T cells in psoriatic lesional skin that survive conventional therapy with NB-UVB radiation display reduced IFN-γ expression

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**Abstract**

Type 1 T cell-derived cytokine interferon (IFN)-γ is overexpressed in psoriatic lesional skin. Recently, we showed that a single high erythematous dose of broad-band ultraviolet B (UVB) irradiation reduces type 1 and favors type 2, i.e. interleukin (IL)-4, cytokine expression in normal and psoriatic skin. In this study, we wanted to see whether conventional narrow-band UVB (NB-UVB) therapy (i.e. repetitive exposure to non-erythematous doses) also affects type 1/ type 2 cytokine expression of T cells present in chronic plaque type psoriasis lesions. Staining of cryostat sections showed decreased expression of both IFN-γ and IL-4 *in situ* after NB-UVB therapy. Primary dermal T cell lines, derived from psoriatic lesional skin, displayed significantly decreased intracellular IFN-γ expression in CD4⁺ dermal T cells during and after the NB-UVB therapy as compared to pre-treatment values. Intracellular IL-4 expression was increased in most patients after the therapy. Analysis of the supernatants of these stimulated dermal T cells revealed that IFN-γ production decreased significantly following NB-UVB therapy whereas IL-4 expression increased in most patients in the T cell supernatants, confirming the intracellular determinations. In addition, IL-10 and transforming growth factor-β levels in the supernatants appeared to be increased in the majority of patients upon UVB therapy. Apart from the well-known killing effect of UVB on T cells, our results show that the improvement of psoriatic skin upon NB-UVB therapy is also due to a reduced capacity of the surviving dermal T cells to express the pro-inflammatory cytokine IFN-γ.
Introduction

Psoriasis is commonly regarded as a disease with a disturbed balance between type 1 T cells (expression of interferon (IFN)-γ) and type 2 T cells (expression of interleukin (IL)-4). The majority of investigations report a predominance of type 1 T cells in peripheral blood and in skin lesions although some controversial results were published as well 1-7. The view that type 1 T cells have a prominent role in psoriasis disease activity is supported by the successful application of anti-psoriatic therapies with type 1 cytokine suppressing capability 8-11.

Ultraviolet B (UVB) irradiation is known to influence the composition of the immunocompetent cells in skin. It depletes Langerhans cells and T cells from the epidermis 12,13, but on the other hand, it causes the influx of, in the order of appearance, neutrophils 14,15, macrophages 16, T cells 13 and Langerhans cells 17. These cells, together with the irradiated keratinocytes, act co-operatively to change the microenvironment in UVB-irradiated skin to cause a shift from type 1 to type 2 cytokine responses 18. T cells which infiltrate normal skin after UVB exposure have a decreased expression of IFN-γ and an increased expression of IL-4 19. This UVB-induced type 2 skewing is accomplished amongst others by neutrophils, migrating into the skin after UVB irradiation and having a high expression of IL-4 15. IL-4 is known to promote type 2 and inhibit type 1 T cell development. Macrophages invading the UVB-exposed skin express IL-10 in high amounts 20. Like IL-4, IL-10 inhibits type 1 immune responses, suppresses the synthesis of pro-inflammatory cytokines and counteracts the antigen presentation 21-23. In this way, IL-10 is, at least partially, responsible for the immunosuppressive effects of UVB 18. Expression of transforming growth factor-β (TGF-β), another important immunosuppressive cytokine, increases after in vitro UVB irradiation 24 and TGF-β may also contribute to the UVB-induced suppression of delayed type hypersensitivity 25.

