Cells with UV-Specific DNA Damage Are Present in Murine Lymph Nodes After *In Vivo* UV Irradiation

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Ultraviolet radiation is absorbed in the skin, especially in the epidermis. After ultraviolet irradiation the number of major histocompatibility complex class II$^+$, adenosine triphosphatase$^+$ Langerhans cells and Thy-1$^+$ dendritic epidermal cells in the epidermis decreases. Whether this decrease is due to migration of these cells or to loss of membrane markers is not clear. To address this question we have used the monoclonal antibody H3 directed against cyclobutyl thymine dimers—a form of DNA damage that is specifically induced by ultraviolet radiation—to investigate whether H3$^+$ cells are present in the draining lymph nodes of the skin after ultraviolet irradiation of hairless, inbred mice (HRA/Skh).

After a single dose of ultraviolet radiation (Westinghouse FS40, 1.5 kJ/m^2), H3$^+$ cells were present in the paracortex of the draining lymph nodes. No positive cells were found in the blood of irradiated mice. These results suggest that the H3$^+$ cells in the lymph nodes originate from the skin. The number of H3$^+$ cells in the draining lymph nodes increased the first 24 h after irradiation and then stabilized. Immunohistochemical double staining revealed that all H3$^+$ cells were major histocompatibility complex II$^+$, and that only a fraction of the cells were NLDC-145 positive. No Vγ3 T-cell receptor bearing cells could be found in the lymph nodes after UV irradiation of the skin. ***Key words: thymine dimers/migration/photoimmunology. J Invest Dermatol 104:734–738, 1995***

The immunosuppressive properties of ultraviolet radiation (UVR) have been known for several years. However, the mechanisms by which this immunosuppression is induced are still unclear. In the mouse it has been shown that after UV irradiation of the skin, a cascade of events takes place that leads to altered antigen presentation, ultimately resulting in activation of suppressor T lymphocytes. This in turn prevents immunologic rejection of UVR-induced neoplastic transformed cells, which allows the outgrowth of highly antigenic UVR-induced tumors [1,2]. Moreover, delayed-type hypersensitivity reactions against a variety of antigens are systemically suppressed [3–6].

Langerhans cells (LC) and Thy-1$^+$ dendritic epidermal cells (DEC) are constituents of the murine skin immune system. LC are epidermal antigen-presenting cells expressing major histocompatibility complex (MHC) class II and CD1, which are capable of initiating a specific T-lymphocyte–mediated immune response [7]. Thy-1$^+$ DEC express CD3 and the γ/δ T-lymphocyte receptors [8,9] and hence are T cells. However, their function within the epidermis is unknown. UVR is known to affect the number and morphology of both LC [10–12] and Thy-1$^+$ DEC [13] in murine epidermis: the number of Ia$^+$ LC and of Thy-1$^+$ DEC decreases [13–16], and Ia$^+$ LC lose their dendrites and assume a round shape [15] after UV irradiation of the skin. Whether the decrease in cell number is due to cell death, loss of surface markers, or migration, e.g., to the draining lymph nodes is not yet clear. The best evidence thus far that the UVR-induced decrease of Ia$^+$ LC and Thy-1$^+$ DEC is due to actual migration of cells from the skin to the draining lymph nodes was provided by Moodycliffe *et al* [17]. These authors found a significant increase in the number of dendritic cells in the draining lymph nodes 24 h after UV irradiation of the skin, and they suggested that this increase was due to migration of dendritic cells from the irradiated skin to the draining lymph nodes. Although this was a reasonable explanation, it could not be proved that actual migration of dendritic cells from the skin took place.

In addition to its immunosuppressive effects, another major effect of UVR is the induction of lesions in cellular DNA, among which cyclobutyl pyrimidine dimers and (6-4)photoproducts predominate [18]. UVR-induced DNA damage is thought to play an important role in the initiation of skin cancer and, after UV irradiation of the skin, all epidermal cells and some cells in the upper parts of the dermis contain cyclobutyl thymine dimers (T<>T) [19]. In this study we use the monoclonal antibody H3 that is specifically directed against T<>T [20], to investigate whether UV irradiation of murine skin results in the appearance of T<>T-containing cells in the draining lymph nodes.

This study describes the presence and the localization of T<>T$^+$ cells in the draining lymph nodes of UV-irradiated mice. After a series of trials, we were able to establish the phenotype of these cells by double staining of membrane markers and the nuclear UV damage.

