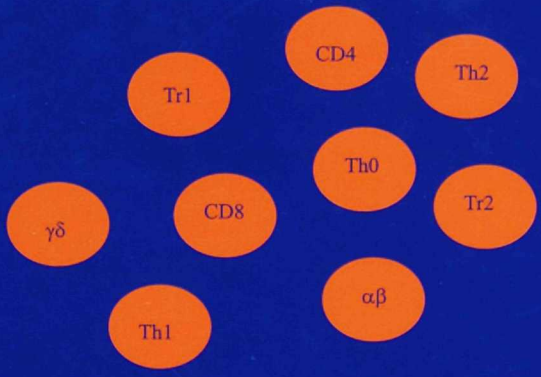
Effects of Ultraviolet Radiation on Cutaneous T cells



Sergio Di Nuzzo

Stellingen

1. A single exposure to ultraviolet radiation results in intraepidermal T cells depletion of normal human skin, which may be seen as a mechanism of protective immunosuppression (this thesis).

2. UVB radiation preferentially induces recruitment of memory $CD4^+$T cells in normal human skin that may be of importance for eradication of UV-induced neo-antigens (this thesis).

3. The expression of the $CD4^+$T-cell chemoattractant psoriasin *in vivo* is strongly upregulated both at mRNA level and at protein level in UVB-exposed skin, which correlate with the selective influx of $CD4^+$ T cells (this thesis).

4. The abundant use of many vowels (including double vowels), as well as the construction of long word makes the Dutch language, in its written form, looks like someone sat on a typewriter! (The Dutch Courier, Australia).

5. Science, as such, cannot prove God exists, but neither can it prove there is no God.

6. Since the Dutch are so proficient with languages, non è giusto che la tesi possa essere difesa in lingua olandese, inglese, francese e tedesca ma non in lingua italiana.

Stellingen behorende bij het proefschrift "Effects of Ultraviolet radiation on cutaneous T cells"
Sergio Di Nuzzo
Amsterdam, Mei 2000

Effects of Ultraviolet Radiation on Cutaneous T cells

SAMENVATTING

De mens staat in toenemende mate bloot aan ultraviolette (UV) straling door een veranderend leefpatroon (meer vakantie(s) in zonnige landen en meer vrije tijdsbesteding buitenshuis), door de onjuiste opvatting dat een bleke huidskleur is geassocieerd met ziekte (men roostert zich op zonnebanken) en door verdunning van de ozon laag, welke nodig is om deze schadelijke straling, afkomstig van de zon, tegen te houden. Het is bekend dat UV-straling leidt tot een verzwakking van het immuunsysteem, met name in de huid maar bij hoge doseringen ook in het gehele lichaam. Het immuunsysteem is een samenspel van verschillende cellen waardoor een organisme in staat is zich te beschermen tegen infecties en uitgroei van tumor cellen. De dermatoloog gebruikt onder gecontroleerde condities UV straling als therapie om bepaalde huidziekten (bijvoorbeeld psoriasis of atopisch dermatitis) met succes te behandelen. Het werkingsmechanisme is echter onbekend.

T cellen zijn een zeer belangrijke groep van cellen die zorgen dat het immuunsysteem goed functioneert. Ook in de epidermis en dermis komen T cellen voor. In dit onderzoek hebben we het lot van de T cellen in de huid na blootstelling aan UV bestudeerd, om meer inzicht te krijgen hoe UV straling het immuunsysteem in de huid moduleert en om te bepalen wat de rol van de T cel hierin is. Hiertoe hebben we huidbiopten genomen van gezonde vrijwilligers na een eenmalige blootstelling aan een fysiologische dosis kunstmatige zonnestraling (SSR: "solar-simulated radiation"; welke UVA en UVB bevat), UVB of UVA. Een biopt van onbestraalde huid werd als controle gebruikt.

We vonden dat gedurende enkele dagen na een enkele bestraling met SSR, UVB of UVA de T cellen in de epidermis sterk in aantal afnam of zelf verdwenen (Hoofdstuk 2) . Deze bevinding bevestigde onze hypothese dat een fysiologische dosis UV de T cellen in de epidermis kan doden. Er is door anderen gepostuleerd dat T cellen een rol zouden spelen in de bewaking tegen het ontstaan van huidtumoren en aanhoudende infecties. Het verminderen of verdwijnen van T cellen na UV bestraling zou kunnen worden gezien als een oorzaak van het ontstaan van UV-geïnduceerde huidkanker of infectie. In tegenstelling tot de resultaten in de epidermis, werd in dermis geen afname van het aantal T cellen waargenomen, maar juist een toename van het aantal T cellen, met name rondom de bloedvaten in de papillaire dermis, na bestraling met SSR en UVB, maar niet met UVA. Deze toename van T cellen werd veroorzaakt door selectieve infiltratie van $CD4^+$ T cellen (Hoofdstuk 2). Nadere analyse wees uit dat deze T cellen niet-geactiveerde "memory" T cellen zijn en dat ze $\alpha\beta$ T cel-receptoren dragen (Hoofdstuk 3). Bij de migratie van cellen vanuit het bloed naar de weefsels zijn diverse moleculen betrokken (bijvoorbeeld adhesie moleculen en chemotactische cytokinen), waarvan de aan- of afwezigheid nauwgezet gereguleerd dient te worden. Correlatie tussen de UV-geïnduceerde expressie van ICAM-1 door endotheel cellen en de toename van LFA-1 positieve T cellen in de dermis suggereert dat het adhesie molecuul-paar LFA-1/ICAM-1 betrokken zou kunnen zijn bij de UV-geïnduceerde infiltratie van T cellen (Hoofdstuk 4). Tevens wordt in dit hoofdstuk aangetoond dat de expressie van het molecuul psoriasin, selectief chemotactisch voor $CD4^+$ T cellen, geïnduceerd wordt door UV en qua locatie en tijdstip na bestraling correleert met de geïnfiltreerde $CD4^+$ T cellen, hetgeen suggereert dat mogelijk psoriasin verantwoordelijk is voor de selectieve migratie van $CD4^+$ in de UV-bestraalde huid. Wat betreft het effect van UVB straling op de balans van type 1 T cellen [interferon-γ producerend] en type 2 T cellen [interleukine-4 (IL-4)producerend] in de huid vonden we dat deze straling de respons van type 1 cellen verminderde en tegelijkertijd de ontwikkeling van type 2 T cellen bevorderde en dat dit mogelijk gerelateerd is aan het door UV-veranderde dermale micromilieu (Hoofdstuk 5). Opmerkelijk was de bevinding dat er IL-4 positieve cellen in UVB-bestraalde huid verschenen (Hoofdstuk 5), welke geen kenmerken hebben voor T cellen (CD3), mest cellen (tryptase), "natural killer" cellen (CD56) en macrofagen (CD36), maar wel kenmerken (CD11b en CD15) van granulocyten bezitten (Hoofdstuk 6).

Concluderend kunnen we stellen dat een eenmalige blootstelling aan UV straling leidt tot een langdurige verandering in het aantal en samenstelling van verschillende subpopulaties van T cellen in de epidermis en dermis, waarbij tevens door het locale micromilieu de ontwikkeling van type 2 T cellen bevorderd wordt ten opzichte van type 1 T cellen. Deze veranderingen aan de T cellen kunnen een relevante rol spelen in de UV-geïnduceerde remming van het immuunsysteem in de huid.

Effects of Ultraviolet Radiation on Cutaneous T Cells

Printing of this thesis was financially supported by: Huidstichting Chanfleury van IJsselsteijn, European Immunodermatology Society, Stiefel Laboratories, Janssen-Cilag, University of Amsterdam, Abbott, Galderma Nederland, Roche Nederland, UCB Pharma, Smith & Nephew, Bipharma, Van der Bend, Sanofi-Synthelabo, Novartis Pharma, Bauerfeind.

Di Nuzzo, Sergio
Effects of Ultraviolet Radiation on Cutaneous T cells/S. Di Nuzzo
Thesis University of Amsterdam.- With ref.
ISBN 90-9013659-2
NUGI 743
Subjects headings: Ultraviolet radiation/T-cell/Skin/Humans
A copy of the CIP entry for this book is available upon request from the Koninklijke Bibliotheek, Den Haag

Printed by PrintPartners Ipskamp, Enschede

Effects of Ultraviolet Radiation on Cutaneous T Cells

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam,
op gezag van de Rector Magnificus
Prof. Dr. J.J.M. Franse
ten overstaan van een door het college voor promotie ingestelde
commissie in het openbaar te verdedingen in de Aula der Universiteit

op dinsdag 16 mei 2000 te 10.00 uur

door

Sergio Di Nuzzo

geboren te Pietramelara (CE), Italië

Promotiecommissie

Promotor:	Prof. dr. J.D. Bos
Co-promotores:	dr. M.B.M. Teunissen
	dr. M.A. de Rie
Overige leden:	Prof. F. Allegra
	Prof. dr. R.J.M. ten Berge
	Prof. dr. S.J.H. van Deventer
	dr. F.R. de Gruyl
	Prof. dr. M.L. Kapsenberg
	dr. H. van Loveren
	Prof. dr. R. Willemze

Faculteit Geneeskunde

The studies described in this thesis were conducted at the Department of Dermatology (Laboratory of Experimental Dermatology and Laboratory of Neurosciences), Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

A mia madre

Abbreviations

AEC	3-amino-9-ethylcarbazole
AP	Alkaline Phosphatase
APC	Antigen presenting cell
CAM	Cell adhesion molecule
CH	Contact hypersensitvity
CLA	Cutaneous lymphocyte-associated antigen
CRTH2	Chemoattractant receptor-homologous molecule expressed on type 2 T-cell
DN	Double negative
DNCB	2,4 dinitrochlorobenzene
EC	Endothelial cell
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldeyde 3-phosphate dehydrogenase
IFN	Interferon
IL	Interleukin
IL-R	Interleukin-receptor
LC	Langerhans cell
LFA	Lymphocyte function-associated antigen
mAb	Monoclonal antibody
MED	Minimal erythema dose
MIP	Macrophage inflammatory protein
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PHA	Phytohemagglutinin
RT-PCR	Reverse-Transcriptase Polymerase chain reaction
SIS	Skin immune system
SSR	Solar-simulated radiation
TCC	T-cell clones
TCR	T-cell receptor
TNF	Tumor necrosis factor
Tr	T regulatory cell
UV	Ultraviolet
VLA	Very late activation antigen

Contents

Chapter 1

Introduction

1. Photoimmunology and Photoimmunodermatology

Photoimmunology is an area of biomedical research that investigates the effects of ultraviolet (UV) radiation (200-400 nm) on the immune system. Interest in this area expanded in the mid-1970's, when the immunosuppressive effect of UV was demonstrated in animal studies of UV carcinogenesis (1). Induction of skin tumors by repetitive exposure to UVB (280-320 nm) proved to be due to a combination of genotoxic effects and a decrease in immunosurveillance against the tumor cells. Along with these findings, studies on the function of Langerhans cells (LC), which are the primary antigen-processing and antigen-presenting cells of the human epidermis, also provided evidence that UV has immunosuppressive effects (2). Studies on the use of blood and blood-product transfusions and on transplantation of both solid organs and several types of cellular transplants corroborated and expanded the knowledge in this field (3-5). Nowadays, photoimmunology attracts the attention mainly of photobiologists, immunologists, haematologists and dermatologists.

Photoimmunodermatology is a specialised subdiscipline that describes the effects of UV on the skin immune system (SIS), which is defined as the complexity of immune response-associated cells and humoral factors present in normal human skin (6). Appreciation of the role of the skin as an active part of the immune system is relatively recent and UV-induced immunosuppression is a phenomenon that offers a fascinating opportunity to unravel the complex processes of the immune responses in the skin. Interest in this area of experimental dermatology is not a mere academic exercise: it is important because of its potential implications for human health. In this regard, the decrease in immunosurveillance against tumor cells is suspected of having a major role in the development of human skin cancer (7). Indeed, studies with skin-cancer patients suggested that the immunosuppression of contact hypersensitivity (CH) induced by UV-exposure is a risk factor for development of skin-cancer in humans, because 90% of patients with non-melanoma skin cancers (8) and 100% of patients with melanomas (9) were shown to be susceptible to UVB-induced immunosuppression. Since the first observation, the association between skin cancer and UV-induced immunosuppression has been the leading driving force behind the efforts of many investigators to unravel the mechanisms by which UV affects skin immune responses. However, exposure to sunlight and to artificial sources of UV may also have other implications for human health. Intense UV exposure may exacerbate skin infectious diseases

(10-12). Abnormal cutaneous reactivity to UV is a feature of a group of diseases called photodermatoses (13). Moreover, modulation of the SIS by UV is the basis of phototherapy of various dermatological disorders, such as psoriasis and atopic dermatitis, which are characterised by hyperactivity of the SIS (14,15).

2. Experimental evidence of immunosuppression by UV in humans.

A CH response is a T-cell mediated inflammatory reaction which can be induced by epicutaneous application of an allergenic chemical hapten. It is presumed to be prototypical of immune responses generated against neoantigens expressed by cutaneous malignancies and by infected skin cells. The pathophysiology of CH consists of two different phases: the sensitization phase and the elicitation phase. The sensitization phase is referred to as induction phase or afferent phase and occurs at the first contact of the skin with the hapten. This contact has no clinical consequences, except in some cases, in which the first application of a sensitizing dose of a hapten results in an intense inflammatory response that appears at the application site within 8 to 14 days (primary allergic reaction). In most cases, the second and subsequent contact of the skin with the allergenic hapten lead to development of a reproducible and characteristic inflammatory response (elicitation phase, also known as the challenge phase or the efferent phase of CH) .

In recent years, many studies have addressed the effects of UV-exposure on CH in humans (16). Interest in this area of research is based on the speculation that tumor antigens may, like allergenic haptens, be inappropriately presented through UV-exposed skin, resulting in down-regulatory mechanisms that inhibit immune responses against the incipient tumor and allow it to grow (17). Exposing human skin to UV has profound effects on the induction of CH to the contact sensitizer 2,4 dinitrochlorobenzene (DNCB). When a universally sensitizing dose of DNCB was directly painted onto acute UVB-exposed skin of healthy human volunteers within one hour after the last exposure, CH failed to develop in some (40%), whereas other subjects (60%) developed CH (8). The ability of UVB irradiation to impair induction of CH to DNCB in humans has also been reported by Cooper *et al.* (18). Furthermore, these two studies have shown that a subset of healthy volunteers who failed to develop CH when hapten was encountered through UVB-exposed skin, also became unable to generate a contact sensitivity response after repeated sensitization. In other words, a state of tolerance was induced. In addition, these and other studies revealed that UVB-exposed skin

fails to develop both the primary allergic reaction (8,18-20) and the elicitation reaction (21). Thus, in humans, UVB-exposure is deleterious both for the afferent and for the efferent phases of CH. In addition to the well-established immunosuppressive effects of UVB, recent studies reported a similar effect on CH in humans after a single exposure to different UV sources, such as solar-simulated radiation (SSR) (290-400 nm) (22-24) and UVA-II (320-340 nm) (25). However, exposure to UV does not always lead to immunosuppression. A recent study showed that UVA-I (340-400 nm) irradiation did not affect the immunisation rate in normal human volunteers (26). Moreover, Tie *et al.* (21) found that subjects who were sensitized through normal skin and challenged via UVB-exposed skin exhibited enhanced CH. Thus, UV-exposure may have suppressive or enhancing effects on the expression of CH in humans, depending on wavelength and sensitization protocol.

Various speculations on the physiological role of UV-induced immunosuppression in human health have been made by several investigators. One generally accepted hypothesis suggests two sides of the coin (27-31). On the one side, the teleological hypothesis that UV-induced suppression of T-cell mediated immunity may have arisen in the course of evolution as a way to protect the organism's integrity against autoimmune responses that are induced as a consequence of UV-mediated skin injury. In this view, photodermatoses, such as polymorphous light eruption, have been suggested as models of impaired UV-induced immunosuppression (31). On the other side, the hypothesis that the undesirable consequences of this physiological protective response to UV is observed both in occurrence of skin cancers and in exacerbation of skin infectious diseases.

3. Cellular targets of UV: emphasis on cutaneous T cells.

Exposure to UV induces a number of changes in the cellular and humoral components of the SIS, which are crucial for the generation of UV-induced immunosuppression (32-36). The aim of this chapter is to review the changes in human cutaneous T-cell populations upon exposure to physiological doses of UV. The first part focuses on effects of UV on cutaneous and circulating T cells *in vivo* and *in vitro*. The second part concentrates on the role of Langerhans cells, macrophages, endothelial cells (EC), keratinocytes, mast cells and neutrophils in cutaneous immune/inflammatory responses to UV in humans, in particular on the ability of these cells to modulate both functional properties and recirculation of cutaneous

T cells.

3.1. Effects of UV on T cells.

3.1.1. Distribution and characteristics of T cells in normal human skin.

Since the 1950's, it has been known that some lymphocytes are localized in the epidermal compartment of nonlesional, clinically normal human skin (37). In the past few decades, advances in cellular and molecular immunology have allowed us to reveal that virtually all the intraepidermal lymphocytes are T cells (38) and to describe the heterogeneous phenotype of these cells (39). Intraepidermal T cells account for approximately 2% of the total number of cutaneous $CD3^+$ cells (38). The vast majority of intraepidermal T cells express the T-cell receptor (TCR) $\alpha\beta$, only a minor portion (1-15%) carries the TCR $\gamma\delta$ (40-46). The intraepidermal T-cell population is primarily represented by single positive CD4 or CD8 cells, with skewing towards the CD8 subset (38,42,47). Rare double negative (DN; $CD4^-$ $CD8^-$) T cells, mostly carrying the TCR $\gamma\delta$, have been observed (46,48). The vast majority of intraepidermal T lymphocytes express the memory/effector phenotype defined by expression of CD45RO and lack of CD45RA (42,46,49,50). The expression of markers which indicate recent activation, such as interleukin-2 receptor α (IL-2Rα) and HLA-DR, is still controversial. In one study, the majority of intaepidermal T cells lacked IL-2Rα and HLA-DR and therefore these T lymphocytes were regarded as being in a "resting state" (42). In two other studies, these two markers of recent activation were found in a large proportion of intraepidermal T cells (38,46). However, data from different research groups showed consistently that only a few intraepidermal T lymphocytes expressed the HNK-1 epitope (CD57) and the very late activation antigen-1 (VLA-1), both markers that appear late after activation (42,46). While some T cells may migrate into normal skin as "passenger leukocytes", it is possible that many localize in the epidermis as "resident cells". However, the biological role(s) of such resident epidermal T-cell populations is(are) still unknown. The predominance of memory T lymphocytes over naïve T lymphocytes might reflect the chronicity of antigenic exposure challenging this tissue and suggests a specific role for T-cell mediated immunoresponses in reply to this challenge. It has been hypothesized that the intraepidermal T cell populations may play a role in continuous immunosurveillance against the development of cutaneous cancers and persistent infection with intracellular pathogens (51,52). Szabo *et al.* recently demonstrated that human intraepidermal T cells produce

interferon (IFN)-γ (53), which forms the basis of a tumor-suppressor mechanism (54), thus corroborating the above-mentioned hypothesis. Furthermore, given the cytotoxic potential of TCR $\gamma\delta^+$ DN ($CD4^-CD8^-$) T cells and TCR $\alpha\beta^+$ $CD8^+$ T cells, these two cell populations would be good candidates for the role of effector cells in the postulated intraepidermal immunosurveillance. Thus, activated intraepidermal T cell subpopulations may injure antigen-bearing target cells (e.g., keratinocytes, melanocytes) by either the release of cytokines, or MHC-restricted cytotoxicity.

Dermal T cells are preferentially (90%) clustered around postcapillary venules of the superficial plexus, and within the connective tissue sheaths of adnexal appendages (38,49); the remaining 8% are "free" in the connective tissue of the reticular dermis (38). The dermal T-cell population consists of $CD4^+CD8^-$ and $CD4^-CD8^+$ cells; in the perivascular compartment either an equal distribution is seen (38,44,55,56) or preference for the $CD4^+$ subset (49), and "free" in the papillary and reticular dermis a preference for the $CD8^+$ subset (38). Only few dermal DN $CD4^-CD8^-$ T cells have been observed (48). The majority of dermal T cells (90%) express the TCR αβ, and only a minor portion (7-9%) carries the TCR γδ (40,43-45). Most dermal T cells belong to the $CD45RO^+$ memory population (38,49,50). The memory phenotype and close apposition to either macrophages (57) or endothelial cells (38), which might induce and regulate cytolytic T-cell differentiation (58), suggest that dermal T cells might be involved in a response against exogenous and/or endogenous antigens. Thus, dermal T cells as well as epidermal T cells may perform a local sentinel function by ensuring rapid responses upon encountering cutaneous recall antigens.

Renal-transplant recipients provide indirect evidence for the role of cutaneous T cells in the immunosurveillance against skin cancer and skin infections. These patients, who usually receive long-term immunosuppressive therapy in order to maintain the viability of the transplanted kidney, have an increased risk of getting skin tumors, such as squamous cell carcinoma (59,60), and of viral opportunistic skin infections such as warts (59). In a recent study (61), it was found that skin specimens from renal-transplant recipients, who did not have any skin diseases, had fewer T cells in both the epidermal and the dermal compartments than age-matched healthy volunteers. Moreover, *in situ* the depletion of cutaneous T cells increased during the entire duration of the immunosuppressive therapy, which is in line with the clinical evidence that the longer the immunosuppression is present, the more skin cancers and skin infections develop (60).

3.1.2. In situ effects of UV on cutaneous T-cell populations.

The appearance of a dermal inflammatory cell infiltrate, mainly composed of T lymphocytes, after exposure to a single dose of either UVB or UVA has been well documented (62-67). As observed in biopsy specimens stained with hematoxylin and eosin, dermal lymphocytic infiltrates, mainly located in the perivascular area, were present from day 1 up to day 3 after UVB-exposure, and declined by day 7. A single exposure to erythematogenic doses of UVA elicited a dermal inflammatory infiltrate indistinguishable in cell type and location from that described for the UVB-exposed skin specimens, but the former was greater in terms of both quantity and depth of cell infiltrate than the latter (62,63). More recently, Norris *et al.* (68) and Van der Vleuten *et al.* (69) observed an increase in the number of perivascular $CD3^+$ or $CD2^+$ T cells, respectively, up to 1 day after a single exposure to UVB and both group saw progressive dwindling thereafter. Margolis *et al.* (70) and Lavker *et al.* (71) found that the majority of the UVA-induced perivascular mononuclear cells were $CD3^+$ T cells, mostly of the $CD4^+$ subpopulation (70). Lavker *et al.* (71) observed that repeated exposure to suberythematogenic doses of either UVA or SSR given once per day for 28 days also induced an increase in inflammatory cells, mainly lymphocytes, primarly in a perivenular location. The UVA exposures resulted in significantly more cellularity than the SSR exposure. Similar results were obtained in another study (72), in which exposure to either broad UVA or UVA-I was repeated at 24h intervals for 8 days, resulting in perivascular accumulation of T lymphocytes. The degree of cellularity was directly related to the daily UVA doses.

Upon exposure to UV a relevant quota of incident light reaches the basal layer of the epidermis (73). It has been speculated that intraepidermal T cells, which are mainly located in the basal layer, could easily be killed upon exposure to physiological doses of UV (74). However, so far, no attempt has been made to describe quantitative and qualitative alterations *in situ* of the intraepidermal T-cell populations upon single UV-exposure.

3.1.3. In vivo effects of UV on circulating T cells.

In humans, the effects of single and repetitive whole-body exposures to UV on peripheral blood lymphocytes have been investigated in many studies, and were reviewed by Morison (75-77). With the exception of two reports (78,79), changes in circulating T-cell subsets have been consistently found. A single exposure to UVB induced a dose-dependent

decrease in the proportion of circulating T cells (75) and significant changes of the CD4/CD8 ratio (80). Repetitive exposure to the mid-day sunlight (1h per day for 12 days) was followed by a significant decrease in the number of circulating $CD3^+$ T cells (81). The CD4/CD8 ratio was also reduced, due to a significant increase in circulating $CD8^+$ T cells and a decrease in circulating $CD4^+$ T cells. Moreover, subjects exposed repetitively to UV (mainly UVA-I) in a commercial solarium showed a reduction in the numbers of circulating $CD3^+$ and $CD4^+$ T cells as well as in the CD4/CD8 ratios (82,83).

The mechanism by which *in vivo* exposure to either SSR, UVB or UVA-I alters the proportion of circulating T lymphocytes is unknown. It has been suggested that the decrease in peripheral $CD3^+$ and $CD4^+$ T cells might be due to migration into lymphoid tissue at other body sites (81). As described in the previous paragraphs, exposure to either SSR, UVB or UVA-I induces accumulation of $CD3^+$ T cells, likely of the $CD4^+$ subset, into the dermal perivascular area, which suggests that redistribution of specific subpopulations of circulating T cells into the skin compartment might contribute to changes in circulating T-cell subsets. Interestingly, repetitive exposure to incremental doses of narrow-band UVB (311 nm) given three times per week for 4 to 5 weeks did not induce either perivascular accumulation of T lymphocytes (84) or changes in proportion of circulating T cells (85,86). This is in contrast with all the other studies but may indicate that changes in circulating and cutaneous T cells upon UV-exposure depend on the applied wavelength.

3.1.4. In vitro effects of UV on viability of T-cell subsets.

A large number of investigations have been performed concerning the effects of UV on human T cells *in vitro*, excluding effects mediated via other cells. Early studies, reviewed by Morison (76,77,87) consistently found a dose-dependent decrease in viability (80,87-92). The sensitivity of T cells to UV also proved to be dependent on the applied wavelength (87,90-92); UVC (200-280 nm) was more lethal than UVB, which in turn was more lethal than UVA. More recent reports suggest that the UV-induced killing of T cells is due to a combination of effects on different cellular targets, such as DNA and plasma-membrane receptors, activating a cascade of events eventually leading to cell death. Apoptosis is a physiological form of cell death characterised by nuclear condensation and cell shrinkage with preservation of an intact plasma membrane. UVB has been shown to be a particularly potent inducer of apoptosis in peripheral human T cells (93,94). The mechanism is not

entirely clear, but may involve DNA damage (93), and alteration of the Fas/FasL signalling pathway (95).

The reports concerning the effects of UV on viability of $CD4^+$ or $CD8^+$ T-cell subsets are controversial. In one study, the investigators found that $CD8^+$ T cells were more sensitive to UVB than $CD4^+$ T cells (80). In contrast, two other studies showed that the CD4/CD8 ratio was not altered upon SSR (96) or UVB (74), indicating that UV had no selective effect on either of these two T-cell subsets. Human activated T cells may also be roughly divided into two polarized subsets (i.e. type-1 and type-2 T cells) based on the pattern of cytokine they secrete, on the immune response they participate in, and on the preferential expression of some surface antigens (97,98). Type-1 T cells produce predominantly IFN-γ, which activates cytotoxic functions of effector cells such as macrophages and $CD8^+$ cytotoxic T cells. In contrast, type-2 T cells are characterized by predominant production of IL-4, which inhibits several macrophage functions and promotes humoral immunity. Upon UVB-exposure *in vitro* T-cell production of cytokines such as IL-2, IL-4, IL-5, IFN-γ and TNF-α, was reduced in an identical dose-dependent way for all cytokines tested (74). Moreover, a strong correlation was found between loss of viability and the reduction in cytokine productions, which suggests that the fall in production of cytokines was due to cell death.

3.2. Impact of UV on the cellular components of the SIS: effects on functions and dynamics of cutaneous T cells as end-points.

3.2.1. Upon UV-exposure cutaneous antigen presenting cells (APCs) induce preferential activation of type-2 T-cell responses.

Cutaneous APCs, such as LC and macrophages, may have a central role in determining the differentiation of T-cell subtypes and the cytokine-production pattern of the activated T cells in the skin. This T-cell stimulatory function of APCs can be affected by UV radiation, and may ultimately result in a different T-cell response.

The effects of UV on epidermal LC, the "professional" APC of the human epidermis, have been studied for many years since the first reports in 1981 on the deleterious effects of UV on epidermal LC population in humans (64,99). Exposure to SSR (100), UVB (99) and UVA (66) induces a decrease in the number of LC within human skin. Depletion of LC from the epidermis after UV exposure is probably due to a combination of cell death (101) and

enhanched migration to regional lymph nodes (102). In addition to the effects on LC numbers, UV-exposure induces functional alterations of this cell populations. A common theme found in a number of studies reviewed in (103-105) is the UV-exposed LC activation of type-2 T cells accompanied by the induction of type-1 T-cell tolerance. Because type-1 T cells generally help cell-mediated immune responses in CH and tumor rejection, the suppression of this particular cutaneous immuneresponse fits with the UV-induced immunosuppression described above.

At the nadir of depletion of epidermal $CD1a^+$ LC after UV exposure, $CD1a^-$, $HLA\text{-}DR^+$, $CD11b^+$, $CD36^+$ epidermal macrophages appear (106). These macrophages are distinct from LC, amongst others, in their high production of IL-10 (107,108) and in their inability to induce early up-regulation of T-cell IL-2Rα (109). The UV-induced macrophages activate $CD4^+$ $CD45RA^+$ suppressor inducer T cells that induce maturation of $CD8^+$ suppressor effector T cells, which in turn suppress T-cell mediated responses (110,111). It is unknown whether UV-induced macrophages, in analogy to UV-exposed LC, will preferentially activate a type 2 T-cell response.

The reversal of APC population from LC into macrophages in the UV-irradiated site and the altered capacity to activate type 1/type-2 T-cell responses have been proposed to be the key cellular mechanisms of UV-induced immunosuppression (33). However, recent studies indicate that LC and epidermal macrophages alone are not responsible for the UV-induced suppression of cell-mediated immunity, although it is likely that they are involved in the process (112).

3.2.2. Microvascular EC are the key gate-keepers for circulating T-cell migration into UV-exposed skin.

EC are essential elements in the recruitment of circulating T cells into sites of cutaneous inflammation. Upon activation, EC express cell-surface proteins and glycoproteins known as cell adhesion molecules (CAM), such as E-selectin, ICAM-1 and VCAM-1, that allow circulating T cells to bind to activated EC. A transient interaction between the cutaneous lymphocyte-associated antigen (CLA) and its ligand E-selectin expressed on dermal EC has been suggested as the first step in the extravasation of skin-homing T cells into a skin inflammatory site (113). Subsequently, the circulating T cells, which express or have

locally been induced to express lymphocyte function-associated antigen (LFA)-1 and/or very late activation antigen (VLA)-4, will more steadily interact with ICAM-1 and/or VCAM-1 on dermal EC (114). In humans, upregulation of the dermal vascular E-selectin and ICAM-1 has been described to occur *in vivo* after a single exposure to UVB (68,115,116) or to UVA (115,117). The extent of infiltration of neutrophils and macrophages into UVB-irradiated sites has been correlated with E-selectin upregulation on dermal EC (68,116,118). However, accumulation of T cells did not correlate with the expression of E-selectin on dermal EC in UVB-exposed sites (68), suggesting that CLA-independent transmigration, possibly involving interaction of LFA-1 with ICAM-1 (113), may recruit T cells into UVB-exposed skin. Furthermore, CLA/E-selectin interaction appeared to be irrelevant *in vitro* in UVA-induced T-cell adhesion to human dermal microvascular EC (119). Several investigators consistently reported that expression of VCAM-1 on dermal EC did not change upon exposure to UV (68,115,116), and suggested that VLA-4/VCAM-1 interaction does not play any role in recruiting circulating T cells into UV-exposed skin.

3.2.3. Keratinocytes and mast cells are the major sources of pro-inflammatory cytokines and chemokines relevant for T-cell recruitment into UV-exposed skin.

Human keratinocytes are known to produce a wide variety of soluble peptide mediators (120,121). These humoral factors, produced locally within the epidermal microenviroment and termed "epidermal cytokines", play an essential role during induction, amplification, and resolution of inflammation and skin immune responses. According to Baker *et al.* (122,123), keratinocytes act principally as pro-inflammatory signal transducers, responding to nonspecific external stimuli with the production of inflammatory cytokines, which influences leukocyte accumulation and subsequent immunological events. Indeed, exposure to UV has been shown to trigger keratinocytes to produce and/or release several pro-inflammatory cytokines, such as IL-1 (124-126), IL-6 (127,128), IL-8 (129) and tumor necrosis factor (TNF)-α (126,130,131).

In normal human skin, mast cells are preferentially located in the perivascular area of the papillary dermis (132,133). A recent study, using the human leukaemic mast cell line HMC-1, which exhibits a number of phenotypic and functional properties typical for tissue mast cells, has shown that these cells can be induced to express the genes for IL-1, IL-6, IL-8,

TNF-α and to release these cytokines (134). Moreover, mast cells seem to be unable to produce cytokines which support type 1 T-cell responses, such as IL-12 and IFN-γ, whereas they produce cytokines possibly involved in type 2 T-cell responses, such as IL-4 and IL-10. Although such a broad but specific spectrum of cytokine secretion makes the mast cell a candidate for playing an important role in UV-induced immunosuppression, only limited data have been generated on the effect *in vivo* of UV on human dermal mast cells. *In vivo*, immediately after a single exposure to UVA or UVB human dermal mast cells degranulate (64-66,135), and release a variety of pre-formed mediators, such as histamine (64,65) and TNF-α (136).

UV-induced cytokines influence lymphocyte accumulation by at least two different mechanisms. First, cytokines modulate expression of adhesion molecules on endothelial cells. There is no doubt about the involvement of IL-1 and TNF-α in EC-mediated recruitment of T lymphocytes into inflammatory sites. In vitro, IL-1 and TNF-α induced a time-dependent increase of adhesion molecules on human EC monolayers (137) and increased EC adhesiveness for T lymphocytes obtained from normal human subjects (138). Moreover, direct intradermal injection of the two cytokines led to similar patterns of expression of adhesion molecules on the dermal vasculature of normal human volunteers, and induced a dermal leukocyte infiltrate (139,140).

