Effects of ultraviolet radiation on cutaneous T cells
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Chapter 5
UVB radiation induces expression of interleukin-4 in normal human skin, and favors the development of type 2 responses in dermal T cells.

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Submitted

Abstract
In humans, exposure to physiological doses of UVB induces the appearance of a dermal cell infiltrate, mainly composed of CD4⁺ T lymphocytes. In this study, we intended to determine the type 1 or type 2 nature of these UVB-induced T cells on the bases of cytokine production or cell surface marker expression. After UVB-exposure, a considerable number of IL-4⁺ cells appeared in the dermis and epidermis whereas IFN-γ expression decreased as assessed by RT-PCR and immunohistochemistry. IL-4 was not expressed by the UVB-induced dermal T cells, leaving the identity of the actual IL-4 source still open. Concerning the type 1 (CCR5, CXCR3) and type 2 (CCR3, CRTH2) cell surface markers, the UVB-induced dermal T cells did not express any of these markers in situ. Because we could not discern between type 1 or type 2 T cells in situ, additional in vitro experiments were necessary. In contrast to the non-irradiated controls, T cells present in primary dermal cell cultures from irradiated skin preferentially produced IL-4 over IFN-γ. However, dermal T cells directly cloned from irradiated skin (in the absence of surrounding dermal cells) did not show such skewing to type 2, but were mainly IFN-γ producers, like the T-cell clones from nonexposed skin. Taken together, these findings show that UVB exposure might induce a change in the dermal microenvironment, shifting the development of T-cell responses towards significant higher numbers of type 2 T cells.
Introduction

In humans, following a single exposure to physiological dose of UV, a variety of immunomodulatory factors, such as TNF-α, IL-1, IL-1 receptor antagonist and IL-10, are induced and released into the irradiated skin (1). Exposure to UV induces also the appearance of a dermal inflammatory cell infiltrate, mainly composed of T lymphocytes (2). After UV exposure, CD4+ T lymphocytes, mostly of the memory/effector T-cell subtype as judged by their expression of the marker CD45RO, accumulate into the irradiated skin, peaking in the dermal perivascular area at day 2 after exposure and dwindling thereafter (3). Recruitment of a specific subset of memory/effector T cells may be important for the outcome of the inflammatory/immune reaction. Indeed, inflammatory T cells can regulate locally the function of other cells in the site of inflammation via secretion of a discrete subset of cytokines.

Human memory /effector T cells may be roughly divided into two polarised subtypes (i.e. type 1 and type 2 T cells) based on the pattern of cytokines they secrete, and the immune response they participate in (4). Type 1 T cells produce predominantly IFN-γ, which activates cytotoxic functions of effector cells such as macrophages and CD8+ cytotoxic T cells, and are involved in cell-mediated immune responses. In contrast, type 2 T cells are characterised by predominant production of IL-4, which inhibits several macrophage functions, and promotes humoral immunity. Recently, new types of T-cell subsets were introduced, the so-called T regulatory cells (Tr) 1 (5) and Tr2 (6), which may shut down ongoing inflammatory responses via a mechanism of bystander suppression, likely mediated by the production of the soluble immunosuppressive molecule IL-10 (7).

Polarised human T-cell subsets not only produce different sets of cytokines, but they also preferentially express certain cell surface markers (8). CD30 is a member of tumor necrosis factor receptor family that is expressed by activated T cells in the presence of IL-4 (9); the expression of this marker on T cells appears to be associated with type 2 responses in vivo (10). Human T cells acquire distinct profiles of chemokine receptors after polarisation induced by cytokines, a phenomenon that has been correlated with specific tissue-migration of T-cell subsets (11,12). CXCR3 and CCR5 are preferentially expressed at high levels on type 1 T cells (11-13). In contrast, type 2 T cells preferentially express CCR3, CCR4 and CCR8 (11,12,14-16). A novel member of the G protein-coupled leukocyte chemoattractant receptor family, which is selectively expressed in type 2 but not type 1 T cells, thereby named
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CRTH2 (chemoattractant receptor-homologous molecule expressed on type 2 T cells) has recently been described (17).

In connection to the type 1 / type 2 T-cell paradigm of immune regulation, UV-induced immunomodulation can be considered as a local and systemic dysregulation of the balance between pro-inflammatory type 1 cytokines and anti-inflammatory type 2 cytokines (18). In this view we asked whether the natural balance of the two discriminating cytokines IL-4 and IFN-γ would be modulated in normal human skin upon UVB-exposure, and in addition, we wondered about the nature of the cytokine production by T cells in the UVB-induced infiltrate. To study this, we took skin biopsies from healthy human volunteers who were locally exposed to an erythematogenic dose of UVB. We analysed the effects of UVB on the expression of IL-4 and IFN-γ at mRNA level, using RT-PCR techniques, and at protein level by immunohistochemistry. Further, we established dermal T-cell bulk cultures and T-cell clones (TCC) from irradiated and unirradiated control skin and examined their cytokine pattern by intracellular cytokine staining or ELISA and their phenotypes (type-1 versus type-2 marker expression) by flow cytometry.