MATERIALS AND METHODS

Mice Inbred albino HRA/Skh hr/hr mice (female, 8–12 weeks old) were bred and housed at the central animal facilities of Utrecht University,

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Abbreviations: DEC, dendritic epidermal cell; T<>T, cyclobutyl thymine dimer.

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Utrecht, The Netherlands. Mice were housed in separate cages with free access to standard mouse chow and tap water.

UV Irradiation of Mice The UVR source was a Westinghouse FS40 sunlamp. This lamp had an irradiance of 0.38 W/m^2, as monitored with a Robertson-Berger meter (Solar Light Company, Philadelphia, PA) in combination with a microampmeter; this combination of lamps and Robertson-Berger meter was calibrated with a Kipp E11 thermopile. For localization studies, a single dose of 1.5 kJ/m^2 (≈0.9 minimal edemal dose) was delivered to the backs of the mice. Control animals were not exposed to UVR.

For kinetics and phenotype studies, higher UV doses, approximately six minimal erythemal doses were used, because the lower UV dose used only resulted in a very faint staining of thymine dimers. Higher doses induced more DNA damage, thus giving a better thymine dimer staining.

Preparation of Lymph Node Cryostat Sections and Lymph Node and Blood Cytospins At various time points after UV irradiation mice were ether anesthesized, blood was collected from the retro-orbital veneus plexus, and mice were subsequently sacrificed by cervical dislocation. Axillary and inguinal lymph nodes were removed. The heparinized blood samples were incubated in buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM ethylenediaminetetraacetic acid, pH 7.4) for 20 min at 4°C to lyse the erythrocytes and from the resulting suspension cytospin preparations were made.

For cryostat sections, lymph nodes were snap-frozen in liquid nitrogen and stored at −70°C until further use. Cryostat sections (8 μm) were cut and mounted on 3-aminopropyl triethoxysilane (Sigma, St. Louis, MO)–coated slides. For cytospin preparations, lymph nodes were cut into small pieces and incubated in 0.5 mg/ml collagenase (type IV, Sigma), 0.02 mg/ml DNAse (Boehringer Mannheim, FRG) in Dulbecco's modified Eagle's medium (Gibco BRL, Paysley, UK) supplemented with 2% fetal bovine serum (Gibco BRL), for 30 min at 37°C. The resulting suspensions were brought to a concentration of 5×10^6/ml and were enriched for dendritic cells using a metrizamide gradient (14.5% metrizamide, Sigma). Cytospin preparations were made of the fraction enriched for dendritic cells as well as the other fraction.

Immunohistochemical Staining

Membrane Staining Cytospin preparations of axillar and inguinal lymph nodes were fixed in acetone for 10 min at room temperature. After three washes in phosphate-buffered saline (PBS) over 15 min the slides were incubated for 60 min with either α-MHC II (clone M5/114 [21], a kind gift of Dr. W. van Ewijk, Department of Immunology, Erasmus University, Rotterdam, The Netherlands), α-Vγ3 T-cell receptor (TCR) [22] (Pharmingen, San Diego, CA, USA), both conjugated with fluorescein isothiocyanate (FITC), or non-lymphocyte dendritic cell marker (NLDC) 145 (a kind gift from Dr G. Kraal, Department of Histology, Free University, Amsterdam, The Netherlands) [23] at room temperature. After washing in PBS, slides that were treated with FITC-conjugated antibodies were incubated with sheep-Fab–anti-FITC antibodies conjugated with alkaline phosphatase (Boehringer Mannheim) for 60 min, and slides that were treated with unconjugated NLDC 145 were incubated with rabbit–anti-rat antibodies conjugated with biotin (DAKO, Glostrup, Denmark) for 60 min, followed by incubation with avidin-biotin complex conjugated with alkaline phosphatase (DAKO) for 60 min. After three washes in PBS over 15 min, in all slides alkaline phosphatase activity was visualized by incubation in a freshly made solution of BCIP-NBT (37.5 μl 5-bromo-4-chloro-3-indolyl phosphate [50 mg/ml in dimethylformamide] and 50 μl 4-nitro blue tetrazolium chloride [100 mg/ml in 70% dimethylformamide] in 10 ml 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$ and 2.5 mg Levamisole) for 5–30 min, resulting in a blueish-black, water- and ethanol-insoluble membrane staining, which has to endure the following aggressive fixation and denaturing treatment that is necessary for the T<>T staining.