Narrow-band (NB) UVB is the treatment of choice for patients with psoriasis who do not sufficiently respond to topical therapies 26,27. As compared to broad-band (BB)-UVB therapy, it is less erythemogenic 26. Moreover, the cumulative doses needed in the treatment of psoriasis are less than that of BB-UVB decreasing the risk of carcinogenesis 28. Similar to BB-UVB therapy, it has immunosuppressive effects which likely participate in the clinical improvement. Considering the crucial role of T cells in the pathogenesis of psoriasis 29, it seems to be an important mechanism that NB-UVB causes T cell depletion in psoriatic skin, even more effectively than BB-UVB 30. Jones and co-workers showed that the peripheral blood mononuclear cells from psoriasis patients have less lymphoproliferative activity upon stimulation after NB-UVB therapy 31. They also found that these cells express less IFN-γ after NB-UVB therapy. We recently observed that a local single high dose of BB-
UVB irradiation of lesional skin of psoriasis patients causes a decrease in IFN-γ and an increase in IL-4 expression as determined by immunohistochemical staining in skin sections in situ and by intracellular staining in dermal T cells in vitro. Here we describe that upon therapeutic low-dose NB-UVB exposure both IFN-γ and IL-4 expression was reduced in situ. Furthermore, in vitro stimulated dermal T cells from NB-UVB-irradiated psoriatic lesions express markedly decreased IFN-γ as compared to pretreatment dermal T cells indicating that NB-UVB therapy leads to a reduction of type 1 characteristics in dermal T cells in psoriatic skin.

Materials and Methods

Patients, NB-UVB therapy and skin biopsies. Thirteen patients with chronic plaque type psoriasis were included in this study which was approved by the local ethical committee. Patients with a history of photo-aggravated disease, with cutaneous malignancy or using phototoxic medications were excluded. Systemic anti-psoriatic medications and topical treatments other than emollients were discontinued for a minimum of 4 weeks and 2 weeks, respectively. Patients received the standard NB-UVB therapy which was given thrice weekly in a TL-01 lamp (Philips, The Netherlands) equipped cabinet. The initial UVB dose was 0.08 and 0.1 J/cm² for skin phototypes II and III, respectively. Dose was increased by $3\sqrt{2}$ times at each subsequent exposure. In case of UVB-induced skin irritation, previous dose was applied or treatment was stopped according to the severity of the reaction. Psoriasis area and severe index (PASI) was determined before, during and after the therapy. Five mm punch biopsies were obtained before treatment, at the third week of treatment and after treatment from the same lesion. Biopsies were either transferred to sterile PBS containing 100 mg/ml gentamycin (Duchefa, Haarlem, The Netherlands) for subsequent T cell cultures or were snap frozen and stored at −80 °C for immunohistochemistry.

Immunohistochemistry. Cryostat sections (5 mm) were stained with mouse anti-human CD3 (Dako, Glostrup, Denmark), mouse anti-human IFN-γ (R&D Systems, Minneapolis, MN), and mouse anti-human IL-4 (Immunex, Seattle, WA) as described before. Shortly, primary antibodies and isotype controls were applied to acetone fixed sections followed by incubation with biotinylated goat-anti-mouse (Dako) and streptavidin peroxidase (Dako). Peroxidase activity was detected as red colour using the chromogen 3-amino-9-ethylcarbazole (Sigma, St Louise, MO). Hematoxylin staining was applied to observe the cell nuclei. The thickness of the epidermis was measured at the thickest point. The length
of the 3 sections from each biopsy was measured and positively stained cells were counted blindly. Positive cells in epidermis and dermis were counted separately and were expressed as cell numbers per 1 mm².

Dermal T cell cultures. Biopsies were incubated with 0.3 % dispase II (Boehringer Mannheim, Mannheim, Germany) overnight at 4 °C to enable separation of the epidermis and dermis. Dermis was minced and incubated with 0.2 % collagenase D (Boehringer Mannheim), 40 U/ml DNase I (Boehringer Mannheim) and 2 % fetal calf serum in PBS for 2 h at 37 °C while shaking. After that, the mix was filtered and the cell suspension was placed in a 96-well plate (1-2 x 10⁵ cells in 200 ml per well) containing Iscove’s modified Dulbecco’s medium (IMDM; Gibco Laboratories, Detroit, MI) with 10 % human pooled serum, 50 U/ml human IL-2 (Cetus Corp., Emmeryville, CA) and 1:1000 phytohemagglutinin (PHA; Difco Laboratories, Detroit, MI) to favor T cell growth.