Materials and Methods

Subjects, UV irradiations and biopsies. Five healthy Caucasian volunteers of sun-reactive skin types II or III were enrolled in this study. All participants gave informed consent according to the guidelines of the Medical Ethical Committee of the hospital. UVB irradiations were performed with a 1000 W xenon-arc lamp (Oriel, Stratford, CT) equipped with a 303-nm interference filter (Jenaer Glaswerke, Schott & Gen., Mainz, Germany). One week before the experiment, the individual MED was determined. A single dose of 4 MED of UVB was then given to multiple sites on the buttocks 14 and 2 days before taking biopsies. Unirradiated sites were used as controls. Five-millimetre punch biopsies were obtained under local anaesthesia and most of the reticular dermis was eliminated with scissors. The specimens used for immunohistochemistry were immersed in OCT freezing medium (Miles, Elkhart, IL), snap frozen in liquid nitrogen, and stored at −80 °C. The punch biopsies used for the preparation of dermal T-cell bulk cultures and dermal T-cell clones were transferred into sterile PBS.
RNA isolation and Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) for detection of IL-4 and IFN-γ mRNA. Ten cryostat sections (10 μm each) were homogenised in 600 μl of TRIzol Reagent (Gibco, Paisley, U.K.). Subsequently, the RNA was isolated according to the manufacturer’s protocol. The extracted total cellular RNA was reverse-transcribed in a reaction volume of 20 μl, and 1 μl of the resulting cDNA was amplified by cytokine-specific PCR. The PCR conditions and electrophoresis of the PCR products are described in detail elsewhere (19). The following specific primer sets were synthesized in our laboratory by an oligosynthesizer: GAPDH forward primer 5’-CGAGATCCCTCCAAAATCAA-3’ (nt 298-317) and GAPDH reverse primer 5’-AGGTAGGGTCCACCACCTGAC-3’ (nt 799-780); IL-4 forward primer ACTCTGTGCACCGAGTTGACCGTAA and IL-4 reverse primer TCTCATGATCGTCTTTAGCCTTTCC; IFN-γ forward primer GACTTCGACCCGAGATTGACGCTAA and IFN-γ reverse primer ACAGTCACAGGATATAGGAA. The PCR products were of the expected size and the specificity was confirmed by sequence analysis. The ethidium bromide-stained PCR products were scanned by EAGLE EYE R® II Still video system (Stratagene, La Jolla, Ca), and the signal strength was integrated to obtain a densitometric value for each PCR product. To allow semiquantitative analysis, in each sample the densitometric values of IL-4 and IFN-γ were related to the signal of the house-keeping gene GAPDH, which is assumed to be produced at constant rate. Two independent RT-PCR experiments per time-point per volunteer were performed, to obtain a duplicate determination of the cytokine mRNA densitometric signal per time-point.

Monoclonal antibodies (mAb). For immunohistochemistry and flow cytometric analysis, the following primary antibodies were used: mouse anti-human CD3 (Becton Dickinson, Mountain View, CA), fluorescein isothiocyanate (FITC)-labelled mouse anti-human CD3 (Becton Dickinson), FITC-labelled mouse anti-human CD4 and phycoerythrin (PE)-labeled mouse anti human CD8 dual color reagent (Dako, Glostrup, Denmark), mouse anti-human CCR5 (Becton Dickinson), mouse anti-human CXCR3 (R&D Systems Europe Ltd, Abingdon, UK), rat anti-human CCR3 (R&D Systems Europe Ltd), mouse anti-human CD30 (Immunotech S.A., Marseille, France), rat anti-human CRTH2 (clone BM16, a gift from Dr. K. Nagata, BioMedical Laboratories, Kawagoe, Saitama, Japan), mouse anti-human IL-4 (Genzyme, Cambridge, MA), mouse anti-human IL-4 (clone 1-41-1, a gift from Dr. Kalthoff, Novartis, Vienna, Austria), mouse anti-human IFN-γ (Genzyme).
**Immunohistochemistry.** Sections of 6 µm thickness were cut from the frozen skin biopsies at -24°C, after which they were allowed to dry overnight, and stored at -80°C. The sections were fixed in acetone for 10 min at 4°C before staining. Immunohistochemical single-staining was performed according to a protocol based on a two-step indirect peroxidase technique as described previously (20). Briefly, the cryostat sections were incubated with primary mAb mouse anti-human CD3, followed by an incubation with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako). Before counterstaining with haematoxilin, the horseradish peroxidase activity was detected with H₂O₂ as substrate and AEC (Sigma, St Louis, MO) as chromogen.