T<>T Staining Cryostat sections and cytospin preparations of axillary and inguinal lymph nodes that were already stained for membrane markers or cytospin preparations of blood were fixed in acetic acid 25% (v/v) with 0.02% H_2O_2 for 10 min at room temperature. After three washes in PBS, slides were stained for the detection of T<>T as described previously [24,25] with modifications. In brief, the slides were incubated with 0.07 N NaOH in ethanol 70% at room temperature for 2 min. After washing in PBS, slides were incubated with 10% normal mouse serum (NMS) in PBS for 30 min at room temperature to reduce background staining, immediately followed by incubation with the mouse anti-thymine dimer monoclonal antibody (H3) conjugated with biotin. The appropriate dilution of H3-biotin was made in PBS containing 10% NMS, and slides were incubated for 60 min at room temperature. After three washes in PBS, the slides were incubated with an avidin-biotin complex conjugated with HRP (DAKO) for 60 min. Peroxidase reactivity was visualized by incubation in a freshly made solution of 3-amino-9-ethylcarbazole (AEC, Sigma) (20 mg/100 ml) in 50 mM sodium acetate buffer, pH 5.0, containing 5% (v/v) dimethylformamide and 0.03% (v/v) H_2O_2.

RESULTS

Presence and Localization of T<>T^+ Cells in Draining Lymph Nodes of UV-Irradiated Mice Mice were irradiated with UVR on their backs, and cryostat sections and cytospin preparations of the draining lymph nodes as well as cytospin preparations of blood were stained for T<>T^+ cells. Immediately after irradiation, no positive cells could be detected in the draining lymph nodes, but as early as 1 h after irradiation, T<>T^+ cells were present in both axillary and inguinal lymph nodes. The T<>T^+ cells were located in the paracortical area of the draining lymph nodes, a site where dendritic cells and T lymphocytes reside (data not shown). They could also be detected in cytospin preparations of lymph-node suspensions enriched for dendritic cells taken at 1, 4, 24, and 48 h after irradiation. No positively stained cells were found in the cytospin preparations depleted of dendritic cells or in cytospin preparations of the blood of UV-irradiated animals (data not shown).

The number of T<>T^+ cells in the draining lymph nodes varied in time, with increasing numbers up to 4 h after irradiation, followed by a non-significant decrease at 12 h. At 24 h, the cell number again increased and was the same as at 48 h after irradiation. **Figure 1** shows the exact numbers of T<>T^+ cells in the cytospin preparations enriched for dendritic cells at different time points. The intensity of the T<>T staining was highest in cytospin preparations from lymph nodes taken at 1 h after irradiation, and decreased at later timepoints.

T<>T^+ Cells in the Draining Lymph Nodes Contain Different Amounts of T<>T The intensity of the anti-T<>T staining varied among the positively stained cells in the same cryostat section, indicating that the amount of T<>T in these cells was not the same **(Fig 2)**. This could be due to a different dose of UVR the cells received, which agrees with the hypothesis that T<>T^+ cells in the lymph nodes originate from the skin. The amount of UVR that cells receive in the skin is dependent on their location: cells residing in the most superficial layers contain more T<>T after UV irradiation than cells located deeper [19]. Another possible explanation could be that the rate of repair is not the same in all T<>T^+ cells.

Phenotype of T<>T^+ Cells in the Lymph Nodes To reveal the phenotype of the cells that migrated from the skin to the draining lymph nodes, we used monoclonal antibodies α-MHC II, NLDC 145, and α-Vγ3 T-cell receptor. In an immunohistochemical double staining together with the H3 antibody, these antibodies

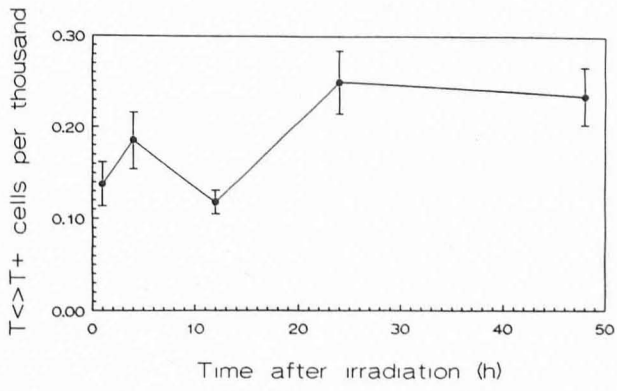

Figure 1. Diagram showing the total number of T<>T^+ cells in the metrizamide interphases of lymph node suspensions taken at different time points. N = 3 per group. Data are expressed as mean ± SEM.

