bearing, FITC$^+$ cells were isolated with anti-FITC-conjugated magnetic microbeads (18). The dendritic nature of the cells isolated was determined by direct microscopic examination (Fig. 1) and was confirmed by flow cytometry. The data indicate isolation of a population devoid of T and B cells and enriched for DC/Mϕ (Fig. 2).

Detection of IL-12p70 and -p40 homodimers by ELISA

Defective APC function by these cells was confirmed by the lack of contact hypersensitivity induction when they were used to immunize normal mice, as described previously (21) (data not shown). The FITC$^+$ cells were then cultured with LPS in vitro to induce IL-12 secretion. Cytokine-specific ELISA was used to mea-

sure IL-12 content in the supernatant fluid. The data (Fig. 3) indicate no significant effect of prior UV exposure on IL-12 production. These results were at first puzzling because it is well established that these cells do not present Ag to Th1 cells (9, 21). Significant production of IL-12 was not consistent with these findings. These data forced us to re-evaluate the ELISA used to measure IL-12 production. Biologically active IL-12 is a heterodimeric protein consisting of two covalently linked subunits, p35 and p40. The genes encoding these two proteins reside on different chromosomes (chromosome 5 for p40 and chromosome 3 for p35). In addition, IL-12p40 monomers and IL-12p40 heterodimers do exist in nature and are secreted by activated DC/Mϕ (22). Because the Ab pairs used in the ELISA depicted in Fig. 3 detect different determinants on the IL-12p40 subunit, this ELISA can detect biologically active IL-12p70, IL-12p40 monomers, and/or IL-12p40 homodimers. We suspected overproduction of IL-12p40 homodimer, which is a natural antagonist to IL-12p70 (23–26), by DC/Mϕ isolated from UV-irradiated mice.

To determine whether this was the case, supernatants were obtained from DC/Mϕ cultured with and without LPS, and an ELISA specific for IL-12p40 homodimer was used. As shown in Fig. 4, unstimulated cells (both nonirradiated and UV-irradiated cells) failed to produce any IL-12p40 homodimer. While stimulation of DC/Mϕ from nonirradiated mice with LPS resulted in little IL-12p40 homodimer production, LPS activation of DC/Mϕ from UV-irradiated mice resulted in a significant increase in the production of IL-12p40 homodimer. This result demonstrates that UV irradiation of the dorsal skin causes an increase in DC IL-12p40 homodimer production.

Biological activity of DC-derived IL-12

The findings presented in Figs. 3 and 4 suggest that the majority of the IL-12 secreted by the DC/Mϕ isolated from UV-irradiated mice is the IL-12p40 homodimer. If correct, then little or no IL-12 biological activity should be found in these supernatant fluids. We measured IL-12 biologic activity in these supernatants by measuring IFN-γ production by normal spleen cells. Different amounts of rIL-12p70 were added to cultures of normal spleen cells, and the IFN-γ secreted into culture supernatant was measured. From these
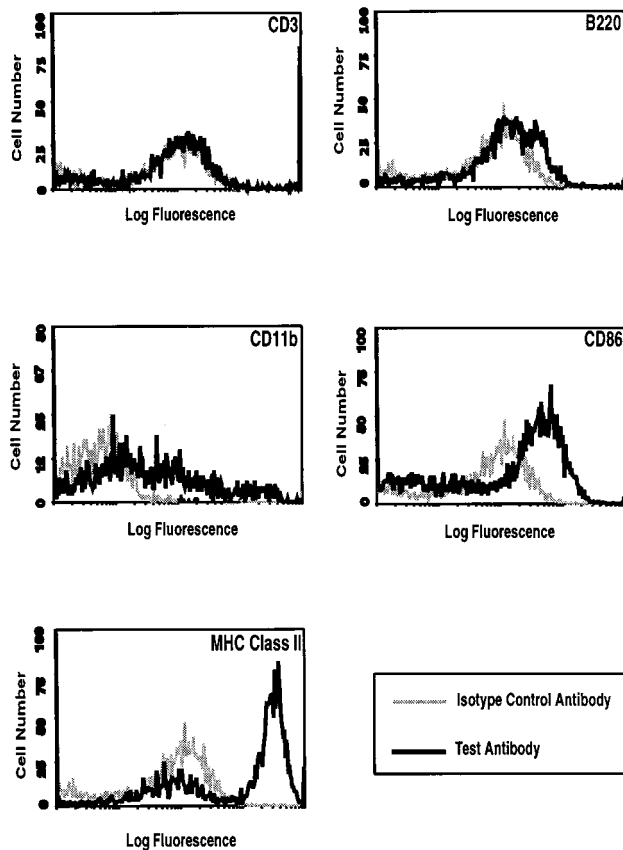