Second, T-cell migration through the dermal perivascular area to the target microenvironment, such as epidermis, is driven by chemotactic cytokines (i.e. chemokines). In this respect, IL-8 and IL-15 have been shown to be chemotactic factors for T cells (141,142) and recently Duthie et al. (16) speculated that macrophage-derived IL-15 could play an important role in attracting T cells into UV-exposed sites. However, it is still unknown which specific chemokine(s) is (are) involved in the selective accumulation of $CD4^+$ T cells in the skin in response to UV. Psoriasin is a low-molecular-mass calcium-binding protein that is synthesised (albeit at low levels), and is partially secreted by noncultured unfractionated keratinocytes from normal human skin (143). A recent study indicates that psoriasin is a potent and selective chemotactic protein for $CD4^+$ T cells in vitro (144). Also IL-16, a selective chemoattractant for $CD4^+$ T cells (145), which is stored in a bioactive form in mast cell granules (146), might be released upon UV-exposure and therefore participate in $CD4^+$ T-cell chemoattraction.

3.2.4. Neutrophils participate in the switch in leukocyte infiltration from a predominantly acute neutrophil response to a chronic mononuclear cell response in UV-exposed sites.

Neutrophils are most prominent early in the UV-induced cellular recruitment from the blood stream into UV-irradiated human skin. Dermal neutrophils appear immediately after UV-exposure, with an increase in number up to 14 h and progressive dwindling thereafter (67). E-selectin, IL-8 and TNF-α appear to be involved in the UV-induced recruitment of neutrophils (118). The function of neutrophils in UV-irradiated sites is unclear. The ability of neutrophils to generate several chemokines, such as IL-8, macrophage inflammatory protein (MIP)1-α and MIP-1β (147,148), indicates that they might be involved in leukocyte infiltration and might switch the acute neutrophil response to a chronic mononuclear cell response (i.e. by macrophages and T cells) in the UV-exposed site, a phenomenon observed over time (68).

4. Aims of the Studies

In humans, following exposure to single doses of UV, the appearance of a dermal inflammatory cell infiltrate, mainly composed of T lymphocytes, has been well documented in the past. Since T cells play a pivotal role in cutaneous immunology, particularly in the course of inflammatory skin conditions (149,150), it is surprising that, so far, few data have been generated concerning phenotype, mechanism of recruitment, and functional properties of the UV-induced dermal T cells. The aim of this thesis was to further investigate the effects *in situ* of a single physiological dose of either SSR, UVB or UVA on the dermal T-cell population. Moreover, we focused on changes in the epidermal T-cell populations. Although UV irradiation has only limited depth penetration in human skin (151), epidermal T lymphocytes might be directly affected upon exposure to physiological doses of UV. In the initial time-course study, described in **chapter 2**, we locally irradiated healthy human volunteers with graded doses of SSR. Quantitative changes in the total cutaneous $CD3^+$ T-cell population, as well as changes in the $CD4^+$ and $CD8^+$ subsets, were assessed by immunohistochemistry. In **chapter 3**, in which a similar protocol was used, are described studies to determine in more detail the kinetics of the $CD4^+$ and $CD8^+$, subpopulations, as well as the phenotype (such as memory versus naïve, activated versus resting, or TCRαβ versus TCRγδ) of the T lymphocytes that infiltrated the human skin after a single exposure to

SSR or UVB. In order to gain a better understanding of the mechanism by which UVB induces recruitment of T cells into the irradiated site, we investigated the expression of adhesion molecules on infiltrating T cells and related this to the expression of their counterreceptors on EC (**chapter 4**). We further assessed the effect of UVB on the two chemokines, psoriasin and IL-16 at mRNA level with RT-PCR techniques and at protein level with immunohistochemistry. To further analyze the characteristics of UV-induced T cells, in the study described in **chapter 5** we isolated T-cell clones from irradiated skin and examined their phenotypes with respect to the expression of type-1 versus type-2 markers; the results were compared with those obtained *in situ* using immunohistochemistry. Effect of UV on the two discriminating cytokines IL-4 and IFN-γ were also investigated. Serendipity led us to discover that UVB-exposure induces the appareance of $CD3^-$ $IL\text{-}4^+$ cells into the irradiated site. **Chapter 6** describes a study that was conducted in order to identify this previously unknown UVB-induced cellular source of IL-4.

References

1. Kripke, M.L. 1984. Immunological unresponsiveness induced by ultraviolet radiation. *Immunol.Rev.* 80:87.

2. Streilein, J.W. and P.R. Bergstresser. 1984. Langerhans cells: antigen presenting cells of the epidermis. *Immunobiology* 168:285.

3. Hardy, M.A., J. Chabot, and G. Tannenbaum. 1986. Immunomodulation by ultraviolet irradiation. In Transplantation: approaches to graft rejection. H.T. Meryman, editor. Liss, New York. 119-138.

4. Deeg, H.J. 1988. Ultraviolet irradiation in transplantation biology. Manipulation of immunity and immunogenicity. *Transplantation* 45:845.

5. Pamphilon, D.H., A.A. Alnaqdy, and T.B. Wallington. 1991. Immunomodulation by ultraviolet light: clinical studies and biological effects. *Immunol.Today* 12:119.

6. Bos, J.D. and M.L. Kapsenberg. 1993. The skin immune system: progress in cutaneous biology. *Immunol.Today* 14:75.

7. Streilein, J.W., J.R. Taylor, V. Vincek, I. Kurimoto, J. Richardson, C. Tie, J.P. Medema, and C. Golomb. 1994. Relationship between ultraviolet radiation-induced immunosuppression and carcinogenesis. *J.Invest.Dermatol.* 103:107S.

8. Yoshikawa, T., V. Rae, W. Bruins-Slot, J.W. van den Berg, J.R. Taylor, and J.W. Streilein. 1990. Susceptibility to effects of UVB radiation on induction of contact hypersensitivity as a risk factor for skin cancer in humans. *J.Invest.Dermatol.* 95:530.

9. Streilein, J.W., J.R. Taylor, V. Vincek, I. Kurimoto, T. Shimizu, C. Tie, and C. Golomb. 1994. Immune surveillance and sunlight-induced skin cancer. *Immunol.Today* 15:174.

10. Chapman, R.S., K.D. Cooper, E.C. De Fabo, J.E. Frederick, K.N. Gelatt, S.P. Hammond, P. Hersey, H.S. Koren, R.D. Ley, and F. Noonan. 1995. Solar ultraviolet radiation and the risk of infectious disease: summary of a workshop. *Photochem.Photobiol.* 61:223.

11. Vermeer, B.J. and J.N. Bouwes Bavinck. 1998. Relevance of photo-immunosuppression for viral infections (i.e. human papilloma virus). *Eur.J.Dermatol.* 8:205.

12. Norval, M. and A.A. El-Ghorr. 1998. UV-induced immunosuppression in virus infections. *Mutat.Res.* 422:131.

13. Taylor, C.R. and A.J. Sober. 1996. Sun exposure and skin disease. *Annu.Rev.Med.* 47:181.

14. De Rie, M.A. and J.D. Bos. 1997. Photo(chemo)therapeutic modulation of the skin immune system. In Skin Immune System (SIS). J.D. Bos, editor. CRC Press LLC, Boca Raton, Florida. 671-685.

15. Krutmann, J. and A. Morita. 1999. Mechanisms of ultraviolet (UV) B and UVA phototherapy. *J.Invest.Dermatol.Symposium Proceedings* 4:70.

16. Duthie, M.S., I. Kimber, and M. Norval. 1999. The effects of ultraviolet radiation on the human immune system. *Br.J.Dermatol.* 140:995.

17. Noonan, F.P., E.C. De Fabo, and M.L. Kripke. 1981. Suppression of contact hypersensitivity by UV radiation and its relationship to UV-induced suppression of tumor immunity. *Photochem.Photobiol.* 34:683.

18. Cooper, K.D., L. Oberhelman, T.A. Hamilton, O. Baadsgaard, M. Terhune, G.J. LeVee, T. Anderson, and H. Koren. 1992. UV exposure reduces immunization rates and promotes tolerance to epicutaneous antigens in humans: relationship to dose, $CD1a^{-}DR^{+}$ epidermal macrophage induction, and Langerhans cell depletion. *Proc.Natl.Acad.Sci.U.S.A.* 89:8497.

19. Tseng, C., B. Hoffman, I. Kurimoto, T. Shimizu, G.J. Schmieder, J.R. Taylor, and J.W. Streilein. 1992. Analysis of effects of ultraviolet B radiation on induction of primary allergic reactions. *J.Invest.Dermatol.* 98:871.

20. Whitmore, S.E. and W.L. Morison. 1995. Ultraviolet-induced suppression of the primary allergic reaction. Four times the minimal erythema dose of ultraviolet B administered 24 hours prior to sensitization. *Photodermatol.Photoimmunol.Photomed.* 11:159.

21. Tie, C., C. Golomb, J.R. Taylor, and J.W. Streilein. 1995. Suppressive and enhancing effects of ultraviolet B radiation on expression of contact hypersensitivity in man. *J.Invest.Dermatol.* 104:18.

22. Kelly, D.A., S.L. Walker, J.M. McGregor, and A.R. Young. 1998. A single exposure of solar simulated radiation suppresses contact hypersensitivity responses both locally and systemically in humans: quantitative studies with high-frequency ultrasound. *J.Photochem.Photobiol.B.* B - Biology. 44:130.

23. Serre, I., J.P. Cano, M.C. Picot, J. Meynadier, and L. Meunier. 1997. Immunosuppression induced by acute solar-simulated ultraviolet exposure in humans: prevention by a sunscreen with a sun protection factor of 15 and high UVA protection. *J.Am.Acad.Dermatol.* 37:187.

24. Kelly, D.A., A.R. Young, J.M. McGregor, P.T. Seed, C.S. Potten, and S.L. Walker. 2000. Sensitivity to sunburn is associated with susceptibility to ultraviolet radiation-induced suppression of cutaneous cell-mediated immunity. *J.Exp.Med.* 191:561.

25. LeVee, G.J., L. Oberhelman, T. Anderson, H. Koren, and K.D. Cooper. 1997. UVA II exposure of human skin results in decreased immunization capacity, increased induction of tolerance and a unique pattern of epidermal antigen-presenting cell alteration. *Photochem.Photobiol.* 65:622.

26. Skov, L., H. Hansen, J.N. Barker, J.C. Simon, and O. Baadsgaard. 1997. Contrasting effects of ultraviolet-A and ultraviolet-B exposure on induction of contact sensitivity in human skin. *Clin.Exp.Immunol.* 107:585.

27. Cruz, P.D., Jr. 1992. Langerhans cells are initiators of the immunosuppressive effect of ultraviolet B radiation. *Springer Semin.Immunopathol.* 13:281.

28. Streilein, J.W. 1993. Sunlight and skin-associated lymphoid tissues (SALT): if UVB is the trigger and TNF alpha is its mediator, what is the message? *J.Invest.Dermatol.* 100:47S.

29. Vermeer, B.J. and M. Hurks. 1994. The clinical relevance of immunosuppression by UV irradiation. *J.Photochem.Photobiol.B.* B - Biology. 24:149.

30. Beissert, S. and R.D. Granstein. 1997. Physiology and pathology of skin photoimmunology. In Skin Immune System (SIS). J.D. Bos, editor. CRC Press LLC, Boca Raton, FL. 399-416.

31. Kölgen, W., H. van Weelden, S. Den Hengst, K.L.H. Guikers, R.C.M. Kiekens, E.F. Knol, C.A. Bruijnzeel-Koomen, W.A. van Vloten, and F.R. de Gruijl. 1999. $CD11b^{+}$ cells and ultraviolet-B-resistant $CD1a^{+}$ cells in skin patient with polymorphous light eruption. *J.Invest.Dermatol.* 113:4.

32. Ullrich, S.E. 1995. The role of epidermal cytokines in the generation of cutaneous immune reactions and ultraviolet radiation-induced immune suppression. *Photochem.Photobiol.* 62:389.

33. Cooper, K.D. 1996. Cell-mediated immunosuppressive mechanisms induced by UV radiation. *Photochem.Photobiol.* 63:400.

34. Mukhatar, H. 1996. Photocarcinogenesis: mechanisms, models and human health implication. *Photochem.Photobiol.* 63:355.

35. Takashima, A. and P.R. Bergstresser. 1996. Impact of UVB radiation on the epidermal cytokine network. *Photochem.Photobiol.* 63:397.

36. Boonstra, A. and H.F. Savelkoul. 1997. The role of cytokines in ultraviolet-B induced immunosuppression. *Eur.Cytokine Netw.* 8:117.

37. Andrew, W. and N.W. Andrew. 1949. Lymphocytes in the normal epidermis of the rat and of the man. *Anat.Rec.* 104:217.

38. Bos, J.D., I. Zonneveld, P.K. Das, S.R. Krieg, C.M. van der Loos, and M.L. Kapsenberg. 1987. The skin immune system (SIS): distribution and immunophenotype of lymphocyte subpopulations in normal human skin. *J.Invest.Dermatol.* 88:569.

39. Foster, C.A. and A. Elbe. 1997. Lymphocyte Subpopulation of the Skin. In Skin Immune System (SIS). J.D. Bos, editor. CRC Press LLC, Boca Raton, New York. 85-108.

40. Groh, V., S. Porcelli, M. Fabbi, L.L. Lanier, L.J. Picker, T. Anderson, R.A. Warnke, A.K. Bhan, J.L. Strominger, and M.B. Brenner. 1989. Human lymphocytes bearing T cell receptor gamma/delta are phenotypically diverse and evenly distributed throughout the lymphoid system. *J.Exp.Med.* 169:1277.

41. Bucy, R.P., C.L. Chen, and M.D. Cooper. 1989. Tissue localization and CD8 accessory molecule expression of T gamma delta cells in humans. *J.Immunol.* 142:3045.

42. Foster, C.A., H. Yokozeki, K. Rappersberger, F. Koning, B. Volc-Platzer, A. Rieger, J.E. Coligan, K. Wolff, and G. Stingl. 1990. Human epidermal T cells predominantly belong to the lineage expressing alpha/beta T cell receptor. *J.Exp.Med.* 171:997.

43. Bos, J.D., M.B.M. Teunissen, I. Cairo, S.R. Krieg, M.L. Kapsenberg, P.K. Das, and J. Borst. 1990. T-cell receptor gamma delta bearing cells in normal human skin. *J.Invest.Dermatol.* 94:37.

44. Dupuy, P., M. Heslan, S. Fraitag, T. Hercend, L. Dubertret, and M. Bagot. 1990. T-cell receptor-gamma/delta bearing lymphocytes in normal and inflammatory human skin. *J.Invest.Dermatol.* 94:764.

45. Alaibac, M., J. Morris, R. Yu, and A.C. Chu. 1992. T lymphocytes bearing the gamma delta T-cell receptor: a study in normal human skin and pathological skin conditions. *Br.J.Dermatol.* 127:458.

46. Spetz, A.L., J. Strominger, and V. Groh-Spies. 1996. T cell subsets in normal human epidermis. *Am.J.Pathol.* 149:665.

47. Smolle, J., R. Ehall, and H. Kerl. 1985. Inflammatory cell types in normal human epidermis: an immunohistochemical and morphometric study. *Acta Derm.Venereol.* 65:479.

48. Groh, V., M. Fabbi, F. Hochstenbach, R.T. Maziarz, and J.L. Strominger. 1989. Double-negative ($CD4^-$ $CD8^-$) lymphocytes bearing T-cell receptor alpha and beta chains in normal human skin. *Proc.Natl.Acad.Sci.U.S.A.* 86:5059.

49. Rowden, G., D. Davis, D. Luckett, and L. Poulter. 1988. Identification of $CD4^+$, $2H4^+$ ($T8\ gamma^+$) suppressor-inducer cells in normal human epidermis and superficial dermis. *Br.J.Dermatol.* 119:147.

50. Bos, J.D., C. Hagenaars, P.K. Das, S.R. Krieg, W.J. Voorn, and M.L. Kapsenberg. 1989. Predominance of "memory" T cells ($CD4^+$, $CDw29^+$) over "naive" T cells ($CD4^+$, $CD45R^+$) in both normal and diseased human skin. *Arch.Dermatol.Res.* 281:24.

51. Streilein, J.W. 1978. Lymphocyte traffic, T-cell malignancies and the skin. *J.Invest.Dermatol.* 71:167.

52. Streilein, J.W. 1983. Skin-associated lymphoid tissues (SALT): origins and functions. *J.Invest.Dermatol.* 80:12s.

53. Szabo, S.K., C. Hammerberg, Y. Yoshida, Z. Bata-Csorgo, and K.D. Cooper. 1998. Identification and quantitation of interferon-gamma producing T cells in psoriatic lesions: localization to both $CD4^+$ and $CD8^+$ subsets. *J.Invest.Dermatol.* 111:1072.

54. Kaplan, D.H., V. Shankaran, A.S. Dighe, E. Stockert, M. Aguet, L.J. Old, and R.D. Schreiber. 1998. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. *Proc.Natl.Acad.Sci.U.S.A.* 95:7556.

55. Atkinson, K., V. Munro, E. Vasak, and J. Biggs. 1986. Mononuclear cell subpopulations in the skin defined by monoclonal antibodies after HLA-identical sibling marrow transplantation. *Br.J.Dermatol.* 114:145.

56. Davis, A.L., J.L. McKenzie, and D.N. Hart. 1988. HLA-DR-positive leucocyte subpopulations in human skin include dendritic cells, macrophages, and CD7-negative T cells. *Immunology* 65:573.

57. McLellan, A.D., A. Heiser, R.V. Sorg, D.B. Fearnley, and D.N. Hart. 1998. Dermal dendritic cells associated with T lymphocytes in normal human skin display an activated phenotype. *J.Invest.Dermatol.* 111:841.

58. Biedermann, B.C. and J.S. Pober. 1998. Human endothelial cells induce and regulate cytolytic T cell differentiation. *J.Immunol.* 161:4679.

59. Brown, J.H., T. Hutchison, A.M. Kelly, and M.G. McGeown. 1988. Dermatologic lesions in a transplant population. *Transplantation* 46:530.

60. Hartevelt, M.M., J.N. Bouwes Bavinck, A.M. Kootte, B.J. Vermeer, and J.P. Vandenbroucke. 1990. Incidence of skin cancer after renal transplantation in The Netherlands. *Transplantation* 49:506.

61. Galvao, M.M., M.N. Sotto, S.M. Kihara, E.A. Rivitti, and E. Sabbaga. 1998. Lymphocyte subsets and Langerhans cells in sun-protected and sun-exposed skin of immunosuppressed renal allograft recipients. *J.Am.Acad.Dermatol.* 38:38.

62. Willis, I. and L. Cylus. 1977. UVA erythema in skin: is it a sunburn? *J.Invest.Dermatol.* 68:128.

63. Rosario, R., G.J. Mark, J.A. Parrish, and M.C. Mihm. 1979. Histological changes produced in skin by equally erythemogenic doses of UV-A, UV-B, UV-C and UV-A with psoralen. *Br.J.Dermatol.* 101:299.

64. Gilchrest, B.A., N.A. Soter, J.S. Stoff, and M.C. Mihm, Jr. 1981. The human sunburn reaction: histologic and biochemical studies. *J.Am.Acad.Dermatol.* 5:411.

65. Gilchrest, B.A., J.S. Stoff, and N.A. Soter. 1982. Chronologic aging alters the response to ultraviolet-induced inflammation in human skin. *J.Invest.Dermatol.* 79:11.

66. Gilchrest, B.A., N.A. Soter, J.L. Hawk, R.M. Barr, A.K. Black, C.N. Hensby, A.I. Mallet, M.W. Greaves, and J.A. Parrish. 1983. Histologic changes associated with ultraviolet A-induced erythema in normal human skin. *J.Am.Acad.Dermatol.* 9:213.

67. Hawk, J.L., G.M. Murphy, and C.A. Holden. 1988. The presence of neutrophils in human cutaneous ultraviolet-B inflammation. *Br.J.Dermatol.* 118:27.

68. Norris, P., R.N. Poston, D.S. Thomas, M. Thornhill, J.L. Hawk, and D.O. Haskard. 1991. The expression of endothelial leukocyte adhesion molecule-1 (ELAM-1), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) in experimental cutaneous inflammation: a comparison of ultraviolet B erythema and delayed hypersensitivity. *J.Invest.Dermatol.* 96:763.

69. van der Vleuten, C.J.M., E.J. Kroot, E.M. de Jong, and P.C. van de Kerkhof. 1996. The immunohistochemical effects of a single challenge with an intermediate dose of ultraviolet B on normal human skin. *Arch.Dermatol.Res.* 288:510.

70. Margolis, R.J., M. Sherwood, D.J. Maytum, R.D. Granstein, M.A. Weinstock, J.A. Parrish, and R.W. Gange. 1989. Longwave ultraviolet radiation (UVA, 320-400 nm)-induced tan protects human skin against further UVA injury. *J.Invest.Dermatol.* 93:713.

71. Lavker, R.M., G.F. Gerberick, D. Veres, C.J. Irwin, and K.H. Kaidbey. 1995. Cumulative effects from repeated exposures to suberythemal doses of UVB and UVA in human skin. *J.Am.Acad.Dermatol.* 32:53.

72. Lavker, R.M., D.A. Veres, C.J. Irwin, and K.H. Kaidbey. 1995. Quantitative assessment of cumulative damage from repetitive exposures to suberythemogenic doses of UVA in human skin. *Photochem.Photobiol.* 62:348.

73. Bruls, W.A.G., H. Slaper, J.C. van der Leun, and L. Berrens. 1984. Transmission of human epidermis and stratum corneum as a function of thickness in the ultraviolet and visible wavelenghts. *Photochem.Photobiol.* 40:485.

74. Teunissen, M.B.M., R.M. Sylva-Steenland, and J.D. Bos. 1993. Effect of low-dose ultraviolet-B radiation on the function of human T lymphocytes in vitro. *Clin.Exp.Immunol.* 94:208.

75. Morison, W.L., J.A. Parrish, K.J. Bloch, and J.I. Krugler. 1979. In vivo effect of UV-B on lymphocyte function. *Br.J.Dermatol.* 101:513.

76. Morison, W.L. 1984. Photoimmunology. *Photochem.Photobiol.* 40:781.

77. Morison, W.L. 1989. Effects of ultraviolet radiation on the immune system in humans. *Photochem.Photobiol.* 50:515.

78. Mutzhas, M.F., V. von Arnim, H.-D. Hentschel, E. Volger, and H.A. Stickl. 1991. Effects of UV-A1 light on immune status in man. In Biological responses to ultraviolet A radiation. F. Urbach, editor. Valdenmar Publishing Company, Overland Park, Kansas. 249-256.

79. Gilmour, J.W., J.P. Vestey, and M. Norval. 1993. The effect of UV therapy on immune function in patients with psoriasis. *Br.J.Dermatol.* 129:28.

80. McGrath, H., W.A. Wilson, and E. Scopelitis. 1986. Acute effects of low-fluence ultraviolet light on human T- lymphocyte subsets. *Photochem.Photobiol.* 43:627.

81. Hersey, P., G. Haran, E. Hasic, and A. Edwards. 1983. Alteration of T cell subsets and induction of suppressor T cell activity in normal subjects after exposure to sunlight. *J.Immunol.* 131:171.

82. Hersey, P., M. Bradley, E. Hasic, G. Haran, A. Edwards, and W.H. McCarthy. 1983. Immunological effects of solarium exposure. *Lancet* 1:545.

83. Rivers, J.K., P.G. Norris, G.M. Murphy, A.C. Chu, G. Midgley, J. Morris, R.W. Morris, A.R. Young, and J.L. Hawk. 1989. UVA sunbeds: tanning, photoprotection, acute adverse effects and immunological changes. *Br.J.Dermatol.* 120:767.

84. Viac, J., C. Goujon, L. Misery, V. Staniek, M. Faure, D. Schmitt, and A. Claudy. 1997. Effect of UVB 311 nm irradiation on normal human skin. *Photodermatol.Photoimmunol.Photomed.* 13:103.

85. Gilmour, J.W., J.P. Vestey, S. George, and M. Norval. 1993. Effect of phototherapy and urocanic acid isomers on natural killer cell function. *J.Invest.Dermatol.* 101:169.

86. Guckian, M., C.D. Jones, J.P. Vestey, E.J. Cooper, R. Dawe, N.K. Gibbs, and M. Norval. 1995. Immunomodulation at the initiation of phototherapy and photochemotherapy. *Photodermatol.Photoimmunol.Photomed.* 11:163.

87. Morison, W.L., J.A. Parrish, R.R. Anderson, and K.J. Bloch. 1979. Sensitivity of mononuclear cells to UV radiation. *Photochem.Photobiol.* 29:1045.

88. Evans, R.G. and A. Norman. 1968. Radiation stimulated incorporation of thymidine into the DNA of human lymphocytes. *Nature* 217:455.

89. Horowitz, S., D. Cripps, and R. Hong. 1974. Selective T cell killing of human lymphocytes by ultraviolet radiation. *Cell Immunol.* 14:80.

90. Cripps, D.J., S. Horowitz, and R. Hong. 1978. Spectrum of ultraviolet radiation on human B and T lymphocyte viability. *Clin.Exp.Dermatol.* 3:43.

91. Pretell, J.O., J. Wimberly, D.J. McAuliffe, and J.A. Parrish. 1984. Comparison of effects of UVA, UVB and UVC on growth and viability of mitogen-stimulated human peripheral blood cells. *Photochem.Photobiol.* 39:369.

92. Walther, T., W. Gast, M. Rytter, U.F. Haustein, and U. Biella. 1990. Effects of monochromatic ultraviolet irradiation (300-400 nm) on viability of human white blood cells. *Photodermatol.Photoimmunol.Photomed.* 7:88.

93. Bazar, L.S. and H.J. Deeg. 1992. Ultraviolet B-induced DNA fragmentation (apoptosis) in activated T-lymphocytes and Jurkat cells is augmented by inhibition of RNA and protein synthesis. *Exp.Hematol.* 20:80.

94. Yaron, I., R. Yaron, S.F. Oluwole, and M.A. Hardy. 1996. UVB irradiation of human-derived peripheral blood lymphocytes induces apoptosis but not T-cell anergy: additive effects with various immunosuppressive agents. *Cell Immunol.* 168:258.

95. Caricchio, R., E.A. Reap, and P.L. Cohen. 1998. Fas/Fas ligand interactions are involved in ultraviolet-B-induced human lymphocyte apoptosis. *J.Immunol.* 161:241.

96. Lederman, M.M., B. Schacter, M.J. LeVine, and J.J. Ellner. 1986. Inhibition of human lymphocyte proliferation by ultraviolet radiation: effects of ultraviolet B (290 to 320 nm) on T-lymphocytes, monocyte accessory function, and induction of suppressor mechanisms. *J.Lab.Clin.Med.* 107:66.

97. Carter, L.L. and R.W. Dutton. 1996. Type 1 and type 2: a fundamental dichotomy for all T-cell subsets. *Curr.Opin.Immunol.* 8:336.

98. Romagnani, S. 1997. The Th1/Th2 paradigm. *Immunol.Today* 18:263.

99. Aberer, W., G. Schuler, G. Stingl, H. Honigsmann, and K. Wolff. 1981. Ultraviolet light depletes surface markers of Langerhans cells. *J.Invest.Dermatol.* 76:202.

100. Laihia, J.K. and C.T. Jansen. 1997. Up-regulation of human epidermal Langerhans' cell B7-1 and B7-2 co-stimulatory molecules in vivo by solar-simulating irradiation. *Eur.J.Immunol.* 27:984.

101. Aberer, W., N. Romani, A. Elbe, and G. Stingl. 1986. Effects of physicochemical agents on murine epidermal Langerhans cells and Thy-1-positive dendritic epidermal cells. *J.Immunol.* 136:1210.

102. Sontag, Y., C.L. Guikers, A.A. Vink, F.R. de Gruijl, H. van Loveren, J. Garssen, L. Roza, M.L. Kripke, J.C. van der Leun, and W.A. van Vloten. 1995. Cells with UV-specific DNA damage are present in murine lymph nodes after in vivo UV irradiation. *J.Invest.Dermatol.* 104:734.

103. Simon, J.C., J. Krutmann, C.A. Elmets, P.R. Bergstresser, and P.D. Cruz, Jr. 1992. Ultraviolet B-irradiated antigen-presenting cells display altered accessory signaling for T-cell activation: relevance to immune responses initiated in skin. *J.Invest.Dermatol.* 98:66S.

104. Ullrich, S.E. 1995. Modulation of immunity by ultraviolet radiation: key effects on antigen presentation. *J.Invest.Dermatol.* 105:30S.

105. Ullrich, S.E. 1996. Does exposure to UV radiation induce a shift to a Th-2-like immune reaction? *Photochem.Photobiol.* 64:254.

106. Cooper, K.D., G.R. Neises, and S.I. Katz. 1986. Antigen-presenting $OKM5^+$ melanophages appear in human epidermis after ultraviolet radiation. *J.Invest.Dermatol.* 86:363.

107. Kang, K., C. Hammerberg, L. Meunier, and K.D. Cooper. 1994. $CD11b^+$ macrophages that infiltrate human epidermis after in vivo ultraviolet exposure potently produce IL-10 and represent the major secretory source of epidermal IL-10 protein. *J.Immunol.* 153:5256.

108. Kang, K., A.C. Gilliam, G. Chen, E. Tootell, and K.D. Cooper. 1998. In human skin, UVB initiates early induction of IL-10 over IL-12 preferentially in the expanding dermal monocytic/macrophagic population. *J.Invest.Dermatol.* 111:31.

109. Stevens, S.R., A. Shibaki, L. Meunier, and K.D. Cooper. 1995. Suppressor T cell-activating macrophages in ultraviolet- irradiated human skin induce a novel, TGF-beta-dependent form of T cell activation characterized by deficient IL-2r alpha expression. *J.Immunol.* 155:5601.

110. Baadsgaard, O., D.A. Fox, and K.D. Cooper. 1988. Human epidermal cells from ultraviolet light-exposed skin preferentially activate autoreactive $CD4^+2H4^+$ suppressor-inducer lymphocytes and $CD8^+$ suppressor/cytotoxic lymphocytes. *J.Immunol.* 140:1738.

111. Baadsgaard, O., B. Salvo, A. Mannie, B. Dass, D.A. Fox, and K.D. Cooper. 1990. In vivo ultraviolet-exposed human epidermal cells activate T suppressor cell pathways that involve $CD4^+CD45RA^+$ suppressor- inducer T cells. *J.Immunol.* 145:2854.

112. Skov, L., H. Hansen, H.C. Dittmar, J.N. Barker, J.C. Simon, and O. Baadsgaard. 1998. Susceptibility to effects of UVB irradiation on induction of contact sensitivity, relevance of number and function of Langerhans cells and epidermal macrophages. *Photochem.Photobiol.* 67:714.

113. Santamaria Babi, L.F., R. Moser, M.T. Perez Soler, L.J. Picker, K. Blaser, and C. Hauser. 1995. Migration of skin-homing T cells across cytokine-activated human endothelial cell layers involves interaction of the cutaneous lymphocyte-associated antigen (CLA), the very late antigen-4 (VLA- 4), and the lymphocyte function-associated antigen-1 (LFA-1). *J.Immunol.* 154:1543.

114. Butcher, E.C. and L.J. Picker. 1996. Lymphocyte homing and homeostasis. *Science* 272:60.

115. Heckmann, M., B. Eberlein-Konig, A. Wollenberg, B. Przybilla, and G. Plewig. 1994. Ultraviolet-A radiation induces adhesion molecule expression on human dermal microvascular endothelial cells. *Br.J.Dermatol.* 131:311.

116. Rhodes, L.E., M. Joyce, D.C. West, I. Strickland, and P.S. Friedmann. 1996. Comparison of changes in endothelial adhesion molecule expression following UVB irradiation of skin and a human dermal microvascular cell line (HMEC-1). *Photodermatol.Photoimmunol.Photomed.* 12:114.

117. Treina, G., C. Scaletta, A. Fourtanier, S. Seite, E. Frenk, and L.A. Applegate. 1996. Expression of intercellular adhesion molecule-1 in UVA- irradiated human skin cells in vitro and in vivo. *Br.J.Dermatol.* 135:241.

118. Strickland, I., L.E. Rhodes, B.F. Flanagan, and P.S. Friedmann. 1997. TNF-alpha and IL-8 are upregulated in the epidermis of normal human skin after UVB exposure: correlation with neutrophil accumulation and E-selectin expression. *J.Invest.Dermatol.* 108:763.

119. Heckmann, M., M. Pirthauer, and G. Plewig. 1997. Adhesion of leukocytes to dermal endothelial cells is induced after single-dose, but reduced after repeated doses of UVA. *J.Invest.Dermatol.* 109:710.

120. Luger, T.A., S. Beissert, and T. Schwarz. 1997. The epidermal cytokine network. In Skin Immune System (SIS). J.D. Bos, editor. CRC Press LLC, Boca Raton, Florida. 271-307.

121. Pasch, M.C., N.H.A. van den Bosch, M.R. Daha, J.D. Bos, and S.S. Asghar. 2000. Synthesis of complement components C3 and factor B in human keratinocytes is differentially regulated by cytokines. *J.Invest.Dermatol.* 114:78.

122. Barker, J.N., R.S. Mitra, C.E. Griffiths, V.M. Dixit, and B.J. Nickoloff. 1991. Keratinocytes as initiators of inflammation. *Lancet* 337:211.

123. Barker, J.N. 1997. Epidermis as a pro-inflammatory organ. In Skin Immune System (SIS). J.D. Bos, editor. CRC Press LLC, Boca Raton; Florida. 339-345.

124. Ansel, J.C., T.A. Luger, and I. Green. 1983. The effect of in vitro and in vivo UV irradiation on the production of ETAF activity by human and murine keratinocytes. *J.Invest.Dermatol.* 81:519.

125. Kupper, T.S., A.O. Chua, P. Flood, J. McGuire, and U. Gubler. 1987. Interleukin 1 gene expression in cultured human keratinocytes is augmented by ultraviolet irradiation. *J.Clin.Invest.* 80:430.

126. Barr, R.M., S.L. Walker, W. Tsang, G.I. Harrison, P. Ettehadi, M.W. Greaves, and A.R. Young. 1999. Suppressed alloantigen presentation, increased TNF-alpha, IL-1, IL-1Ra, IL-10, and modulation of TNF-R in UV-irradiated human skin. *J.Invest.Dermatol.* 112:692.

127. Urbanski, A., T. Schwarz, P. Neuner, J. Krutmann, R. Kirnbauer, A. Kock, and T.A. Luger. 1990. Ultraviolet light induces increased circulating interleukin-6 in humans. *J.Invest.Dermatol.* 94:808.

128. Kirnbauer, R., A. Kock, P. Neuner, E. Forster, J. Krutmann, A. Urbanski, E. Schauer, J.C. Ansel, T. Schwarz, and T.A. Luger. 1991. Regulation of epidermal cell interleukin-6 production by UV light and corticosteroids. *J.Invest.Dermatol.* 96:484.

