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Differential Modulation of Human Epidermal Langerhans Cell Maturation by Ultraviolet B Radiation

Satoshi Nakagawa, Cock W. Koomen, Jan D. Bos, and Marcel B. M. Teunissen

UVB irradiation of the skin causes immunosuppression and Ag-specific tolerance in which Langerhans cells (LC) are involved. We tested the effect of UVB on LC that had migrated out of cultured epidermal sheets derived from the skin that was irradiated ex vivo (200, 400, 800, or 1600 J/m²). Two separate subpopulations of LC were distinguished: large-sized LC with high HLA-DR expression, and HLA-DR-low, small LC. UVB stimulated the maturation of the former LC subset as demonstrated by enhanced up-regulation of CD80, CD86, CD54, CD40, and CD83 and reduced CD1a expression in comparison with unirradiated controls. In the latter LC subset, no up-regulation of these molecules except for high CD1a expression and high binding of annexin V, indicating that they were apoptotic, although their CD95 expression was relatively low. Stimulation of enriched LC with CD40 ligand-transfected cells and IFN-γ revealed that the release of IL-1β, IL-6, IL-8, and TNF-α was enhanced by UVB. In comparison with HLA-DR-low LC, HLA-DR-high LC were the principal IL-8 producers as demonstrated by intracellular cytokine staining, and they retained more accessory function. There was no detectable secretion of IL-12 p70, and IL-18 production was neither affected by any stimulus nor by UVB. These results suggest a dual action of UVB on LC when irradiated in situ: 1) immunosuppression by preventing maturation and inducing apoptotic cell death in part of LC, and 2) immunopotentiation by enhancing the up-regulation of costimulatory molecules and the production of proinflammatory cytokines in another part. The Journal of Immunology, 1999, 163: 5192–5200.

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2 Abbreviations used in this paper: LC, Langerhans cells; DC, dendritic cells; SAC, Staphylococcal aureus Cowan strain I; PI, propidium iodide; KC, keratinocytes; MFI, mean fluorescence intensity; CD40L, CD40 ligand; FSC, forward scatter; SSC, side scatter.

Materials and Methods

Culture medium, Abs, and reagents

Cells were cultured in IMDM (Life Technologies, Paisley, U.K.) supplemented with 10% heat-inactivated FCS (HyClone, Logan, UT) and gentamicin (50 µg/ml; Sigma, St. Louis, MO). The mAb against the following Ags were used: CD80 (clone IGH160) and CD86 (clone IGH226) (Innogenetics, Ghent, Belgium); CD54 (clone RR1/1) and CD58 (LFA-3) (clone TS2/9) (a gift from Dr. T. A. Springer, Harvard Medical School, Boston, MA); CD50 (ICAM-3) (clone HP2/19) (a gift from Dr. F. Sanchez-Madrid, Hospital de la Princesa, Madrid, Spain); CD33 (clone HH-15a) (a gift from Dr. T. F. Tedder, Duke University Medical Center, Durham, NC); CD1a (clone SK9) (Becton Dickinson, San Jose, CA); CD40 (clone 5D12) (a gift from Dr. M. de Boer, Tanox Pharma BV, Amsterdam, The Netherlands);
CD40L (clone DX-2) (Biosource, Camarillo, CA); as well as isotype controls (IgG1, IgG2a, and IgG2b) (Becton Dickinson). Anti-HLA-DR (clone L243, unconjugated and FITC-conjugated) (Becton Dickinson) was used to identify LC, and PE-conjugated goat anti-mouse IgG (Southern Biotechnol-ology Associates, Uithoorn, The Netherlands) was used as a secondary Ab. FITC-conjugated annexin V was a kind gift from Dr. C. P. Reutelings- perger (University of Limburg, Maastricht, The Netherlands). J558L hy-bridoma cells transfected with CD40 ligand (CD40L) as well as the un-transfected control cell line were a gift from Dr. P. Lane (University of Birmingham, Birmingham, U.K.). Staphylococcal aureus Cowan strain I (SAC) was purchased from Calbiochem (San Diego, CA). Human rIFN-γ was purchased from Boehhringer Mannheim (Mannheim, Germany).

**UVB source**
Philips TL12 lamps (Philips, Eindhoven, The Netherlands) were used as a source of UVB, emitting UV in the range of 250–400 nm primarily in the UVB region (290–320 nm) with a peak at 315 nm. The UVB output was monitored with an SEE1240 UV photodetector and IL443 phototherapy radiometer (International Light, Newburyport, MA) and was adjusted to 2.0 W/m² at the target spot.