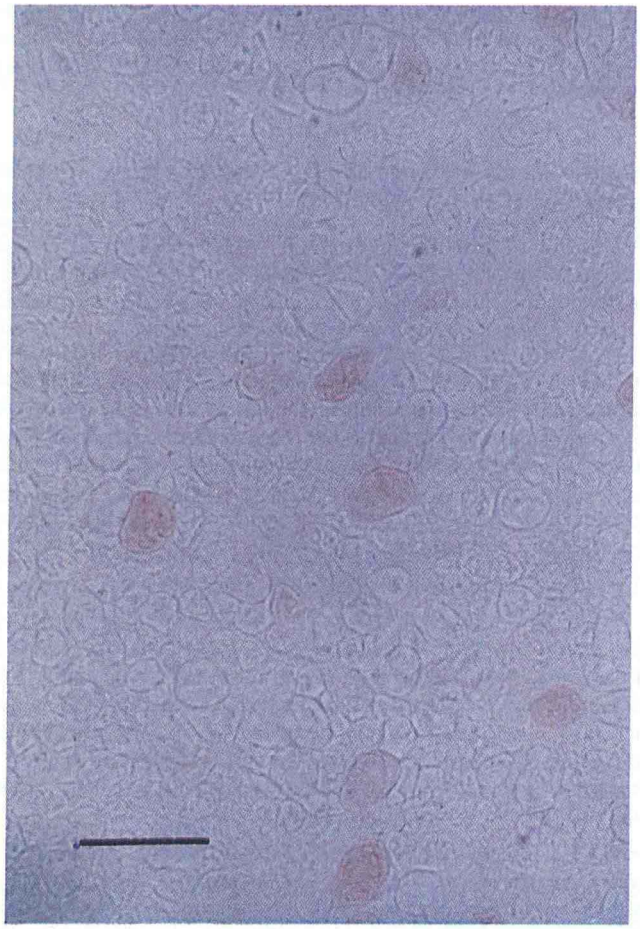

Figure 2. Cryostat section of an axillary lymph node obtained from a UV-irradiated hairless mouse (1.5 kJ/m^2 UV) 24 h after irradiation. The section was immunohistochemically stained for T<>T$^+$ cells (red). Positive cells show different staining intensities, reflecting different amounts of T<>T. *Scale bar*, 20 μm.