FIGURE 2. FACS analysis of DC/Mϕ. A population of cells enriched for DC/Mϕ was isolated from normal or UV-irradiated hapten-sensitized mice by anti-FITC-conjugated magnetic microbeads and directly stained with PE-conjugated anti-CD3, PE-conjugated anti-B220, PE-conjugated anti-CD11b, PE-conjugated anti-CD86, or PE-conjugated anti-MHC class II. Staining with a PE-conjugated isotype-matched control Ab is shown in the gray lines. The profiles shown are DC/Mϕ isolated from UV-irradiated mice; the profiles of DC/Mϕ isolated from normal mice were identical (data not shown).

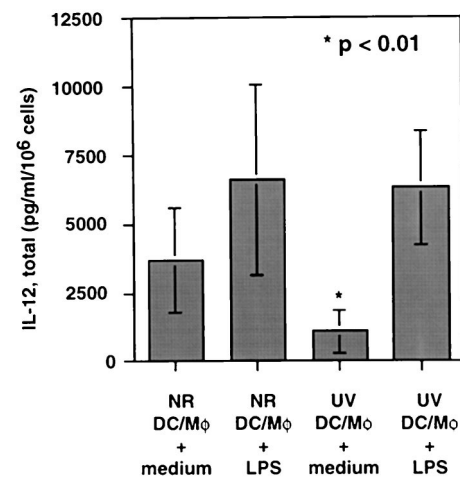**FIGURE 3.** Total IL-12 (p70 plus p40 monomer plus p40 homodimer) levels in the supernatant of DC/Mϕ obtained from normal or UV-irradiated mice. DC/Mϕ isolated from either normal or UV-irradiated mice are stimulated in the presence or the absence of LPS. Total IL-12 levels were determined by ELISA. The amount of total IL-12 produced by either population of LPS-stimulated cells was not significantly different.
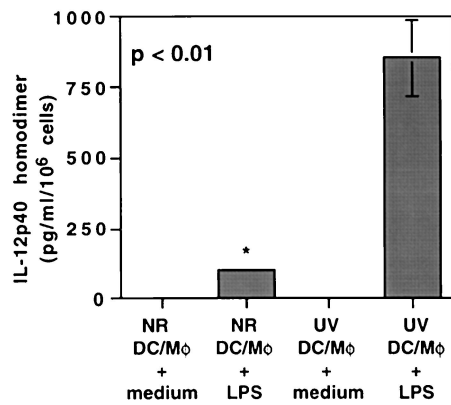
FIGURE 4. IL-12p40 homodimer levels in the supernatant from DC/Mϕ isolated from normal and UV-irradiated mice. While unstimulated DC/Mϕ from either population do not secrete IL-12p40 homodimer, LPS-stimulated DC/Mϕ from UV-irradiated mice produced increased amounts of IL-12p40 homodimer. In contrast, LPS-stimulated DC/Mϕ from normal mice produced little or no IL-12p40 homodimer ($p < 0.01$, by Student's t test).

data a standard curve was generated, which was then used to calculate the amount of biologically active IL-12 present in the DC/Mϕ supernatants. When normal DC/Mϕ were stimulated with LPS, biologically active IL-12 was induced, as measured by secretion of IFN-γ by the treated spleen cells (Fig. 5). When, however, the spleen cells were cultured with supernatants from LPS-stimulated UV-DC/Mϕ, little or no IFN-γ was detected. These findings indicate that DC from UV-irradiated mice cannot be stimulated to secrete biologically active IL-12 in vitro.