Immunohistochemical double-staining of T cells was performed to determine the simultaneous expression of CD3 and subset-specific markers. After incubating the sections with 10% normal goat serum (Dako) for 20 min, the following steps were performed at room temperature: (i) incubation for 60 min with one of the five mAbs, mouse anti-human CCR5, mouse anti-human CXCR3, rat anti-human CCR3, mouse anti-human CD30 or rat anti-human CRTH2; (ii) incubation for 30 min with a biotin-conjugated goat anti-mouse (Dako); (iii) incubation for 30 min with horseradish peroxidase-conjugated streptavidin (Dako); (iv) incubation with normal mouse serum (Dako) for 20 min; (v) incubation for 60 min with FITC-labelled mouse anti-human CD3; (vi) incubation for 15 min with a rabbit anti-FITC Ig (Dako); (vii) incubation for 30 min with alkaline phosphatase (AP) conjugated goat anti-rabbit Ig (Dako); (viii) AP activity was subsequently detected as a blue colour, using naphthol-AS-MX-phosphate (Sigma) as substrate and fast blue BB (Sigma) as azo dye; (ix) peroxidase activity was detected as an orange-red colour, using the chromogen AEC. Double-stained cells could be recognized by their purple colour. The double-staining procedure used to detect IL-4 and IFN-γ was the same described above except for the step (i), in which one of the two primary mouse anti-human IL-4 mAbs or mAb mouse anti-human IFN-γ were incubated overnight at 4°C.

The identification on the object glasses was covered before counting to enable blind quantification by three different investigators. Subdivision of the skin into two horizontal layers, i.e. epidermis and dermis, and counting of the stained cells was performed as described before (21). The numbers of clearly stained cell bodies were counted in three different serial sections per time point. The values of each biopsy specimen were adjusted to 10-mm horizontal section values by dividing with the horizontal width multiplied by 10. The total
mean of cell numbers per time-point was calculated from the mean values of corresponding
time-points of each volunteer.

**Preparation of single dermal cell suspensions from skin biopsy specimens and dermal**
**T-cell bulk cultures.** Because the UVB-induced T cell accumulation in the dermis had a
maximum at day two post irradiation (3) we used biopsies taken at this time-point.
Unirradiated skin was used as control. Epidermis and dermis were separated after overnight
incubation in 0.3 % Dispase (Boehringer Mannheim, Germany) at 4°C. The dermal tissue was
minced and incubated in PBS (2 h at 37°C) containing 0.2 % collagenase D (Boehringer
Mannheim), 40 U/ml DNase I (Boehringer Mannheim) and 2 % fetal bovine serum. The
dermal cell suspension was filtered in order to remove tissue debris and after the second wash
the cells were resuspended in culture medium, consisting of Iscove’s modified Dulbecco’s
medium (IMDM; Gibco) plus 5% pooled normal human serum. The dermal cells were seeded
in 200 µl culture medium in round-bottom 96-well plates (Costar, Cambridge, MA) at 2 x 10^5
cells per well, and 1 µl/ml phytohemagglutinin (PHA; Difco Laboratories, Detroit, Michigan)
plus 50 U/ml recombinant human IL-2 (Cetus Corp., Emmeryville, CA) was added to
stimulate T cell growth.

**Cytokine staining.** To determine intracellular cytokine expression, T cells were
stimulated for 4 h with PMA (Sigma) and ionomycin (Sigma) in the presence of 3 µg/ml
brefeldin A (Sigma), according to the protocol of Becton Dickinson. The cells were washed
and stained with allophycocyanin-labelled CD4. Intracellular staining with PE-labelled anti-
IL-4 and FITC-labelled anti-IFN-γ was exactly performed according to the protocol of Becton
Dickinson, using the mAb and isotype controls of the same manufacturer. The triple-stained
cells were measured with a FACScalibur and analyzed with CellQuest software (Becton
Dickinson).