**UVB irradiation of epidermal sheets ex vivo**
Full-thickness human skin specimens were obtained from healthy female Caucasian subjects undergoing plastic surgery of the breast or abdomen. These specimens were shaved into slices of 0.2 mm thickness by using a dermatome, then the slices were rinsed three times with PBS. The skin slices were floated dermal side down on 10 ml of PBS in petri dishes. They were exposed to 200, 400, 800, or 1600 J/m² of UVB, or were left unirradiated as controls. They were then transferred to petri dishes containing 10 ml of PBS with 0.2% dispase II (Boehringer Mannheim) and incubated for 30 min at 37°C to enable separation of the epidermis from the dermis. Epidermal sheets were rinsed in PBS and cultured in culture medium for 18 or 42 h. Cells that had migrated out of the epidermal sheets were collected from the culture medium. The number of cells was counted and were further processed for flow cytometry or cytokine production stud-ies. For the determination of the viability of the migrated LC, cells were stained with FITC-conjugated anti-HLA-DR to identify LC and analyzed by flow cytometry adding propidium iodide (PI) to discriminate dead cells. The viability LC was calculated as follows: viability = (number of HLA-DR+ PI− cells) / (total number of HLA-DR− cells) × 100%

**Flow cytometric analysis of cell-surface markers**
Epidermal sheet-derived cells were successively stained with the primary mAb, PE-conjugated secondary Ab, and FITC-labeled anti-HLA-DR Ab at 4°C for 30 min. Following each step, the cells were washed with washing buffer (PBS containing 1% FCS and 0.1% NaN₃). After the final wash, cells were resuspended in a small volume of washing buffer for analysis. The three-color flow cytometric measurements were performed with a FACScalibur (Becton Dickinson). Dead cells determined as PI-positive cells were electronically gated out using the FL3 channel; the gated cells were computed in a list mode and analyzed with CellQuest software (Becton Dickinson).

For the annexin V binding assay, the cells were first stained with anti-HLA-DR Ab and PE-conjugated secondary Ab as above. Cells were then washed with a buffer containing 10 ml HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 1.8 mM CaCl₂ supplemented by 1% FCS to enable binding of annexin V to the apoptotic cell surface, then incubated with 5 μg/ml of FITC-conjugated annexin V for 20 min at 4°C. Cells were washed again and resuspended in a small volume of the same buffer. PI was added just before FACS analysis. As a negative control, cells washed with PBS containing 1% FCS and 2 mM EDTA were also analyzed similarly. The annexin V-positive cells that were PI-negative were considered to be apoptotic.

**Stimulation of LC and cytokine analysis by ELISA**
Cells that had migrated from epidermal sheets of (un)irradiated skin were collected after 18 h of culture. LC were enriched from these suspensions by means of anti-HLA-DR-coupled paramagnetic microbeads (magnetic cell separation system (MACS), Miltenyi Biotec, Gladbach, Germany) according to the manufacturer’s protocol. The enriched LC (5.0 ×10⁶well), as well as the same number of cells from LC-depleted fraction for con-trols, were stimulated in 200 μl in a 96-well microtiter plate (Costar, Cam-bridge, MA) with 50 μl transduced murine plasmacytoma cells (J558-CD40L) (22) (ratio 1:5) or untransfected J558 in the presence of 1000 U/ml IFN-γ for 18 or 72 h. In some series of experiments, SAC (75 μg/ml) was used instead of J558-CD40L. Culture supernatants were harvested and stored at −20°C until use. The concentrations of the following cytokines were measured by ELISA: IL-1β (Endogen, Woburn, MA; sensitivity 5 pg/ml), IL-6 (Medegenix, Fleurs, Belgium; sensitivity 20 pg/ml), IL-8 (Medegenix; sensitivity 28 pg/ml), IL-12 (PharMingen, San Diego, CA; sensitivity 32 pg/ml), IL-18 (R&D Systems, Abingdon, U.K.; sensitivity 40 pg/ml), and TNF-α (Biosource, Camarillo, CA; sensitivity 20 pg/ml).