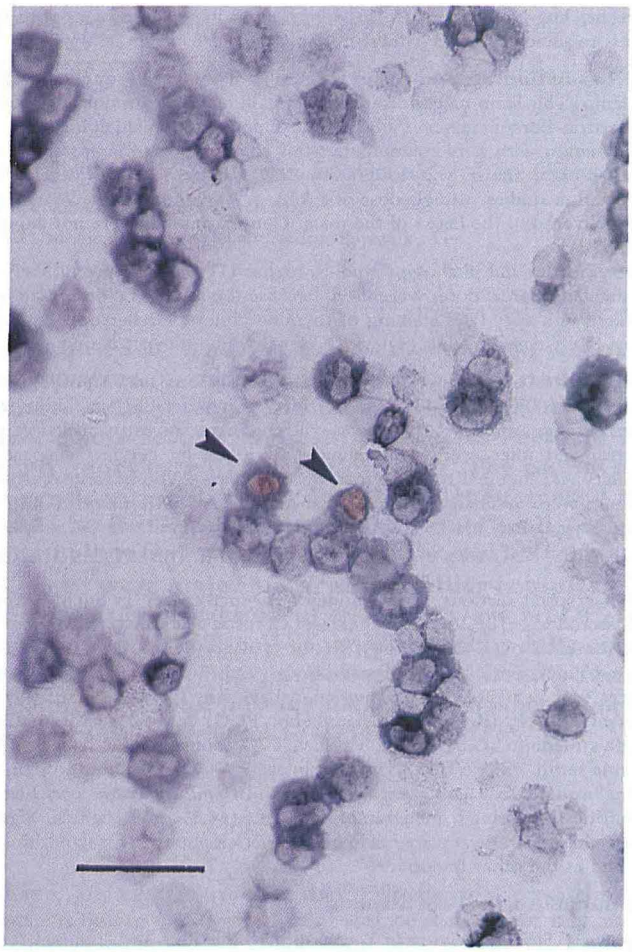

Figure 3. Detail of a cytospin preparation enriched for dendritic cells from an axillary lymph node taken from a hairless mouse 24 h after UV irradiation stained for the presence of T<>T$^+$ cells (red) and MHC II$^+$ cells (blue). T<>T$^+$ cells are positive for MHC II (*arrowheads*). *Scale bar*, 20 μm.

served to investigate whether the T<>T$^+$ cells were Langerhans cells (MHC II$^+$, NLDC 145$^+$) or Thy-1 DEC (Vγ3 TCR$^+$). Because the fixation used for membrane staining does not allow T<>T staining and vice versa, a blue precipitate on the positive membranes had to be formed before staining for T<>T (see *Materials and Methods*). All cells that were positive for T<>T were positive for MHC II **(Fig 3)**. Some, but not all of the cells were positive for NLDC 145, and T<>T$^+$ cells that stained only weakly for NLDC 145 were also present **(Fig 4)**. In the draining lymph nodes, no Vγ3 TCR$^+$ could be found. Hence, the phenotypes of the T<>T$^+$ cells in the draining lymph nodes after UV irradiation of the skin is MHC II$^+$ NLDC 145$^+$, MHC II$^+$ NLDC 145$^-$, and MHC II$^+$ NLDC 145$^{+/-}$.

DISCUSSION

A monoclonal antibody against UVR-specific DNA damage, i.e., thymine dimers (H3), has been used to obtain evidence for migration of skin cells to the draining lymph nodes upon UV irradiation. H3$^+$ cells were present in the paracortex of both axillary and inguinal lymph nodes as early as 1 h after irradiation. The number of H3$^+$ cells increased up to 4 h after irradiation, and then decreased non-significantly at 12 h after irradiation **(Fig 1)**. At 24 h after irradiation, the number of H3$^+$ cells had increased further and remained at that level at 48 h after irradiation. The staining intensity was highest at 1 h after irradiation, and then decreased. The increase in cell number indicates that the influx of cells is probably UV driven. The decrease in staining intensity can be explained by DNA repair. No positive cells were present in lymph node sections from animals that were not UV irradiated, indicating that the H3 antibody gave no a-specific background staining. Because T<>T are only induced in cells directly exposed to UVR, and because UVR cannot penetrate to the depth of the lymph nodes [25], this finding indicates that the T<>T$^+$ cells in the lymph nodes originate from the skin. The failure to find positive cells in the blood immediately after UVR indicates that T<>T$^+$ cells are probably not blood cells exposed to UVR during transit through dermal capillaries, and that the blood cells did not receive appreciable amounts of UVR. The results imply that T<>T$^+$ cells in the lymph nodes originate from cells present in the dermal or epidermal tissues and that they actively migrate to the draining lymph nodes via the afferent lymphatics after UV irradiation. However, it cannot be ruled out that the presence of T<>T$^+$ cells in the draining lymph nodes of the skin results from the normal recirculation of dendritic cells, e.g., veiled cells [26].

The reason for the migration of cells from the skin to the draining lymph nodes upon UV irradiation is unclear. Application of antigen to the skin results in rapid accumulation of antigen-bearing, Ia$^+$ epidermal cells in the draining lymph nodes [27,28]. Antigenic alterations have been detected in UV-irradiated murine skin [29,30] and it is well known that UVR-induced skin cancers express strong tumor-specific antigens [31]. Therefore, it is possible that such antigens are the stimulus for migration. Alternatively, UV irradiation itself may activate a pathway, which is not antigen-mediated,