**Generation of T-cell clones (TCC).** The number of T cells in the dermal cell
suspension was assessed by flow cytometric analysis after staining with FITC-labelled anti-
human CD3. Viable cells were identified as propidium iodide negative cells. TCC were
generated by seeding the dermal cells at 0.3 or 3 CD3^+^ cells per well in flat-bottom 96-well
plates (Costar) in the presence of “feeder” cells (300 Rad γ-irradiated peripheral blood
mononuclear cells from two unrelated donors [1 x 10^5 each] and 1 x 10^4 Epstein-Barr virus-
transformed B cells per well). The cells were maintained in culture medium supplemented
with 10 U/ml rh IL-2 and 1 µl/ml PHA. To expand the T-cell clones, they were transferred to
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24-well tissue culture plates (Costar) and were maintained in culture medium with 20 U/ml recombinant human IL-2. The medium was refreshed twice a week and the cell cultures were split if necessary.

Estimation of IL-4, IFN-γ and IL-10 production. TCC were tested for the production of cytokines 10-12 days after the last restimulation. The cells were washed 3 times and were transferred to a 96-well flat bottom culture plate, using 5 x 10^4 TCC in 200 µl culture medium per well. Triplicate cultures of TCC were stimulated either with the soluble antibody pair CD3/CD28 (CLB, Amsterdam, The Netherlands) or with 25 ng/ml PMA plus 1 µg/ml ionomycine. After 24 h, cell-free supernatant (100 µl) was collected. The remainder cells were cultured for another day in the presence of 0.3 µCi/well of [3H] TdR (Amersham, UK) to estimate the proliferation of the TCC as control for stimulation.

The amount of IL-4 and IFN-γ in the culture supernatants was determined with specific sandwich ELISA techniques, as described in details elsewhere (22). To determine the IL-10 concentration, we used the coating and biotinylated detecting anti-human IL-10 antibodies and the the IL-10 standard from PharMingen (San Diego, CA).

Flow cytometric analysis of TCC cell-surface markers. To determine the cell-surface markers, T cells (2 x 10^5/sample) were stained with primary mAb and PE-labelled secondary Ab, using PBS containing 1 % FCS and 0.1% NaN₃ as incubation and washing buffer. The flow cytometric measurements of minimal 10^4 cells per sample were performed with a FACScan and LYSIS II software from Becton Dickinson.

Statistical analysis. The unpaired student's t test was used for statistical evaluation taking p < 0.05 (two-tailed) as the level of significance.

Results

IL-4 expression in skin is enhanced by UVB exposure, whereas IFN-γ expression is decreased. In order to study whether UVB radiation could disturb the natural balance of IFN-γ (type 1) and IL-4 (type 2) cytokine expression in normal human skin, we performed RT-PCR analysis on RNA extracted from cryostat sections of biopsies derived from UVB-exposed or non-exposed skin. IL-4 mRNA was expressed at a very low level in unirradiated skin (Fig. 1). UVB exposure induced a significant increase in IL-4 mRNA expression at day 2 and day 14 after exposure. The IL-4 mRNA expression at day 14 was significantly higher
than the expression of IL-4 at day 2. In contrast, IFN-γ mRNA, which was readily detectable in unirradiated skin, showed a significant reduction in its expression at both day 2 and day 14 after UVB-exposure.

Figure 1. UVB exposure induced an increase in IL-4 mRNA and a decrease in IFN-γ mRNA expression in normal human skin. Exposures to 4 MED of UVB were performed 14 and 2 days before collecting skin biopsies. Unirradiated skin served as a control. RNA was isolated from cryostat sections and was subjected to semiquantitative IL-4 or IFN-γ specific RT-PCR. The densitometric values of the cytokine mRNA were related to the signal strength of GAPDH mRNA, which was considered to be a constantly produced product. Each bar represents the mean value of 6 determinations (i.e. 2 separate experiments in 3 volunteers). Data are expressed as the relative densitometric value of cytokine mRNA ± SD (* indicates p < 0.05).