Intracellular cytokine staining. Resting T cells were stimulated in IMDM containing 5 % human serum, 25 ng/ml phorbo l 12-myristate 13-acetate (PMA; Sigma), 1 mg/ml ionomycin (Sigma) and 3 mg/ml brefeldin A (Sigma) for 4 h at 37 °C, according to the protocol for intracellular cytokine staining of Becton Dickinson (San Diego, CA). The cells were first stained either with allophycocyanin conjugated mouse anti-human CD4 (Becton Dickinson) or anti-human CD8 (Becton Dickinson) for 15 min at RT. Upon permeabilization for 10 min at RT, they were incubated with fluorescent isothiocyanate-conjugated mouse anti-human IFN-γ (Becton Dickinson) and phycoerythrin-conjugated mouse anti-human IL-4 (Becton Dickinson) for 30 min at RT. The stained cells were fixed with 1 % paraformaldehyde and analysis was performed by FACScalibur equipment and CellQuest software (Becton Dickinson).

Cytokine detection by ELISA. T cells cultured from the dermal cell suspensions were stimulated with soluble 1:1000 diluted anti-human CD3 and anti-human CD28 (both from Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) in 200 ml IMDM per well in a 96-well plate. After 24, 48 and 72 hours the supernatants were collected and stored at -20 °C. The concentration of cytokines was determined by specific sandwich ELISA’s using antibodies to IFN-γ (gift of Dr. P.H. van der Meide, U-cytech, Utrecht, The Netherlands), IL-4 (BD Pharmingen), IL-10 (BD Pharmingen) and TGF-β (R&D Systems), following the instructions of manufacturers.
Statistics All the data were analysed for normal distribution first. Accordingly, the means of data from psoriasis area severity index (PASI) and immunohistochemical staining, obtained before, during and after NB-UVB therapy, were compared by paired Student’s t-test. Flow cytometry and ELISA results were presented as medians. The differences before, during and after NB-UVB therapy were analysed by the Mann-Whitney U-test and $p < 0.05$ was considered as statistically significant.

Results

Improvement of psoriasis after NB-UVB therapy. The psoriasis patients (7 male and 6 female; age 28 - 77 years, mean 53 ± 15) included in this study had a PASI between 4.4 - 13.2 (mean 9.7 ± 2.2). The duration of the NB-UVB treatment was between 9 -11 weeks (mean 10 ± 0.6 weeks). The PASI of all patients decreased significantly 3 weeks after the start of the therapy (mean 6.5 ± 1.9) and a further significant decrease of the PASI was appreciated after therapy (mean 2.0 ± 1.6, $p < 0.001$; Figure 1).

The clinical healing of psoriatic lesions was accompanied by decreased epidermal thickness (66 % decrease, $p < 0.05$) and a reduction of T cell numbers in epidermis (88 % decrease, $p < 0.05$) and in the dermis (60 % decrease, $p < 0.05$; Figure 2).

Reduced expression of IFN-γ and IL-4 in psoriatic skin in situ after NB-UVB therapy. Abundant expression of IFN-γ was detected in sections from lesional psoriatic skin before NB-UVB therapy (6.5 - 37.5 cells / mm², mean 17.3 ± 5.4). This expression was mainly confined to the cellular infiltrate in the papillary dermis. Double staining showed that IFN-γ expression co-localized with CD3+ T cells (data not shown). This result was in agreement with our earlier results. After NB-UVB, IFN-γ expression decreased significantly
Reduced percentage of IFN-γ⁺CD4⁺ T cells present in psoriatic dermis after NB-UVB therapy. We have shown that most IFN-γ expression in situ was in the papillary dermis in psoriatic skin and that dermal T cells were only partially depleted after NB-UVB. Next we wanted to know whether the T cells, remaining in the dermis after NB-UVB, differed in their IFN-γ and IL-4 expression as compared to the dermal T cells in unirradiated lesional skin. For this purpose, dermal cell suspensions were prepared from psoriatic skin before, during and after NB-UVB therapy and these cells were cultured for T cell growth. Intracellular IFN-γ expression of CD4⁺ dermal T cells was between 4.3 - 61.5 % (median 12.8 %) before therapy, between 3.1 - 36.8 % (median 14.8 %) after 3 weeks of therapy and between 0.7 - 15.6 % (median 9.7 %) after therapy. The reduction of IFN-γ expression in this group of 10 psoriasis patients was significant after treatment as compared to the pre-treatment values (Figure 5A). The percentage of CD8⁺IFN-γ⁺ T cells was between 12.7 - 67.7 % (median 31.9 %) before therapy, between 0.7 - 64.1 % (median 34.2