129. Kondo, S., T. Kono, D.N. Sauder, and R.C. McKenzie. 1993. IL-8 gene expression and production in human keratinocytes and their modulation by UVB. *J.Invest.Dermatol.* 101:690.

130. Kock, A., T. Schwarz, R. Kirnbauer, A. Urbanski, P. Perry, J.C. Ansel, and T.A. Luger. 1990. Human keratinocytes are a source for tumor necrosis factor alpha: evidence for synthesis and release upon stimulation with endotoxin or ultraviolet light. *J.Exp.Med.* 172:1609.

131. Wolf, H., H. Maier, R.R. Müllegger, C.A. Chadwick, R. Hofmann-Wellenhof, H.P. Soyer, J. Smolle, M. Horn, L. Cerroni, D. Yarosh, J. Klein, C. Bucana, K. Dunner Jr, C.S. Potten, H. Honigsmann, H. Kerl, and M.L. Kripke. 2000. Topical treatment with liposomes containing T4 endonuclease V protects human skin from ultraviolet-induced upregulation of interleukin-10 and tumor necrosis factor-α. *J.Invest.Dermatol.* 114:149.

132. Dvorak, H.F. and M.C. Mihm, Jr. 1972. Basophilic leukocytes in allergic contact dermatitis. *J.Exp.Med.* 135:235.

133. Benyon, R.C. 1989. The human skin mast cell. *Clin.Exp.Allergy* 19:375.

134. Moller, A., B.M. Henz, A. Grutzkau, U. Lippert, Y. Aragane, T. Schwarz, and S. Kruger-Krasagakes. 1998. Comparative cytokine gene expression: regulation and release by human mast cells. *Immunology* 93:289.

135. Kumakiri, M., K. Hashimoto, and I. Willis. 1977. Biologic changes due to long-wave ultraviolet irradiation on human skin: ultrastructural study. *J.Invest.Dermatol.* 69:392.

136. Walsh, L.J. 1995. Ultraviolet B irradiation of skin induces mast cell degranulation and release of tumour necrosis factor-alpha. *Immunol.Cell Biol.* 73:226.

137. Bevilacqua, M.P. 1993. Endothelial-leukocyte adhesion molecules. *Annu.Rev.Immunol.* 11:767.

138. Watson, C., S. Whittaker, N. Smith, A.J. Vora, D.C. Dumonde, and K.A. Brown. 1996. IL-6 acts on endothelial cells to preferentially increase their adherence for lymphocytes. *Clin.Exp.Immunol.* 105:112.

139. Groves, R.W., M.H. Allen, E.L. Ross, J.N. Barker, and D.M. MacDonald. 1995. Tumour necrosis factor alpha is pro-inflammatory in normal human skin and modulates cutaneous adhesion molecule expression. *Br.J.Dermatol.* 132:345.

140. Groves, R.W., E. Ross, J.N. Barker, J.S. Ross, R.D. Camp, and D.M. MacDonald. 1992. Effect of in vivo interleukin-1 on adhesion molecule expression in normal human skin. *J.Invest.Dermatol.* 98:384.

141. Santamaria Babi, L.F., B. Moser, S.M. Perez, R. Moser, P. Loetscher, B. Villiger, K. Blaser, and C. Hauser. 1996. The interleukin-8 receptor B and CXC chemokines can mediate transendothelial migration of human skin homing T cells. *Eur.J.Immunol.* 26:2056.

142. Wilkinson, P.C. and F.Y. Liew. 1995. Chemoattraction of human blood T lymphocytes by interleukin-15. *J.Exp.Med.* 181:1255.

143. Madsen, P., H.H. Rasmussen, H. Leffers, B. Honore, K. Dejgaard, E. Olsen, J. Kiil, E. Walbum, A.H. Andersen, B. Basse, J.B. Lauridsen, G.P. Ratz, A. Celis, J. Vandekerckhove, and J.E. Celis. 1991. Molecular cloning, occurrence, and expression of a novel partially secreted protein "psoriasin" that is highly up- regulated in psoriatic skin. *J.Invest.Dermatol.* 97:701.

144. Jinquan, T., H. Vorum, C.G. Larsen, P. Madsen, H.H. Rasmussen, B. Gesser, M. Etzerodt, B. Honore, J.E. Celis, and K. Thestrup-Pedersen. 1996. Psoriasin: a novel chemotactic protein. *J.Invest.Dermatol.* 107:5.

145. Center, D.M., H. Kornfeld, and W.W. Cruikshank. 1996. Interleukin 16 and its function as a CD4 ligand. *Immunol.Today* 17:476.

146. Rumsaeng, V., W.W. Cruikshank, B. Foster, C. Prussin, A.S. Kirshenbaum, T.A. Davis, H. Kornfeld, D.M. Center, and D.D. Metcalfe. 1997. Human mast cells produce the $CD4^+$ T lymphocyte chemoattractant factor, IL-16. *J.Immunol.* 159:2904.

147. Kunkel, S.L., N. Lukacs, and R.M. Strieter. 1995. Expression and biology of neutrophil and endothelial cell-derived chemokines. *Semin.Cell Biol.* 6:327.

148. Cassatella, M.A. 1999. Neutrophil-derived proteins: selling cytokines by the pound. *Adv.Immunol.* 73:369.

149. Baadsgaard, O., G.J. Fisher, J.J. Voorhees, and K.D. Cooper. 1990. Interactions of epidermal cells and T cells in inflammatory skin diseases. *J.Am.Acad.Dermatol.* 23:1312.

150. Shiohara, T. and N. Moriya. 1997. Epidermal T cells: their functional role and disease relevance for dermatologists. *J.Invest.Dermatol.* 109:271.

151. Everett, M.A., E. Yeargers, R.M. Sayre, and R.L. Olson. 1966. Penetration of epidermis by ultraviolet rays. *Photochem.Photobiol.* 5:533.

Chapter 2

Solar-simulated ultraviolet irradiation induces selective influx of $CD4^+$ T lymphocytes in normal human skin

Sergio Di Nuzzo[1], Menno A. de Rie[1], Chris M. van der Loos[2], Jan D. Bos[1], and Marcel B.M. Teunissen[1]

[1]Department of Dermatology and [2]Department of Cardiovascular Pathology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

Photochemistry and Photobiology (1996) 64: 988-993.

Abstract

The proportion and composition of the human cutaneous $CD3^+$ T lymphocyte population was determined *in situ* following a single exposure to physiological, erythema-inducing doses of simulated solar radiation, mainly consisting of UV radiation. Biopsies were taken 1, 2 and 7 days after local irradiation of normal volunteers with 1, 2, and 4 MED by a Xenon-arc lamp and immunohistochemistry was performed on cryostat sections. UV radiation caused an initial decrease of intraepidermal $CD3^+$ T cell numbers or even could lead to T-cell depletion 24 and 48 hours post-irradiation, and this was followed by an infiltration of T cells in the epidermis as determined 1 week after UV exposure. The number of dermal $CD3^+$ T cells was increased 24 hours after irradiation, reached a maximum at 48 hours and subsequently declined at day 7, though remained significantly higher than the unirradiated control. Double staining demonstrated that the $CD3^+$ T cells, which immigrated the (epi)dermis upon UV exposure, co-expressed CD4 but not CD8. Therefore the CD4/CD8 ratio in skin was markedly increased during the first week upon UV exposure. Our time course study shows that UV radiation affects the T cell population within the human skin by depleting the majority of epidermal T cells and initiating a selective influx of $CD4^+$ T cells.

Introduction

Acute exposure of human skin to an adequate dose of UV radiation provokes an inflammatory-like response, followed by tanning and epidermal thickening (1,2). The histologic features of inflammation upon UV exposure may be evident in hematoxylin/eosin-stained sections of biopsy specimens as early as 8 to 12 h post-irradiation. The epidermis is the portion of the body that is most extensively affected by UV, displaying spongiosis and formation of sunburn cells. Due to the limited capacity of UV radiation to penetrate the epidermis, the dermis shows very little change except for mild to moderate vascular dilatation and the presence of perivascular infiltrates, predominantly comprized of mononuclear cells and few polymorphonuclear cells.

T lymphocytes play a dominant role in the regulation of immune responses and it is generally believed that the T-cell phenotype is related to its function. Considerable numbers of T cells reside in normal dermis and epidermis (3,4). Upon UVB irradiation, T lymphocytes can occasionally be noticed in the epidermis 24 and 72 h post-irradiation (5,6) and the appearance of perivascular T lymphocyte infiltrates is evident (7-9). Apparently, exposure of skin to UV radiation leads to changes in the number, and probably also composition, of the cutaneous T cell population. In addition to this local effect, systemic alterations of the T cell population can be appreciated, as exemplified by the reported decrease of the proportion of circulating T cells (10) and the change of CD4/CD8 ratio upon acute or chronic UV exposure (11,12).

Further knowledge concerning the (in)direct effects of UV radiation on the different populations of the different T cell subsets in human skin is lacking. Having in mind that UVB irradiation induces suppression of immune responses and that T cells are dominant regulators of these responses, we felt it necessary to determine the fate of the cutaneous T cell subtypes *in situ* upon irradiation, in order to get a better insight in the mechanism of UV-induced immunosuppression. In this initial time course study we focussed on UV-induced quantitative alterations of the total T cell population ($CD3^+$), as well as the CD4 and CD8 subsets, in skin sections of biopsies from volunteers exposed to simulated solar radiation, predominantly consisting of UV radiation. We found that a single exposure to physiological doses of UV radiation caused a selective influx of $CD3^+CD4^+$, but not $CD3^+CD8^+$, T cells in the dermis and epidermis, thereby increasing the CD4/CD8 ratio.

Materials and Methods

Subjects In this investigation we used five healthy volunteers, who had given their informed consent, according to the guidelines of the Medical Ethical Committee of the hospital. They were four caucasian males and one female with skin type II or III, and their mean age was 27.8 (range 18 - 47 years). None suffered from any skin disease nor from light sensitivity. One week before the experiment, the individual minimal erythema dose (MED) was determined by irradiating separate small areas of skin with increasing UV doses. Results were read at 24 h post-exposure and the lowest dose in the series which just produced erythema was taken as 1 MED.

UV irradiation and biopsies UV irradiations were performed with a 1000 Watt Xenon-arc solar-simulator lamp (Oriel, Stratford, CT), as described in detail elsewhere (13). This lamp emitted a broad band UV spectrum (250 - 400 nm) with a peak at 350 nm and was used in combination with a WG 305 cut-off filter (Schott Glaswerke, Mainz, Germany). In order to minimize the heat-causing infra-red radiation, the radiation beam was passed through a water filter of 7 cm pathway and was guided via a dichroic mirror (13). Then the radiation beam was focussed and passed through a light guide (Oriel), enabling the fixation of the irradiation spot on the skin during the exposure time. In this way a circular spot of 2.54 cm^2 was formed at 7 cm distance from the exit aperture of the light guide. The UV-radiation intensity was measured by a 550-1 radiometer (EG&G, Salem, Mass.) fitted with a silicon detector probe with a neutral density filter. The UV radiation intensity at a target distance of 7 cm was 32 J/m^2. Three spots on the lower back of each volunteer received 1, 2 and 4 MED respectively. One, two and seven days post-irradiation three-millimeter punch biopsies were obtained under 2% Xylocaine local anesthesia. Control biopsies were taken from unirradiated back skin. All biopsies were orientated in O.C.T. compound (Tissue Teck) and immediately frozen in liquid nitrogen and stored in -70°C until use.

Single staining procedure Cryostat sections (6 mm) were allowed to dry before fixing in acetone for 10 min at 4°C. Endogenous peroxidase activity was blocked with 0.1% sodium azide and 0.3% H_2O_2 in phosphate buffered saline (PBS) for 20 min at room temperature. Sections were stained at room temperature by sequential incubation with primary mAb mouse anti-human CD3 (1:50; Becton Dickinson, Mountain View, CA) and horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (1:50; Dakopatts, Glostrup, Denmark). The first incubation was done in PBS supplemented with 1% bovine serum albumin and the

second with additional 10% normal human serum. Enzyme activity was detected using the chromogen 3-amino-9-ethylcarbazole (AEC; Sigma, St. Louis, MO) in acetate buffer (pH 4.9) giving a orange-red reaction product. The staining reaction was visually controlled and stopped by washing. Sections were counterstained with Mayer's hematoxylin and mounted with glycerin-gelatin (Dakopatts).

Double staining procedure Double staining of cryostat sections (6mm) was based on two primary antibodies of different animal origin (14). The subsequent steps at room temperature were performed: (1) an incubation of 60 min with a cocktail of rabbit anti-human CD3 (1:50; Dakopatts) plus either mouse anti-human CD4 (1:100; Becton Dickinson) or mouse anti-human CD8 (1:50; Becton Dickinson); (2) an incubation of 30 min with a cocktail of biotin-conjugated goat anti-mouse (1:200; Dakopatts) plus alkaline phosphatase-conjugated goat anti-rabbit (1:10; Dakopatts) (3) an incubation of 30 min with horseradish peroxidase-conjugated streptavidin (1:400; Dakopatts). (4) Alkaline phosphatase activity was detected as a blue color, using naphtol-AS-MX-phosphate (Sigma) as substrate and Fast Blue BB (Sigma) as azo dye. (5) Peroxidase activity was detectect as a orange-red color, using the chromogen AEC. Double stained cells could be appreciated by their purple color. Sections were rinsed three times in Tris buffered saline (pH 7.8) in between successive incubations. The staining reaction was visually controlled and stopped by washing. Sections were finally fixed in formaldehyde (4%) and mounted with glycerin-gelatin without counterstaining.

Lymphocyte enumeration and statistical analysis Enumeration and subdivision of skin in different compartments was performed as described before (3). The number of stained cells of three different sections per irradiation dose per time point were counted. These three values of each biopsy specimen were adjusted to 10 mm horizontal section values by deviding with the horizontal width multiplied by 10. The mean value of each biopsy was then used for the determination of total mean median and ranges of lymphocyte numbers. Horizontal width of sections ranged from 1.00 - 2.5, with a mean of 1.51 mm. The unpaired Student's *t*-test was used for statistical evaluation taking $p < 0.05$ (two-tailed) as the level of significance

Results

Kinetics of $CD3^+$ T cells upon UV radiation exposure in situ. Small areas on the lower back of 4 volunteers were UV-irradiated with 1, 2, or 4 MED. Local erythema development was clearly present at all three irradiated spots after 24 h, whereas maximal erythema

occurred after 48 h. This erythema was completely resolved at day 7 and pigmentation could be observed in the 4 MED irradiated site only.

Punch biopsies were taken 1, 2, and 7 days post-irradiation and cryostat sections were single stained for CD3. Examination of the epidermis revealed that all three applied doses of UV radiation provoked a significant ($p < 0.004$) reduction of the number of $CD3^+$ T cells at day 1 and day 2 (Fig. 1). In some sections, epidermal $CD3^+$ cells were even not detectable. This initial reduction of T cell numbers was followed by a clear significant ($p < 0.0001$) increase as observed one week after irradiation (Figs. 1 and 2C). A different response of the $CD3^+$ T cell population was found in the dermis (Fig. 1). The number of dermal T cells (predominantly perivascular) was considerably increased 24 h after irradiation and was maximal after 48 h (Fig. 2B; $p < 0.0001$). The number of T cells was declined by day 7, but was still significant higher than the unirradiated control (Fig. 2A). The time-course of the T cells in the epidermis and dermis showed a dose-dependent tendency (Fig. 1). Statistic analysis revealed that this tendency was however not significant, except for the dermal T cell numbers found after 4 MED exposure, which significantly outnumbered those after 1 MED exposure at all three time points.

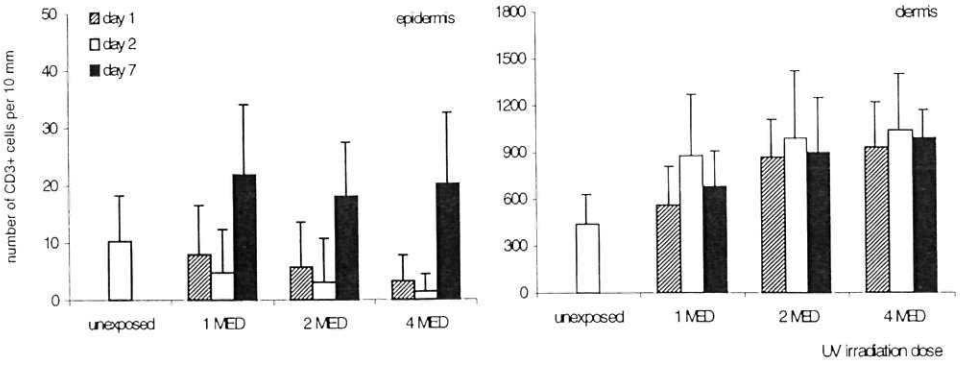


Figure 1. Kinetics of the $CD3^+$ T cell population in the epidermis and dermis following a single exposure to 1, 2, or 4 MED of UV radiation. Biopsies were taken 1, 2 and 7 days following UV-exposure. Unirradiated skin served as control (grey bar). Each bar represents mean a value of 15 determinations (i.e. triplo countings in 5 volunteers). Data are expressed as number of T cells per 10 mm horizontal cryostat section ± SD.

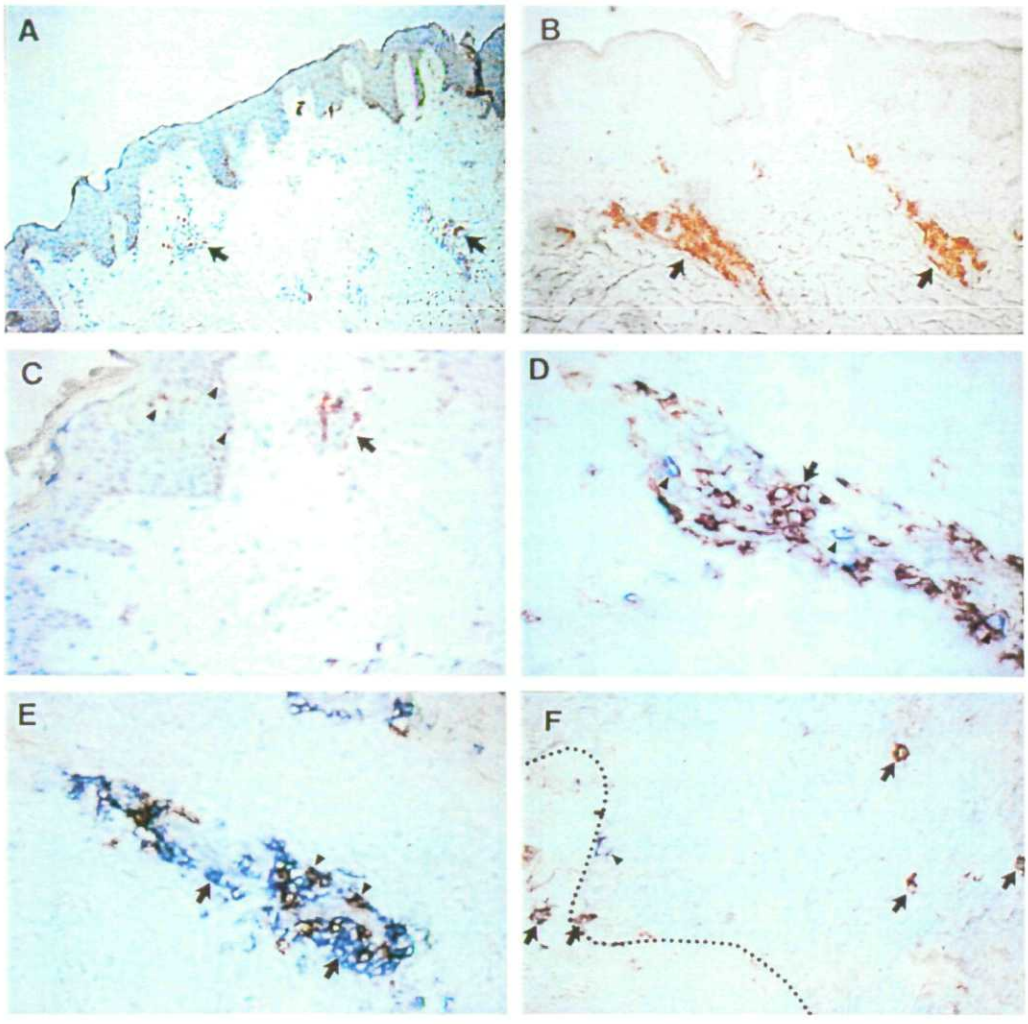


Figure 2. The T cell population in human skin upon exposure to UV radiation. A) CD3 (red) stained T cells in normal human skin are located predominantly perivascular in the dermis (arrow). B) Large infiltrates of $CD3^+$ (red) cells in the dermis can be observed 2 days after exposure to 2 MED (arrows). C) Increased numbers of $CD3^+$ (red) T cells can be found in the epidermis (arrowheads) and in the dermis (arrow) 7 days after exposure to 2 MED. D) CD3 (blue) and CD4 (red) double-staining of an infiltrate 2 days after exposure to 2 MED. Note that the majority of the cells are $CD3^+CD4^+$ (purple; arrow) and that few cells are $CD3^+CD4^-$ (blue; arrowheads). E) CD3 (blue) and CD8 (red) double-staining of an infiltrate 2 days after exposure to 2 MED. Note that the majority of the cells are $CD3^+CD8^-$ (blue; arrows) and that few cells are $CD3^+CD8^+$ (purple; arrowheads). F) CD3 (blue) and CD4 (red) double-staining of the epidermis 7 days after exposure to 2 MED. Majority of the cells are $CD3^+CD4^+$ (purple; arrows) and a single cell is $CD3^+CD4^-$ (blue; arrowhead). Dotted line represents the epidermal-dermal border.

Cutaneous $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell subpopulations differentially respond to UV radiation. The $CD3^+$ T cell population can roughly be divided into $CD4^+$ and $CD8^+$ subpopulations. In order to study whether these two major T cell subpopulations in normal skin are similarly affected by UV irradiation *in vivo*, cryostat sections were stained for double expression of either CD3 and CD4 or CD3 and CD8. This approach allowed us to exclude interference of Langerhans cells, monocytes and macrophages which are also able to express CD4. Analysis of the double stained frozen sections revealed that the (epi)dermal $CD3^+CD4^+$ cells (Figs. 2D and 3) showed quite similar kinetics as the single stained $CD3^+$ cells (Fig 1) in response to simulated solar radiation. The number of $CD3^+CD4^+$ cells was significantly ($p < 0.001$) decreased at 48 h post-irradiation and followed by a marked increase at day 7 (Figs. 2F and 3; $p < 0.04$). By contrast, the $CD3^+CD8^+$ cells did not show the marked influx at day 2 (dermis; Fig. 2E) and day 7 (epidermis). The epidermal $CD3^+CD8^+$ cells only returned to normal numbers at day 7 after initial depletion (Fig. 4), whereas the dermal $CD3^+CD8^+$ cells were not significantly altered in number at all (Fig. 4). The double staining data at day 1, 2, and 7 did not shown any statistical significant difference between the three applied UV doses.

In normal skin the CD4/CD8 ratio in the epidermis and dermis was 0.77 and 0.90, respectively, which is in agreement with previous observations (3,4). The effect of UV irradiation on the CD4/CD8 ratio in epidermis and dermis is summarized in Table 1. We found that the cutaneous CD4/CD8 ratio *in situ* was increased in all biopsies from UV-exposed skin as consequence of raised $CD3^+CD4^+$ and unaltered $CD3^+CD8^+$ numbers.

Control biopsies ($n = 2$) were taken from unirradiated skin at the same distance and time-points as with the irradiated skin. All control biopsies showed a normal distribution and number of $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells. As another control ($n = 2$), instead of taking three biopsies within one irradiated spot, the samples were taken from separate irradiated spots. The distance between the spots was a couple of centimeters, which is certainly large enough to avoid interference by wound healing. We found that this latter approach showed similar results as the former approach (data not shown). Both control experiments confirmed that the initial depletion of T cells in the epidermis and the successive selective influx of $CD3^+CD4^+$ T cells at the exposed skin site is a genuine UV effect.

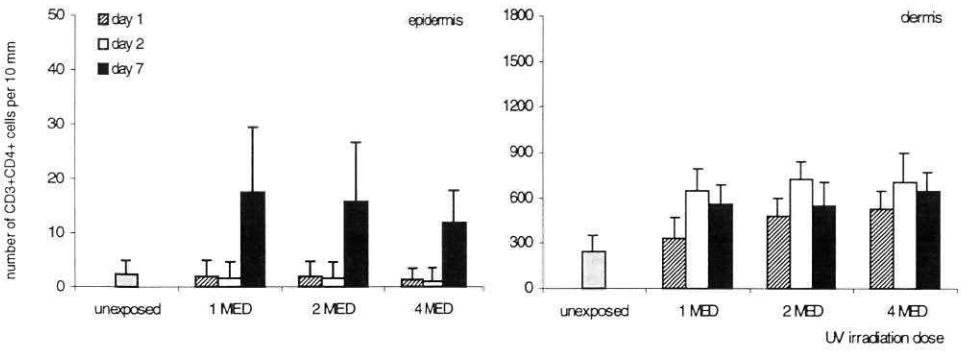


Figure 3. Kinetics of the $CD3^+CD4^+$ T cell population in the epidermis and dermis following a single exposure to 1, 2, or 4 MED of UV radiation. Biopsies were taken 1, 2 and 7 days following UV-exposure. Unirradiated skin served as control (grey bar). Each bar represents a mean value of 15 determinations (i.e. triplo countings in 5 volunteers). Data are expressed as number of $CD3^+CD4^+$ double positive T cells per 10 mm horizontal cryostat section ± SD.

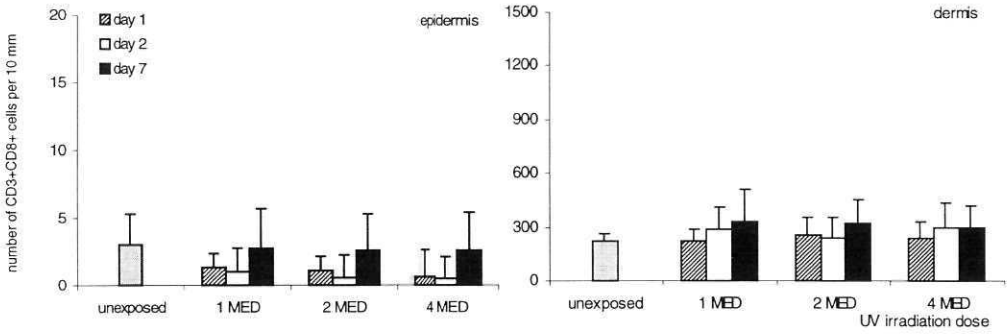


Figure 4. Kinetics of the $CD3^+CD8^+$ T cell population in the epidermis and dermis following a single exposure to 1, 2, or 4 MED of UV radiation. Biopsies were taken 1, 2 and 7 days following UV-exposure. Unirradiated skin served as control (grey bar). Each bar represents a mean value of 15 determinations (i.e. triplo countings in 5 volunteers). Data are expressed as number of $CD3^+CD8^+$ double positive T cells per 10 mm horizontal cryostat section ± SD.

Table 1. CD4/CD8 ratio in human skin after exposure to UV radiation[1].

CD4/CD8 ratio	Epidermis			Dermis		
	1 day[2]	2 days	7 days	1 day	2 days	7 days
1 MED[3]	1.45 ± 0.84[4]	1.55 ± 1.14	6.39 ± 4.68	1.51 ± 1.97	2.22 ± 1.16	1.70 ± 0.66
2 MED	1.73 ± 1.60	2.82 ± 2.15	6.04 ± 2.92	1.85 ± 1.24	3.06 ± 0.98	1.71 ± 1.17
4 MED	2.07 ± 1.32	2.08 ± 1.96	4.53 ± 1.71	2.20 ± 1.34	2.56± 1.03	2.18 ± 0.99
Normal		0.77 ± 0.27			0.90 ± 0.64	

[1]Human volunteers (n = 5) were locally exposed to 1, 2 or 4 MED of UV radiation. Skin sections were double stained for either $CD3^+CD4^+$ or $CD3^+CD8^+$. The CD4/CD8 ratio was calculated from the total numbers of the two T-cell populations. [2] Time lapse between irradiation and removal of skin biopsies. [3] Applied dose of UV radiation. [4] Mean CD4/CD8 ratio ± SD of five volunteers.

Discussion

In this study we demonstrated that the proportion and composition of cutaneous T cells markedly changed upon a single exposure to a physiological, erythema-inducing dose of simulated solar radiation, which predominantly consists of UV radiation. The number of $CD4^+$ and $CD8^+$ T cells in the epidermis was considerably decreased in the first few days after irradiation. The most likely explanation for this loss of T cells is that they are depleted due to the phototoxic effect of UV radiation. In a previous study (15) we have shown that the viability of T cells is hypersensitive to relatively low doses of UV radiation as compared to keratinocytes (15), monocytes (16), fibroblasts (17) or B lymphocytes (16,18). Taking into account that UV radiation hardly penetrates the epidermis and that T cells are highly susceptible, we have hypothesized that an exposure of skin to 1 MED UV radiation could nevertheless be sufficient to kill T cells residing in the epidermis (15). The decline of T cell numbers after acute irradiation reported here provides circumstantial evidence to support this postulation, although as an unlikely alternative, migration of T cells out of the epidermis can not entirely be excluded. Following this initial depletion of epidermal T cells a significant increase of T cell numbers took place, which was mainly attributed to the apparent infiltration of $CD4^+$ T cells. The epidermal $CD8^+$ T cell population just returned to the normal pre-irradiation level. It thus appeared that UV radiation can induce a selective immigration of $CD4^+$ cells into the epidermis and as a result the $CD4^+$ T cells outnumbered the $CD8^+$ T cells. In this respect it is important to note that in normal unirradiated epidermis $CD8^+$ T cells outnumber $CD4^+$ T cells (3,4).

In contrast to the epidermal data, the level of dermal T cell numbers showed an initial raise instead of an initial decline. Absence of this decrease might be explained by the assumption that the dose of UV radiation (especially UV-B) that reached the dermis was not sufficient to be toxic. Like in epidermis, the raise of dermal T cells was due to a selective influx of $CD4^+$, but not $CD8^+$ T cells. Our results demonstrated that the immigration of T cells into the epidermis started later than the influx of T cells into the dermis. We reasoned that this might be due to the fact that it takes some time for the cells to travel from the perivascular infiltrates, through the dermis and the basal membrane, to the epidermis. Our study revealed that the UV-induced inflammatory response in the skin *in vivo* was characterized by a preferential influx of $CD4^+$ T cells. Since many cutaneous inflammatory disorders (19) , but also a mild injury like as a suction blister (20), show a $CD4^+$ T cell dominated infiltrate, the

UV-induced migration of $CD4^+$ T cells into the skin can therefore not considered to be specific. As such, we are observing a general inflammatory principle in the skin immune system (21).

Chemotactic cytokines (22) are essentially involved in triggering and mediating the complex process of migration. At present it is obscure, however, which specific cytokine(s) and sequence of events are involved in the accumulation of $CD3^+CD4^+$ T cells in the skin in response to exposure to UV radiation and which restrain $CD3^+CD8^+$ T cells to enter. Recent studies indicate that the peptides psoriasin (23) and IL-16 (24) are specific chemotactic for $CD4^+$, but not for $CD8^+$ T cells. It might be relevant to investigate whether one of these proteins is the responsible $CD4^+$ T cell-attracting cytokine in irradiated skin.

Our finding that UV radiation induced perivascular infiltrates of considerable numbers of T cells is in accordance with the previously published observations of immigration of mononuclear cells (lymphocytes) in the dermis after UVB irradiation (6-8). However, all these investigations were only descriptive or were carried out with semi-quantitative scoring techniques (e.g. cell number per high power magnification field), using hematoxylin-eosin stained sections, except for Norris *et al* who used a monoclonal antibody against CD3 (9). The importance of our time course study is that we have performed careful quantitative analysis (cell number per 10 mm horizontal section), and in addition, we went one step further by identifying the response to UV exposure of the individual $CD4^+$ and $CD8^+$ cutaneous T cell subpopulations. As yet, the significance of our finding that $CD4^+$ T cells are preferentially immigrating into the skin following UV irradiation is not known. One might suggest that this phenomenon is a reflection of increased immunosurveillance. Ongoing research on these accumulated $CD4^+$ T cells *in situ* will provide a more precize description of the phenotype and function of these cells in the near future and will possibly provide some insight in the role these T cells play in the immunomodulatory mechanism of UV radiation.

Acknowledgements

We thank Arthur Kammeyer for providing expert assistance with the solar-simulator.

References

1. Soter, N.A. 1990. Acute effects of ultraviolet radiation on the skin. *Semin.Dermatol.* 9:11.

2. Hruza, L.L. and A.P. Pentland. 1993. Mechanisms of UV-induced inflammation. *J.Invest.Dermatol.* 100:35S.

3. Bos, J.D., I. Zonneveld, P.K. Das, S.R. Krieg, C.M. van der Loos, and M.L. Kapsenberg. 1987. The skin immune system (SIS): distribution and immunophenotype of lymphocyte subpopulations in normal human skin. *J.Invest.Dermatol.* 88:569.

4. Foster, C.A., H. Yokozeki, K. Rappersberger, F. Koning, B. Volc-Platzer, A. Rieger, J.E. Coligan, K. Wolff, and G. Stingl. 1990. Human epidermal T cells predominantly belong to the lineage expressing alpha/beta T cell receptor. *J.Exp.Med.* 171:997.

5. Gilchrest, B.A., N.A. Soter, J.S. Stoff, and M.C. Mihm, Jr. 1981. The human sunburn reaction: histologic and biochemical studies. *J.Am.Acad.Dermatol.* 5:411.

6. Gilchrest, B.A., J.S. Stoff, and N.A. Soter. 1982. Chronologic aging alters the response to ultraviolet-induced inflammation in human skin. *J.Invest.Dermatol.* 79:11.

7. Rosario, R., G.J. Mark, J.A. Parrish, and M.C. Mihm. 1979. Histological changes produced in skin by equally erythemogenic doses of UV-A, UV-B, UV-C and UV-A with psoralen. *Br.J.Dermatol.* 101:299.

8. Hawk, J.L., G.M. Murphy, and C.A. Holden. 1988. The presence of neutrophils in human cutaneous ultraviolet-B inflammation. *Br.J.Dermatol.* 118:27.