In the next series of experiments we determined the expression of IL-4 and IFN-γ at protein level in the same skin biopsies. The skin sections were double-stained with mAb against CD3 and one of the two different mAbs against IL-4, which have been shown to be suitable for immunohistochemical staining (23). We found that in unirradiated control biopsies, IL-4 protein was not expressed. At day 2 after UVB exposure, numerous IL-4+ cells were found in the irradiated skin (Fig. 2A). The IL-4+ cells were most often seen in the papillary dermis and, to a lesser extent, in the suprabasal epidermal compartment. The cells that expressed IL-4 protein were not clustered, but rather had a scattered distribution. Using the anti-IL-4 from Genzyme, we found 300 ± 75 IL-4+ cells/10 mm section in the dermis and about 59 ± 18 IL-4+ cells in the epidermis (n=4). Only few of the CD3+ cells co-expressed IL-4 (2% of the total CD3+ T cells). These double-positive cells were located in the perivascular area (Fig. 2B). We could not detect any IL-4+ cells in cryostat sections obtained 14 days after UVB-exposure. However, an increased number of intraepidermal CD3+IL4- cells was observed in the
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Figure 2. IL-4 protein expression was induced in normal human skin at day 2 after UVB-irradiation, whereas IFN-γ expression was lost. Healthy volunteers were exposed to 4 MED of UVB 14 and 2 days before collecting skin biopsies. IL-4 (A-C) and IFN-γ (D) expression in T cells was determined by immunohistochemical double-staining, using anti-IL-4 or anti-IFN-γ mAbs (both from Genzyme) and CD3 to identify T cells. IL-4⁺ (black arrowhead) and IFN-γ⁺ cells are stained in red, CD3⁺ cells are stained in blue (open arrowhead), and double-stained cells can be recognized by their purple colour (asterisk). (A, B) Two days after UVB. (C) Fourteen days after UVB. (D) Only few cells in control unirradiated skin were CD3⁺IFN-γ⁺ (asterisk) and were located in the perivascular area. Most of the dermal perivascular CD3⁺ T cells are single stained (open arrowhead).

Figure 4. CXCR3 and CCR3 expression in normal and UVB exposed skin. Cryostat sections were double stained with mAb against CXCR3 or CCR3 (red) and CD3 (blue), using normal human skin or skin obtained two days after exposure to 4 MED of UVB. A) Among the CD3⁺ T cell dermal population (open arrowhead) in normal human skin only few CD3⁺CXCR3⁺ T cells (black arrowhead) are present. B) In the UVB-exposed skin occasional CD3⁺CCR3⁺ T cells (black arrowhead) can be found.
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We found that the CD4/CD8 ratio in the cultures derived from control skin was 0.79, but this ratio was increased to 1.9 in the dermal cell cultures from irradiated skin (n=2), reflecting our earlier observation that a preferential influx of CD4+ T cells occurs in UVB exposed skin (3). The CD4+ T cells in our bulk cultures were further analysed for the intracellular expression of IL-4 and IFN-\(\gamma\) (Fig. 3). In all dermal T-cell cultures, irrespective their history, the T-cell population consisted mainly of a mixture of single-positive IL-4 producing type 2 T cells and IFN-\(\gamma\) producing type 1 T cells. Only a minimal number of IL-4 / IFN-\(\gamma\) double-positive cells were found. Remarkably, the number of IL-4+ cells was 3-6 times higher in the CD4+ T cells derived from UVB-exposed skin, as compared to the unirradiated control cultures (Fig.3). Moreover, concomitantly the expression of IFN-\(\gamma\) of the UVB-exposed skin-derived T cells was considerably reduced in comparison to the control T cells.
in one of the two experiments. These results indicate that UVB radiation can shift the development of T-cell responses in the skin towards considerable higher numbers of type 2 T cells in the responding dermal T-cell population.

Figure 3. Development of type 2 T cells is enhanced in primary cultures of bulk dermal cells from UVB-exposed skin. Dermal bulk cultures were obtained from skin biopsies taken two days after irradiation with 4 MED, using unirradiated skin as control. Primary dermal cell cultures were stimulated with PMA and ionomycin in the presence of brefeldin A for 4 hours and subsequently stained for CD4 and intracellular expression of IL-4 and IFN-γ. The CD4 positive cells were electronically selected and the IL-4 and IFN-γ expression is shown in the dot plots.

UVB-induced dermal T cells in situ do not express markers related to type 1 or type 2 T cells. Type 1 T cells and type 2 T cells may be discriminated by the differential expression of surface molecules: type 1 T cells were reported to express CCR5 and CXCR3, whereas type 2 T cells, exhibit CCR3, CRTH2 and CD30. If this concept of mutually exclusive expression of type 1 or type 2 specific markers is valid, then this could be a way to determine the relative amount of each T-cell type in a given immune/inflammatory response in situ. Based on this concept, we attempted to assess the type 1 or type 2 nature of the T-cell infiltrate in UVB-exposed skin in situ by immunohistochemistry. Cryostat skin sections were double-stained with CD3 and one of each of the above mentioned markers. We found that only a few of the dermal CD3⁺ cells (207 ± 22 cells/10 mm section, n=2) coexpressed CXCR3 (less than 1% of the total CD3⁺ T cells) in unirradiated skin (Fig. 4A), whereas the T
cells were negative for all the other markers tested. There was no reason to believe that the stainings were not successful due to technical deficiency or unsuitable mAbs, because in sections from the lesional skin of patients with psoriasis vulgaris and atopic dermatitis, and in cryostat sections of lymph nodes, we were able to obtain clear specific positive staining (not shown). Analysis of the UVB-induced dermal CD3$^+$ cells (485 ± 56 cells/10 mm section) revealed that a few T cells coexpressed CCR3 (less than 1% of the total CD3$^+$ T cells) (Fig. 4B) and that the vast majority did not coexpress any of the other markers, at least not at detectable levels.