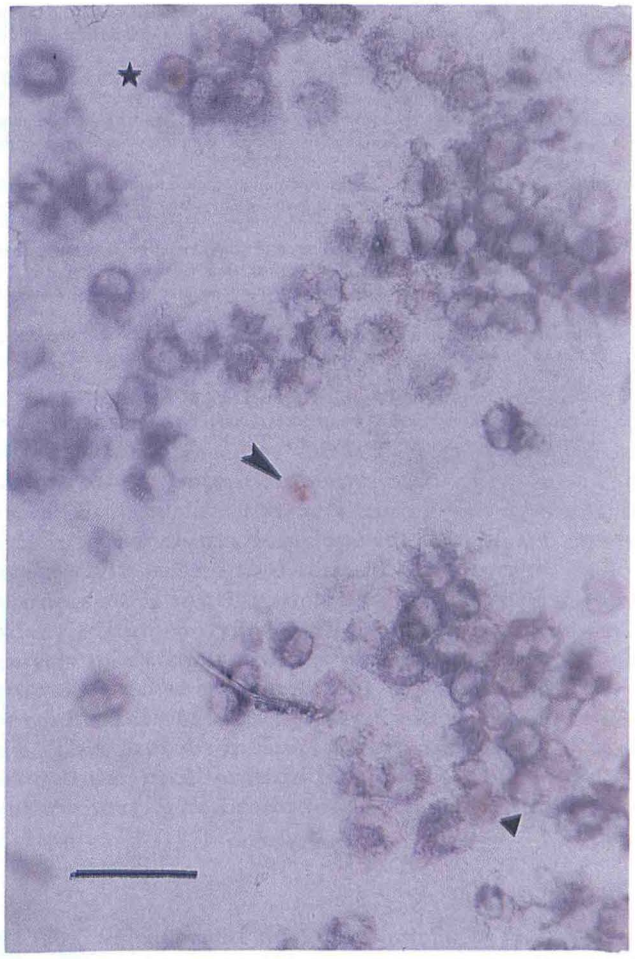

Figure 4. Detail of a cytospin preparation enriched for dendritic cells from an axillary lymph node taken from a hairless mouse 24 h after UV irradiation stained for the presence of T<>T$^+$ cells (red) and NLDC 145$^+$ cells (blue). T<>T$^+$ cells are positive (★), negative (➤), or weakly positive (▶) for NLDC 145. *Scale bar*, 20 μm.

and then results in the migration of lymphoid cells from the skin, e.g., by altering expression of adhesion molecules [32].

The phenotypes of the migrating epidermal cells are MHC II$^+$ NLDC 145$^+$, MHC II$^+$ NLDC 145$^-$, and MHC II$^+$ NLDC 145$^{+/-}$. It is reasonable to speculate that the migrating cells are Langerhans cells, because they are the cells that normally present epicutaneously applied antigens to T lymphocytes in the draining lymph nodes [27,28]. However, immunohistochemical double staining with NLDC 145 revealed that not all the cells that contained T<>T were NLDC 145 positive. NLDC 145 is a monoclonal antibody that reacts with Langerhans cells in the skin as well as with veiled cells and with interdigitating cells in the draining lymph nodes [23]. The fact that not all the T<>T$^+$ cells are positive for NLDC 145 makes it tempting to speculate that some of the migrating cells are not Langerhans cells, but other cells that are present in the epidermis or dermis. Dermal macrophages should be good candidates. However, it cannot be ruled out that the T<>T$^+$ cells are Langerhans cells that have lost the NLDC 145 marker.

Another cell type that might be able to migrate from the skin to the draining lymph nodes is the Thy-1$^+$ DEC. When these cells are hapten conjugated and injected intraveneously in syngeneic animals, they are able to down-regulate the immune response [33]. It could well be possible that after UVR, Thy-1$^+$ DEC migrate to the draining lymph nodes and down-regulate the immune response, resulting in systemic immunosuppression. However, no Vγ3 TCR-positive cells could be detected in the draining lymph nodes after UVR. This indicates that these cells do not migrate from the epidermis to the draining lymph nodes after UV irradiation.

We conclude from this study that upon UV irradiation of the skin, cells are migrating to the draining lymph nodes. The phenotypes of the cells strongly suggests that the migrating cells are Langerhans cells, and that some of them might have lost the NLDC 145 marker. Another possibility is that dermal macrophages that are MHC II$^+$ NLDC 145$^-$ also migrate to the draining lymph nodes upon UV irradiation of the skin. It is well conceivable that these damaged antigen-presenting cells may condition the immune system to bias subsequent processing of an antigen toward suppression instead of activation of a normal cellular response.

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