9. Norris, P., R.N. Poston, D.S. Thomas, M. Thornhill, J.L. Hawk, and D.O. Haskard. 1991. The expression of endothelial leukocyte adhesion molecule-1 (ELAM-1), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) in experimental cutaneous inflammation: a comparison of ultraviolet B erythema and delayed hypersensitivity. *J.Invest.Dermatol.* 96:763.

10. Morison, W.L., J.A. Parrish, K.J. Bloch, and J.I. Krugler. 1979. In vivo effect of UV-B on lymphocyte function. *Br.J.Dermatol.* 101:513.

11. Hersey, P., G. Haran, E. Hasic, and A. Edwards. 1983. Alteration of T cell subsets and induction of suppressor T cell activity in normal subjects after exposure to sunlight. *J.Immunol.* 131:171.

12. McGrath, H., W.A. Wilson, and E. Scopelitis. 1986. Acute effects of low-fluence ultraviolet light on human T- lymphocyte subsets. *Photochem.Photobiol.* 43:627.

13. Kammeyer, A., M.B.M. Teunissen, S. Pavel, M.A. De Rie, and J.D. Bos. 1995. Photoisomerization spectrum of urocanic acid in human skin and in vitro: effects of simulated solar and artificial ultraviolet radiation. *Br.J.Dermatol.* 132:884.

14. van der Loos, C.M., A.E. Becker, and J.J. van den Oord. 1993. Practical suggestions for successful immunoenzyme double- staining experiments. *Histochem.J.* 25:1.

15. Teunissen, M.B.M., R.M. Sylva-Steenland, and J.D. Bos. 1993. Effect of low-dose ultraviolet-B radiation on the function of human T lymphocytes in vitro. *Clin.Exp.Immunol.* 94:208.

16. Kremer, I.B , J.D. Bos, and M.B.M. Teunissen. 1995. Reduced antigen-presenting function of human Epstein-Barr virus (EBV)-B cells and monocytes after UVB radiation is accompanied by decreased expression of B7, intercellular adhesion molecule-1 (ICAM-1) and LFA-3. *Clin.Exp.Immunol.* 101:461.

17. Arlett, C.F., J.E. Lowe, S.A. Harcourt, A.P. Waugh, J. Cole, L. Roza, B.L. Diffey, T. Mori, O. Nikaido, and M.H. Green. 1993. Hypersensitivity of human lymphocytes to UV-B and solar irradiation. *Cancer Res.* 53:609.

18. Deeg, H.J., L. Bazar, M. Sigaroudinia, and M. Cottler-Fox. 1989. Ultraviolet B light inactivates bone marrow T lymphocytes but spares hematopoietic precursor cells. *Blood* 73:369.

19. van Joost, T., J.D. Bos, and T.M. Starink. 1993. T cell-mediated dermatose: an antology of certain T cell-mediated inflammatory dermatoses. Bohn Stafleau Van Loghum, Houten.

20. Reusch, M.K., J.N. Mansbridge, B.J. Nickoloff, and V.B. Morhenn. 1991. Immunophenotyping of skin cells during healing of suction blister injury. *Dermatologica* 183:179.

21. Barker, J.N., R.S. Mitra, C.E. Griffiths, V.M. Dixit, and B.J. Nickoloff. 1991. Keratinocytes as initiators of inflammation. *Lancet* 337:211.

22. Oppenheim, J.J., C.O. Zachariae, N. Mukaida, and K. Matsushima. 1991. Properties of the novel proinflammatory supergene "intercrine" cytokine family. *Annu.Rev.Immunol.* 9:617.

23. Jinquan, T., H. Vorum, C.G. Larsen, P. Madsen, H.H. Rasmussen, B. Gesser, M. Etzerodt, B. Honore, J.E. Celis, and K. Thestrup-Pedersen. 1996. Psoriasin: a novel chemotactic protein. *J.Invest.Dermatol.* 107:5.

24. Laberge, S., W.W. Cruikshank, D.J. Beer, and D.M. Center. 1996. Secretion of IL-16 (lymphocyte chemoattractant factor) from serotonin-stimulated $CD8^+$ T cells in vitro. *J.Immunol.* 156:310.

Chapter 3

UVB radiation preferentially induces recruitment of memory $CD4^+$ T cells in normal human skin: long-term effect after a single exposure

Sergio Di Nuzzo[1], Regien M.R. Sylva-Steenland[1], Menno A. de Rie[1], Pranab K. Das[1,2], Jan D. Bos[1] and Marcel B.M. Teunissen[1].

[1]Department of Dermatology and [2]Department of Pathology,
Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

Journal of Investigative Dermatology (1998) 110: 978-981.

Abstract

Acute, low-doses of ultraviolet-B radiation affect the immune competent cells of the skin immune system. In this study, we examined the time-dependent changes of the cutaneous T cell population in normal human volunteers following a single local exposure to UV. Solar-simulated UV radiation caused an initial decrease in intraepidermal T cells numbers, even leading to T cell depletion at day 4, whereupon a considerable infiltration of T cells in the epidermis occurred which peaked at day 14. In the dermis the number of T cells was markedly increased at day 2 (peak) and 4 after irradiation, and subsequently declined to the non-irradiatiated control values at day 10. Double-staining with several T cell markers showed that the T cells, infiltrating the (epi)dermis upon UV exposure, were almost exclusively $CD4^+$ $CD45RO^+$ T cells, expressing an α/β type T cell receptor, but lacking the activation markers HLA-DR, VLA-1 and IL-2R. Application of UVB radiation resulted in similar dynamics of T cells, indicating that the UVB wavelengths within the solar-simulated UV radiation were responsible for the selective influx of $CD4^+$ T cells. In conjunction with UVB-induced alterations in the type and function of antigen-presenting cells (i.e. Langerhans cells and macrophages), the changes of the cutaneous T cell population may also contribute to UVB-induced immunosuppression at skin level in man.

Introduction

Low-dose UVB radiation can impair cutaneous immunity in humans as illustrated by the reduction in an effective sensitisation and the promotion of tolerance to antigens epicutaneously applied onto UVB-exposed skin (1,2). The exact mechanisms of this low-dose UVB-induced local immunosuppression have not yet been elucidated (3). Upon irradiation the Langerhans cells disappear from the epidermis in a dose-dependent manner (4) and their capacity as antigen-presenting cells to stimulate T cells is reduced (5). Concurrently, the epidermis is infiltrated by a population of macrophages (6), which possess the ability to stimulate $CD4^+$ suppressor-inducer T cells (7). This reversal of the antigen-presenting cell population into the UVB-irradiated site has been proposed as one of the cellular mechanisms of UVB-induced immunosuppression.

In a recent report we demonstrated that in normal human skin the T cell population is also altered by low-doses of UV treatment (8). This preliminary study showed that a single exposure to physiological erythema-inducing doses of solar-simulated UV radiation caused a reduction or even depletion of intraepidermal T cells at day 1 and 2 after irradiation, whereas at day 7 postirradiation remarkably high numbers of $CD4^+$, but not $CD8^+$ T cells, were found in the epidermis. In the dermis, a selective infiltration of $CD4^+$ T cells was observed during the first few days after exposure. Although these findings clearly demonstrate a preferential recruitment of $CD4^+$ T cells upon UV exposure, the number of time points studied were limited. Consequently, it was not known at what time-point the influx of $CD4^+$ T cells reaches its maximum and when the T cells numbers recede to preirradiation values. Further, it is also not clear whether this UV-induced cutaneotropic $CD4^+$ T cell represents a specific subtype of T cell, such as naive or memory, α/β T cell receptor (TCR) or γ/δ TCR, or whether these T cells are activated or not. As concerns the $CD8^+$ T cells we may have missed a possible influx because we have looked at limited array of time points.

Therefore, the present investigation was undertaken to determine in more detail the kinetics of the $CD4^+$ and $CD8^+$ T cells, as well as the phenotype of the T lymphocytes that infiltrate the human skin after a single exposure to solar-simulated UV radiation. We observed that UV radiation caused a selective influx of $CD4^+$, α/β TCR^+, non-activated, memory T cells in the skin that reached a maximum in the dermis at day 2 and in the epidermis at day 14. In addition, we found that the UVB region within the solar simulated radiation source was responsible for this phenomenon.

Materials and Methods

Subjects. Ten healthy volunteers (9 male, 1 female) participated in this investigation. All subjects were caucasian with skin type II or III. The age ranged from 18 - 48 years (mean 26,3). None suffered from any skin disease nor from light sensitivity and they were on no chronic or intermittent medication. All participants gave informed consent.

UV irradiation and biopsies. A 1000 W xenon-arc lamp (Oriel, Stratford, CT) was used as the UV-source for the induction of skin erythema. In order to reduce heat (infrared radiation) the light beam was passed through a waterfilter, reflected by a dichroic (cold) mirror and filtered by a UG 11 filter (Jenaer Glaswerke Schott & Gen., Mainz, Germany). A 3 mm WG 305 filter (Jenaer Glaswerke Schott & Gen.) was used to adjust the short wave side of the xenon-arc emission for the solar irradiance at sea level. Alternatively, narrow band UVB irradiations were performed with a 303 nm interference filter (Jenaer Glaswerke Schott & Gen.), the WG 305 and UG11 filters were removed.

Several weeks before the experiment, the individual MED was determined. Single doses of 1 or 4 MED were then given to multiple sites on the opposite buttocks at various time periods before the biopsies. Control biopsies were taken from unirradiated skin. Three millimeter punch biopsies were obtained under 2% local anesthesia. All biopsies were oriented in O.C.T. compound (Tissue Teck), immediately frozen in liquid nitrogen and stored at -70°C until use.

Immunohistochemistry. Immunohistochemical single-staining was performed by using two different protocols. The first protocol was based on a two-step indirect peroxidase technique as described previously (8). Briefly, the cryostat sections (6 mm) were incubated with primary monoclonal antibody mouse anti-human CD3 (Becton Dickinson, Mountain View, CA), followed by an incubation with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako, Glostrup, Denmark). To detect TCR(s) a second protocol was applied using the streptavidin-biotin-complex (streptABC) method. The sections were incubated with primary monoclonal antibodies mouse anti-human TCR δ1 chain (T Cell Diagnostics, Woburn, MA) or mouse anti-human TCR β chain (T Cell Diagnostics), followed by an incubation with biotinylated goat anti-mouse IgG, then treated with the peroxidase-streptABC solution (Dako). In both protocols before counterstainig with haematoxilin, the horseradish peroxidase activity was detected with H_2O_2 as substrate and AEC (Sigma, St Louis, MO) as chromogen.

Immunohistochemical double-staining was performed using different, previous described protocols based on combination of a polyclonal rabbit and a monoclonal mouse primary antibodies (9). The subsequent steps at room temperature were performed: (1) an incubation of 60 min with a cocktail of rabbit anti-human CD3 (Dako) plus one of each of the mouse antibodies anti-human CD4, CD8 (Becton Dickinson), CD45RA, CD45RO, CD25, HLA-DR (Dako), CD49a (Serotec, Oxford, England); (2) an incubation of 30 min with a cocktail of biotin-conjugated goat anti-mouse (Dako) plus either alkaline phosphatase-conjugated goat anti-rabbit (Dako) (protocol 1) or horseradish peroxidase-conjugated goat anti-rabbit (Dako) (protocol 2) and (3) an incubation of 30 min with either horseradish peroxidase-conjugated streptavidin (Dako) (protocol 1) or alkaline phosphatase-conjugated streptavidin (Dako) (protocol 2). (4) Alkaline phosphatase activity was detected as blue color, using naphthol-AS-MX-phosphate (Sigma) as a substrate and fast blue BB (Sigma) as azo dye. (5) peroxidase activity was detected as an orange-red color, using the chromogen AEC. Double-stained cells could be appreciated by their purple color.

Lymphocyte enumeration and statistical analysis. Enumeration and subdivision of the skin in different compartments was performed as described before (10). Briefly, the skin was divided into two compartments: epidermis and dermis. The identifications on the object glasses were covered before counting to enable blind quantification. The number of single or double clearly stained cell bodies of three up to six different serial sections per irradiation dose per time point were counted. The values of each biopsy specimen were adjusted to 10 mm horizontal section values by dividing with the horizontal width multiplied by 10. The mean value of each biopsy was then used for the determination of total mean of lymphocyte numbers. The unpaired Student's t-test was used for statistical evaluation taking $P< 0.05$ (two-tailed) as the level of significance.

Results

Prolonged disturbance of the cutaneous T cell population upon a single exposure to solar-simulator UV radiation. Analysis of single-stained skin sections revealed that both doses of UV radiation provoked a significant reduction of the number of intraepidermal $CD3^+$ cells at day 2 and day 4 (Fig. 1a). This initial reduction in T-cell numbers was followed by a significant increase at day 10 and was maximal after 14 days (4 MED). Although, the T-cell kinetics after 1 MED treatment showed a similar tendency, the increase of T cell numbers at

day 10 and 14 was not significant as compared to pre-irradiation values, but was significant when compared to day 2 and 4 values. The number of T cells returned to pre-irradiation values at day 21, showing that a single exposure to a physiological erythema-inducing dose of simulated-solar radiation can cause a long term effect (about 3 weeks) on intraepidermal T lymphocytes. A different response of the $CD3^+$ cell population was found in the dermis (Fig. 1b).

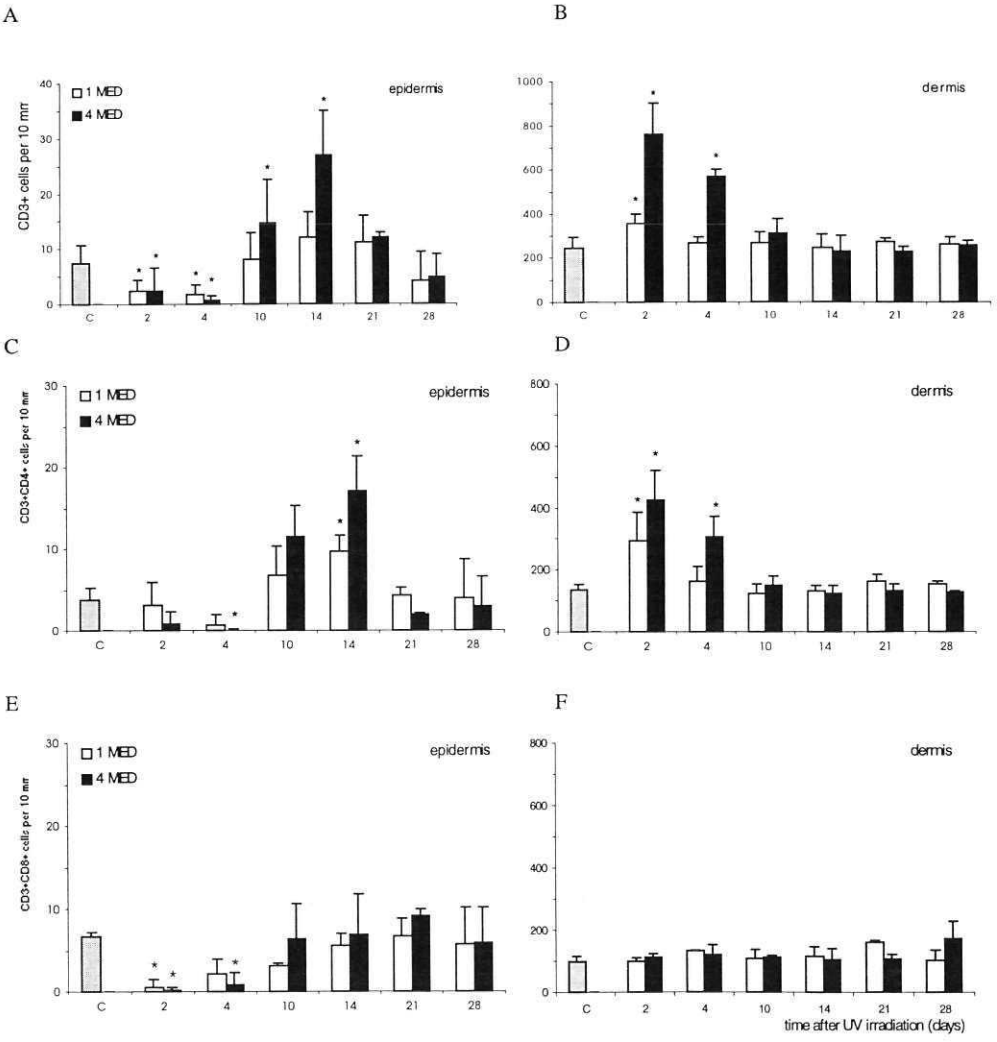


Figure 1. Solar-simulated ultraviolet radiation induces prolonged disturbance of the cutaneous T cell population. Kinetics of the $CD3^+$ (Fig. 1A and 1B) , $CD3^+CD4^+$ (Fig. 1C and 1D) and $CD3^+CD8^+$ (Fig. 1E and 1F) T cell populations in the epidermis and dermis following a single exposure to 1 (open bar) or 4 (black bar) MED of solar-simulated UV radiation. Exposures to UV were performed at various time periods (-28,-21,-14,-10,-4,-2 days) before collecting biopsies. Unirradiated skin served as control (gray bar). Each bar represents the mean value of 9 determinations (i.e. triple countings in 3 volunteers, n=3). Data are expressed as a number of T cells per 10 mm horizontal section + SD (* indicates $p<0.05$).

The number of dermal T cells (predominantly perivascular) was considerably increased 2 days after irradiation (both applied doses). The number was still significantly higher at day 4 and returned to pre-irradiations values at day 10 (4 MED). The time-course of the $CD3^+$ cells in the epidermis and dermis showed a dose-dependent tendency. Statistic analysis revealed that this tendency was only significant for the dermal T-cell numbers at day 2 and 4 but not for the epidermal T cell numbers.

$CD3^+$ cells migrating into the (epi)dermis upon solar-simulated ultraviolet irradiation coexpressed CD4 but not CD8. Examination of the double-stained sections revealed that the (epi)dermal $CD3^+CD4^+$ cells (Fig. 1c and 1d) showed similar kinetics as the single-stained $CD3^+$ cells in response to solar-simulated radiation. The number of intraepidermal $CD3^+CD4^+$ cells was decreased at day 2, reached the minimum at day 4 for both the applied doses, subsequently followed by an increase which was maximal after 14 days and receded to pre-irradiation values at day 21 (Fig. 1c). The dermal $CD3^+CD4^+$ cells were significantlly increased at day 2 and 4 (both applied doses), and reached the preirradiation values at day 10 (Fig. 1d). By contrast, the intraepidermal $CD3^+CD8^+$ cells only returned to preirradiation numbers at day 14 after initial depletion (Fig. 1e). The dermal $CD3^+CD8^+$ cells were not significantly altered in number at all (Fig. 1f). The double-staining data showed a dose-dependent tendency, but this was however not significant.

UVB wavelenghts within the solar-simulated UV radiation are responsible for the changes of the cutaneous T cells. The solar-simulated radiation from our xenon-arc lamp setup mainly consisted of UVA, UVB and visible light. We assumed that the UVB wavelenghts within our solar-simulated UV radiation spectrum were responsible for the changes of the cutaneous T cells after exposure. Like the solar-simulated radiation, UVB radiation significantly reduced the intraepidermal T cells at day 2 (Fig. 2a), and the numbers of dermal $CD3^+$ and $CD3^+CD4^+$, but not $CD3^+CD8^+$ cells were significantly increased at day 2 in the dermis (Fig. 2b). The numbers of intraepidermal single-stained $CD3^+$ and double-positive $CD3^+CD4^+$ cells at day 14 were significantly higher as compared to non-irradiated control values (Fig. 2a), and the number of intraepidermal double-stained $CD3^+CD8^+$ cells only receded to pre-irradiation values. These results indicate that indeed the UVB wavelenghts within the solar-simulated radiation caused the observed modulation of the cutaneous T cell population.

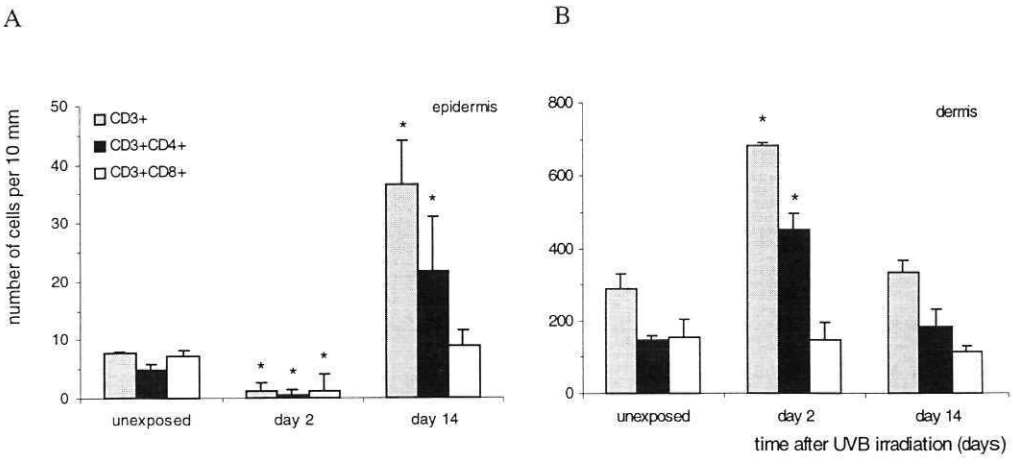


Figure 2. Differential effect of UVB on cutaneous CD4⁺ and CD8⁺ T cell subpopulations. Quantitative analysis of T cell subpopulations in the epidermis (Fig. 2A) and dermis (Fig. 2B) after single exposure of 4 MED of UVB. Exposures to UVB were performed at 2 time points (-14,-2 days) before collecting biopsies. Unexposed skin served as control. Each bar represents the mean value of 12 determinations (i.e. 6 countings in 2 volunteers, n=2) of the number of $CD3^+$ (gray bar), $CD3^+CD4^+$ (black bar), $CD3^+CD8^+$ (open bar) T cells. Data are expressed as a number of T cells per 10 mm horizontal section + SD (* indicates $p<0.05$).

T cells infiltrating the UV irradiated skin are predominantly helper/inducer, TCR αβ⁺, resting memory T cells. At day 2 the number of double-stained epidermal T cells was reduced for all markers (Fig. 3A, 3C and 3E), which is in line with above findings. The dermis showed a predominant influx of memory T cells ($CD3^+CD45RO^+$) and a small but significant increase in naive T cells ($CD3^+CD45RA^+$) (Fig. 3B). The majority of the infiltrating T cells expressed α/β TCR (Fig. 3F) and did not express markers of recent activation, like HLA-DR and IL-2R (CD25), nor of late activation, such as VLA-1(CD49a) (Fig. 3D). The UV-induced intraepidermal T cell at day 14 expressed a similar phenotype; i.e. they were predominantely non-activated, TCR α/β^+, memory T cells (Fig. 3C, 3E and 3A respectively).

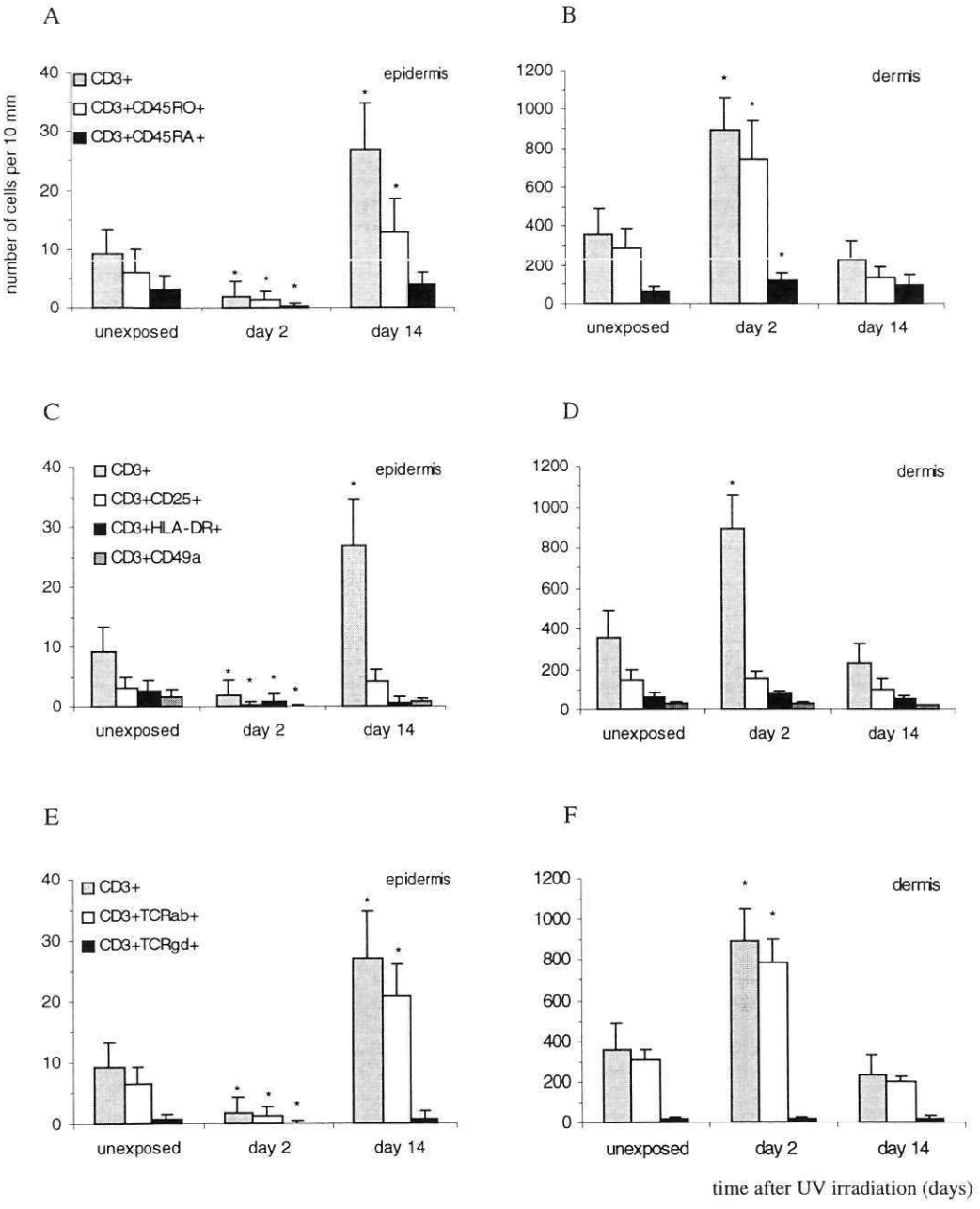


Figure 3. Solar-simulated ultraviolet radiation preferentially induces influx of memory, non-activated, TCR $\alpha\beta^+$ T cells in normal human skin. Quantitative analysis of T cell subpopulations in the epidermis (Fig. 3A, 3C and 3E) and dermis (Fig. 3B, 3D, 3F) after single exposure of 4 MED of solar-simulated UV radiation. Exposures were performed at 2 time points (-14,-2 days) before collecting biopsies. Unexposed skin served as control. Each bar represents the mean value of 24 determinations (i.e. triple countings in 8 volunteers, n=8). Data are expressed as a number of T cells per 10 mm horizontal section + SD (* indicates $p<0.05$).

Discussion

In this study we demonstrated that a single exposure to UV radiation leads to a longterm change in the number and composition of the local T cell population of normal human skin. We found that the numbers of intraepidermal T cells, which can be detected in non lesional, clinically normal human skin (10,11), were significantly reduced in the first few days after UV irradiation. A similar result was found in mice: dendritic epidermal T cells, which are resident epithelial TCR γδ expressing cells found in normal mouse skin, have been depleted from the epidermis by UVB irradiation (12,13). Because human epidermal T lymphocytes have been postulated to provide immunosurveillance against the development of cutaneous neoplasms and persistent infections with intracellular pathogens (14), the disappareance of epidermal T lymphocytes upon single exposure to UV radiation may be considered as a permissive factor for UVB-induced skin tumor and skin infection development.

The dermis showed a perivascular CD4$^+$ T cell-dominated infiltrate during the first fews days after UV irradiation, likely due to influx of T lymphocytes from the blood stream. This is in line with the finding that sun-exposed subjects had a significant decrease in circulating CD4$^+$ cells in the days after exposure to sunlight (15). Apparently, UV-exposure induces an increased homing of CD4$^+$ lymphocytes into the skin, possibly by altering the expression of adhesion molecules. Indeed, E-selectin, thought to be a mediator of lymphocyte trafficking into inflammed skin is induced on endothelial cells at sites of UVB exposed skin (16,17). We found that the initial depletion of intraepidermal T cells is followed by an infiltration of CD4$^+$ T cells into the epidermis. Because these T cells expressed the same phenotype of those infiltrating the dermis we reasoned that this might be due to the fact that T cells travel from the perivascular infiltrates, through the dermis and the basal membrane, to the epidermis, which is in line with the finding that the immigration of T cells into the epidermis started later than the influx of T cells into the dermis.

The function of CD4$^+$ T lymphocytes infiltrating the UVB-irradiated sites is unclear. One might speculate that they are involved in the recognition of neo-antigens induced by UV-exposure. T cells entering the dermis from the microvasculature in UV-exposed skin in vivo, may reasonably expected to encounter infiltrated UV-induced macrophages or UV-exposed Langerhans cells. Taking in mind that the "dermal perivascular unit" is a site of immunological reactivity (18) antigen presentation to T cells could occur directly in UV-

exposed skin without the necessity for T-cell recruitment from a regional lymph node. Presentation of antigens to memory or naive T cells under this circumstances could result in activation of suppressor pathways. The lack of expression of IL-2R on T cells could be in line with the finding that the UV-induced macrophages stimulate $CD4^+$ T cells in a novel form of T cell activation characterized by a deficient IL-2Rα expression (19). Studies conducted in humans after hapten application to UVB-exposed skin have revealed that UVB-irradiated skin acquires sustained immunosuppressive properties (20) and fails to develop the primary allergic reaction (21). The time course of increase in $CD4^+$ epidermal T cell, rising between day 7 and 21, may play a role in the inability of human UV-exposed skin to express immunity. This hypothesis is in line with the fact that T cells of the $CD4^+$ subtype have been proposed to mediate the local low-dose UVB-induced immunosuppression (3). At this moment, immunophenotyping cannot explain the exact mechanism(s) by which these T cells cause immunosuppression.

In conclusion, our results indicate that UVB radiation has long term effects on the local T cell population within human skin by depleting the majority of epidermal T cells and promoting a selective influx of non-activated, memory, $CD4^+$ T cells. We believe that these T cells play a important role in the UVB-induced local immunomodulation. Further studies are required to determine their cytokine profiles and their functional properties in order to gain a better understanding of the role of T cells in local low-dose UVB-induced immuno modulation.

Acknowledgments

Arthur Kammeyer is gratefully acknowledged for his expert assistance with the UV source, and Chris van der Loos for his very helpful discussion in optimizing the immunohistochemical stainings.

References

1. Yoshikawa, T., V. Rae, W. Bruins-Slot, J.W. van den Berg, J.R. Taylor, and J.W. Streilein. 1990. Susceptibility to effects of UVB radiation on induction of contact hypersensitivity as a risk factor for skin cancer in humans. *J.Invest.Dermatol.* 95:530.

2. Cooper, K.D., L. Oberhelman, T.A. Hamilton, O. Baadsgaard, M. Terhune, G.J. LeVee, T. Anderson, and H. Koren. 1992. UV exposure reduces immunization rates and promotes tolerance to epicutaneous antigens in humans: relationship to dose, CD1a$^-$DR$^+$ epidermal macrophage induction, and Langerhans cell depletion. *Proc.Natl.Acad.Sci.U.S.A.* 89:8497.

3. Tokura, Y. 1992. Mechanisms of local, low-dose UVB-induced immunosuppression in contact hypersensitivity. *J.Dermatol.* 19:923.

4. Murphy, G.M., P.G. Norris, A.R. Young, M.F. Corbett, and J.L. Hawk. 1993. Low-dose ultraviolet-B irradiation depletes human epidermal Langerhans cells. *Br.J.Dermatol.* 129:674.

5. Cooper, K.D., P. Fox, G. Neises, and S.I. Katz. 1985. Effects of ultraviolet radiation on human epidermal cell alloantigen presentation: initial depression of Langerhans cell- dependent function is followed by the appearance of T6- Dr+ cells that enhance epidermal alloantigen presentation. *J.Immunol.* 134:129.

6. Cooper, K.D., G.R. Neises, and S.I. Katz. 1986. Antigen-presenting OKM5$^+$ melanophages appear in human epidermis after ultraviolet radiation. *J.Invest.Dermatol.* 86:363.

7. Baadsgaard, O., B. Salvo, A. Mannie, B. Dass, D.A. Fox, and K.D. Cooper. 1990. In vivo ultraviolet-exposed human epidermal cells activate T suppressor cell pathways that involve CD4$^+$CD45RA$^+$ suppressor- inducer T cells. *J.Immunol.* 145:2854.

8. Di Nuzzo, S., M.A. De Rie, C.M. van der Loos, J.D. Bos, and M.B.M. Teunissen. 1996. Solar-simulated ultraviolet irradiation induces selective influx of CD4$^+$ T lymphocytes in normal human skin. *Photochem.Photobiol.* 64:988.

9. van der Loos, C.M., A.E. Becker, and J.J. van den Oord. 1993. Practical suggestions for successful immunoenzyme double- staining experiments. *Histochem.J.* 25:1.

10. Bos, J.D., I. Zonneveld, P.K. Das, S.R. Krieg, C.M. van der Loos, and M.L. Kapsenberg. 1987. The skin immune system (SIS): distribution and immunophenotype of lymphocyte subpopulations in normal human skin. *J.Invest.Dermatol.* 88:569.

11. Foster, C.A., H. Yokozeki, K. Rappersberger, F. Koning, B. Volc-Platzer, A. Rieger, J.E. Coligan, K. Wolff, and G. Stingl. 1990. Human epidermal T cells predominantly belong to the lineage expressing alpha/beta T cell receptor. *J.Exp.Med.* 171:997.

12. Aberer, W., N. Romani, A. Elbe, and G. Stingl. 1986. Effects of physicochemical agents on murine epidermal Langerhans cells and Thy-1-positive dendritic epidermal cells. *J.Immunol.* 136:1210.