The population of dermal TCC generated from irradiated skin and from nonexposed control skin do not differ in their expression of type 1 and type 2 markers. Because we observed that UVB radiation favored the development of type 2 CD4$^+$ T cells in primary dermal cell cultures, the question arose as to whether these T cells were stable type 2 cells. TCC were generated from unexposed and UVB-exposed skin obtained from a healthy volunteer by a direct limiting-dilution protocol in the presence of recombinant human IL-2 and PHA. The cloning efficiency showed no major differences between unexposed and exposed skin. A total of 49 and 52 TCC were generated from unexposed or UVB-exposed skin, respectively. The TCC underwent at least 3 cycles of restimulation before they were tested for the expression of cell surface molecules and cytokine production.

We found that 65% of the total CD3$^+$ TCC from unexposed skin were CD3$^+$CD4$^+$ and 35% were CD3$^+$CD8$^+$ resulting in a CD4/CD8 ratio of 1.8. The $\alpha\beta$ TCR was expressed in 81% of these CD4$^+$ TCC. The majority of the TCC generated from UVB-exposed skin were CD3$^+$CD4$^+$ (85%), and only 15% of the total CD3$^+$ T cells were CD3$^+$CD8$^+$ providing a higher CD4/CD8 ratio of 5.5. Up to 97% of these CD4$^+$ TCC expressed the $\alpha\beta$ TCR. This result is in line with our previous reports in which the CD4/CD8 ratio in situ was increased as a consequence of raised $\alpha\beta$ TCR$^+$CD3$^+$CD4$^+$ and unaltered CD3$^+$CD8$^+$ T-cell numbers in the UV-irradiated site (3,20). The CD4$^+$ TCC were analysed for the expression of type 1 or type 2 cell-surface markers, as well as for IL-4 and IFN-\(\gamma\) secretion upon stimulation. As shown in Fig. 5C, all the TCC derived from the control skin as well as from the UVB exposed skin expressed the type 2 marker CCR3. However, only two TCC generated from unirradiated and irradiated skin, coexpressed CRTH2, the other type 2 marker, whereas all others were negative. Remarkably, many of the CCR3$^+$ TCC also expressed the type 1 marker CCR5 ranging from strong expression (Fig. 5A) to weak expression (Fig. 5B) to no expression.
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Figure 5. Phenotype of TCC generated from unexposed and UVB-exposed normal human skin. TCC were generated from the dermis of biopsies taken from unirradiated control skin or from skin which was exposed to 4 MED of UVB two days earlier. After at least three cycles of restimulation the TCC were stained for type 1 marker CCR5 or type 2 markers CCR3 or CRTH2 and the expression of these markers in one randomly selected TCC from control skin is shown in A). A TCC from UVB-exposed skin is shown in B). The underbroken line in A) and B) depicts the arbitrary borderline between negative and positive. A summary of the expression of CCR5, CCR3 and CRTH2 by TCC from unirradiated (black dot) and irradiated (open dot) skin are given in C).

As concerned the cytokine production, all TCC from unirradiated and irradiated skin could secrete moderate to high amounts of IFN-γ and most of the TCC were able to produce low to moderate levels of IL-4 as well (Fig. 6). There was no TCC which could produce IL-4 but not IFN-γ. As shown in Fig. 6, there was no difference between the two TCC on the basis of cytokine production. We could not find a correlation between any of the three type 1 / type 2 cell surface markers and the level of IL-4 and /or IFN-γ production of the TCC. In a recent publication (6), Shreedhar et al. demonstrated that in mice UVB radiation induced the appearance of a population of Tr2 cells, which produce high quantities of IL-10, but no IL-4 or IFN-γ. This prompted us to investigate whether the UVB-induced CD4+ T cells may represent Tr2 cells. Although IL-10 could be detected in several TCC, none of the TCC generated from unirradiated or irradiated skin produced high levels of IL-10 but no IL-4 or IFN-γ (Fig. 6,7).
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**Figure 6.** The production of IFN-γ and IL-4 by dermal TCC generated from unirradiated (A) and irradiated (B) normal human skin. Dermal TCC were generated by a limiting-dilution protocol from UVB-exposed (4 MED) or unirradiated control skin. The TCC were stimulated overnight with antibody pair CD3 and CD28. Subsequently, culture supernatant was harvested and the cytokine concentration was measured by ELISA. Each dot represents an individual TCC.