Figure 2. Histological evaluation of epidermal thickness and T cell numbers after NB-UVB therapy. Clinical healing was accompanied by histological improvement of the skin lesions. The epidermal thickness was decreased, as well as the number of T cells, mainly in the epidermis and to a lesser extent in the dermis, as determined by CD3 staining of frozen sections (n = 5, * p < 0.05).

to 0 - 25 cells/ mm², mean 5.4 ± 4.9 (68.8 % decrease, p < 0.05; Figure 3). Interestingly, IL-4 was also present in the pre-treatment biopsies, though in low numbers (0.5 - 3 cells/ mm², mean 1.7 ± 0.5), as single positive cells in epidermis and / or dermis, having no preferential localization. After the NB-UVB therapy, no IL-4 expression could be detected in any of the psoriatic sections (Figure 4).
Figure 3. IFN-γ expression before and after NB-UVB therapy. Expression of IFN-γ was significantly decreased upon NB-UVB therapy. Left panel, IFN-γ expression was abundant at papillary dermis before the therapy as seen as red stained cells. Middle panel, this expression markedly decreased following the therapy (magnification x 200). Right panel, the decrease of IFN-γ expression was already significant at 3rd week of therapy (n = 5, * p < 0.05) (≥ page 189).

Figure 4. IL-4 expression before and after NB-UVB therapy. As shown in the left panel, IL-4 occasionally was expressed as single cells (arrows) in epidermis and dermis of lesional skin before NB-UVB therapy. In the middle and right panel is illustrated that, this expression disappeared completely after NB-UVB therapy (n = 5, magnification x 200, * p < 0.05) (≥ page 189).

After 3 weeks of therapy and between 0.7 - 84.5 % (median 11.9 %) after completion of the therapy. Although the median IFN-γ expression of CD8⁺ T cells was decreased upon NB-UVB therapy, this reduction was, however, not significant (Figure 5B). The percentage of IL-4 expressing CD4⁺ T cells was between 0.6 - 26.8 % (median 11.5 %) before the therapy. In 6 out of 10 patients, IL-4 expression was increased after the treatment (Figure 5C), but judged as a group this increase was not significant (2.5 - 77.8 %, median 11.7 %). Similarly, CD8⁺ T cells from 4 patients showed increased expression of IL-4 after the therapy (Figure 5D), although this change, like in the CD4⁺ T cells, did not reach statistical significance (before therapy 1.4 - 29.3 %, median 8.2 % and after therapy 0.6 - 34.3 %, median 8.5 %). As concerned the IL-4 expression at week 3 of the therapy, an insignificant decrease was observed in the percentages of IL-4 expressing CD4⁺ and CD8⁺ T cell lines (6 out of 10 and 7 out of 10) respectively, as compared to pre-treatment values (IL-4⁺CD4⁺ T cells between 1.8 - 18 %, median 6.1 % and IL-4⁺CD8⁺ T cells between 0.9 - 16.9 %, median 6.7 %; Figure 5C and 5D).
Dermal T cells from psoriatic skin display reduced production of IFN-γ after NB-UVB therapy. To estimate the cytokine secretion by the lesional T cells, the dermal T cell lines were stimulated with CD3 and CD28 for 24 h, 48 h, and 72 h after which the supernatants were tested by ELISA. The highest cytokine expression was found in the supernatants yielded at 48 h (data not shown). Because of this, cytokine determinations were done in 48 h-supernatants in all further experiments. Similar to the results of intracellular cytokine determination in dermal T cells from psoriatic skin, the amount of IFN-γ in T cell supernatants decreased significantly after the NB-UVB therapy (mean IFN-γ expression 785 pg/ml after the treatment, p < 0.05; Figure 6A). The presence of IL-4 in T cell supernatants increased in 6 out of 8 patients therapy (mean IL-4 expression before therapy 351.5 pg/ml...
Figure 6. Expression of IFN-γ, IL-4, IL-10, and TGF-β in the supernatants of activated dermal T cells from psoriatic skin lesions. a, IFN-γ expression in the supernatants of anti-CD3 and anti-CD28 activated dermal T cells decreased significantly after NB-UVB therapy (* p < 0.05). b, The supernatants from 6 out of 8 dermal T cell lines contained more IL-4 after NB-UVB therapy, but this change was not consistent. c and d, Expression of IL-10 and TGF-β increased in 5 out of 8 patients, but this was also not a consistent finding (n = 8). Individual bars represent patients over the time points of the treatment.