13. Hammerberg, C., N. Duraiswamy, and K.D. Cooper. 1996. Temporal correlation between UV radiation locally-inducible tolerance and the sequential appearance of dermal, then epidermal, class II MHC$^+$CD11b$^+$ monocytic/macrophagic cells. *J.Invest.Dermatol.* 107:755.

14. Streilein, J.W. 1983. Skin-associated lymphoid tissues (SALT): origins and functions. *J.Invest.Dermatol.* 80:12s.

15. Hersey, P., G. Haran, E. Hasic, and A. Edwards. 1983. Alteration of T cell subsets and induction of suppressor T cell activity in normal subjects after exposure to sunlight. *J.Immunol.* 131:171.

16. Norris, P., R.N. Poston, D.S. Thomas, M. Thornhill, J.L. Hawk, and D.O. Haskard. 1991. The expression of endothelial leukocyte adhesion molecule-1 (ELAM-1), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) in experimental cutaneous inflammation: a comparison of ultraviolet B erythema and delayed hypersensitivity. *J.Invest.Dermatol.* 96:763.

17. Strickland, I., L.E. Rhodes, B.F. Flanagan, and P.S. Friedmann. 1997. TNF-alpha and IL-8 are upregulated in the epidermis of normal human skin after UVB exposure: correlation with neutrophil accumulation and E-selectin expression. *J.Invest.Dermatol.* 108:763.

18. Bos, J.D. and M.L. Kapsenberg. 1993. The skin immune system: progress in cutaneous biology. *Immunol.Today* 14:75.

19. Stevens, S.R., A. Shibaki, L. Meunier, and K.D. Cooper. 1995. Suppressor T cell-activating macrophages in ultraviolet- irradiated human skin induce a novel, TGF-beta-dependent form of T cell activation characterized by deficient IL-2r alpha expression. *J.Immunol.* 155:5601.

20. Tie, C., C. Golomb, J.R. Taylor, and J.W. Streilein. 1995. Suppressive and enhancing effects of ultraviolet B radiation on expression of contact hypersensitivity in man. *J.Invest.Dermatol.* 104:18.

21. Tseng, C., B. Hoffman, I. Kurimoto, T. Shimizu, G.J. Schmieder, J.R. Taylor, and J.W. Streilein. 1992. Analysis of effects of ultraviolet B radiation on induction of primary allergic reactions. *J.Invest.Dermatol.* 98:871.

Chapter 4

Exposure to UVB induces accumulation of LFA-1$^+$ T cells and enhanced expression of the chemokine psoriasin in normal human skin.

Sergio Di Nuzzo[1], Regien M.R. Sylva-Steenland[1], Cornelis W. Koomen[1], Menno A. de Rie[1], Jan D. Bos[1], Pranab K. Das[1,2] and Marcel B.M. Teunissen[1].

[1]Department of Dermatology and [2]Department of Pathology,
Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

Submitted

Abstract

Normal human skin shows preferential (epi)dermal infiltration of CD4$^+$ T cells upon acute UV exposure. To study the mechanism behind this feature we locally exposed healthy volunteers to physiological doses of UV. Expression of integrins on T cells and expression of adhesion molecules on dermal endothelial cells were quantitatively assessed by immunohistochemisty *in situ*. We also investigated the effects of UVB exposure on psoriasin and IL-16, two specific chemoattractant factors for CD4$^+$ T cells, at mRNA level by semiquantitative RT-PCR and at protein level by immunohistochemistry. We found at day 2 after exposure to four minimal erythema doses of UVB predominant recruitment of LFA-1$^+$ CLA$^-$ VLA-4$^-$ T cells in the dermis. Concomitantly the expression of ICAM-1, but not that of E-selectin and VCAM-1, was upregulated on dermal endothelial cells. The increase in the number of dermal T cells was not due to proliferation because only 2% of the UVB-induced dermal T cells expressed the marker of proliferation Ki-67. Whereas exposure to 35 J/cm^2 of UVA, like UVB, induced a loss of intraepidermal T cells at day 2 after exposure, UVA induced neither any influx of T cells into the dermis nor any adhesion molecule upregulation on endothelial cells. In response to UVB exposure, the expression of psoriasin mRNA, but not of IL-16 mRNA, was upregulated; the expression of psoriasin protein was also found to be upregulated. These results suggest that LFA-1/ICAM-1 pathway and psoriasin are both involved in CD4$^+$ T cell recruitment into UVB-irradiated skin.

Introduction

In humans, local exposure to physiological doses of UV, which cause skin inflammatory responses (1), has been shown to modulate skin immune responses such as expression of contact hypersensitivity (2,3). Changes in the composition and activation level of the constituent cells of the skin immune system have been proposed to be cellular key sources of immunomodulation induced by UV. Several studies revealed that human skin *in vivo* responds to an acute injury caused by UVB with an initial reduction in the number of both Langerhans cells (4) and T cells located in the epidermis (5), followed by (epi)dermal (re)infiltration of neutrophils (6), macrophages (7), Langerhans cells (8) and T cells (5). After UVB exposure, $CD3^+$ T lymphocytes, mostly of the $CD4^+$ $CD45RO^+$ subtype (i.e. memory/effector T cells), infiltrate the irradiated skin; in the dermal perivascular space they peak at day 2 post-exposure, and in the epidermis at day 14 (5). A single exposure to a high dose (ranging from 60 to 200 J/cm^2) of UVA also recruits T lymphocytes (9,10) of the $CD4^+$ subpopulation (11) into irradiated human skin.

Passage of circulating memory/effector T cells through the vascular endothelium to inflamed tissue is a multistep process dependent on the expression of integrin molecules on the cell surface of lymphocytes which interact with their counterreceptors on EC in postcapillary venules (12). A transient interaction between the CLA and its ligand E-selectin expressed on dermal EC has been suggested as a first step in the extravasation of skin-homing memory/effector T cells (i.e. CLA^+ $CD4^+$ $CD45RO^+$ T cells) into a skin inflammatory site (13). Subsequently, the circulating T cells, which express or have locally been induced to express LFA-1 and/or VLA-4, will more steadily interact with ICAM-1 and/or VCAM-1 on dermal EC. Consistent with this hypothesis is the finding by Picker *et al.* that 85% of T cells infiltrating cutaneous sites of inflammation expressed CLA (14). This reflects the ability of skin-homing T cells to bind to E-selectin (15), which is upregulated on dermal EC in sites of cutaneous inflammation (16). These observations and more recent investigations (17) have led to the assumption that interaction between CLA and E-selectin plays an essential role in targeting circulating memory/effector T cells to sites of cutaneous inflammation. In humans, upregulation of the dermal vascular E-selectin and ICAM-1 has been described to occur *in vivo* after a single exposure to UVB (18-20) and to a high dose of UVA (19,21). The extent of infiltration of neutrophils and macrophages into UVB-irradiated sites has been correlated with E-selectin upregulation on dermal EC (18,20,22). However, accumulation of T cells did not

correlate with the expression of E-selectin on dermal EC in UVB-irradiated sites (18), suggesting that CLA-independent transmigration, possibly involving interaction of LFA-1 with ICAM-1 (13), may occur in recruitment of T cells into UVB-exposed skin. Furthermore, CLA/E-selectin interaction appears to be irrelevant also in UVA-induced T-cell adhesion to human dermal microvascular EC *in vitro* (23).

Chemotactic cytokines (i.e. chemokines) are essentially involved in triggering and mediating the complex process of leukocyte migration from the blood stream into sites of inflammation (24,25). T lymphocytes have for a long time been considered to be poor targets for chemokines, but recent findings have changed this view; especially the selective chemoattractant activities of several chemokines for lymphocyte subsets make them ideal candidates to play a key role in the targeting of specific T cell subpopulations to sites of inflammation (26). However, it is still unknown which specific chemokine(s) is(are) involved in the selective accumulation of $CD4^+$ T cells in the skin in response to UVB. Psoriasin is a newly described low-molecular-mass calcium-binding protein that is synthesised (albeit at low levels), and partially secreted by noncultured unfractionated keratinocytes from normal human skin (27). Recent studies indicate that psoriasin is a potent and selective chemotactic protein for $CD4^+$ T cells *in vitro* (28). An additional candidate for $CD4^+$ T cell chemoattraction upon UVB exposure is IL-16, which has been shown to be a selective chemoattractant for $CD4^+$ T cells (29). $CD8^+$ T cells and mast cells, which both are constitutionally present in normal human skin, have been shown to release bioactive IL-16 upon various stimuli (29,30).

In our experiments, the first objective was to determine the expression of integrins on UVB-induced T cells and to relate these to the expression of their ligands on dermal EC. Our second objective was to determine whether UVB-exposure is able to induce dermal T-cell proliferation. Our third objective was to assess the effects of a single low dose of UVA (35 J/cm^2) on the cutaneous T cell population and on the expression of adhesion molecules on dermal EC. Our fourth objective was to determine whether psoriasin and IL-16 are upregulated in UVB-exposed skin. To address these questions we took biopsies from healthy human volunteers who were locally exposed to physiological doses of UV. The expression of integrins on T cells, adhesion molecules on dermal EC, and a marker of proliferation in T cells were quantitatively assessed by immunohistochemistry *in situ*. The effects of UVB on

psoriasin and IL-16 were assessed at mRNA level with RT-PCR techniques and at protein level with immunohistochemistry.

Materials and Methods

Subjects, UV irradiations and biopsies. Four healthy Caucasian volunteers of sun-reactive skin types II or III were recruited for our study. All participants gave informed consent according to the guidelines of the Medical Ethical Committee of the hospital. A 1000 W xenon-arc lamp (Oriel, Stratford, CT) was used as UV source. In order to reduce the heat (infrared radiation), the light beam was passed through a waterfilter and reflected by a dichroic (cold) mirror. UVB irradiations were performed with a 303-nm interference filter (Jenaer Glaswerke, Schott & Gen., Mainz, Germany); UVA irradiations were performed with a UG 11 filter (Jenaer Glaswerke, Schott & Gen.) and a WG 335 cut-off filter (Jenaer Glaswerke, Schott & Gen.). The minimal erythema dose of UVB was determined for each volunteer to allow delivery of biologically equivalent doses of UV light. A single dose of four minimal erythema doses of UVB was given to multiple sites on the buttocks 14, 10, 2 and 1 days before the biopsies. A single dose of UVA (35 J/cm^2) was administered to other multiple sites on the buttocks 14 days and 2 days before the biopsies. Unirradiated sites were used as controls. Four-millimetre punch biopsies were obtained under local anaesthesia, immersed in OCT freezing medium (Miles, Elkhart, IL), snap frozen in liquid nitrogen, and stored at –70°C.

Immunohistochemistry. Sections of 6 μm were cut from the biopsies at –24°C and were allowed to dry overnight, after which they were fixed in acetone for 10 min at 4°C, and stored at –20°C. To detect T cells or psoriasin by a two-step indirect peroxidase single staining, sections were incubated with mAb mouse anti-human CD3 (Becton Dickinson, Mountain View, CA) or mAb mouse anti-human psoriasin (hybridoma supernatant of clone 1363; a gift from Dr. J.E. Celis, University of Aarhus, Aarhus, Denmark), followed by incubation with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako, Glostrup, Denmark). Before counterstaining with hematoxilin, the horseradish peroxidase activity was detected with H_2O_2 as substrate and AEC (Sigma, St Louis, MO) as chromogen.

Immunohistochemical double staining of T-cell antigens was performed using two different protocols, based on a combination of a polyclonal rabbit primary antibody and a monoclonal mouse primary antibody, as previously described (5). The following steps were

performed at room temperature: (i) incubation for 60 min with a cocktail of rabbit anti-human CD3 (Dako) plus one of the four mouse antibodies, anti-human CD11a (i.e. the α-chain of the LFA-1 complex) (CLB; Central Laboratory Blood Transfusion Service, Amsterdam, The Netherlands), CDw49d (i.e. the α-chain of the VLA-4 complex) (Immunotech S.A., Marseille, France), Moab HECA-452 (31), or MIB-1 (Immunotech S.A.); (ii) incubation for 30 min with a cocktail of biotin-conjugated goat anti-mouse (Dako) plus either alkaline phosphatase-conjugated goat anti-rabbit (Dako) (protocol 1), or horseradish peroxidase-conjugated goat anti-rabbit (Dako) (protocol 2); (iii) incubation for 30 min with either horseradish peroxidase-conjugated streptavidin (Dako) (protocol 1) or alkaline phosphatase-conjugated streptavidin (Dako) (protocol 2). (iv) For both protocols alkaline phosphatase activity was subsequently detected as a blue colour, using naphthol-AS-MX-phosphate (Sigma) as substrate and fast blue BB (Sigma) as azo dye; (v) peroxidase activity was detected as an orange-red colour, using the chromogen AEC (Sigma). Double-stained cells could be detected by their purple colour.

To determine surface antigens on EC, the following steps were performed: (i) incubation for 60 min with a cocktail of biotinylated Ulex Europaeus agglutinin 1 (Sigma) as a pan-EC marker plus one of the three mouse antibodies, anti-human CD62E (i.e. E-selectin) (R&D System Europe Ltd, Abingdon, UK), CD54 (i.e. ICAM-1) (R&D System Europe Ltd), or CD106 (i.e. VCAM-1) (R&D System Europe Ltd); (ii) incubation for 30 min with a cocktail of alkaline phosphatase-conjugated streptavidin (Dako) and horseradish peroxidase-conjugated rabbit-anti-mouse (Dako); (iii) alkaline phosphatase activity and (iv) peroxidase activity were detected as described above.

Cell counting. The identification on the object glasses were covered before counting to enable blind quantitation. Subdivision of the skin into epidermis and dermis, and counting of stained cells were performed as described before (5). The numbers of clearly stained cell bodies were counted by three independent investigators in three or six different serial sections (section number is stated in the legend of each figure) per irradiation dose 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 mean value of each biopsy was then used to determine the total mean of T cell or EC numbers.

RNA isolation and RT-PCR for detection of cytokine mRNAs. 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 (32). The following specific primer sets were synthesised in our laboratory by an oligosynthesiser: glyceraldeyde 3-phosphate dehydrogenase (GAPDH) forward primer 5'-CGAGATCCCTCCAAAATCAA-3' (nt 298-317) and GAPDH reverse primer 5'-AGGTCAGGTCCACCACTGAC-3' (nt 799-780); psoriasin forward primer 5'-TACTCGTGACGCTTCCCAG-3' (nt 9-27) and psoriasin reverse primer 5'-CTCTGCTTGTGGTAGTCTGTGG-3' (nt 318-297); IL-16 forward primer 5'-TTCACAGAGTGTTTCCAAATGG-3' (nt 599-620) and IL-16 reverse primer 5'-CAGCTGCAAGATTTCATCTCC-3' (nt 1037-1017). The PCR products were of the expected size and the specificity was confirmed by sequence analysis. The radiolabeled PCR products were scanned by a beta imager (B&L Systems, Maarsen, The Netherlands), and the signal strength was integrated to obtain a densitometric value for each application product. To allow semiquantitative analysis, in each sample the densitometric values of psoriasin and IL-16 were related to the signal of the house keeping gene GAPDH, which is assumed to be produced at constant rate. Two values of two independent experiments per volunteer per time point were determined, the mean value of each biopsy was then used to determine the total mean per time point of cytokine mRNA densitometric signal.

Statistical analysis. The unpaired Student's *t*-test was used for statistical evaluation, taking $p < 0.05$ (two-tailed) as the level of significance. Correlations between integrins on T cells and their ligands on EC were performed using the Spearman Rank Correlation.

Results

UVB radiation predominantly induced recruitment of LFA-1$^+$ CD3$^+$ T cells into the dermis of irradiated skin. In unirradiated samples, a few CD3$^+$ T cells were present in the epidermis (Fig. 1A). Two days after exposure to UVB, a significant decrease in the number of intraepidermal CD3$^+$ T cells ($p=0.0001$) was induced, which was followed by a significant ($p=0.0008$) increase in the intraepidermal CD3$^+$ T cell numbers observed 14 days after irradiation. At day 2 after exposure, the numbers of double-stained epidermal T cells were reduced for all markers, which is in line with the above findings. At day 14 after exposure, the numbers of intraepidermal CD3$^+$/CLA$^+$ and CD3$^+$/VLA-4$^+$, but not CD3$^+$/LFA-1$^+$, cells were

significantly (p=0.0033, p=0.0042 and p=0,1855, respectively) higher than the pre-irradiation values. The percentages of double-stained $CD3^+/CLA^+$, $CD3^+/VLA\text{-}4^+$ and $CD3^+/LFA\text{-}1^+$ T cells were 48%, 53% and 48% of the total $CD3^+$ intraepidermal T cells, respectively. A different response of the $CD3^+$ T cell population was found in the dermis (Fig. 1B). At day 2 after UVB exposure, the numbers of dermal $CD3^+$ T cells had increased considerably ($p<0.0001$), and had declined by day 14 to the pre-irradiation values. At day 2 after exposure, examination of the double-stained sections revealed that UVB induced a significant (p=0.0002) influx into the dermis of $CD3^+/LFA\text{-}1^+$ T cells which represented 47% of the total $CD3^+$ T cells (Fig. 2A). UVB also induced a small but significant increase in both $CD3^+/CLA^+$ and $CD3^+/VLA\text{-}4^+$ T cells (p=0.0014 and p=0.0002, respectively), which represented 26% and 22% of the total $CD3^+$ T cells (Fig. 2B and 2C). The number of dermal $CD3^+/CLA^+$ and $CD3^+/LFA\text{-}1^+$ T cells returned to pre-irradiation values at day 14, whereas the number of dermal $CD3^+/VLA\text{-}4^+$ T cells remained slightly, though significantly higher than the pre-irradiation value.

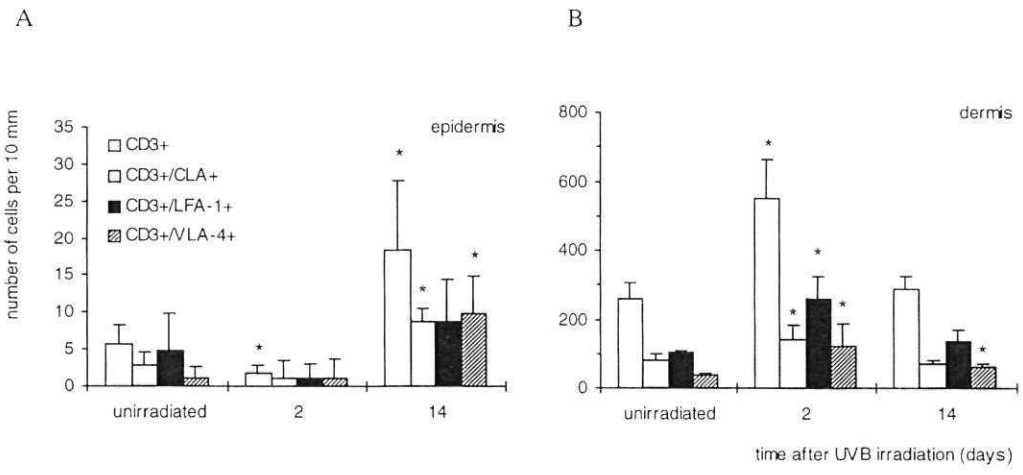
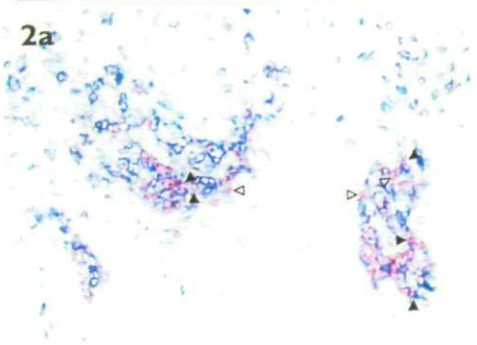
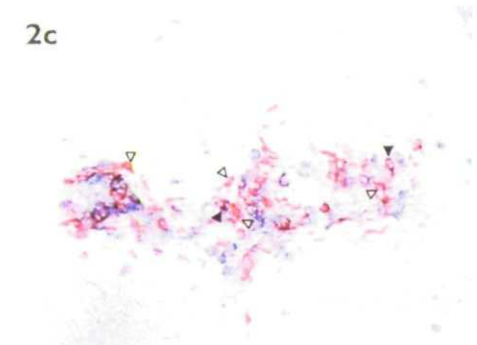
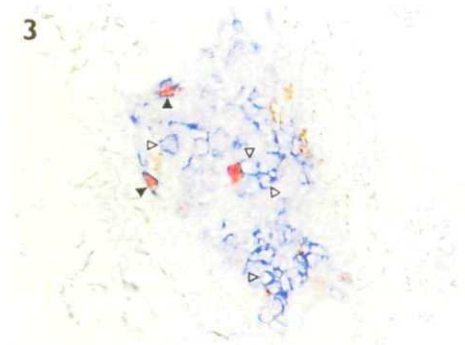


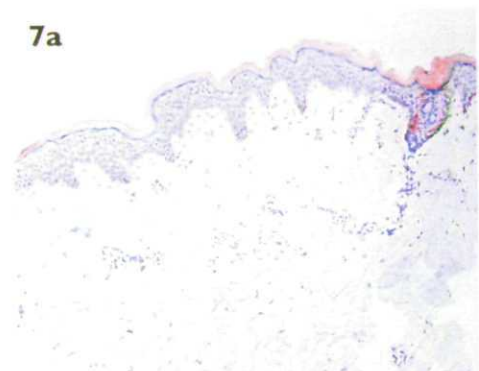
Figure 1. UVB irradiation predominantly induced recruitment of $LFA\text{-}1^+$ $CD3^+$ T cells into the dermis of irradiated skin. Quantitative analysis of T cell subpopulations in the epidermis (A) and dermis (B) after a single exposure to four minimal erythema doses of UVB. Exposures were performed 14 days and 2 days before collecting biopsies. Unirradiated skin served as a control. After immunohistochemical (double)staining of cryostat sections, the expression of CD3, or the double expression of CD3 plus CLA, LFA-1, or VLA-1, was determined. Each bar represents the mean value of 12 determinations (i.e. triple countings in 4 volunteers, n=4). Data are expressed as the number of T cells per 10 mm horizontal section $\pm$ SD (* indicates $p<0.05$).

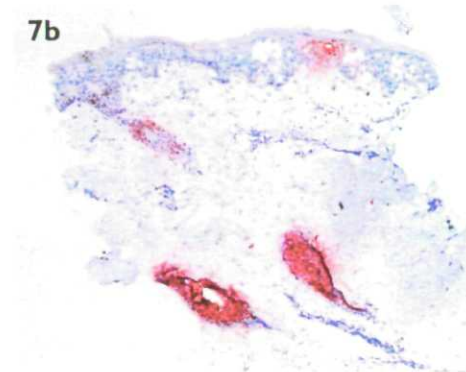

2a

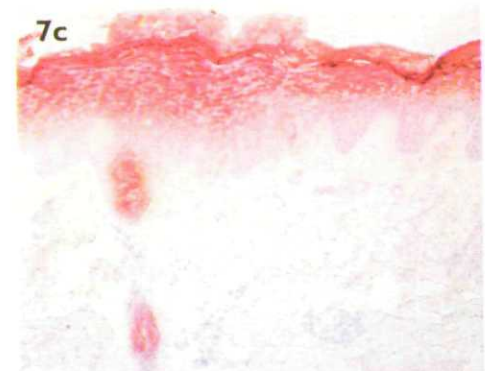

2b


2c


3


7a


7b


7c

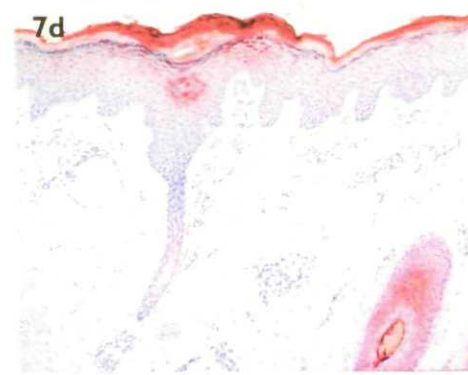

7d

Figure 2. Dermal T cell populations in normal human skin 2 days after exposure to 4 MED of UVB. (A) CD3 (red) and LFA-1 (blue) double staining shows that many $CD3^+$ $LFA\text{-}1^+$ cells (purple; black arrowhead) are present in the dermal T cell infiltrate. $CD3^+LFA\text{-}1^-$ T cells are stained in red (open harrowhead). (B) CD3 (red) and CLA (blue) double staining shows that the majority of the cells is $CD3^+$ CLA^- (red; open arrowhead), and a few cells are $CD3^+$ CLA^+ (purple; black arrowhead). (C) CD3 (red) and VLA-4 (blue) double staining shows that few cells are $CD3^+$ $VLA\text{-}4^+$ (purple; black arrowhead), and the majority is $CD3^+$ $VLA\text{-}4^-$ (red; open arrowhead).

Figure 3. UVB did not induce proliferation of dermal T cells. Human skin was double stained with MoAb against Ki-67 (red) and CD3 (blue) two days after exposure to 4 MED of UVB. Among the $CD3^+$ T cell dermal population (open arrowhead) only few $CD3^+Ki\text{-}67^+$ T cells (black arrowhead) are present.

Figure 7. ***Psoriasin protein expression increased in irradiated skin after UVB-irradiation.*** Healthy volunteers were exposed to four minimal erythema doses of UVB 14, 10 and 2 days before collecting skin biopsies. (A-D) Immunohistochemical single staining of psoriasin was based on a two-step indirect peroxidase technique, using the anti-psoriasin mAb 1363. (A) Control unirradiated skin. (B) Two days after UVB. (C) Ten days after UVB. (D) Fourteen days after UVB.

UVB did not induce proliferation of dermal T cells. A recent study showed that the UVB-induced dermal expansion of macrophages is not only due to infiltration but to proliferation *in situ* as well (33). The increased numbers of dermal T cells found upon UVB exposure might also be caused, at least in part, by proliferation *in situ* of perivascular T cells, which reside normally in unirradiated skin. To assess this hypothesis, we performed double staining with a mAb against CD3 plus a mAb against Ki-67, which is a marker of proliferation (34). At day 2 after UVB exposure, only a few double-positive cells (2% of the total $CD3^+$ T cells) were present (Fig. 3), mostly located in the perivascular area. This result excludes the possibility that the UVB-induced increased numbers of dermal T cells was due to proliferation.

ICAM-1, but not E-selectin and VCAM-1, was upregulated on dermal endothelial cells 2 days after UVB irradiation. In unirradiated control biopsies, $Ulex^+$/$ICAM\text{-}1^+$ and $Ulex^+$/$E\text{-}selectin^+$ cells were constitutively present in the dermis (Fig. 4); VCAM-1 expression was either undetectable or just positive with weak staining of a few EC. Two days after UVB irradiation, a significant increase in $Ulex^+$/$ICAM\text{-}1^+$ cells was seen in the superficial and mid dermis; at day 14, the number of $Ulex^+$/$ICAM\text{-}1^+$ cells had returned to the pre-irradiation value. By contrast, at day 2 the number of $Ulex^+$/$E\text{-}selectin^+$ cells was slightly reduced compared to the pre-irradiation value. A small but significant ($p=0.0117$) increase in $Ulex^+$/$E\text{-}selectin^+$cells was observed in the superficial and mid dermis at day 14. No change in microvascular expression of VCAM-1 was observed over the time course in any of the biopsies. There was a significant ($p<0.0001$) direct correlation between the numbers of $Ulex^+$/$ICAM\text{-}1^+$ cells and the numbers of dermal $CD3^+$/$LFA\text{-}1^+$ cells over the time course ($r = 0.66$). No correlation was found between the numbers of $Ulex^+$/$E\text{-}selectin^+$ and the numbers of dermal $CD3^+$/CLA^+ and between the numbers of $Ulex^+$/$VCAM\text{-}1^+$ cells and the numbers of dermal $CD3^+$/$VLA\text{-}4^+$ cells.

Single exposure to a low dose of UVA induced a loss of intraepidermal T cells, but neither induced an influx of T cells into the (epi)dermis nor upregulation of adhesion molecules on EC. At day 2, upon exposure to a low dose of UVA (35 J/cm^2), a significant decrease in the number of intraepidermal $CD3^+$ T cells ($p<0.0001$) was induced (Fig. 5A). Fourteen days after exposure to UVA, the epidermal $CD3^+$ T cells had returned to the pre-irradiation numbers. With respect to the T cell population in the dermis, we did not find any significant change in dermal T cells numbers upon UVA exposure (Fig. 5B). Also no

significant change in microvascular expression of E-selectin, ICAM-1 nor VCAM-1 was observed over the time course after UVA exposure (data not shown).

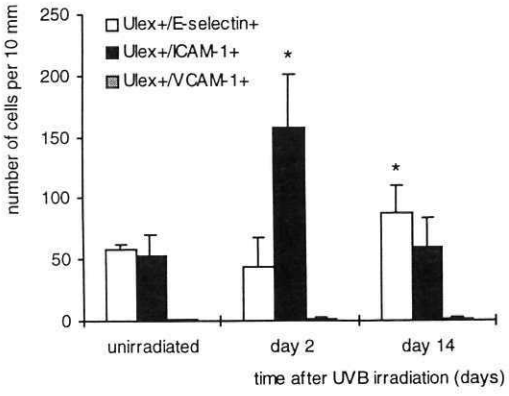


Figure 4. ICAM-1, but not E-selectin and VCAM-1, was upregulated on dermal endothelial cells 2 days after UVB irradiation. The expression of E-selectin, ICAM-1 and VCAM-1 was determined on dermal endothelial cells in cryostat sections derived from UVB-exposed skin, using Ulex to identify the endothelial cells. Exposures to a single dose of four minimal erythema doses of UVB were performed 2 and 14 days before taking biopsies. Unexposed skin served as a control. Each bar represents the mean value of 12 determinations (i.e. triple countings in 4 volunteers, n=4). Data are expressed as the number of endothelial cells per 10 mm horizontal section ± SD (* indicates $p<0.05$).

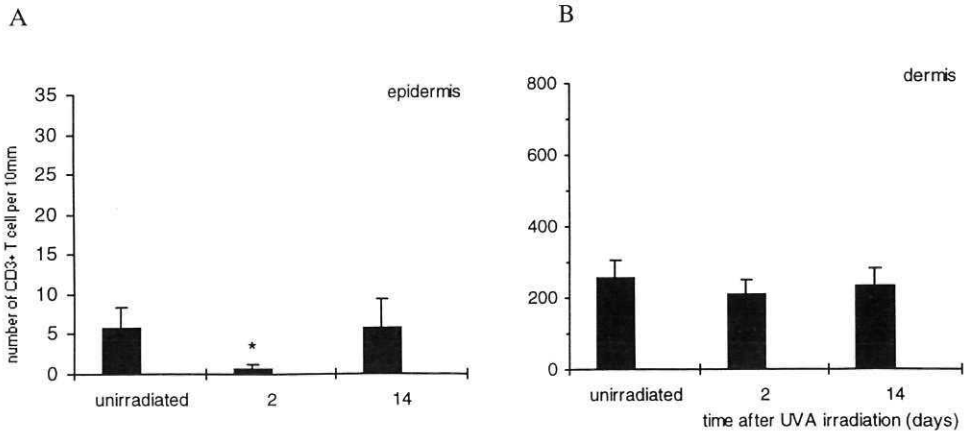


Figure 5. Single exposure to UVA induced a loss of intraepidermal T cells, but did not induce any influx of T cells into the (epi)dermis. Effects of a single dose of UVA (35 J/cm^2) on cutaneous CD3$^+$ T cells in the epidermis (A) and dermis (B). Exposures were performed 14 days and 2 days before collecting biopsies. Unirradiated skin served as a control. Each bar represents the mean value of 18 determinations (i.e. 6 countings in 3 volunteers, n=3). Data are expressed as the number of CD3$^+$ T cells per 10 mm horizontal section ± SD (* indicates $p<0.05$).

Psoriasin was upregulated into the UVB exposed skin. Psoriasin mRNA and IL-16 mRNA were constitutively expressed in unirradiated normal skin, albeit the former at very low levels, as detected by RT-PCR (Fig. 6). At day 1 after exposure to UVB, the psoriasin mRNA expression was considerably increased ($p<0.0001$) and this expression remained high till day 10 post-irradiation ($p<0.0001$). A marked decrease in the psoriasin mRNA signal was found 14 days after irradiation. By contrast, no significant changes in skin expression of IL-16 mRNA were observed over the time course in any of the biopsies.

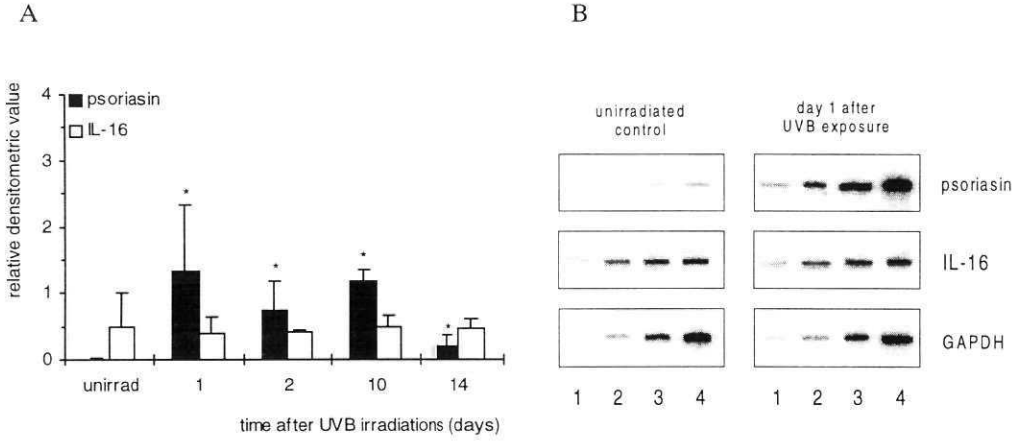


Figure 6. UVB exposure induced upregulation of psoriasin mRNA into irradiated skin. (A) Semiquantitative analysis of psoriasin mRNA and IL-16 mRNA after exposure to four minimal erythema doses of UVB. Exposures were performed 14, 10, 2 and 1 days before collecting skin biopsies. Unirradiated skin served as a control. RNA was isolated from cryostat sections and was subjected to cytokine-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 4 determinations (i.e. 2 experiments in 2 volunteers, n=2). Data are expressed as the relative densitometric value of cytokine mRNA ± SD (* indicates $p<0.05$). (B) After electrophoresis on an acrylamide gel, the PCR products were scanned by a phosphor imager. Lane 1 through 4 represents the different cycle numbers: for psoriasin the cycle numbers were 20, 22, 24, 26; for IL-16 the cycle numbers were 38, 40, 42, 44 and for GAPDH these numbers were 26, 28, 30, 32.