**RNA isolation and RT-PCR analysis**
LC were harvested, enriched, and stimulated with CD40L plus IFN-γ as described above. After 4 h of stimulation, cells were lysed in 0.5 ml TRIzol reagent (Life Technologies), and RNA was isolated according to the manufac-turer’s protocol. The reverse transcription of the extracted RNA and PCR conditions have been described in detail previously (23). The specific primer sets were synthesized in our laboratory by an oligosynthesizer: IL-12 p35 forward primer 5′-GTCAACACTGTCCTGAGAA-3′ (nt 345–364), IL-12 p35 reverse primer 5′-TCATGGTCTGAACTC CACC-3′ (nt 628–608); IL-12 p40 forward primer 5′-ACAGAGACAGT GAGGCTTGTAGG-3′ (nt 201–222), IL-12 p40 reverse primer 5′-CAAG GAGCCTCTGCTGCTT-3′ (nt 503–484); GAPDH forward primer 5′- CGAGATCCTCCTACAAATCAA-3′ (nt 298–317), and GAPDH reverse primer 5′-AGGTCAAGTCCACCAGCAG-3′ (nt 799–780). The PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis. The products were of the expected size, and the specificity was confirmed by sequence analysis.

**Flow cytometric analysis of intracellular cytokines**
LC were harvested, enriched, and stimulated with CD40L plus IFN-γ as described above in the presence of 3 μg/ml of brefeldin A (Sigma). After 18 h of culture, cells were harvested, washed in PBS, and stained with FITC-conjugated anti-HLA-DR for 15 min on ice. Intracellular cytokine staining was performed using anti-IL-6 PE, anti-IL-8 PE as well as IgG1 PE isotype control (Becton Dickinson) according to the manufacturer’s protocol (Becton Dickinson). In the analysis of the acquired data, LC were electronically separated from contaminating J558-CD40L cells using FL1-gate settings as HLA-DR+ cells as well as forward scatter (FSC)/side scatter (SSC) gatings.

**FACS of the two LC subpopulations and the mixed lymphocyte-LC reaction**
Skin sheets were irradiated and the epidermis was cultured as described above. After 42 h of culture, migrating cells were harvested and stained with FITC-anti-HLA-DR for 20 min at 4°C. After washing with PBS containing 2 mM EDTA and 1% FCS, cells were sorted using FACS Vantage SE (Becton Dickinson). Gate settings were made using FSC/FL1 dot plot, and, after sorting, the sorted cells were reanalyzed for purity as well as viability using PI. Allogeneic T cells were purified from PBMC using MACS pan T cell isolation kit (Miltenyi Biotec) reaching a purity of >97% CD3+ and <3% HLA-DR+. Graded numbers of viable LC (0.03–1.0 ×10⁶) were cultured with 1.5 ×10⁶ T cells for 5 days in round-bottom 96-well plates, and, during the last 6 h, 0.3 μCi of [³H]Thy (Amersham, Aylesbury, U.K.) was added to each well. Incorporation of the isotope was measured by liquid scintillation counter.

**Statistics**
Statistical analysis was performed using the Student’s t test for unpaired samples, and a value of p < 0.05 was considered significant.

**Results**

The dose of UVB determines the number of LC migrating from epidermal sheets
In the first series of experiments, we investigated whether UVB irradiation affects the number and viability of LC, which spontaneously migrated from epidermal sheets during culture. To test this, skin was irradiated ex vivo with serial single doses of UVB (0, 200, 400, 800, or 1600 J/m²), and, after dispase digestion, epidermal sheets were cultured for 18 or 42 h while floating on the medium with the dermal-side down. Cells that had migrated from epidermal sheets into culture media were collected and counted, and the percentage of LC (estimated as HLA-DR-positive cells) as well as viability were analyzed by flow cytometry. The different components consisted of 5–10% CD3+ T cells, <1% CD11b+ cells, and CD3+ CD11b+ HLA-DR+ cells, probably keratinocytes (Kc)
as judged by morphology. Although there was some variation between the experiments, the number of viable LC showed a tendency to increase by low doses of UVB irradiation compared with unirradiated controls, but these numbers decreased after high doses (Table I). In cases of low doses of UVB as well as unirradiated controls, the number of viable LC increased 42 h after the irradiation in comparison with that after 18 h, but higher doses of irradiation rendered the number of LC to be lower after 42 h of culture. Due to a large variation, no clear effect on the viability of the migrated LC was observed 18 h after irradiation, but at 42 h postirradiation, a clear dose-dependent decrease of LC viability was found.