and after therapy 748 pg/ml; Figure 6B). Because the anti-inflammatory cytokines IL-10 and TGF-β may participate in immunomodulation upon UVB therapy, we decided to test the T cell supernatants for these cytokines as well. The IL-10 expression in 5 out of 8 patients increased after the therapy (mean IL-10 concentration before therapy 2507 pg/ml and after therapy = 4707 pg/ml; Figure 6C). The expression of TGF-β was quite low as compared to the level of the other cytokines in the supernatants of dermal T cell lines. This expression also increased in 5 of 8 patients after the NB-UVB therapy (mean TGF-β concentration before therapy 62.5 pg/ml and after therapy 108.5 pg/ml; Figure 6D). Thus, the production of IL-4, IL-10, as well as TGF-β, all capable to counteract the production of IFN-γ, showed a tendency to be increased by NB-UVB therapy, although this enhancement was not significant.
In this study we demonstrated that NB-UVB therapy resulted in a significant reduction of IFN-γ expression in the psoriatic lesional skin in situ. This decrease was not simply due to the reduction of T cell numbers upon NB-UVB irradiation (88 % in epidermis and 60 % in dermis). As compared to pre-treatment lesional dermal T cells, dermal T cells present in the dermis after the NB-UVB therapy produced less IFN-γ, as based on intracellular flow cytometry and ELISA determinations. Therefore, another crucial effect of NB-UVB therapy on the T cell population in psoriatic skin is, apart from the depletion of T cells, clear inhibition of IFN-γ expression in the dermal T cells surviving NB-UVB.

The results of this study and studies of other researchers showed that the decrease in number of T cells after UVB therapy is mostly confined to the epidermis, while this decrease is less pronounced in the dermis. This is likely due to the limited capacity of UVB to penetrate through the epidermis. Nevertheless, UVB irradiation can cause changes in the cellular composition of the dermis. The effects of UVB exposure on dermal T cells can be a direct effect of UVB as approximately 9 % of UVB with a wavelength of 313 nm can penetrate the skin. Because T cells can be killed with doses of UVB as little as 1-10 % of estimated MED for a Caucasian individual, UVB dose reaching the dermis is still high enough to have phototoxic effects on dermal T cells. On the other hand, cutaneous T cells may receive indirect effects of UVB via alterations in the levels of cytokines (e.g. IL-1, IL-8, TNF-α and many others) in the irradiated skin which likely are produced by keratinocytes. Interestingly, UVB-exposed Langerhans cells lose their capacity to stimulate Th1 cells, but not Th2 cells, which could explain the switch from type 1 to type 2 cytokine responses in UVB-irradiated skin. This UVB-caused immunosuppression in the skin can be transmitted to the draining lymph nodes by migration of immunocompetent cells and altered cytokine levels, eventually leading to the dermal and systemic failure of the immune system.