Biological function of IL-12p40 homodimers secreted by DC isolated from UV-irradiated mice

The IL-12p40 homodimer is antagonistic to IL-12p70 by virtue of its ability to bind to the IL-12 receptor on Th1 cells and block the
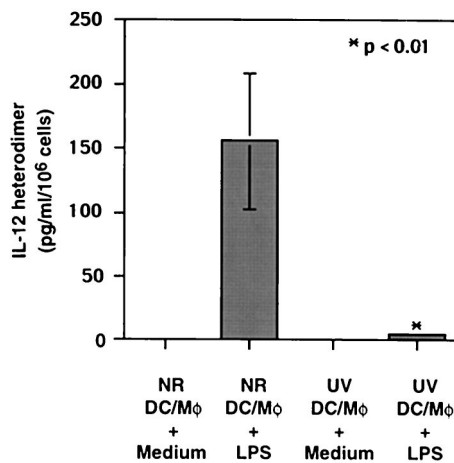
subsequent binding and signaling activity by biologically active IL-12p70 (24). Therefore, we determined whether the IL-12p40 homodimers found in the supernatants of DC/Mϕ isolated from UV-irradiated mice blocked IL-12p70 biological activity. A fixed amount of recombinant IL-12p70 was added to spleen cells in the presence or the absence of supernatants from the DC/Mϕ cultures. The effect this treatment had on IFN-γ production was measured (Fig. 6). As expected, adding rIL-12 to normal spleen cells induced IFN-γ secretion. The specificity of the reaction for IL-12 was demonstrated by complete suppression of IFN-γ production when monoclonal anti-IL-12 was added to the cultures. Adding supernatants from normal nonactivated DC/Mϕ, LPS-activated normal DC/Mϕ, or nonactivated DC/Mϕ isolated from UV-irradiated mice resulted in enhanced production of IFN-γ. Whether this enhancement was due to the presence of other IFN-γ-inducing cytokines, (i.e., IL-18) in these culture supernatants remains to be seen. However, when DC/Mϕ isolated from the UV-irradiated mice were stimulated with LPS, and these supernatants were added to IL-12-stimulated whole spleen cells, complete and total inhibition of IFN-γ secretion was observed. Similarly, complete suppression of IFN-γ production was noted when rIL-12p40 homodimer (IL-12 (p40)$_2$) was added to the IL-12-stimulated whole spleen cells. These data indicate that the IL-12p40 homodimer secreted by DC/Mϕ isolated from UV-irradiated mice is active and is capable of blocking rIL-12 biological activity.

Discussion

Although it has been recognized for ~20 years that total body UV exposure will impair APC function of DC/Mϕ residing in distant nonirradiated lymph nodes, the mechanism involved is not clear. More recent findings have indicated that the APC defect induced by total body UV exposure is selective, in that presentation to Th1 cells is severely depressed, whereas Ag presentation to Th2 cells occurs normally. Here also the mechanism involved, particularly the mechanism by which APC from UV-irradiated mice distinguish between Th1 and Th2 cells, is not clear. Based on the proposed role of CD86 in stimulating Th2 cells (27), some have suggested an effect of UV radiation on APC costimulatory molecule
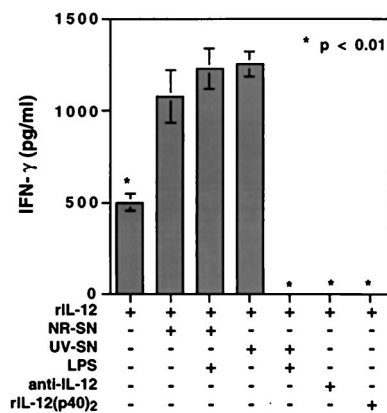

FIGURE 5. Levels of biologically active IL-12 in the supernatant of DC/Mϕ isolated from normal or UV-irradiated mice. DC/Mϕ from normal or UV-irradiated mice were cultured in the presence or the absence of LPS, and the biological activity of IL-12 was measured by inducing normal spleen cells to secrete IFN-γ. IL-12 in the supernatant fluid was calculated by culturing normal spleen cells with rIL-12p70, measuring IFN-γ secretion, and constructing a standard curve. Although LPS-stimulated DC/Mϕ from normal mice secreted significant amounts of biologically active IL-12, prior UV exposure significantly suppressed IL-12 biological activity ($p < 0.01$, by Student's t test).

FIGURE 6. Supernatants from DC isolated from UV-irradiated contain IL-12p40 homodimer biological activity. Normal spleen cells were cultured with rIL-12p70 (250 pg/ml) in the presence or the absence of supernatants from DC isolated from normal (NR-SN) or UV-irradiated (UV-SN) mice. Adding supernatant from LPS-stimulated UV-irradiated DC (UV-SN + LPS), but not LPS-stimulated normal DC (NR-SN + LPS), significantly suppressed IL-12-induced IFN-γ production ($p < 0.01$, by Student's t test). The addition of monoclonal anti-IL-12 Ab or rIL-12p40 homodimers to the spleen cells also significantly suppressed IFN-γ production, demonstrating the specificity of the reaction.

expression. Although it is clear that direct irradiation of APCs by UV will modulate the expression of costimulatory molecules (28), CD80/CD86 expression on APC isolated from the lymphoid organs of UV-irradiated mice is normal (29). Moreover, more recent findings question the absolute requirement for signaling through CD86 in the activation of Th2 cells (30, 31). This suggests that an effect of UV radiation on costimulation may not be involved.