Two different subpopulations of LC appear after UVB irradiation, which differ in their state of phenotypical maturation

The next aim was to determine whether UVB irradiation affects the phenotypical maturation of LC, which normally occurs during in vitro culture, by means of flow cytometry (Fig. 1). We found that after irradiation two subpopulations of LC could be distinguished as judged by HLA-DR expression: one was exhibiting a reduced HLA-DR expression whereas the other retained a normal high HLA-DR level. The relative number of HLA-DR-low LC increased in UVB dose-dependent way, and that of the HLA-DR-high LC decreased concomitantly. These two LC subsets could most clearly be distinguished 42 h after irradiation, especially upon exposure to high doses of UVB. The FSC/SSC profiles (Fig. 1) showed that the former LC subset was smaller in size as compared with the latter LC subset and the unirradiated controls.

We then compared several cell-surface maturation markers between the two LC subpopulations by flow cytometry (Fig. 2). For that purpose, we selected 42 h after irradiation, because at that time point the subdivision into two populations was most prominent. Each of the two subpopulations was gated electronically by cell size and the intensity of HLA-DR expression and analyzed separately for the coexpression of their cell-surface molecules. In the HLA-DR-low LC subpopulation as compared with HLA-DR-high LC, the expression of costimulatory molecules such as B7-1, B7-2, ICAM-1, and CD40 as well as CD83 was markedly low, whereas that of ICAM-3 and LFA-3 was similar between the two LC subsets, and that of CD1a was higher (Fig. 2). These profiles of the HLA-DR-low LC subset were similar to those of freshly isolated LC (data not shown), illustrating UVB inhibited the maturation of these LC. By contrast, the HLA-DR-high LC subset showed a clear up-regulation of the costimulatory molecules such as B7-1, B7-2, ICAM-1, and CD40 as well as the maturation marker CD83 (Fig. 2). Moreover, the up-regulation of costimulatory molecules B7-2, ICAM-1, and CD40 was enhanced upon exposure to low doses of UVB as the mean fluorescence intensity (MFI) was compared with unirradiated controls, but it was hampered at the highest doses (Fig. 3). These results suggest that low doses of UVB promote the maturation of the HLA-DR-high subpopulation of LC.

HLA-DR-low LC subpopulation exhibits UVB-induced apoptosis

The above results indicated that LC either failed to mature or were stimulated in their maturation after UVB irradiation. Because UVB irradiation is known to induce apoptosis of epidermal cells, for instance via CD95/CD95L pathway, we compared if there was any difference in the stage of apoptosis between the two LC subpopulations, using annexin V binding as indicator for apoptosis (Fig. 4). The HLA-DR-low LC subpopulation were induced to express

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Table I. The number of viable LC and the viability of LC migrating from epidermal sheets after UVB irradiation