We have recently demonstrated that irradiation of normal and psoriatic lesional skin with a single high erythemal dose of BB-UVB induced or increased IL-4 expression in situ. In contrast however, present study revealed a decreased in situ expression of IL-4 in the skin sections after the NB-UVB therapy (repetitive non-erythemal doses). It is unlikely that this discrepancy was caused by the different UVB sources, because a very recent study revealed similar immunological effects after exposure to equal erythemal doses of NB-UVB or BB-UVB. Our observation that a nonerythemogenic dose of UVB does not induce IL-4 expression in situ in normal skin (Teunissen, unpublished data), makes us believe that the differences between our earlier and present studies could be explained by high dose
versus low dose UVB exposure. A high dose (4 times the minimal erythema dose) of BB-UVB causes inflammation and the infiltration of, amongst others, neutrophils that happen to be IL-4+ 15,40. In case of repetitive non-erythema low dose NB-UVB there is no inflammation and no infiltration of immunocompetent cells, hence the absence of IL-4 in the chronic NB-UVB exposed skin is related to a lack of neutrophil influx. The few IL-4 expressing cells in untreated psoriatic skin of some patients were identified as neutrophils 40 and they were not found in UVB-treated skin, probably due to the remission of the local inflammatory activity. We did not study the fate of these intralesional neutrophils after exposure to NB-UVB, but it is reasonable to think that they died (because it is well known that these cells have a short life) rather than they recirculated.

Concerning the effect on IFN-γ and IL-4 production in primary cultures of T cells from NB-UVB exposed skin, we demonstrated a reduced IFN-γ but increased IL-4 production in most T cell lines. This effect of repetitive non-erythema UVB irradiation is quite similar to the effect of one single exposure to a high erythema dose of UVB, as we reported before 40. In case of high dose BB-UVB treatment inflammation occurs, and related to this, infiltration of IL-4+ neutrophils and IL-10+ macrophages takes place, both creating conditions for type 2 T cell skewing. In case of NB-UVB therapy non-erythema doses are used which do not provoke inflammation and no influx of neutrophils and macrophages, but nevertheless the intralesional T cells also display significantly reduced IFN-γ expression and increased expression of IL-4 in most T cells. In line with our results, it was recently reported that NB-UVB therapy causes decreased IFN-γ and increased IL-4 expression in psoriatic skin lesions 41. Although the enhancement of IL-4 expression in dermal T cells was less pronounced after repetitive low dose NB-UVB than observed after high dose BB-UVB, it is important to note that these different UVB irradiation protocols both lead to a significant decrease in the expression of proinflammatory cytokine IFN-γ. Suppression of type 1 cytokine response and induction of type 2 cytokine response as a result of exposure to UVB can play a prominent role in the healing of psoriasis lesions. This view is supported by the effectivity of treatments promoting the type 2 immune responses e.g. IL-4 11. In clinical trials, IL-10 was also found to be effective as a treatment for psoriasis 10,42. Interestingly, we found that the IL-10 expression was increased in most patients after NB-UVB therapy. Another cytokine of interest being produced by T cells was TGF-β 43, because it has anti-proliferative actions on keratinocytes 44,45. This expression increased in most patients after the NB-UVB therapy, but the amount was low as compared to the other measured cytokines and
it is not clear whether TGF-β expression from T cells contributes to the immunosuppressive effects of UVB.

The results of the present study emphasize that immunological changes occur in the psoriatic skin after NB-UVB therapy. It is clear that the depletion of epidermal T cells expressing IFN-γ contributes to the therapeutic effect of UVB therapy as these cells are known to be involved in the pathogenesis of psoriasis. Our in vitro results show that the intralesional T cells surviving NB-UVB therapy have a clearly different phenotype in terms of their cytokine expression profile than T cells in the untreated lesional skin. The former have a diminished capacity to express IFN-γ and an increased capacity to express IL-4, IL-10 and TGF-β in lesions of most psoriasis patients. This loss of type 1 phenotype and skewing to a type 2 phenotype could be another immunological mechanism in therapeutic effectiveness of NB-UVB-radiation on psoriatic lesions showing that the changes after NB-UVB therapy are not only confined to epidermis. It is not yet known, however, whether this change is mediated directly through the effect of UVB radiation on T cells or, indirectly, through the alteration of environmental conditions of these cutaneous T cells.

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References