We suggest depression of IL-12 secretion by APC as a more likely mechanism. It is clear that production of IL-12 by APC is an absolute requirement for the activation of Th1 clones (32–34). Equally clear is the IL-12-independent activation of Th2 cells (13, 35). The inability of DC/Mϕ isolated from UV-irradiated mice to secrete biologically active IL-12, as demonstrated here, would explain why Th1 cells are not activated when UV-APC are used to present Ag. In addition, the secretion of IL-12p40 homodimers and the binding of this inhibitory molecule to IL-12R on Th1 cells would further serve to depress the activation of this subset of T cells. Secretion of IL-12p40 homodimers may also help to explain why rather large amounts of rIL-12 are needed to overcome UV-induced immune suppression in vivo (15). Furthermore, because IL-12 is a critical factor in the activation of NK cells, the UV-induced suppression of IL-12p70 secretion and the enhancement of IL-12p40 homodimer secretion may provide a mechanism by which UV exposure down-regulates NK cell activity (36, 37), potentially contributing to skin cancer progression.

DC appear to have two different developmental stages that are defined by their function. Immature DC are efficient at Ag uptake and processing, but are poor stimulators of T cell activity. Some have suggested that DC at this stage of development may induce tolerance (38). Immature DC express low levels of MHC class II and costimulatory molecules, such as CD40 and CD86. Upon uptake of Ag, DC maturation begins. The cells increase surface expression of MHC class II molecules, increase expression of the costimulatory molecules CD40 and CD86, and become potent APCs. The mature DC acquire migratory activity and carry the Ag to the lymph node, where they produce IL-12p70 during the activation of T cells (39). It is entirely possible that any one of these steps can be targeted by UV irradiation or the cytokines induced by UV. However, because it is clear that the UV-induced APC defect occurs 3–5 days after exposure (9), an effect of UV exposure on DC maturation seems unlikely. We suggest that a more reasonable target is the mature DC. For example, Liu and colleagues recently described a DC (DC-1) that normally secretes IL-12 and promotes the activation of Th1 cells (40). We propose that UV radiation, or the cytokines that are secreted in response to UV exposure target IL-12 production by DC-1. If this is the case, one would predict that the other class of DC, derived from a plasmacytoid precursor (DC-2), a cell that promotes the activation and differentiation of Th2 cells, would not be affected by UV exposure.

These observations may have implications beyond the effect of UV radiation on cancer immunobiology. IL-12p40 homodimers have been shown to suppress the immune response to a variety of Ags, including pathogenic bacteria (25, 26, 41–43). Similarly, exposure to UV radiation suppresses the immune response to bacterial Ags, particularly to those Ags in which the induction of a Th1 reaction is protective (6, 44–46). The data presented here suggest the suppression of biologically active IL-12 as a potential mechanism.

Although these findings provide a mechanism to explain how DC from UV-irradiated mice distinguish between and provide different activation signals for Th1 and Th2 cells, how the suppressive signal is transmitted from the skin to the draining lymph nodes is still not clear. Because UV radiation does not penetrate further than the dermal-epidermal junction of the skin, direct irradiation of

the APC is clearly not involved. However, cytokines and immune regulatory factors are released following UV irradiation, and previous studies indicated that a cascade of events, including the release of PGE$_2$, IL-4, and IL-10, is involved (19). As these factors are reported to affect IL-12 production by DC/Mϕ (35, 47–49), we suggest that cytokine release in response to UV exposure is the signal that triggers the suppression of IL-12p70 secretion and the overproduction of IL-12p40 homodimers. Because UV exposure induces cytokine production in humans (50–52) and human biologically active (antagonistic) IL-12p40 has been described (24), it is entirely possible that a similar mechanism is contributing to UV-induced immune suppression in humans. Finally, the findings presented here, production of an immune modulatory cytokine (IL-12p40 homodimer) in response to an environmental insult (UV radiation), support the emerging concept that in addition to being the primary initiator of the immune response, the DC can also contribute to immune regulation.

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