**Figure 7.** The expression of IL-10 and IL-4 by dermal TCC generated from unirradiated (A) and irradiated (B) normal human skin. One healthy volunteer was locally exposed to four MED of UVB and skin biopsies were taken two days after irradiations. Unirradiated skin served as a control. Dermal TCC were generated by a direct limiting-dilution protocol and after 3 cycles of restimulation, TCC were stimulated overnight with CD3 / CD28 antibodies. The cytokine level in the culture supernatant was assessed by ELISA. Each dot represents an individual TCC.
Discussion

A single exposure to a physiological dose of UVB induces a decrease in the number of intraepidermal T cells and a selective accumulation of perivascular CD4+ T cells in normal human skin during the first few days after irradiation (3). In this study, we asked whether the natural balance of type 1 / type 2 T cells would be modulated in this way by UVB radiation. We could detect only a very low signal for IL-4 mRNA and no IL-4 protein in normal human skin, which is in line with the findings of other researchers (24-26). These findings suggest that activated IL-4 producing type 2 T cells are commonly not present in normal skin. We showed that exposure to UVB induced the expression of IL-4, both at mRNA level and protein level, in the irradiated site. There is evidence from in vivo experiments in the murine system that IL-4 plays a key role in systemic immunosuppression following UV exposure (27-29). IL-4 is a potent pleiotropic immunomodulatory cytokine (30), which could locally affect EC and inflammatory cells, such as neutrophils, macrophages and T cells, which infiltrate UVB irradiated site over a period of time (3,31,32). For example, because IL-4 can downregulate the induced E-selectin expression on dermal EC (33), the augmented IL-4 expression might inhibit the UVB-induced E-selectin expression on dermal EC (34), thus limiting the recruitment of early inflammatory cells, such as neutrophils and macrophages (35). Further, IL-4 might enhance locally the phagocytic activity of infiltrated neutrophils (36) and macrophages (37), perhaps in order to facilitate the removal of the UVB-induced skin damage. IL-4 may also modulate the cytokine production of infiltrating neutrophils; it might synergize with TNF-α and induce IL-1 receptor antagonist production (38), which is known to be induced in the UVB-irradiated site (1).

Perhaps due to the application of different techniques, reports on the expression of IFN-γ in normal human skin are controversial. While some investigators have reported lack of IFN-γ, by RT-PCR (24,39) or by immunohistochemistry (26,40), low numbers of IFN-γ producing T cells were found, by flow cytometry, in the epidermal and dermal cell suspension obtained from healthy volunteers (25). It has been hypothesized that the cutaneous T cell populations may play a role in continuous immunosurveillance against the development of cutaneous neoplasm and persistent infection with intracellular pathogens (41,42). The findings of Szabo et al. (25) and our observation that a subset of human cutaneous T cells express IFN-γ, which may form the basis of a tumor-suppressor mechanism (43), corroborate the above hypothesis. In this view, the UVB-induced downregulation of IFN-γ expression in
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irradiated sites found in this study may be considered as a permissive factor for UVB-induced skin tumor and skin infection development. UVB may reduce the IFN-\(\gamma\) expression in skin through at least three different mechanisms. First, exposure to UVB causes a decrease in the number of intraepidermal IFN-\(\gamma\) producing T cells, which is probably a phototoxic effect. Second, IFN-\(\gamma\) production by dermal perivascular T cells may be suppressed in a paracrine manner by IL-10, which is locally produced by UVB-induced infiltrating macrophages (44). Third, UVB-induced IL-4 may synergize with IL-10 and further downregulate IFN-\(\gamma\).

As compared to the T cells from control skin, T-cell lines and TCC grown from irradiated skin biopsies were mostly of the CD4 TCR\(\alpha\)\(\beta\) T-cell subtype. This is in line with our earlier study where we showed that exposure to UVB induced an accumulation of TCR \(\alpha\)\(\beta\)^+CD4^+ T cells in the dermal compartment of the irradiated skin (3). In a previous study (manuscript submitted), we have found that the chemokine psoriasin was upregulated in normal human skin upon exposure to a single dose of UVB. Since psoriasin has been shown to be a selective chemokine for CD4^+ but not for CD8^+ T cells (45) we speculated that upregulation of this chemokine might be responsible for the selective accumulation of CD4^+ T cells into the irradiated site. Also other chemokine factors, such as IL-8, are released into irradiated skin (46). However, IL-8 is chemotactic for both CD4 and CD8 T-cell subsets (47). Interestingly, although IL-4 is not chemotactic for T lymphocytes, it inhibits only CD8^+ but not CD4^+ T cells chemotaxis towards IL-8 (47), thus providing another explanation for the selective accumulation of CD4^+ T cells.