In unirradiated control biopsies, psoriasin protein was either undetectable or was weakly positive in a few isolated keratinocytes located in the upper part of the interfollicular epidermis (Fig. 7A). Two days after exposure to UVB, expression of psoriasin protein was induced in the deep follicular epithelium, and increased focal staining of keratinocytes located in the interfollicular epidermis was noticed (Fig. 7B). The psoriasin expression was markedly enhanced in the granular layer of the interfollicular epidermis 10 days after exposure (Fig.

7C). A marked decrease in the psoriasin expression both in the interfollicular epidermis and in the follicular epithelium was found 14 days after exposure (Fig. 7D).

Discussion

In our experiments, we studied the effects of physiological doses of UV on the integrin expression on cutaneous T cells, and related these to the effects of UV exposure on the counterreceptors on dermal EC. In line with an earlier report (5), exposure to UVB recruited $CD3^+$ T cells into irradiated skin at day 2 after exposure. Only 26% of these infiltrating T cells expressed CLA, which suggests that the interaction between CLA and E-selectin is not critically involved in the recruitment of the majority of dermal UVB-induced T cells. Our finding that at day 2 after exposure E-selectin was not up-regulated on dermal EC agrees with the previous observation of transient up-regulation *in vivo* of E-selectin upon UVB exposure (18). These investigators found that after exposure to UVB maximal induction of E-selectin occurred at 24 h, followed by a decline toward basal level by 72 h; concurrently, they detected neutrophil accumulation in the dermis, which preceded lymphocyte recruitment. Thus, the expression pattern of E-selectin on dermal EC upon UVB exposure is to some extent related to the influx of neutrophils, which are most prominent early in the UVB-induced cellular recruitment (6), but it is not related to the later influx of dermal T cells which peak in the dermal perivascular compartment at a time point when the down-regulation of E-selectin is taking place (5). The UVB-induced dermal T cells that expressed CLA might have been recruited in the early stages of UVB-induced inflammation. In our study, upregulation of E-selectin on dermal EC was seen at day 14 after exposure to UVB; as yet the significance of this finding is unclear. One might speculate that this phenomenon is related to the increase in the number of intraepidermal CLA^+ T cells, which occurred at same time. The kinetics of the number of dermal LFA-1^+ T cells correlated with the number of ICAM-1^+ EC upon UVB exposure, both increased at day 2 and subsequently declined to pre-irradiation value at day 14. It is likely that the recruitment of dermal T cells into UVB-irradiated sites is mediated by LFA-1/ICAM-1 interaction. However, to validate this hypotesis functional studies are needed. Interestingly, other studies showed that *in vitro* UVB irradiation selectively upregulates ICAM-1, but does not upregulate E-selectin or VCAM-1, cell-surface expression in human dermal microvascular EC (20,35). Furthermore, they found that upon UVB-exposure *in vivo* the increased expression of ICAM-1 on dermal EC was still present 48 h and 72 h after

irradiation (35). Thus, the extended UVB-induced upregulation of ICAM-1 on dermal EC, compared to the short-term UVB-induced upregulation of E-selectin, might be related to the T cell recruitment. However, in our experiments only 47% of the UVB-induced dermal T cells expressed LFA-1. Since only 2% of the UVB-induced dermal T cells expressed a marker of proliferation, a likely explanation is that other receptor/ligand pairs may be involved in the recruitment of circulating T cells into UVB-exposed sites. Our observation that the expression of VCAM-1 on dermal EC did not change upon exposure to UVB *in vivo* corroborates a previous report suggesting no role for the VLA-4/VCAM-1 interaction in recruiting circulating T cells into UV-exposed skin (18). However, an increased expression of VCAM-1 on dermal endothelial cells might have been missed since this study was restricted to few time points. Early induction of VCAM-1 on dermal endothelial cells upon UVB-exposure might explaine the increase in dermal $CD3^+/VLA\text{-}4^+$ T cell numbers found at day 2 after exposure. Alternatively, since VLA-4 binds to fibronectin (36), one can speculate that VLA-4 on UVB-induced T cells might play a role for a minor subpopulation of lymphocytes in extravasation (e.g. binding to the endothelial basement membrane) and in extravascular migration into UVB-exposed skin.

Normal unirradiated human epidermis harbours few scattered T cells (37,38). In the current study, we found that a low dose of UVA induced a decrease in intraepidermal T cell numbers at day 2 after exposure. The most likely explanation for this loss of intraepidermal T cells is that they are depleted due to a phototoxic effect of the UVA radiation. Assuming that 20-50% of UVA radiation incident on the skin is transmitted to the viable layer of the epidermis (39), it is to be expected that intraepidermal T lymphocytes might receive 7-17 J/cm^2 UVA during exposure to 35 J/cm^2; this is precisely the range in which *in vitro* the viability of lymphocytes at day 2 after exposure proved to be considerably decreased by UVA exposure (40). Since the exact role of human intraepidermal T cells is not yet known, it is difficult to speculate on the biological consequences of UV-induced depletion of these cells. We have proposed that the disappearance of epidermal T lymphocytes upon exposure to solar-simulated UV radiation (41) or to UVB radiation (5) may be considered to be a permissive factor for development of UV-induced skin tumors and infections; the results of the present study show that UVA alone can have a deleterious effect on intraepidermal T cells. We found that exposure to a low dose of UVA did not recruit $CD3^+$ T cells into irradiated skin, which is in line with the finding that the dose of UVA applied did not induce

any upregulation of adhesion molecules on dermal EC. However, *in vivo* exposure to a high dose of UVA, which has been shown to induce an influx of $CD3^+$ T cells into the dermis of the irradiated site (11), was able to upregulate ICAM-1 on dermal EC (19,21). Furthermore, *in vitro* experiments using a high dose of UVA showed that T cell adhesion to dermal EC depends on UVA-induced expression of ICAM-1 (23). Taken together, these findings suggest that LFA-1/ICAM-1 interaction is essential for recruiting T cells into UVA-exposed skin *in vivo*.

The preferential skin infiltration of $CD4^+$ T cells observed in UVB-exposed skin prompted us to investigate the effects of UVB exposure *in vivo* on psoriasin and IL-16, which have been shown to be specific chemoattractant factors for $CD4^+$ T cells. We found that the expression of psoriasin was upregulated both at mRNA level and at protein level in UVB-exposed skin *in vivo* for a prolonged time (1 to 10 days after exposure), suggesting a role for the keratinocytes-derived psoriasin in T-cell recruitment into UVB irradiated sites. If psoriasin is involved, then the infiltrating $CD4^+$ T cells should carry receptors for psoriasin, but so far the absence of specific mAb against the psoriasin receptor makes it impossible to investigate this. Concerning the expression of IL-16, we found that UVB exposure did not induce any significant change in its mRNA, suggesting no role of this molecule in UVB-induced $CD4^+$ T cell recruitment. In line with these observations is that dermal $CD8^+$ T cells, which might be a source of IL-16, are not altered in number upon UVB exposure (5). However, it should be mentioned that IL-16 secretion from $CD8^+$ T cells, which store preformed bioactive IL-16, may be independent of both transcription and translation (29). Furthermore, preformed IL-16 has been found in human mast cells (30), which are known to degranulate upon UV exposure (42); thus a relevant question to be answered might be whether preformed bioactive IL-16 is secreted by $CD8^+$ T cells and mast cells upon UVB exposure.

Regardless of the mechanisms involved in T cell recruitment into UVB-irradiated human skin, this phenomenon is strikingly different from that induced by acute UVB exposure on dermal T cell populations in mice. Upon a single exposure to UVB, dermal T cells, normally present within mouse skin, showed a rapid reduction in cell numbers which reached a nadir between day 1 and day 2, after which recovery occurred 3 days after exposure (43). The difference between the responses of mice and human dermal T cells upon UVB exposure might suggest that in mice circulating T cells preferentially migrate into lymph

nodes upon UVB exposure (44), while in humans UVB exposure apparently redistributes circulating T cells partially into the dermis. Bearing in mind that the "dermal perivascular unit" is a site of immunologic reactivity (45,46), recruitment of T cells into this compartment might be biologically relevant. In this respect Butcher and Picker (12) proposed an important role for competitive niche homing (e.g. lymph node vs dermal perivascular unit) in controlling lymphocytes homeostasis and shaping the immune response. Thus, in humans, recruitment of T cells into UVB-exposed skin might create an additional adaptive immunological compartment, which is not present in nocturnal animals such as mice.

In conclusion, we observed that both a single exposure to UVB and a single exposure to a low dose UVA induced a decrease in intraepidermal T cell numbers, but only exposure to UVB was followed by infiltration of T lymphocytes into UV-irradiated skin. LFA-1/ICAM-1 pathway and probably psoriasin are involved in T-cell recruitment into UVB-irradiated skin. Regardless of the mechanisms involved, the human dermal perivascular unit might be an important site of immunological reactivity upon UV exposure. Further work is required to test this hypothesis critically, and to apply the results to photo-immuno-mediated skin diseases.

Acknowledgments

Arthur Kammeyer is gratefully acknowledged for his expert assistance with the UV source, Chris van der Loos for his very helpful discussion in optimising the immunohistochemical stainings, and Wendy van Noppen for her critical reading of the manuscript.

References

1. Soter, N.A. 1990. Acute effects of ultraviolet radiation on the skin. *Semin.Dermatol.* 9:11.

2. Tie, C., C. Golomb, J.R. Taylor, and J.W. Streilein. 1995. Suppressive and enhancing effects of ultraviolet B radiation on expression of contact hypersensitivity in man. *J.Invest.Dermatol.* 104:18.

3. LeVee, G.J., L. Oberhelman, T. Anderson, H. Koren, and K.D. Cooper. 1997. UVA II exposure of human skin results in decreased immunization capacity, increased induction of tolerance and a unique pattern of epidermal antigen-presenting cell alteration. *Photochem.Photobiol.* 65:622.

4. Aberer, W., G. Schuler, G. Stingl, H. Honigsmann, and K. Wolff. 1981. Ultraviolet light depletes surface markers of Langerhans cells. *J.Invest.Dermatol.* 76:202.

5. Di Nuzzo, S., R.M. Sylva-Steenland, M.A. De Rie, P.K. Das, J.D. Bos, and M.B.M. Teunissen. 1998. UVB radiation preferentially induces recruitment of memory $CD4^+$ T cells in normal human skin: long-term effect after a single exposure. *J.Invest.Dermatol.* 110:978.

6. Hawk, J.L., G.M. Murphy, and C.A. Holden. 1988. The presence of neutrophils in human cutaneous ultraviolet-B inflammation. *Br.J.Dermatol.* 118:27.

7. Cooper, K.D., G.R. Neises, and S.I. Katz. 1986. Antigen-presenting $OKM5^+$ melanophages appear in human epidermis after ultraviolet radiation. *J.Invest.Dermatol.* 86:363.

8. Gilliam, A.C., I.B. Kremer, Y. Yoshida, S.R. Stevens, E. Tootell, M.B.M. Teunissen, C. Hammerberg, and K.D. Cooper. 1998. The human hair follicle: a reservoir of $CD40^+$ B7-deficient Langerhans cells that repopulate epidermis after UVB exposure. *J.Invest.Dermatol.* 110:422.

9. Rosario, R., G.J. Mark, J.A. Parrish, and M.C. Mihm. 1979. Histological changes produced in skin by equally erythemogenic doses of UV-A, UV-B, UV-C and UV-A with psoralen. *Br.J.Dermatol.* 101:299.

10. Gilchrest, B.A., N.A. Soter, J.L. Hawk, R.M. Barr, A.K. Black, C.N. Hensby, A.I. Mallet, M.W. Greaves, and J.A. Parrish. 1983. Histologic changes associated with ultraviolet A-induced erythema in normal human skin. *J.Am.Acad.Dermatol.* 9:213.

11. Margolis, R.J., M. Sherwood, D.J. Maytum, R.D. Granstein, M.A. Weinstock, J.A. Parrish, and R.W. Gange. 1989. Longwave ultraviolet radiation (UVA, 320-400 nm)-induced tan protects human skin against further UVA injury. *J.Invest.Dermatol.* 93:713.

12. Butcher, E.C. and L.J. Picker. 1996. Lymphocyte homing and homeostasis. *Science* 272:60.

13. Santamaria Babi, L.F., R. Moser, M.T. Perez Soler, L.J. Picker, K. Blaser, and C. Hauser. 1995. Migration of skin-homing T cells across cytokine-activated human endothelial cell layers involves interaction of the cutaneous lymphocyte-associated antigen (CLA), the very late antigen-4 (VLA- 4), and the lymphocyte function-associated antigen-1 (LFA-1). *J.Immunol.* 154:1543.

14. Picker, L.J., S.A. Michie, L.S. Rott, and E.C. Butcher. 1990. A unique phenotype of skin-associated lymphocytes in humans. Preferential expression of the HECA-452 epitope by benign and malignant T cells at cutaneous sites. *Am.J.Pathol.* 136:1053.

15. Berg, E.L., T. Yoshino, L.S. Rott, M.K. Robinson, R.A. Warnock, T.K. Kishimoto, L.J. Picker, and E.C. Butcher. 1991. The cutaneous lymphocyte antigen is a skin lymphocyte homing receptor for the vascular lectin endothelial cell-leukocyte adhesion molecule 1. *J.Exp.Med.* 174:1461.

16. Groves, R.W., M.H. Allen, J.N. Barker, D.O. Haskard, and D.M. MacDonald. 1991. Endothelial leucocyte adhesion molecule-1 (ELAM-1) expression in cutaneous inflammation. *Br.J.Dermatol.* 124:117.

17. Tietz, W., Y. Allemand, E. Borges, D. von Laer, R. Hallmann, D. Vestweber, and A. Hamann. 1998. $CD4^+$ T cells migrate into inflammed skin only if they express ligands for E- and P-selectin. *J.Immunol.* 161:963.

18. Norris, P., R.N. Poston, D.S. Thomas, M. Thornhill, J.L. Hawk, and D.O. Haskard. 1991. The expression of endothelial leukocyte adhesion molecule-1 (ELAM-1), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) in experimental cutaneous inflammation: a comparison of ultraviolet B erythema and delayed hypersensitivity. *J.Invest.Dermatol.* 96:763.

19. Heckmann, M., B. Eberlein-Konig, A. Wollenberg, B. Przybilla, and G. Plewig. 1994. Ultraviolet-A radiation induces adhesion molecule expression on human dermal microvascular endothelial cells. *Br.J.Dermatol.* 131:311.

20. Rhodes, L.E., M. Joyce, D.C. West, I. Strickland, and P.S. Friedmann. 1996. Comparison of changes in endothelial adhesion molecule expression following UVB irradiation of skin and a human dermal microvascular cell line (HMEC-1). *Photodermatol.Photoimmunol.Photomed.* 12:114.

21. Treina, G., C. Scaletta, A. Fourtanier, S. Seite, E. Frenk, and L.A. Applegate. 1996. Expression of intercellular adhesion molecule-1 in UVA- irradiated human skin cells in vitro and in vivo. *Br.J.Dermatol.* 135:241.

22. Strickland, I., L.E. Rhodes, B.F. Flanagan, and P.S. Friedmann. 1997. TNF-alpha and IL-8 are upregulated in the epidermis of normal human skin after UVB exposure: correlation with neutrophil accumulation and E-selectin expression. *J.Invest.Dermatol.* 108:763.

23. Heckmann, M., M. Pirthauer, and G. Plewig. 1997. Adhesion of leukocytes to dermal endothelial cells is induced after single-dose, but reduced after repeated doses of UVA. *J.Invest.Dermatol.* 109:710.

24. Schall, T.J. and K.B. Bacon. 1994. Chemokines, leukocyte trafficking, and inflammation. *Curr.Opin.Immunol.* 6:865.

25. Furie, M.B. and G.J. Randolph. 1995. Chemokines and tissue injury. *Am.J.Pathol.* 146:1287.

26. Moser, B., M. Loetscher, L. Piali, and P. Loetscher. 1998. Lymphocyte responses to chemokines. *Int.Rev.Immunol.* 16:323.

27. Madsen, P., H.H. Rasmussen, H. Leffers, B. Honore, K. Dejgaard, E. Olsen, J. Kiil, E. Walbum, A.H. Andersen, B. Basse, J.B. Lauridsen, G.P. Ratz, A. Celis, J. Vandekerckhove, and J.E. Celis. 1991. Molecular cloning, occurrence, and expression of a novel partially secreted protein "psoriasin" that is highly up- regulated in psoriatic skin. *J.Invest.Dermatol.* 97:701.

28. Jinquan, T., H. Vorum, C.G. Larsen, P. Madsen, H.H. Rasmussen, B. Gesser, M. Etzerodt, B. Honore, J.E. Celis, and K. Thestrup-Pedersen. 1996. Psoriasin: a novel chemotactic protein. *J.Invest.Dermatol.* 107:5.

29. Center, D.M., H. Kornfeld, and W.W. Cruikshank. 1996. Interleukin 16 and its function as a CD4 ligand. *Immunol.Today* 17:476.

30. Rumsaeng, V., W.W. Cruikshank, B. Foster, C. Prussin, A.S. Kirshenbaum, T.A. Davis, H. Kornfeld, D.M. Center, and D.D. Metcalfe. 1997. Human mast cells produce the $CD4^+$ T lymphocyte chemoattractant factor, IL-16. *J.Immunol.* 159:2904.

31. Duijvestijn, A.M., E. Horst, S.T. Pals, B.N. Rouse, A.C. Steere, L.J. Picker, C.J. Meijer, and E.C. Butcher. 1988. High endothelial differentiation in human lymphoid and inflammatory tissues defined by monoclonal antibody HECA-452. *Am.J.Pathol.* 130:147.

32. Teunissen, M.B.M., C.W. Koomen, R. de Waal Malefyt, E.A. Wierenga, and J.D. Bos. 1998. Interleukin-17 and interferon-γ synergize in the enhancement of proinflammatory cytokines production by human keratinocytes. *J.Invest.Dermatol.* 111:101.

33. Meunier, L., Z. Bata-Csorgo, and K.D. Cooper. 1995. In human dermis, ultraviolet radiation induces expansion of a $CD36^+$ $CD11b^+$ $CD1^-$ macrophage subset by infiltration and proliferation; $CD1^+$ Langerhans-like dendritic antigen-presenting cells are concomitantly depleted. *J.Invest.Dermatol.* 105:782.

34. Cattoretti, G., M.H. Becker, G. Key, M. Duchrow, C. Schluter, J. Galle, and J. Gerdes. 1992. Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB 1 and MIB 3) detect proliferating cells in microwave- processed formalin-fixed paraffin sections. *J.Pathol.* 168:357.

35. Cornelius, L.A., N. Sepp, L.J. Li, K. Degitz, R.A. Swerlick, T.J. Lawley, and S.W. Caughman. 1994. Selective upregulation of intercellular adhesion molecule (ICAM- 1) by ultraviolet B in human dermal microvascular endothelial cells. *J.Invest.Dermatol.* 103:23.

36. Wayner, E.A., A. Garcia-Pardo, M.J. Humphries, J.A. McDonald, and W.G. Carter. 1989. Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. *J.Cell Biol.* 109:1321.

37. Bos, J.D., I. Zonneveld, P.K. Das, S.R. Krieg, C.M. van der Loos, and M.L. Kapsenberg. 1987. The skin immune system (SIS): distribution and immunophenotype of lymphocyte subpopulations in normal human skin. *J.Invest.Dermatol.* 88:569.

38. Foster, C.A., H. Yokozeki, K. Rappersberger, F. Koning, B. Volc-Platzer, A. Rieger, J.E. Coligan, K. Wolff, and G. Stingl. 1990. Human epidermal T cells predominantly belong to the lineage expressing alpha/beta T cell receptor. *J.Exp.Med.* 171:997.

39. Bruls, W.A.G., H. Slaper, J.C. van der Leun, and L. Berrens. 1984. Transmission of human epidermis and stratum corneum as a function of thickness in the ultraviolet and visible wavelenghts. *Photochem.Photobiol.* 40:485.

40. Morison, W.L., J.A. Parrish, R.R. Anderson, and K.J. Bloch. 1979. Sensitivity of mononuclear cells to UV radiation. *Photochem.Photobiol.* 29:1045.

41. Di Nuzzo, S., M.A. De Rie, C.M. van der Loos, J.D. Bos, and M.B.M. Teunissen. 1996. Solar-simulated ultraviolet irradiation induces selective influx of $CD4^+$ T lymphocytes in normal human skin. *Photochem.Photobiol.* 64:988.

42. Gilchrest, B.A., N.A. Soter, J.S. Stoff, and M.C. Mihm, Jr. 1981. The human sunburn reaction: histologic and biochemical studies. *J.Am.Acad.Dermatol.* 5:411.

43. Hammerberg, C., N. Duraiswamy, and K.D. Cooper. 1996. Temporal correlation between UV radiation locally-inducible tolerance and the sequential appearance of dermal, then epidermal, class II MHC^+CD11b^+ monocytic/macrophagic cells. *J.Invest.Dermatol.* 107:755.

44. Spangrude, G.J., E.J. Bernhard, R.S. Ajioka, and R.A. Daynes. 1983. Alterations in lymphocyte homing patterns within mice exposed to ultraviolet radiation. *J.Immunol.* 130:2974.

45. Bos, J.D. and M.L. Kapsenberg. 1993. The skin immune system: progress in cutaneous biology. *Immunol.Today* 14:75.

46. Murphy, G.F. and L. Vincent. 1997. The dermal immune system. In Skin Immune System (SIS). J.D. Bos, editor. CRC Press, Boca Raton. 347-363.

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.

Sergio Di Nuzzo[1], Regien M.R. Sylva-Steenland[1], Cornelis W. Koomen[1], Satoshi Nakagawa[1], Marielle van Breemen[1], Menno A. de Rie[1], Jan D. Bos[1] , Pranab K. Das[1,2] and Marcel B.M. Teunissen[1].

[1]Department of Dermatology and [2]Department of Pathology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

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 $IL\text{-}4^+$ 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

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'-CGAGATCCCTCCAAA ATCAA-3' (nt 298-317) and GAPDH reverse primer 5'-AGGTCAGGTCCACCACTGAC-3' (nt 799-780); IL-4 forward primer ACTCTGTGCACCGAGTTGACCGTAA and IL-4 reverse primer TCTCATGATCGTCTTTAGCCTTTCC; IFN-γ forward primer GACTTCGAAAA GCTGACTAA 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 H_2O_2 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 ionomycine (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

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 [^{3}H] 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% NaN_3 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.

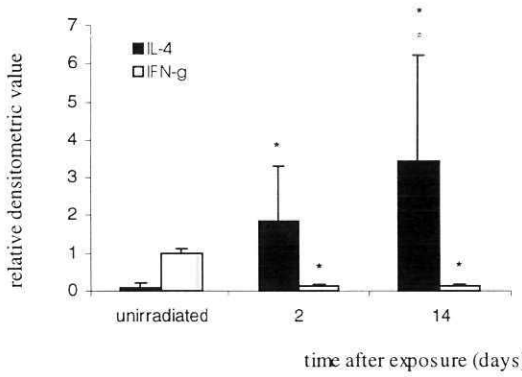
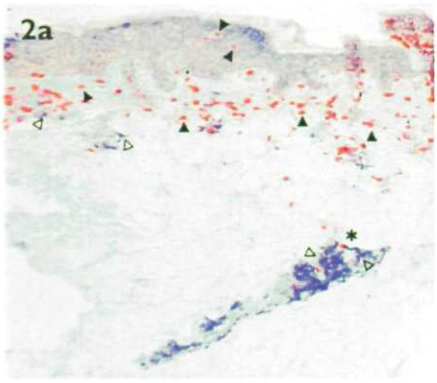
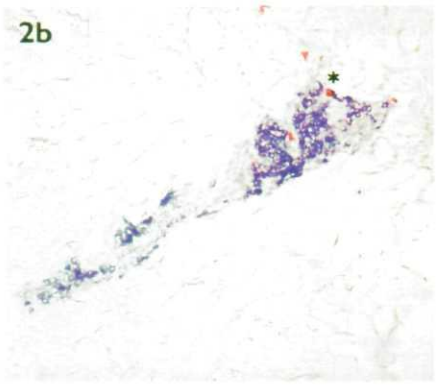


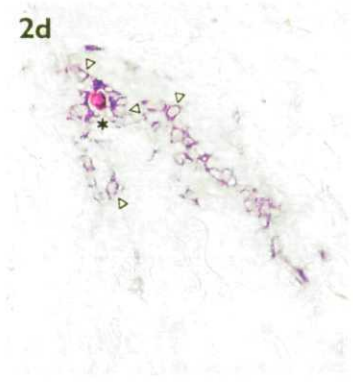
Figure 1. UVB exposure induced an increase in IL-4 mRNA and a decrease in IFN-γ mRNA expression in normal human skin. Exposures to 4 MED of UVB were performed 14 and 2 days before collecting skin biopsies. Unirradiated skin served as a control. RNA was isolated from cryostat sections and was subjected 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 $p < 0.05$).

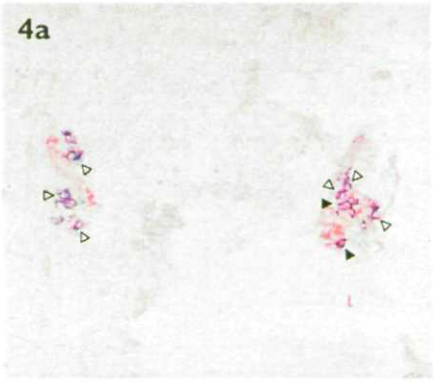
In the next series of experiments we determined the expression of IL-4 and IFN-γ at protein level in the same skin biopsies. The skin sections were double-stained with mAb against CD3 and one of the two different mAbs against IL-4, which have been shown to be suitable for immunohistochemical staining (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 obseved in the


2a


2b


2c


2d


4a

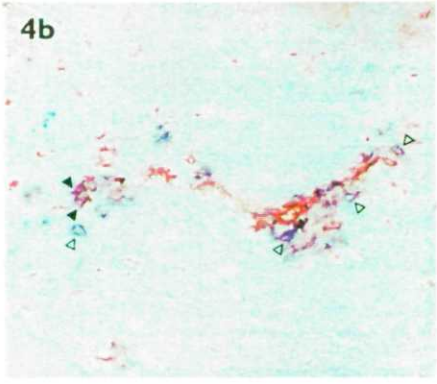

4b

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 harrowhead).

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.

epidermal compartment (Fig. 2C), which is in line with our previous report (3). The anti-IL-4 mAb from Genzyme provided a strong staining of the IL-4$^+$ cells *in situ*, whereas a similar though weaker staining was found at day 2 post-irradiation using the other mAb against IL-4, resulting in smaller numbers of IL-4$^+$ cells (data not shown). With respect to IFN-γ protein expression in unirradiated control skin, we found few perivascular CD3$^+$IFN-γ$^+$ (2% of the total CD3$^+$ T cells; Fig. 2D). At day 2 and 14 after exposure to UVB, IFN-γ protein was not detectable in any of the specimens tested (n=3; not shown).

UVB radiation favors the development of type 2 T cells. As outlined above, we were not successful to characterize *in situ* the IL-4 and IFN-γ expression of the vast majority of the infiltrated T cells in the UVB irradiated skin. To be able to determine the cytokine production in these T cells we had to use a different approach. We prepared dermal cell suspensions of the biopsies from UVB-exposed skin 2 days post-irradiation and from nonexposed control skin. The yield of dermal cells was small (about 10^5 cells/biopsy) and contained approximately 10 % T cells. To get enough dermal T cells for cytokine analysis, we stimulated the dermal cell suspension with the polyclonal T-cell stimulus PHA and added recombinant human IL-2 to support T-cell growth. In order to make it possible to determine simultaneously the IL-4 and IFN-γ production in each individual T cell in the dermal T-cell bulk cultures, we decided to assess the intracellular cytokine expression by flow cytometry. Three weeks after setting up the dermal cell bulk cultures, the cells were stimulated for 4 h with PMA and ionomycin in the presence of brefeldin A, before the cells were stained with fluorescent-labelled mAbs.

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-γ (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-γ producing type 1 T cells. Only a minimal number of IL-4 / IFN-γ 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-γ 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 .

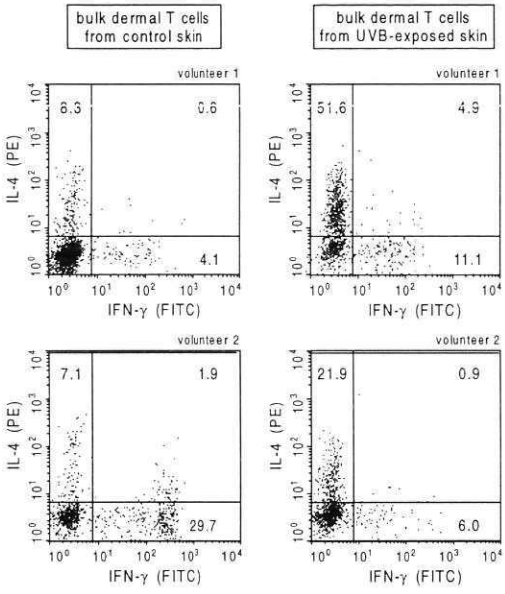


Figure 3. Development of type 2 T cells is enhanced in primary cultures of bulk dermal cells from UVB-exposed skin. Dermal bulk cultures were obtained from skin biopsies taken two days after irradiation with 4 MED, using unirradiated skin as control. Primary dermal cell cultures were stimulated with PMA and ionomycin in the presence of brefeldin A for 4 hours and subsequentely 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, exibit 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 $\pm$ 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 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-γ 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.

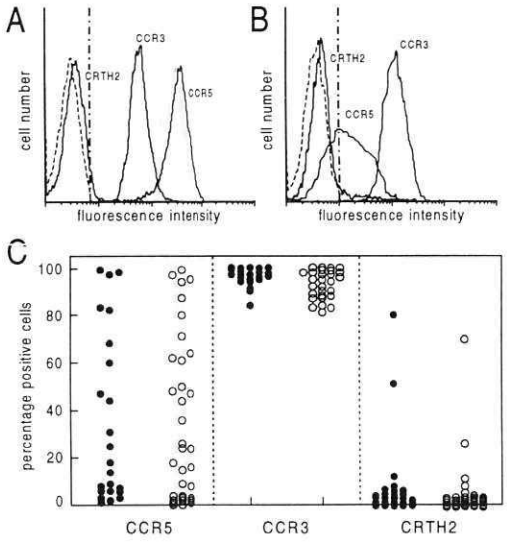


Figure 5. Phenotype of TCC generated from unexposed and UVB-exposed normal human skin. TCC were generated from the dermis of biopsies taken from unirradiated control skin or from skin which was exposed to 4 MED of UVB two days earlier. After at least three cycles of restimulation the TCC were stained 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).

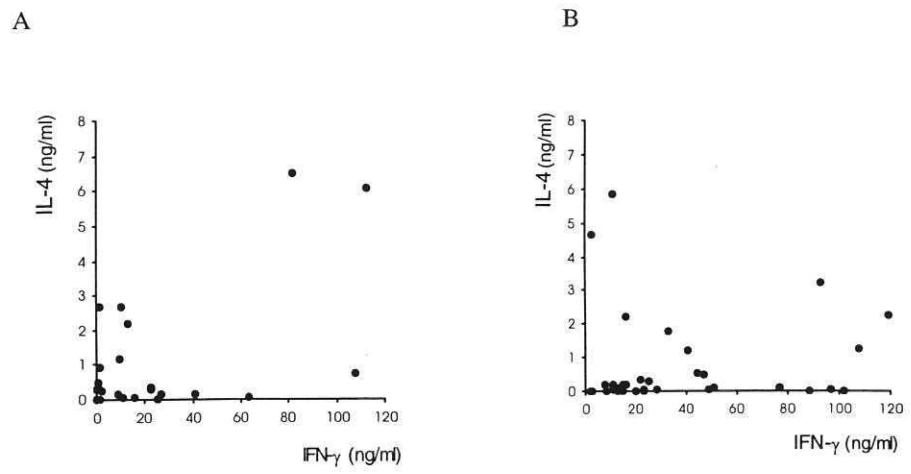


Figure 6. The production of IFN-γ and IL-4 by dermal TCC generated from unirradiated (A) and irradiated (B) normal human skin. Dermal TCC were generated by a limiting-dilution protocol from UVB-exposed (4 MED) or unirradiated control skin. The TCC were stimulated overnight with antibody pair CD3 and CD28. Subsequentely, culture supernatant was harvested and the cytokine concentration was measured by ELISA. Each dot represents an individual TCC.

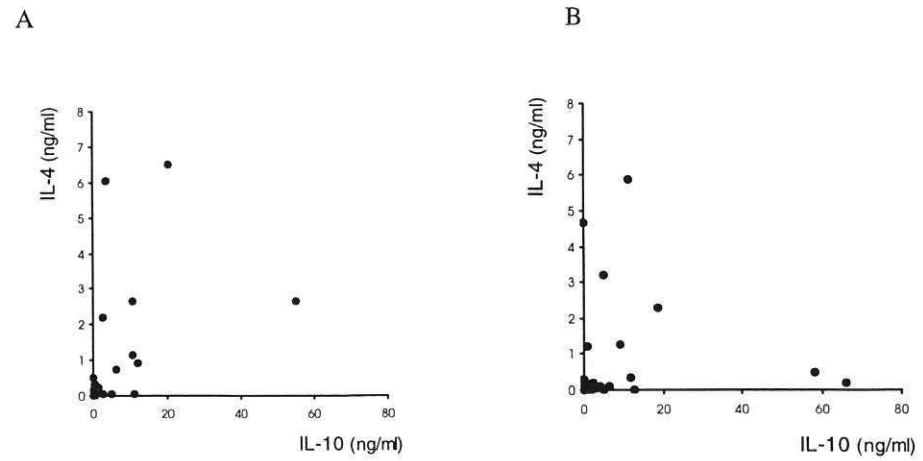


Figure 7. The expression of IL-10 and IL-4 by dermal TCC generated from unirradiated (A) and irradiated (B) normal human skin. One healthy volunteer was locally exposed to four MED of UVB and skin biopsies were taken two days after irradiations. Unirradiated skin served as a control. Dermal TCC were 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 immunosuppresion 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 dowregulation of IFN-γ expression in

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-γ expression in skin through at least three different mechanisms. First, exposure to UVB causes a decrease in the number of intraepidermal IFN-γ producing T cells, which is probably a phototoxic effect. Second, IFN-γ 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-γ.