We further investigated whether the UVB-induced T cells were the actual source of the UVB-induced IL-4. As assessed by immunohistochemical double staining, the vast majority of CD3^+ UVB-induced dermal T cells did not coexpress IL-4. Apparently, the major source of IL-4 found in the epidermal and dermal compartment of the irradiated site is not a T-cell. Investigation is currently performed to identify this UVB-induced cutaneous cellular source of IL-4. However, it is believed that IL-4 can not be easily detected by immunohistochemistry in T cells, because after synthesis, it is rapidly transported and does not accumulate in sufficient concentration to be detected with such a method (48). Moreover, we may also have underestimated the number of potential IL-4-expressing T cells since the UVB-induced T cells are likely in a resting state. Most of the infiltrated T cells expressed neither markers of recent activation, such as IL-2R and HLA-DR, nor the marker of late activation VLA-1 (3), and T cells in a resting state hardly express cytokines.
To solve this last issue, we have started in vitro cultures of dermal T cells, which enabled us to activate the cells, forcing them to show their cytokine-production potential and thus exhibiting their type 1 or type 2 nature. We found that dermal T cells in primary cultures from irradiated skin preferentially produce IL-4 over IFN-γ. This phenomenon might be due to the presence of IL-4+ cells, which appeared in the dermal perivascular area of the irradiated skin. At the start of the bulk cultures about 10% of the dermal cells consisted of T cells. Probably, the as yet unidentified IL-4+ cells were present in these cultures as well. The cytokine environment has been put forward as a major variable influencing T-cell skewing into type 1 or type 2 T-cell subpopulations. In this respect it is important to note that IL-4 can influence the differentiation of memory/effector T cells, especially those not dramatically polarized to a type 1 or type 2 pattern, to become polarized towards secretion of type 2 cytokines (49). The presence of IL-4+ dermal cells could be responsible for the enhancement of the development of the type 2 T cells in UVB-exposed skin. Although we did not test for it, one can assume that the dermal cell suspension from UVB-exposed skin, but not from control skin, also contained UVB-induced infiltrating macrophages, which produce large amounts of IL-10 (50). This cytokine, known to skew T-cell responses towards type 2, could also have contributed to the higher numbers of type 2 T cells in the dermal cells derived from irradiated skin. Another explanation might be that the UVB-induced T cells are intrinsically committed to produce IL-4 upon stimulation. In other words, the T cells that infiltrate the dermal perivascular area of the irradiated site are stable type 2 T cells. Apparently this is not the case since in this study, we found that the UVB-induced dermal T cells did not preferentially express any markers related to either type 1 or type 2 T-cell subpopulations, as assessed in situ by immunohistochemistry and in vitro by cytofluorometric analysis of TCC. However, it has to be taken into consideration that little is known about the stability of T-cell cytokine profile in vivo in humans (51,52), and identification of a specific cell-surface marker for type 1 or type 2 cytokine-producing T cells is difficult, since none of the markers identified so far is widely considered a truly selective marker of type 1 or type 2 T cells.

Recently, new types of T-cell regulatory subsets have been described in vivo experiments in the murine system. Trl cells are induced by chronic activation of T cells in the presence of IL-10, and they produce IL-10 and IFN-γ but not IL-4 (5). Tr2 cells, which have been found in lymph nodes from UVB-irradiated mice, produce IL-10 but not IL-4 or IFN-γ (6). We found that a small percentage of the TCC generated from irradiated skin as
well as from unirradiated control skin produced high levels of IL-10. However, all the IL-10 producing TCC also produced IFN-γ and moderate or low amounts of IL-4, indicating that such Tr1 or Tr2 cells were not induced in normal human skin by UVB.

In conclusion, exposure to a single physiological dose of UVB resulted in changes in the development of type 1 / type 2 cytokine T-cell responses in normal human skin. The UVB-induced T cells, which apparently are not committed to either type 1 or type 2 when they enter in the irradiated site, showed a skewing towards type 2 cytokine production in primary cultures in vitro when dermal cells are present. These findings suggest that the UVB-irradiated skin provides a microenvironment that is conductive to the development of type 2 T-cell.

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References


UVB favors the development of type 2 T-cell responses


38. Marie, C., C. Pitton, C. Fitting, and J.M. Cavaillon. 1996. IL-10 and IL-4 synergize with TNF-alpha to induce IL-1ra production by human neutrophils. Cytokine 8:147.