As compared to the T cells from control skin, T-cell lines and TCC grown from irradiated skin biopsies were mostly of the CD4 TCRαβ 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 chemoattractant psoriasin was upregulated in normal human skin upon exposure to a single dose of UVB. Since psoriasin has been shown to be a selective chemoattractant 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 chemoattractant 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 immunohistochemisty 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. Tr1 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 type1 / 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 microenviroment that is conductive to the development of type 2 T-cell.

Acnowledgments

Arthur Kammeyer is gratefully acknowledged for his expert assistance with the UV source, Mohamed El Haddouchi for his help in optimizing some immunohistochemical stainings, and Wendy van Noppen for her critical reading of the manuscript.

References

1. Barr, R.M., S.L. Walker, W. Tsang, G.I. Harrison, P. Ettehadi, M.W. Greaves, and A.R. Young. 1999. Suppressed alloantigen presentation, increased TNF-alpha, IL-1, IL-1Ra, IL-10, and modulation of TNF-R in UV-irradiated human skin. *J.Invest.Dermatol.* 112:692.

2. Willis, I. and L. Cylus. 1977. UVA erythema in skin: is it a sunburn? *J.Invest.Dermatol.* 68:128.

3. Di Nuzzo, S., R.M. Sylva-Steenland, M.A. De Rie, P.K. Das, J.D. Bos, and M.B.M. Teunissen. 1998. UVB radiation preferentially induces recruitment of memory $CD4^+$ T cells in normal human skin: long-term effect after a single exposure. *J.Invest.Dermatol.* 110:978.

4. Carter, L.L. and R.W. Dutton. 1996. Type 1 and type 2: a fundamental dichotomy for all T-cell subsets. *Curr.Opin.Immunol.* 8:336.

5. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J.E. de Vries, and M.G. Roncarolo. 1997. A $CD4^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389:737.

6. Shreedhar, V.K., M.W. Pride, Y. Sun, M.L. Kripke, and F.M. Strickland. 1998. Origin and characteristics of ultraviolet-B radiation-induced suppressor T lymphocytes. *J.Immunol.* 161:1327.

7. Groux, H. and F. Powrie. 1999. Regulatory T cells and inflammatory bowel disease. *Immunol.Today* 20:442.

8. Annunziato, F., G. Galli, L. Cosmi, P. Romagnani, R. Manetti, E. Maggi, and S. Romagnani. 1998. Molecules associated with human Th1 or Th2 cells. *Eur.Cytokine Netw.* 9:12.

9. Annunziato, F., R. Manetti, L. Cosmi, G. Galli, C.H. Heusser, S. Romagnani, and E. Maggi. 1997. Opposite role for interleukin-4 and interferon-gamma on CD30 and lymphocyte activation gene-3 (LAG-3) expression by activated naive T cells. *Eur.J.Immunol.* 27:2239.

10. D'Elios, M.M., P. Romagnani, C. Scaletti, F. Annunziato, M. Manghetti, C. Mavilia, P. Parronchi, C. Pupilli, G. Pizzolo, E. Maggi, G.F. Del Prete, and S. Romagnani. 1997. In vivo CD30 expression in human diseases with predominant activation of Th2-like T cells. *J.Leukoc.Biol.* 61:539.

11. Sallusto, F., D. Lenig, C.R. Mackay, and A. Lanzavecchia. 1998. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J.Exp.Med.* 187:875.

12. Bonecchi, R., G. Bianchi, P.P. Bordignon, D. D'Ambrosio, R. Lang, A. Borsatti, S. Sozzani, P. Allavena, P.A. Gray, A. Mantovani, and F. Sinigaglia. 1998. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J.Exp.Med.* 187:129.

13. Loetscher, P., M. Uguccioni, L. Bordoli, M. Baggiolini, B. Moser, C. Chizzolini, and J.M. Dayer. 1998. CCR5 is characteristic of Th1 lymphocytes. *Nature* 391:344.

14. Sallusto, F., C.R. Mackay, and A. Lanzavecchia. 1997. Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. *Science* 277:2005.

15. Zingoni, A., H. Soto, J.A. Hedrick, A. Stoppacciaro, C.T. Storlazzi, F. Sinigaglia, D. D'Ambrosio, A. O'Garra, D. Robinson, M. Rocchi, A. Santoni, A. Zlotnik, and M. Napolitano. 1998. The chemokine receptor CCR8 is preferentially expressed in Th2 but not Th1 cells. *J.Immunol.* 161:547.

16. Gerber, B.O., M.P. Zanni, M. Uguccioni, M. Loetscher, C.R. Mackay, W.J. Pichler, N. Yawalkar, M. Baggiolini, and B. Moser. 1997. Functional expression of the eotaxin receptor CCR3 in T lymphocytes co-localizing with eosinophils. *Curr.Biol.* 7:836.

17. Nagata, K., K. Tanaka, K. Ogawa, K. Kemmotsu, T. Imai, O. Yoshie, H. Abe, K. Tada, M. Nakamura, K. Sugamura, and S. Takano. 1999. Selective expression of a novel surface molecule by human Th2 cells in vivo. *J.Immunol.* 162:1278.

18. Ullrich, S.E. 1996. Does exposure to UV radiation induce a shift to a Th-2-like immune reaction? *Photochem.Photobiol.* 64:254.

19. Teunissen, M.B.M., C.W. Koomen, R. de Waal Malefyt, E.A. Wierenga, and J.D. Bos. 1998. Interleukin-17 and interferon-γ synergize in the enhancement of proinflammatory cytokines production by human keratinocytes. *J.Invest.Dermatol.* 111:101.

20. Di Nuzzo, S., M.A. De Rie, C.M. van der Loos, J.D. Bos, and M.B.M. Teunissen. 1996. Solar-simulated ultraviolet irradiation induces selective influx of $CD4^+$ T lymphocytes in normal human skin. *Photochem.Photobiol.* 64:988.

21. Bos, J.D., I. Zonneveld, P.K. Das, S.R. Krieg, C.M. van der Loos, and M.L. Kapsenberg. 1987. The skin immune system (SIS): distribution and immunophenotype of lymphocyte subpopulations in normal human skin. *J.Invest.Dermatol.* 88:569.

22. Kremer, I.B., C.M. Hilkens, R.M. Sylva-Steenland, C.W. Koomen, M.L. Kapsenberg, J.D. Bos, and M.B.M. Teunissen. 1996. Reduced IL-12 production by monocytes upon ultraviolet-B irradiation selectively limits activation of T helper-1 cells. *J.Immunol.* 157:1913.

23. Verhagen, C. E. Immunopathology of leprosy:significance of T cell subsets and antigens. 1998. *Academic (PhD) Thesis.* 89.

24. Llorente, L., Y. Richaud-Patin, C. Alvarado, E. Reyes, J. Alcocer-Varela, and R. Orozco-Topete. 1997. Elevated Th1 cytokine mRNA in skin biopsies and peripheral circulation in patients with erythema nodosum. *Eur.Cytokine Netw.* 8:67.

25. Szabo, S.K., C. Hammerberg, Y. Yoshida, Z. Bata-Csorgo, and K.D. Cooper. 1998. Identification and quantitation of interferon-gamma producing T cells in psoriatic lesions: localization to both $CD4^+$ and $CD8^+$ subsets. *J.Invest.Dermatol.* 111:1072.

26. Thepen, T., E.G. Langeveld-Wildschut, I.C. Bihari, D.F. van Wichen, F.C. van Reijsen, G.C. Mudde, and C.A. Bruijnzeel-Koomen. 1996. Biphasic response against aeroallergen in atopic dermatitis showing a switch from an initial TH2 response to a TH1 response in situ: an immunocytochemical study. *J.Allergy Clin.Immunol.* 97:828.

27. Rivas, J.M. and S.E. Ullrich. 1994. The role of IL-4, IL-10, and TNF-alpha in the immune suppression induced by ultraviolet radiation. *J.Leukoc.Biol.* 56:769.

28. El-Ghorr, A.A. and M. Norval. 1997. The role of interleukin-4 in ultraviolet B light-induced immunosuppression. *Immunology* 92:26.

29. Shreedhar, V.K., T. Giese, V.W. Sung, and S.E. Ullrich. 1998. A cytokine cascade including prostaglandin E2, IL-4, and IL-10 is responsible for UV-induced systemic immune suppression. *J.Immunol.* 160:3783.

30. Brown, M.A. and J. Hural. 1997. Functions of IL-4 and control of its expression. *Crit.Rev.Immunol.* 17:1.

31. Hawk, J.L., G.M. Murphy, and C.A. Holden. 1988. The presence of neutrophils in human cutaneous ultraviolet-B inflammation. *Br.J.Dermatol.* 118:27.

32. Cooper, K.D., G.R. Neises, and S.I. Katz. 1986. Antigen-presenting $OKM5^+$ melanophages appear in human epidermis after ultraviolet radiation. *J.Invest.Dermatol.* 86:363.

33. Etter, H., R. Althaus, H.P. Eugster, L.F. Santamaria Babi, L. Weber, and R. Moser. 1998. IL-4 and IL-13 downregulate rolling adhesion of leukocytes to IL-1 or TNF-alpha-activated endothelial cells by limiting the interval of E-selectin expression. *Cytokine* 10:395.

34. Norris, P., R.N. Poston, D.S. Thomas, M. Thornhill, J.L. Hawk, and D.O. Haskard. 1991. The expression of endothelial leukocyte adhesion molecule-1 (ELAM-1), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) in experimental cutaneous inflammation: a comparison of ultraviolet B erythema and delayed hypersensitivity. *J.Invest.Dermatol.* 96:763.

35. Saleem, S., Z. Dai, S.N. Coelho, B.T. Konieczny, K.J. Assmann, F.K. Baddoura, and F.G. Lakkis. 1998. IL-4 is an endogenous inhibitor of neutrophil influx and subsequent pathology in acute antibody-mediated inflammation. *J.Immunol.* 160:979.

36. Boey, H., R. Rosenbaum, J. Castracane, and L. Borish. 1989. Interleukin-4 is a neutrophil activator. *J.Allergy Clin.Immunol.* 83:978.

37. Montaner, L.J., R.P. da Silva, J. Sun, S. Sutterwala, M. Hollinshead, D. Vaux, and S. Gordon. 1999. Type 1 and type 2 cytokine regulation of macrophage endocytosis: differential activation by IL-4/IL-13 as opposed to IFN-gamma or IL-10. *J.Immunol.* 162:4606.

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.

39. Barker, J.N., G.D. Karabin, T.J. Stoof, V.J. Sarma, V.M. Dixit, and B.J. Nickoloff. 1991. Detection of interferon-gamma mRNA in psoriatic epidermis by polymerase chain reaction. *J.Dermatol.Sci.* 2:106.

40. Livden, J.K., Nilsen, R., Bjerke, J.R., and Matre, R. 1989. In situ localization of inerferons in psoriatic lesions. *Arch.Dermatol.Res.* 281:392.

41. Streilein, J.W. 1978. Lymphocyte traffic, T-cell malignancies and the skin. *J.Invest.Dermatol.* 71:167.

42. Streilein, J.W. 1983. Skin-associated lymphoid tissues (SALT): origins and functions. *J.Invest.Dermatol.* 80:12s.

43. Kaplan, D.H., V. Shankaran, A.S. Dighe, E. Stockert, M. Aguet, L.J. Old, and R.D. Schreiber. 1998. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. *Proc.Natl.Acad.Sci.U.S.A.* 95:7556.

44. Kang, K., C. Hammerberg, L. Meunier, and K.D. Cooper. 1994. $CD11b^+$ macrophages that infiltrate human epidermis after in vivo ultraviolet exposure potently produce IL-10 and represent the major secretory source of epidermal IL-10 protein. *J.Immunol.* 153:5256.

45. Jinquan, T., H. Vorum, C.G. Larsen, P. Madsen, H.H. Rasmussen, B. Gesser, M. Etzerodt, B. Honore, J.E. Celis, and K. Thestrup-Pedersen. 1996. Psoriasin: a novel chemotactic protein. *J.Invest.Dermatol.* 107:5.

46. Strickland, I., L.E. Rhodes, B.F. Flanagan, and P.S. Friedmann. 1997. TNF-alpha and IL-8 are upregulated in the epidermis of normal human skin after UVB exposure: correlation with neutrophil accumulation and E-selectin expression. *J.Invest.Dermatol.* 108:763.

47. Jinquan, T., B. Deleuran, B. Gesser, H. Maare, M. Deleuran, C.G. Larsen, and K. Thestrup-Pedersen. 1995. Regulation of human T lymphocyte chemotaxis in vitro by T cell-derived cytokines IL-2, IFN-gamma, IL-4, IL-10, and IL-13. *J.Immunol.* 154:3742.

48. Bradding, P., I.H. Feather, P.H. Howarth, R. Mueller, J.A. Roberts, K. Britten, J.P. Bews, T.C. Hunt, Y. Okayama, and C.H. Heusser. 1992. Interleukin 4 is localized to and released by human mast cells. *J.Exp.Med.* 176:1381.

49. Swain, S.L. 1993. IL4 dictates T-cell differentiation. *Res.Immunol.* 144:616.

50. Kang, K., A.C. Gilliam, G. Chen, E. Tootell, and K.D. Cooper. 1998. In human skin, UVB initiates early induction of IL-10 over IL-12 preferentially in the expanding dermal monocytic/macrophagic population. *J.Invest.Dermatol.* 111:31.

51. Swain, S.L. 1994. Generation and in vivo persistence of polarized Th1 and Th2 memory cells. *Immunity* 1:543.

52. Doyle, A.G., K. Buttigieg, P. Groves, B.J. Johnson, and A. Kelso. 1999. The activated Type-1-polarized $CD8^+$ T cell population isolated from an effector site contains cells with flexible cytokine profiles. *J.Exp.Med.* 190:1081.

Chapter 6

Ultraviolet-B radiation induces the expression of interleukin-4 in a cutaneous $CD11b^+CD15^+$ cell subset in normal human skin: a preliminary report

Marcel B.M. Teunissen, Sergio Di Nuzzo, Gamze Pişkin, Regien M.R. Sylva-Steenland, Menno A. de Rie, and Jan D. Bos

Department of Dermatology Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

To be submitted

Abstract

There is ample evidence that UVB irradiation causes considerable changes in the cutaneous cell distribution and local cytokine levels in the normal human skin. We show in this report that high numbers of IL-4^+ cells appeared in the dermis, and to a lesser extent in the epidermis, within 24 h upon a single exposure to a physiological dose of UVB. In the next 24 h the number of IL-4^+ cells in the epidermis raised and reached a peak, while in the dermis the numbers remained high. At day 4 the number of IL-4^+ cells was markedly decreased and at later time-points the IL-4 expression was not detectable. Immunohistochemical double-staining experiments revealed that all the cutaneous cells that are known to be able to express IL-4 (i.e. T cells, mast cells, and natural killer cells) were not triggered by UVB to express IL-4. The expression of this cytokine seemed to be associated with $CD11b^+CD15^+CD36^-$ cells, possibly neutrophils, which are known to infiltrate UVB-exposed skin. However, the results so far do not provide conclusive evidence that the IL-4 is actually produced by these cells. Additional experiments are necessary to exclude the possibility that the IL-4 we detect is taken up by IL-4 receptors or phagocytosis and thus derived from other cells.

Introduction

Exposure to UV radiation is inevitable, since it is part of the sunlight that reaches the earth surface. UV radiation, especially UVB, has considerable impact on the natural homeostasis within the skin. Amongst others, the skin immune system is affected, as illustrated by the features of inflammation that develop and the concurrent alterations in the composition and function of different cell types (1). The LC in the epidermis are decreased in density and altered in morphology due to the phototoxic effects of UVB (2). Some of the LC can survive and are still able to migrate and to stimulate T cells, despite the presence of UVB-induced DNA damage (3). Probably depended on the amount of DNA damage, these surviving LC undergo either accelerated apoptotic cell death or potentiated maturation (4). The epidermal T cells are depleted by the deleterious radiation (5), likely by induction of apoptosis (6,7). In the dermis a cellular infiltrate starts to develop upon UVB exposure, beginning few hours after irradiation and peaking a day 2. In the order of entrance, this infiltrate is composed of neutrophils, macrophages (8) and predominantly $CD4^+$ memory T cells (5). During the next few days, these infiltrated cells tend to migrate into the epidermis; first the neutrophils appear, later followed by the macrophages. Three days after UVB irradiation LC start to migrate from the hair follicles to repopulate the epidermis (9), whereas one week post-irradiation a selective influx of $CD4^+$ T cells emerges (5).

In addition to the dynamics of these different cell populations in time, UVB radiation also causes a temporal change in the cutaneous cytokine micro milieu. Keratinocytes are believed to be major sources of all kinds of factors, such as cytokines, chemokines, growth factors and many others (10). The constitutive production of these factors by these cells is rather low, but considerably enhanced by UVB radiation (11). UVB potently induces the release of pro-inflammatory mediators IL-1, IL-6, IL-8, TNF-α, and PGE2 from keratinocytes, likely responsible for the onset of the inflammation and the induction of the chemotaxis of the neutrophils and macrophages into the skin. The infiltrating macrophages have been shown to produce huge amounts of IL-10 (12). UVB also induces a strong transient expression of the chemokine psoriasin, first around the dermal capillaries and subsequently in the epidermis (Chapter 4). Psoriasin is a specific chemoattractant for $CD4^+$ T cells, and the anatomically location of UVB-induced psoriasin expression nicely correlates with the influx of the $CD4^+$ T cells into the irradiated skin site at all time points. As a result of all the changes in the composition and function of the different cutaneous cells and their cytokine-production patterns, the UVB-exposed

skin provides a micro environment that favors the development of type 2 T cell responses (Chapter 5).

In this respect it was interesting to find by RT-PCR and immunohistochemistry that UVB radiation induced a strong expression of IL-4 mRNA and protein in normal human skin *in situ* two days post-irradiation, while reducing the expression of IFN-γ (Chapter 5). The majority of the IL-4$^+$ cells were found in the papillary dermis and to a lesser extent in the epidermis, and they had a scattered distribution. Double-staining with CD3 antibody indicated that only 2 % of the IL-4$^+$ cells could be identified as T cell. At day 14 after UVB exposure, as well as in unirradiated control skin, IL-4 protein expression was not found. This study was set up to determine the kinetics of UVB-induced IL-4 expression, using skin biopsies obtained at different time- points after irradiation. To identify the actual cell source of this cytokine we performed double-staining immunohistochemistry, using specific antibodies against cell types known to produce IL-4, such as T cells, mast cells and natural killer cells. In this report we show that UVB induced cutaneous CD11b$^+$ CD15$^+$ cells to express IL-4 and that this expression was maximal at day 1 and 2 post-irradiation.

Materials and Methods

UVB irradiation of the subjects. Four adult Caucasian volunteers participated in this study after informed consent according to the guidelines of the Medical Ethical Committee of the hospital. Their mean age was 26 (range 21-31) years and none suffered neither from any skin disease nor from light hypersensitivity. One month before the start and during the experiment the volunteers had to refrain from excessive sunlight exposure and were prohibited to use tanning lamps. The MED for each donor was determined on the left buttock one week before the experiment by irradiating separate small areas of skin with increasing doses of UVB and reading the results 24 h later. The lowest dose inducing erythema was taken as 1 MED. The irradiations were performed with a 1000 W xenon-arc solar simulator lamp (Oriel, Stratford, CT) in combination with a 303 nm interference filter (Schott Glaswerke, Mainz, Germany), as described previously (5). Single doses of 4 MED were given to separate sites of the right buttock at various time points before taking biopsies. The biopsies were taken under local anesthesia and were immediately frozen in liquid nitrogen and stored at -80°C.

Immunohistochemistry. Series of 6-mm cryostat sections were cut and after drying

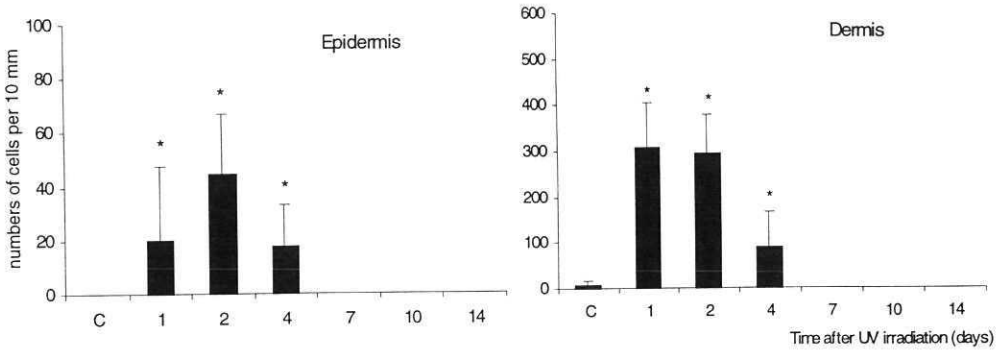
overnight they were separately wrapped in aluminium foil and stored at -80°C until use. The details of the single- and double-staining procedures are described elsewhere (5). In brief, the cryostat sections were thawed, unwrapped and fixed in acetone for 10 min at 4°C. The sections were incubated overnight with mouse anti-human IL-4 antibody (clone M1; Genzyme, Cambridge, MA), followed by an incubation for 30 min with biotin-conjugated goat anti-mouse (Dako, Glostrup, Denmark), and another incubation for 30 min with horseradish peroxidase-conjugated streptavidin (Dako). The peroxidase activity was visualized as a orange-red color by incubation with AEC (Sigma, St Louis, MO) plus H_2O_2. In the double-staining experiments we used FITC- or AP-conjugated primary antibodies to allow simultaneous detection of IL-4 and several cluster of differentiation markers. The binding of the FITC-labeled antibodies was detected by AP-conjugated goat anti-FITC immunoglobulin (Dako) and the AP activity was visualized as a blue color by incubation with naphthol-AS-MX-phosphate (Sigma) plus fast blue BB (Sigma). The following FITC-labeled antibodies were used: CD3 (Becton Dickinson, Mountain View, CA), CD11b (Immunotech, Marseille, France), CD15 (Dako), CD36 (Immunotech), CD56 (Becton Dickinson). The AP-conjugated anti-tryptase was purchased from Chemicon (Temecula, CA).

Enumeration and statistic analysis. The identification labels of all object glasses were covered and the sequence of the glasses was mixed before counting to enable blind quantification by three different investigators. Only clearly stained cell bodies were counted in three different sections per time-point per volunteer. The value of each individual section was adjusted to 10-mm horizontal section values by dividing with the horizontal width multiplied by 10. The total mean of cell numbers of each time-point was calculated from the mean values of corresponding time-points of each volunteer. The unpaired two-sided Student's t test was used to evaluate the results, considering value $p < 0.05$ as statistically significant.

Results

UVB radiation induces a transient expression of IL-4. In our previous study (Chapter 5) we detected by immunohistochemistry a clear expression of IL-4 in UVB-exposed human skin 2 days after irradiation. Because UVB-exposed skin 14 days post-irradiation and unirradiated control skin did not contain IL-4^+ cells, we may conclude that UVB can induce a transient expression of IL-4 in normal human skin. This investigation was aimed to determine the course

of the UVB-induced IL-4 expression. To this end we took skin biopsies from UVB-exposed buttock skin at different time-points after irradiation and stained cryostat sections with an anti-human IL-4 specific antibody, which is suitable for immunohistochemistry. In unirradiated control skin an occasional IL-4$^+$ cell was observed in the dermis, whereas the epidermis was



devoid of such cells (Fig. 1). Already 1 day after UVB exposure, several hundreds (307 ± 98 per 10 mm section) of IL-4$^+$ cells were present in the dermis, and they also started to appear in the epidermis.

Figure 1. Transient expression of IL-4 in human skin after UVB irradiation. Skin biopsies were obtained from buttock skin of 4 volunteers at various times after UVB exposure with 4 MED, using unirradiated skin as control (c). Three cryostat sections per time point were stained for IL-4 and the number of positive cells in the dermis and epidermis was counted by three investigators, so each bar represents the mean of 27 determinations with the SD. The asterisk indicates the time-points at which the number of IL-4$^+$ cells was significantly different from that found in control skin.

At day 2 post-irradiation, the number of IL-4$^+$ cells in the dermis was comparable to the numbers of day 1, but in the epidermis the IL-4 expression had reached a maximum (45 ± 23 per 10 mm section). The IL-4$^+$ cells in the dermis and epidermis had a scattered distribution (Fig. 2A).The number of IL-4$^+$ cells was clearly decreased at day 4 in the dermis and epidermis. The IL-4 expression could not be found at later time-points (Fig. 1).

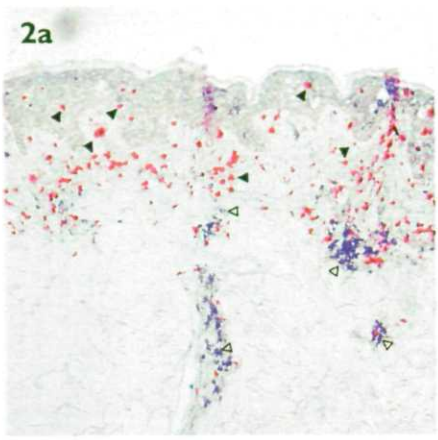
The UVB-induced IL-4$^+$ cells co-express CD11b and CD15. In order to determine which cell-type in the normal human skin was triggered by UVB to express IL-4, we performed double-staining experiments. We used the biopsies taken at day 2 after irradiation for these experiments, because the IL-4 signal was most prominent in both dermis and epidermis at this time-point. The skin sections were stained with anti-IL-4 plus one antibody specific for cell-types that are known to produce IL-4; i.e. CD3 as a marker for T cells, tryptase for mast cells, CD56 for natural killer

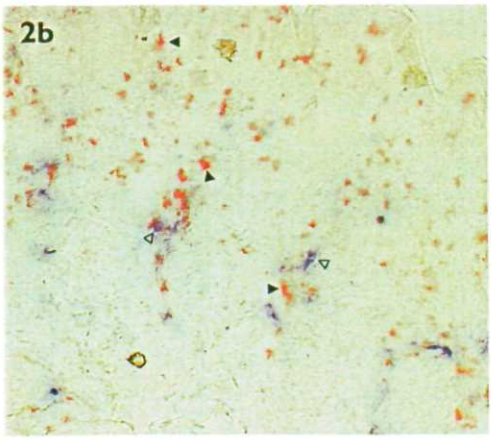
cells. In line with our previous study, we found that about 2 % of the IL-4$^+$ cells co-expressed CD3 (Fig. 2A). Except for an occasional double-positive cell (much less then 1%), the IL-4$^+$ cells were negative for tryptase (Fig. 2B) and for CD56 (not shown). These data indicate that the IL-4 was apparently not induced in a resident skin cell.

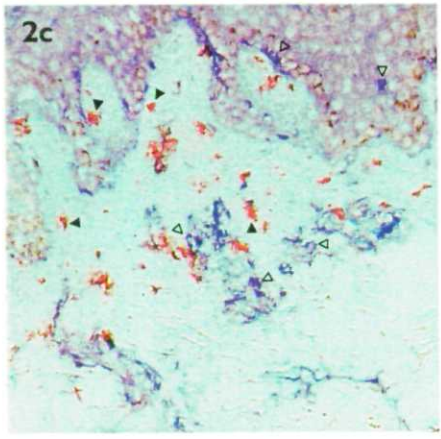
Because the approach mentioned above did not resolve the issue about the identity of the IL-4$^+$ cells, we extended our panel of antibodies with markers for cells that are known to infiltrate the skin after UVB irradiation; i.e. CD11b, CD15, and CD36 for macrophages/granulocytes. Although many CD36$^+$ cells were found in the UVB-exposed skin samples (mainly in the dermis), they did clearly not co-express IL-4 (Fig 2C). Much to our surprise, we found that the majority (75 % ± 11 %) of the IL-4$^+$ cells in the cryostat sections co-expressed CD11b and CD15 (Fig 2D, 2E). In addition, 60 % ± 10 % of the CD11b$^+$ cells and 61 % ± 13 % of the CD15$^+$ cells were also positive for IL-4. The CD11b and CD15 expressing cells had a scattered distribution, like the IL-4 expressing cells. The presence of IL-4 within CD11b$^+$ and CD15$^+$ was confirmed in cytospin preparations of dermal cell suspensions derived from UVB-exposed skin (Fig 2F). These data indicate that UVB-induced IL-4 co-localized with infiltrating granulocytes.

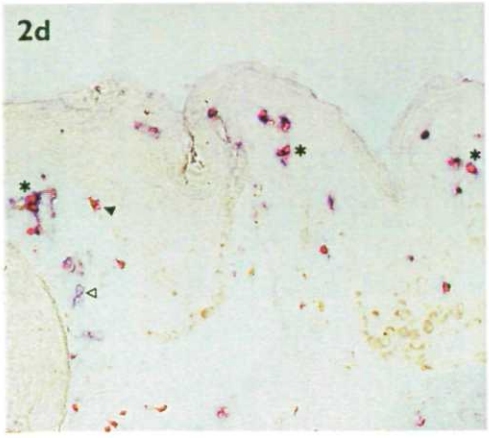
Discussion

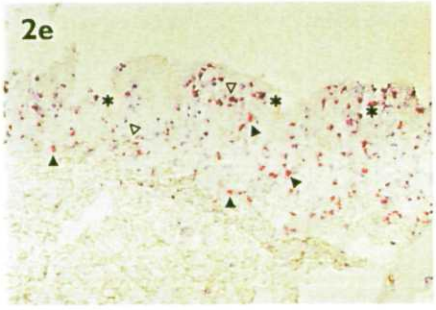
In this study we show that exposure of normal human skin to a physiological dose of UVB induced the transient appearance of numerous IL-4$^+$ cells in the dermis (peak at 24 h and 48 h after irradiation) and in the epidermis (peak at 48 h), having a scattered distribution. Double-staining experiments ruled out that the IL-4 expression was induced in local cutaneous T cells, mast cells or natural killer cells, which are known to possess the capacity to synthesize IL-4 (13). It was quite astounding to discover that the UVB-induced IL-4 expression was associated with infiltrating CD11b$^+$CD15$^+$ cells, because neutrophils (CD11b$^+$CD15$^+$) and macrophages (CD11b$^+$), which do infiltrate UVB-exposed skin, are not known to express IL-4 (13,14). On the other hand, basophils and eosinophils can produce IL-4 (13) and do express CD11b and CD15, but they are not known to infiltrate UVB-exposed normal human skin (15). By means of specific antibodies we can check the possible but unlikely presence of the latter two cell types in irradiated skin. The absence of CD36 and the presence of CD15 on the IL-4$^+$ cells make the macrophage an improbable candidate. Taken all together, our results thus far suggest that the IL-4 expression we detect in UVB-exposed skin is associated with neutrophils, which infiltrate the skin after UVB


2a


2b


2c


2d


2e

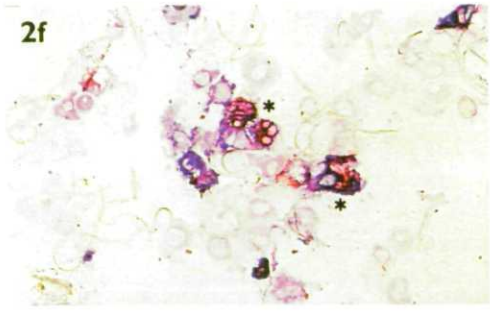

2f

Figure 2. IL-4 present in UVB-induced infiltrating CD11b$^+$CD15$^+$ cells. Skin biopsies were taken at day 2 post-irradiation with 4 MED of UVB and cryostat sections were double-stained to detect IL-4 plus the markers (a) CD3, (b) tryptase, (c) CD36, (d) CD11b, or (e) CD15. The presence of IL-4 can be recognized by a red color (black arrowhead) and the mentioned cell-markers by a blue color (open arrowhead), whereas an asterisk indicates a double-positive cell. In cytospin preparations of dermal cell suspensions derived from UVB-exposed skin (f) double-positive cells for IL-4 and CD15 were present.

irradiation. Additional studies with neutrophil-specific markers, such as elastase, should be performed to confirm this.

Our observation that IL-4 expression is associated with UVB-induced neutrophil-like cells does not necessarily mean that these cells are the actual IL-4 source. In other words, the neutrophil-like cells may be unable to synthesize IL-4, but did acquire this expression through binding and/or uptake of environmental IL-4 by the IL-4 receptor or by phagocytosis. Indeed neutrophils have been shown to exhibit these receptors (16) and thus may have picked up exogenous IL-4. In forthcoming experiments we will isolate the $CD11b^+CD15^+$ cells from the dermal cell suspension derived from UVB-exposed skin, and determine by RT-PCR and ELISA if these cells can express mRNA for IL-4 and can secrete IL-4 protein upon a short-term culture *in vitro*.

The anti-IL-4 antibody we used in this study should be suitable for immunohistochemistry according to the data sheet of the manufacturer. In a previous study in which we discovered the UVB-induced IL-4 expression (Chapter 5), we also used another anti-IL-4 antibody that was selected out of a large panel of anti-IL-4 antibodies for the application in immunohistochemistry. The latter anti-IL-4 antibody confirmed the results obtained with the former. Although both anti-IL-4 antibodies are described as useful tools for the staining of IL-4 in cryostat sections, we cannot exclude the possibility that the antibodies recognize a cross-reactive epitope on an irrelevant molecule in the $CD11b^+CD15^+$ infiltrating cells. The succeeding studies as suggested above will resolve this issue.

Several reports on *in vivo* studies with mice indicate that IL-4 plays an important role in the development of UVB-induced immunosuppression, as convincingly evidenced by the findings that injection of blocking anti-IL-4 can abolish this immunosuppression (17) and that in IL-4 gene knockout mice the delayed-type hypersensitivity response is not suppressed by UVB exposure (18). UVB radiation can induce serum IL-4 in a dose-dependent fashion in mice and this IL-4 seems to be responsible for the subsequent induction of serum IL-10 (19), a cytokine with immunosuppressive properties. Thus far the cellular source of the UVB-induced IL-4 in mice is unknown. Due to its pleiotropic property, IL-4 may be involved in UVB-induced immunosuppression in several ways as described in the discussion of Chapter 5 of this thesis. One important effect of the presence of IL-4 in the cutaneous tissue is that the development of type 2 T-cell responses in skin are promoted, while the type 1 T-cell responses are concomitantly

inhibited, nicely fitting in the model of UVB-induced immunosuppression (20).

In summary, we observed the transient presence of numerous IL-4^+ cells in the dermis and epidermis of UVB-exposed normal human skin. This expression was not induced in resident skin cells, but rather associated with the UVB-induced infiltrating $CD11b^+CD15^+CD36^-$ cells, possibly neutrophils.

Acknowledgements

We thank Arthur Kammeyer for providing expert assistance with the solar-simulator.

References

1. Cooper, K.D. 1996. Cell-mediated immunosuppressive mechanisms induced by UV radiation. *Photochem.Photobiol.* 63:400.

2. Aberer, W., G. Schuler, G. Stingl, H. Honigsmann, and K. Wolff. 1981. Ultraviolet light depletes surface markers of Langerhans cells. *J.Invest.Dermatol.* 76:202.

3. Kremer, I.B., R.M. Sylva-Steenland, J.D. Bos, and M.B.M. Teunissen. 1997. Despite the presence of UVB-induced DNA damage, HLA-DR$^+$ cells from ex vivo UVB-exposed human skin are able to migrate and show no impaired allostimulatory capacity. *J.Invest.Dermatol.* 109:626.

4. Nakagawa, S., C.W. Koomen, J.D. Bos, and M.B.M. Teunissen. 1999. Differential modulation of human epidermal Langerhans cell maturation by ultraviolet B radiation. *J.Immunol.* 163:5192.

5. Di Nuzzo, S., R.M. Sylva-Steenland, M.A. De Rie, P.K. Das, J.D. Bos, and M.B.M. Teunissen. 1998. UVB radiation preferentially induces recruitment of memory CD4$^+$ T cells in normal human skin: long-term effect after a single exposure. *J.Invest.Dermatol.* 110:978.

6. Caricchio, R., E.A. Reap, and P.L. Cohen. 1998. Fas/Fas ligand interactions are involved in ultraviolet-B-induced human lymphocyte apoptosis. *J.Immunol.* 161:241.

7. Ozawa, M., K. Ferenczi, T. Kikuchi, I. Cardinale, L.M. Austin, T.R. Coven, L.H. Burack, and J.G. Krueger. 1999. 312-nanometer ultraviolet B light (narrow-band UVB) induces apoptosis of T cells within psoriatic lesions. *J.Exp.Med.* 189:711.

8. Norris, P., R.N. Poston, D.S. Thomas, M. Thornhill, J.L. Hawk, and D.O. Haskard. 1991. The expression of endothelial leukocyte adhesion molecule-1 (ELAM-1), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) in experimental cutaneous inflammation: a comparison of ultraviolet B erythema and delayed hypersensitivity. *J.Invest.Dermatol.* 96:763.

9. Gilliam, A.C., I.B. Kremer, Y. Yoshida, S.R. Stevens, E. Tootell, M.B.M. Teunissen, C. Hammerberg, and K.D. Cooper. 1998. The human hair follicle: a reservoir of CD40$^+$ B7-deficient Langerhans cells that repopulate epidermis after UVB exposure. *J.Invest.Dermatol.* 110:422.

10. Luger, T.A., S. Beissert, and T. Schwarz. 1997. The epidermal cytokine network. In Skin Immune System (SIS). J.D. Bos, editor. CRC Press LLC, Boca Raton, Florida. 271-307.

11. Takashima, A. and P.R. Bergstresser. 1996. Impact of UVB radiation on the epidermal cytokine network. *Photochem.Photobiol.* 63:397.

12. Kang, K., C. Hammerberg, L. Meunier, and K.D. Cooper. 1994. CD11b$^+$ macrophages that infiltrate human epidermis after in vivo ultraviolet exposure potently produce IL-10 and represent the major secretory source of epidermal IL-10 protein. *J.Immunol.* 153:5256.

13. Brown, M.A. and J. Hural. 1997. Functions of IL-4 and control of its expression. *Crit.Rev.Immunol.* 17:1.

14. Cassatella, M.A. 1999. Neutrophil-derived proteins: selling cytokines by the pound. *Adv.Immunol.* 73:369.

15. Gilchrest, B.A., N.A. Soter, J.S. Stoff, and M.C. Mihm, Jr. 1981. The human sunburn reaction: histologic and biochemical studies. *J.Am.Acad.Dermatol.* 5:411.

16. Girard, D., R. Paquin, and A.D. Beaulieu. 1997. Responsiveness of human neutrophils to interleukin-4: induction of cytoskeletal rearrangements, de novo protein synthesis and delay of apoptosis. *Biochem.J.* 325:147.

17. Rivas, J.M. and S.E. Ullrich. 1994. The role of IL-4, IL-10, and TNF-alpha in the immune suppression induced by ultraviolet radiation. *J.Leukoc.Biol.* 56:769.

18. El-Ghorr, A.A. and M. Norval. 1997. The role of interleukin-4 in ultraviolet B light-induced immunosuppression. *Immunology* 92:26.

19. Shreedhar, V.K., T. Giese, V.W. Sung, and S.E. Ullrich. 1998. A cytokine cascade including prostaglandin E2, IL-4, and IL-10 is responsible for UV-induced systemic immune suppression. *J.Immunol.* 160:3783.

20. Ullrich, S.E. 1996. Does exposure to UV radiation induce a shift to a Th-2-like immune reaction? *Photochem.Photobiol.* 64:254.

Chapter 7

Summary and Discussion

The aim of this thesis was to investigate the fate of the cutaneous T-cell population *in situ* after a single physiological dose of SSR, UVB or UVA. Small areas on the buttock skin of normal human volunteers were exposed to single doses of UV and punch biopsies were taken at several time points after exposure. The effects of UV on cutaneous T cells, on cutaneous cytokines that are known to regulate T-cell function, and on adhesion molecules expressed by EC that regulate T-cell migration, were assessed by immunohistochemistry and RT-PCR. Additional experiments were performed *in vitro* to provide a more precise description of the phenotypes and functions of cutaneous T cells isolated from the irradiated site. Overall, we found that a single exposure to UV led to a long-term changes in the number and composition of the cutaneous T cells, in the cytokine production and in the expression of adhesion molecules on EC in the irradiated skin. Because T cells and cytokines play pivotal roles in the regulation of cutaneous immune responses, we speculated that such changes might have a biologically relevant role in UV-induced immunosuppresion.

1. Effects of UV on the intraepidermal T-cell populations.

In the studies described in this thesis, we consistently found few lymphocytes in the epidermal compartment of non-lesional, clinically normal skin. In line with previous reports (1-3), they were mainly of the CD8 subpopulation bearing an αβ TCR. More than 60% of the total intraepidermal $CD3^+$ T cells were of the memory/effector phenotype. As we previously pointed out (see Introduction), the expression of markers that indicate T-cell activation on the intraepidermal T cells is still controversial. Our finding that about 70 % of the total $CD3^+$ intraepidermal T cells did not express any markers of recent or late activation is in accordance with a previous study by Foster *et al.* (3), in which intraepidermal T cells were regarded as being in a "resting state". Resting T cells might secrete a discrete subset of cytokines upon stimulation, such as IFN-γ (type 1 T cells) or IL-4 (type 2 T cells), and might in this way determine the outcome of the cutaneous immune responses. The nature (type 1 or type 2) of the resident epidermal T-cell populations in normal human skin is still unknown. In our studies, we could detect only a very low signal for IL-4 mRNA and no IL-4 protein in normal human skin, but we did find some IFN-γ^+ T cells. These findings are in line with the findings of other researchers (4-6), and taken together, they might indicate that the distribution over type 1 / type 2 T cells in normal human skin is dominated by the type 1 T-cell subset.

Studies *in vitro* have shown that the viability of T cells is hypersensitive to relatively low doses of UV radiation compared with that of other cutaneous cell populations such as keratinocytes (7), monocytes (8) or fibroblasts (9). Taking into account that significant quantities of UV radiation transverse the epidermis (10,11) and that T cells are highly susceptible, we hypothesized that exposure of skin to physiological doses of UV radiation could kill T cells residing in the epidermis (7). Indeed, we found that the numbers of the intraepidermal T-cell populations were significantly reduced in the first few days after a single physiological dose of SSR, UVB or UVA. However the mechanism by which UV irradiation induces intraepidermal T cells depletion in normal human skin is still obscure. The most likely explanation for this loss of T cells is that they are depleted due to phototoxic effects of UV radiation, although, as an unlikely alternative, migration of T cells out of the epidermis can not entirely be excluded. A decrease in intraepidermal T-cell numbers upon UV exposure is not specific for normal human skin. A similar effect of UV radiation on the intraepidermal T-cell population in lesional skin of psoriatic patients has been found after treatment with UVB (12-15). Successful UVB phototherapy of psoriatic patients induced a reduction in the number of epidermis-infiltrating T cells that are thought to contribute to the pathogenesis of the disease. The mechanism of this reduction is most likely cell death through apopoptosis (15), which might also account for the intraepidermal T-cell depletion found in normal human skin.

Recently, it has been speculated that the increased susceptibility towards UV-induced apoptosis of T cells may be considered to be a protective mechanism towards such environmental trauma (16). UV-damaged T cells are no longer needed and they could become dangerous. Activation of signalling pathways (e.g. FAS/FAS ligand) eventually leading to apoptosis may facilitate the elimination of such cells and may protect the skin from autoimmune reactions. On the other hand, the undesirable consequences of this physiological protective response to UV is the disruption of a tumor suppressor and infection suppressor mechanism that might be represented by the subset of intarepidermal T cells that produce IFN-γ. This possibility provides an additional mechanism that may account for the occurrence of skin cancers and exacerbation of skin infectious diseases upon exposure to sunlight.

2. Effects of UV on the dermal T-cell populations.

2.1. Mechanism of the UV-induced influx of T cells in normal human skin.

In contrast to the epidermal data, the level of perivascular dermal T-cell numbers showed an UV-dose-dependent increase instead of a decrease during the first few days after SSR and UVB irradiation. The numbers of dermal $CD3^+$ T cells were increased at 24 h, were maximal at 48 h, and progressively dwindled thereafter. The $CD3^+$ T-cell population can roughly be divided into $CD4^+$ and $CD8^+$ subpopulations. Our studies revealed that the UV-induced inflammatory response in the skin *in vivo* was characterized by selective accumulation of $CD4^+$ but not $CD8^+$ T cells. Because only 2% of the UV-induced dermal T cells expressed the proliferation marker Ki-67, we reasoned that accumulation of these cells was due to influx of T cells from the blood stream.

Modulation of adhesion molecules on EC is essential in regulating the localization, timing, nature, and progression of T-lymphocyte accumulation in inflammatory responses. In the studies described in this thesis, we studied the integrin expression on the UV-induced infiltrating T cells, and related this to the effects of UV exposure on counterreceptors on dermal EC. Apparently the pathways E-selectin/CLA and VCAM-1/VLA-4 are not essentially involved in the recruitment of the majority of the dermis-infiltrating T cells. Since the kinetics of the number of dermal LFA-1^+ T cells correlated with the kinetics of the number of ICAM-1^+ EC upon UV exposure, it is likely that the recruitment is mediated by LFA-1/ICAM-1 interaction. However, to validate this hypothesis functional studies are needed. Furthermore, since only 47% of the UV-induced dermal T cells expressed LFA-1, it is likely that other receptor/ligand pairs may be involved in this process. In a recent study (17), a newly identified molecule designated lymphocyte endothelial-epithelial cell adhesion molecule (LEEP-CAM) has been shown to mediate T-cell adhesion to EC. It would be of interest to investigate the role of this or of others adhesion molecules, such as P-selectin, in the recruitment of T cells into the UV-irradiated site.

Cytokines play a key role in modulating the expression of adhesion molecules on EC. Exposure to UV triggers keratinocytes and mast cells to produce and/or release IL-1 and TNF-α. Studies on the kinetics of release of UV-induced cytokines in human skin *in vivo* have indicated that IL-1 (18) and TNF-α (19) are late mediators in UV-induced inflammation. In our studies we showed that T cells were recruited in the late phase of UV-induced inflammation. These two cytokines may play a role in T-cell recruitment into UV-irradiated site. However, studies *in vitro* have shown that IL-1 and TNF-α can each increase the adhesiveness of EC for T cell (20,21) and neutrophils (22-24); thus, they do not specifically

promote recruitment of $CD4^+$ T cells. In contrast, IL-4 increases the adhesiveness of EC for T cells but not for neutrophils (25). Furthermore, *in vivo* cytokines are more likely to act in combination at sites of inflammation. Interestingly, it has been shown that the simultaneous presence of IL-4 and TNF-α selectively enhanced EC adhesiveness for T cells (26). In our studies, we found that UV-exposure induced the expression of IL-4 in $CD11b^+CD15^+$ cells in normal human skin. This may be of key importance in determining the dominant recruitment of T lymphocytes into the UV-exposed skin. IL-6, which is upregulated upon UV exposure (27), might further amplify the recruitment of T lymphocytes into the irradiated skin. Indeed, it has been shown that IL-6 acted on human EC increasing their adhesiveness preferentially for T lymphocytes, but not for monocytes or neutrophils (28).

The recirculation of T cells relies not only on the expression of specific adhesion molecules on EC, but also on specific chemokines present in the microenvironment. The chemokine(s) regulating T-cell accumulation into UV-inflamed skin site is(are) unknown. A possible role for the chemokine psoriasin in determining the accumulation and retention of T cells in the UV-irradiated site is supported by the results of our studies. Psoriasin is a newly described low-molecular-mass calcium-binding protein that is synthesized (albeit at low levels), and partially secreted by noncultured unfractionated keratinocytes from normal human skin (29). Additionally, functional studies *in vitro* showed that psoriasin preferentially attracts the $CD4^+$ T-cell subset, whereas it does not attract $CD8^+$ T cells (30). Here, we found *in vivo* that the expression of psoriasin was strongly upregulated both at mRNA level and at protein level in UVB-exposed skin for a prolonged time (1 to 10 days after exposure). The psoriasin protein was predominantly expressed in the deep follicular epithelium at day 2 after exposure, but at day 10 it was highly expressed in the granular layer of the interfollicular epidermis. The close correlation between these skin sites and the location of the accumulated $CD4^+$ T cells over the time course (first in the dermal compartment and then in the epidermal compartment) suggests that psoriasin may play a key role in the infiltration of $CD4^+$ T cells into UV-irradiated skin. Such a possible role of psoriasin as chemoattractant for UV-induced T cells implies the presence of a psoriasin receptor on the recruited $CD4^+$ T cells. So far, the absence of specific mAb against the psoriasin receptor makes it impossible to investigate this. A T cell is likely to encounter several chemokine gradients as it moves in a given microenvironment. Also other chemoattractant factors, e.g. IL-8, are released into irradiated skin (19). However, IL-8 is chemotactic for both CD4 and CD8 T-cell subsets (31).

Interestingly, although IL-4 itself is not chemotactic for T lymphocytes, it inhibits only $CD8^+$ but not $CD4^+$ T-cell chemotaxis towards IL-8 (31). Because in addition to the expression of IL-8, the expression of IL-4 is also induced by UV-exposure, this provides another explanation for the selective accumulation of $CD4^+$ T cells in irradiated skin.

2.2. Speculations on the biological role of the UV-induced recruitment of T cells in the dermal compartment.

Because T cells play a dominant role in the regulation of immune responses and because it is generally believed that the T-cell phenotype is related to its function, we reasoned that our understanding of the biological role of UV-induced T-cell recruitment would be partially explained by investigating the distinctive cell-surface phenotypes of these T cells. We found that the UV-induced T cells were almost exclusively of the memory $CD4^+$ phenotype, and the majority expressed an α/β TCR. Because many cutaneous inflammatory disorders (32,33), but also a mild injury such as a suction blister (34), show infiltrates dominated by memory $CD4^+$ T cells, the UV-induced migration of memory $CD4^+$ T cells into the skin is likely not to be specific (antigen-independent cutaneous inflammation). On the other hand, as an unlikely alternative, given their memory phenotypes, one might speculate that the UV-induced T cells may be involved in the recognition of neo-antigens induced by UV-exposure (antigen-dependent cutaneous inflammation). In this respect, it might be relevant to investigate whether or not the TCR repertoire of infiltrating T cells in the UV-exposed site is restricted and whether these cells expand by a given antigen stimulation. However, acute UV injury is inflammatory and immunomodulatory, and these two are integrally tied together (35,36).

T cells entering the dermis from the microvasculature in UV-exposed skin may reasonably be expected to encounter cutaneous APCs, such as infiltrated UV-induced macrophages or UV-exposed LC; at the same time they might be exposed to a UV-modulated cytokine microenvironment. Presentation of antigens to T cells under these circumstances could result in activation of suppressor pathways such as inhibition of type 1 T-cell responses which will promote the development of type 2 T-cell responses. Although cutaneous APCs in the irradiated site have been suggested to play a major role in inducing preferential activation of type 2 T cells (37-39), the cytokine environment may also represent a major variable that influences T-cell skewing. In the studies presented in this thesis we found that the UV-

induced T cells (which apparently are not committed when they enter the irradiated site) showed a skewing towards type 2 cytokine production in primary cultures, that is only when dermal cells from UV-exposed skin were present, but not with nonirradiated control dermal cells. Although we did not test for it, one may, we think, assume that the dermal cell suspension from UV-exposed skin contained the $CD11b^{+}CD15^{+}IL\text{-}4^{+}$ cells that we observed *in vivo*, as well as UV-induced macrophages that produce a large amount of IL-10 (40). We speculate that the locally produced IL-4 and IL-10, which are both known to skew T-cell responses towards type 2, may contribute to UV-induced immunosuppression.

Regardless of the mechanism involved in the T-cell recruitment into UV-irradiated human skin, this phenomenon was strikingly different from the response of dermal T cell populations in mice after acute UV exposure. Upon a single exposure to UV, dermal T cells (normally present within mouse skin) showed a rapid reduction in cell numbers which reached a nadir between day 1 and day 2, after which recovery occurred 3 days after exposure (41). The difference between the responses of mice and human dermal T cells upon UV exposure might suggest that in mice circulating T cells preferentially migrate into lymph nodes upon UV exposure (42), while in humans UV exposure apparently redistributes circulating T cells partially into the dermis. Bearing in mind that the "dermal perivascular unit" is a site of immunological reactivity (43,44), recruitment of T cells into this compartment might be biologically relevant. In this respect Butcher and Picker (45) proposed an important role for competitive niche homing (e.g. to the lymph node vs the dermal perivascular unit) in controlling lymphocyte homeostasis and in shaping the immune response. Thus, in humans, recruitment of T cells into UVB-exposed skin might create an additional adaptive immunological compartment that is not present in nocturnal animals such as mice.

3. Concluding remarks

The studies presented in this thesis have provided the first evidence, as far as we know, for reduction in the number of intraepidermal T cells upon UV-exposure *in vivo*; concurrently, the dermis of the irradiated skin proved to become infiltrated with non-activated $CD4^{+}$ memory T cells. We believe that the UV-induced disappearance of intraepidermal T cells and the UV-induced accumulation of T cells into the irradiated skin may represent an additional mechanism by which UV mediates immunosuppression. In spite of the results

provided here, several questions remain less than fully answered: i) Is apoptosis the mechanism by which UV radiation induces disappearance of intraepidermal T cells? ii) In addition to psoriasin and LFA-1/ICAM-1, which other chemokine(s) or adhesion molecules is (are) involved in T-cell recruitment upon UV exposure? iii) What is the function of infiltrating T cells and how does this lead to impaired immune response? iv) What is the impact of the cytokine microenvironment in the dermis of UV-exposed skin on the function of the infiltrating T cells? v) What is the identity of the UV-induced $CD11b^{+}CD15^{+}IL\text{-}4^{+}$ cell? vi) And finally what are the effects of repeated UV exposures on cutaneous T cells?

References

1. Smolle, J., R. Ehall, and H. Kerl. 1985. Inflammatory cell types in normal human epidermis: an immunohistochemical and morphometric study. *Acta Derm.Venereol.* 65:479.

2. Bos, J.D., I. Zonneveld, P.K. Das, S.R. Krieg, C.M. van der Loos, and M.L. Kapsenberg. 1987. The skin immune system (SIS): distribution and immunophenotype of lymphocyte subpopulations in normal human skin. *J.Invest.Dermatol.* 88:569.

3. Foster, C.A., H. Yokozeki, K. Rappersberger, F. Koning, B. Volc-Platzer, A. Rieger, J.E. Coligan, K. Wolff, and G. Stingl. 1990. Human epidermal T cells predominantly belong to the lineage expressing alpha/beta T cell receptor. *J.Exp.Med.* 171:997.

4. Llorente, L., Y. Richaud-Patin, C. Alvarado, E. Reyes, J. Alcocer-Varela, and R. Orozco-Topete. 1997. Elevated Th1 cytokine mRNA in skin biopsies and peripheral circulation in patients with erythema nodosum. *Eur.Cytokine Netw.* 8:67.

5. Thepen, T., E.G. Langeveld-Wildschut, I.C. Bihari, D.F. van Wichen, F.C. van Reijsen, G.C. Mudde, and C.A. Bruijnzeel-Koomen. 1996. Biphasic response against aeroallergen in atopic dermatitis showing a switch from an initial TH2 response to a TH1 response in situ: an immunocytochemical study. *J.Allergy Clin.Immunol.* 97:828.

6. Szabo, S.K., C. Hammerberg, Y. Yoshida, Z. Bata-Csorgo, and K.D. Cooper. 1998. Identification and quantitation of interferon-gamma producing T cells in psoriatic lesions: localization to both $CD4^+$ and $CD8^+$ subsets. *J.Invest.Dermatol.* 111:1072.

7. Teunissen, M.B.M., R.M. Sylva-Steenland, and J.D. Bos. 1993. Effect of low-dose ultraviolet-B radiation on the function of human T lymphocytes in vitro. *Clin.Exp.Immunol.* 94:208.

8. Kremer, I.B., J.D. Bos, and M.B.M. Teunissen. 1995. Reduced antigen-presenting function of human Epstein-Barr virus (EBV)-B cells and monocytes after UVB radiation is accompanied by decreased expression of B7, intercellular adhesion molecule-1 (ICAM-1) and LFA-3. *Clin.Exp.Immunol.* 101:461.

9. Arlett, C.F., J.E. Lowe, S.A. Harcourt, A.P. Waugh, J. Cole, L. Roza, B.L. Diffey, T. Mori, O. Nikaido, and M.H. Green. 1993. Hypersensitivity of human lymphocytes to UV-B and solar irradiation. *Cancer Res.* 53:609.

10. Everett, M.A., E. Yeargers, R.M. Sayre, and R.L. Olson. 1966. Penetration of epidermis by ultraviolet rays. *Photochem.Photobiol.* 5:533.

11. Bruls, W.A.G., H. Slaper, J.C. van der Leun, and L. Berrens. 1984. Transmission of human epidermis and stratum corneum as a function of thickness in the ultraviolet and visible wavelenghts. *Photochem.Photobiol.* 40:485.

12. Krueger, J.G., J.T. Wolfe, R.T. Nabeya, V.P. Vallat, P. Gilleaudeau, N.S. Heftler, L.M. Austin, and A.B. Gottlieb. 1995. Successful ultraviolet B treatment of psoriasis is accompanied by a reversal of keratinocyte pathology and by selective depletion of intraepidermal T cells. *J.Exp.Med.* 182:2057.

13. Szabo, S.K., C. Hammerberg, Z. Bata-Csorgo, and K.D. Cooper. 1997. The mechanism of action of UVB in psoriasis: rapidily decreased T cell numbers and preferential reduction of interferon γ (IFN-γ) production. *J.Invest.Dermatol.* 108:558.(Abstract)

14. Gutierrez-Steil, C., T. Wrone-Smith, X. Sun, J.G. Krueger, T. Coven, and B.J. Nickoloff. 1998. Sunlight-induced basal cell carcinoma tumor cells and ultraviolet-B-irradiated psoriatic plaques express Fas ligand (CD95L). *J.Clin.Invest.* 101:33.

15. Ozawa, M., K. Ferenczi, T. Kikuchi, I. Cardinale, L.M. Austin, T.R. Coven, L.H. Burack, and J.G. Krueger. 1999. 312-nanometer ultraviolet B light (narrow-band UVB) induces apoptosis of T cells within psoriatic lesions. *J.Exp.Med.* 189:711.

16. Caricchio, R., E.A. Reap, and P.L. Cohen. 1998. Fas/Fas ligand interactions are involved in ultraviolet-B-induced human lymphocyte apoptosis. *J.Immunol.* 161:241.

17. Shieh, C.C., B.K. Sadasivan, G.J. Russell, M.P. Schon, C.M. Parker, and M.B. Brenner. 1999. Lymphocyte adhesion to epithelia and endothelia mediated by the lymphocyte endothelial-epithelial cell adhesion molecule glycoprotein. *J.Immunol.* 163:1592.

18. Barr, R.M., S.L. Walker, W. Tsang, G.I. Harrison, P. Ettehadi, M.W. Greaves, and A.R. Young. 1999. Suppressed alloantigen presentation, increased TNF-alpha, IL-1, IL-1Ra, IL-10, and modulation of TNF-R in UV-irradiated human skin. *J.Invest.Dermatol.* 112:692.

19. Strickland, I., L.E. Rhodes, B.F. Flanagan, and P.S. Friedmann. 1997. TNF-alpha and IL-8 are upregulated in the epidermis of normal human skin after UVB exposure: correlation with neutrophil accumulation and E-selectin expression. *J.Invest.Dermatol.* 108:763.

20. Cavender, D., Y. Saegusa, and M. Ziff. 1987. Stimulation of endothelial cell binding of lymphocytes by tumor necrosis factor. *J.Immunol.* 139:1855.

21. Cavender, D.E., D.O. Haskard, B. Joseph, and M. Ziff. 1986. Interleukin 1 increases the binding of human B and T lymphocytes to endothelial cell monolayers. *J.Immunol.* 136:203.

22. Bevilacqua, M.P., J.S. Pober, M.E. Wheeler, R.S. Cotran, and M.A. Gimbrone, Jr. 1985. Interleukin 1 acts on cultured human vascular endothelium to increase the adhesion of polymorphonuclear leukocytes, monocytes, and related leukocyte cell lines. *J.Clin.Invest.* 76:2003.

23. Gamble, J.R., J.M. Harlan, S.J. Klebanoff, and M.A. Vadas. 1985. Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. *Proc.Natl.Acad.Sci.U.S.A.* 82:8667.

24. Schleimer, R.P. and B.K. Rutledge. 1986. Cultured human vascular endothelial cells acquire adhesiveness for neutrophils after stimulation with interleukin 1, endotoxin, and tumor-promoting phorbol diesters. *J.Immunol.* 136:649.

25. Thornhill, M.H., U. Kyan-Aung, and D.O. Haskard. 1990. IL-4 increases human endothelial cell adhesiveness for T cells but not for neutrophils. *J.Immunol.* 144:3060.

26. Thornhill, M.H., S.M. Wellicome, D.L. Mahiouz, J.S. Lanchbury, U. Kyan-Aung, and D.O. Haskard. 1991. Tumor necrosis factor combines with IL-4 or IFN-gamma to selectively enhance endothelial cell adhesiveness for T cells. The contribution of vascular cell adhesion molecule-1-dependent and -independent binding mechanisms. *J.Immunol.* 146:592.

27. Urbanski, A., T. Schwarz, P. Neuner, J. Krutmann, R. Kirnbauer, A. Kock, and T.A. Luger. 1990. Ultraviolet light induces increased circulating interleukin-6 in humans. *J.Invest.Dermatol.* 94:808.

28. Watson, C., S. Whittaker, N. Smith, A.J. Vora, D.C. Dumonde, and K.A. Brown. 1996. IL-6 acts on endothelial cells to preferentially increase their adherence for lymphocytes. *Clin.Exp.Immunol.* 105:112.

29. Madsen, P., H.H. Rasmussen, H. Leffers, B. Honore, K. Dejgaard, E. Olsen, J. Kiil, E. Walbum, A.H. Andersen, B. Basse, J.B. Lauridsen, G.P. Ratz, A. Celis, J. Vandekerckhove, and J.E. Celis. 1991. Molecular cloning, occurrence, and expression of a novel partially secreted protein "psoriasin" that is highly up- regulated in psoriatic skin. *J.Invest.Dermatol.* 97:701.

30. Jinquan, T., H. Vorum, C.G. Larsen, P. Madsen, H.H. Rasmussen, B. Gesser, M. Etzerodt, B. Honore, J.E. Celis, and K. Thestrup-Pedersen. 1996. Psoriasin: a novel chemotactic protein. *J.Invest.Dermatol.* 107:5.

31. Jinquan, T., B. Deleuran, B. Gesser, H. Maare, M. Deleuran, C.G. Larsen, and K. Thestrup-Pedersen. 1995. Regulation of human T lymphocyte chemotaxis in vitro by T cell-derived cytokines IL-2, IFN-gamma, IL-4, IL-10, and IL-13. *J.Immunol.* 154:3742.

32. Markey, A.C., M.H. Allen, C. Pitzalis, and D.M. MacDonald. 1990. T-cell inducer populations in cutaneous inflammation: a predominance of T helper-inducer lymphocytes (THi) in the infiltrate of inflammatory dermatoses. *Br.J.Dermatol.* 122:325.

33. van Joost, T., J.D. Bos, and T.M. Starink. 1993. T cell-mediated dermatose: an antology of certain T cell-mediated inflammatory dermatoses. Bohn Stafleau Van Loghum, Houten.

34. Reusch, M.K., J.N. Mansbridge, B.J. Nickoloff, and V.B. Morhenn. 1991. Immunophenotyping of skin cells during healing of suction blister injury. *Dermatologica* 183:179.

35. Barker, J.N., R.S. Mitra, C.E. Griffiths, V.M. Dixit, and B.J. Nickoloff. 1991. Keratinocytes as initiators of inflammation. *Lancet* 337:211.

36. Cooper, K.D. 1996. Cell-mediated immunosuppressive mechanisms induced by UV radiation. *Photochem.Photobiol.* 63:400.

37. Simon, J.C., J. Krutmann, C.A. Elmets, P.R. Bergstresser, and P.D. Cruz, Jr. 1992. Ultraviolet B-irradiated antigen-presenting cells display altered accessory signaling for T-cell activation: relevance to immune responses initiated in skin. *J.Invest.Dermatol.* 98:66S.

38. Ullrich, S.E. 1995. Modulation of immunity by ultraviolet radiation: key effects on antigen presentation. *J.Invest.Dermatol.* 105:30S.

39. Ullrich, S.E. 1996. Does exposure to UV radiation induce a shift to a Th-2-like immune reaction? *Photochem.Photobiol.* 64:254.

40. Kang, K., A.C. Gilliam, G. Chen, E. Tootell, and K.D. Cooper. 1998. In human skin, UVB initiates early induction of IL-10 over IL-12 preferentially in the expanding dermal monocytic/macrophagic population. *J.Invest.Dermatol.* 111:31.

41. Hammerberg, C., N. Duraiswamy, and K.D. Cooper. 1996. Temporal correlation between UV radiation locally-inducible tolerance and the sequential appearance of dermal, then epidermal, class II $MHC^{+}CD11b^{+}$ monocytic/macrophagic cells. *J.Invest.Dermatol.* 107:755.

42. Spangrude, G.J., E.J. Bernhard, R.S. Ajioka, and R.A. Daynes. 1983. Alterations in lymphocyte homing patterns within mice exposed to ultraviolet radiation. *J.Immunol.* 130:2974.

43. Bos, J.D. and M.L. Kapsenberg. 1993. The skin immune system: progress in cutaneous biology. *Immunol.Today* 14:75.

44. Murphy, G.F. and L. Vincent. 1997. The dermal immune system. In Skin Immune System (SIS). J.D. Bos, editor. CRC Press, Boca Raton. 347-363.

45. Butcher, E.C. and L.J. Picker. 1996. Lymphocyte homing and homeostasis. *Science* 272:60.

Curriculum vitae

The author of this thesis, Sergio Di Nuzzo, was born in Pietramelara (CE), Italy on May 1st 1962. After completing his secondary education in Caserta, he studied medicine at the University of Naples "Federico II", Naples, Italy. During his studies he attended an internship of two years at the Department of Dermatology of the same University. He received his M.D. degree in December 1989. The next year was spent as general practitioner in Caserta, and during that year he attended a course of specialisation in vascular diseases at the University of Naples. From January 1991 until December 1995 he was trained as a specialist in dermato-venereology at the University of Parma, Parma, Italy. During this training, he spent 7 months at the Department of Dermatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands. In January 1996 he joined the Photoimmunology Group of the Department of Dermatology at the Academic Medical Center as a research fellow. The studies described in this thesis were performed at this institute. From July 2000 onwards he will work at the Department of Dermatology, University of Parma, Italy.

With thanks to

My co-promotor Marcel Teunissen, who helped me in many different ways: he taught me the basic principles of experimental dermatology, guided and supported me during the period of this research program, and made everything I wrote better. I express my gratitude to my promotor Prof. J.D. Bos for giving me the opportunity to work as a research fellow in his department and for his continuous support. Thanks are further extended to my co-promotor Menno de Rie who also contributed to my research activities.

The excellent technical assistance by Regien Sylva-Steenland was of immense importance to me during these studies. My gratitude also goes out to Cock Koomen, Arthur Kammeyer, Chris van der Loos, Gamze Pişkin, Satoshi Nakagawa, Evert Lamme, Marielle van Breemen and Mohamed El Haddouchi for their technical contributions. I am grateful to my friend Marcel Pasch who helped me in different ways, especially by being just a few months ahead with his thesis.

Also Pran Das deserves my special thanks for his involvement and continuous support. I would like to thank Bert Tiggers, Nike Classen and Cynthia Lehè for their technical guidance at the very beginning of my research in Amsterdam.

I am indebted to Robert Rodenburg and Wilfried Meun for their expertise and technical assistance with the figures and computer work. Jan-Willem van der Wal helped me out in several computer crashes. Wendy van Noppen is gratefully acknowledged for her critical reading of the thesis and correcting my English.

My gratitude and special thanks are also due to Prof. Fulvio Allegra and Prof. Alberto Alinovi who aroused my interest in dermatology. I would like to thank Annalisa Pelagatti, for whom the distance did not matter.

I am deeply indebted to my father who has always been caring and helpful in many aspects throughout my studies.

This thesis is dedicated to my mother, who died from cancer when still young. Without the memory of her this thesis would not have been born.

Thanks to you all, and to the many others whom I didn't mention by name. There is a key to how I managed to write this thesis: it is you.

Sergio Di Nuzzo
April 2000