<table>
<thead>
<tr>
<th>Expt. Time After Irradiation (h)</th>
<th>UVB (J/m²)</th>
<th>0</th>
<th>200</th>
<th>400</th>
<th>800</th>
<th>1600</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 18</td>
<td></td>
<td>1.5 (67%)</td>
<td>3.5 (73%)</td>
<td>6.7 (67%)</td>
<td>2.8 (55%)</td>
<td>7.5 (60%)</td>
</tr>
<tr>
<td>1 42</td>
<td></td>
<td>2.2 (75%)</td>
<td>4.1 (71%)</td>
<td>4.1 (72%)</td>
<td>1.8 (36%)</td>
<td>1.8 (23%)</td>
</tr>
<tr>
<td>2 18</td>
<td></td>
<td>0.36 (53%)</td>
<td>1.3 (58%)</td>
<td>0.75 (43%)</td>
<td>1.9 (59%)</td>
<td>0.25 (31%)</td>
</tr>
<tr>
<td>2 42</td>
<td></td>
<td>3.4 (65%)</td>
<td>2.8 (57%)</td>
<td>0.96 (28%)</td>
<td>1.5 (26%)</td>
<td>0.36 (9.2%)</td>
</tr>
<tr>
<td>3 18</td>
<td></td>
<td>0.81 (43%)</td>
<td>4.6 (69%)</td>
<td>1.0 (41%)</td>
<td>1.1 (50%)</td>
<td>ND</td>
</tr>
<tr>
<td>3 42</td>
<td></td>
<td>6.9 (63%)</td>
<td>15 (61%)</td>
<td>0.93 (22%)</td>
<td>0.93 (17%)</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Skin sheets derived from three different donors were irradiated with UVB, and the epidermal sheets were prepared and cultured for 18 or 42 h. As a control, cells from unirradiated skin (0 J/m²) were also analyzed. Cells migrated from the sheets were collected and analyzed by flow cytometry. The number of migrating LC was calculated by multiplying the total number of migrated cells per cm² of epidermal sheet with the percentage of PI–HLA-DR+ LC. Viability of LC was determined as described in Materials and Methods.
strongly annexin V binding (MFI: 1200) in a UVB dose-dependent manner at 18 h postirradiation, although part of HLA-DR-low LC showed a very weak expression of annexin V (MFI: 10–16). HLA-DR-high LC also shifted slightly positive for annexin V (MFI: 12–20) as compared with unirradiated control (MFI: 8), demonstrating that UVB caused apoptotic effect on this LC subset as well. Some part of HLA-DR$^-$ KC also showed annexin V binding after irradiation. To check if the Fas/FasL pathway was involved in UVB-induced apoptosis of the HLA-DR-low LC subset, we studied the expression of CD95. As shown in Fig. 4B, CD95 expression was already present in unirradiated LC (MFI: 40) and was up-regulated by UVB irradiation in HLA-DR-high LC subset (MFI: 80). In addition, most of HLA-DR$^-$ KC were clearly positive for CD95. In contrast, CD95 remained low in some of the HLA-DR-low LC subset (like freshly isolated LC, data not shown). Similar changes were observed 42 h after irradiation except for the observation that the annexin V binding of LC from unirradiated controls became slightly positive (data not shown). We also determined if there was any difference in the viability of each of the migrating LC subsets (Table II). Although the dose of UVB irradiation did not seem to affect the viability in either HLA-DR-high or HLA-DR-low LC subset 18 h after irradiation, high doses of UVB decreased the viability of both LC subsets 42 h after irradiation. The HLA-DR-low LC showed significantly less viability than the other LC subset at both 18 and 42 h upon UVB exposure ($p < 0.0001$ in both cases). These results indicate that the HLA-DR-low LC subset was more susceptible to apoptotic cell death than HLA-DR-high LC after UVB irradiation and that this effect was not likely to be mediated through Fas/Fas ligand pathways.

UVB enhances the release of proinflammatory cytokines by LC

We next determined the effect of UVB on the cytokine-producing capacity of LC. For that purpose, LC were enriched from the cells that had migrated from the epidermal sheets using paramagnetic
microbeads coated with anti-HLA-DR Ab. The purity of the enriched LC ranged between 71 and 97% (data from 11 independent experiments, not shown). Thereafter these LC were stimulated with a CD40L-transfected cell line in the presence of IFN-γ. As shown in Table III, there was no detectable production of IL-1β, IL-6, IL-12, and TNF-α in the 18-h culture supernatant of unstimulated LC from the unirradiated epidermis, whereas IL-8 and IL-18 were clearly present. Stimulation of these LC with CD40L-transfected cells in the presence of IFN-γ induced the production of IL-6 and TNF-α and the enhancement of IL-8 production by LC, but IL-1β remained undetectable. The IL-18 secretion was not affected by this stimulation.

In the culture supernatant of LC derived from UVB-irradiated skin, not only IL-8 and IL-18 but also IL-1β, IL-6, and TNF-α could be detected in unstimulated LC cultures. Moreover, the up-regulation of the release of IL-6, IL-8, and TNF-α, after stimulation by CD40L plus IFN-γ, was enhanced by a low-dose (400 J/m²) UVB treatment as compared with the release by unirradiated LC, but IL-1β release was not affected by this stimulation. A higher dose (800 J/m²) of UVB seemed to perturb the secretion of these cytokines, although there was an increased secretion of IL-1β. Neither the stimulation of LC with CD40L nor UVB irradiation had any clear effect on the IL-18 release by LC, and the level

Table III. Release of cytokines from the (un)irradiated LC after stimulation with CD40L in the presence of IFN-γ

<table>
<thead>
<tr>
<th>Exp. 1</th>
<th>LC (unirradiated)</th>
<th>LC (400 J/m²)</th>
<th>LC (800 J/m²)</th>
<th>KC (unirradiated)</th>
<th>KC (400 J/m²)</th>
<th>KC (800 J/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;7</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>IL-6</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>232</td>
<td>20</td>
<td>&lt;20</td>
<td>&lt;20</td>
</tr>
<tr>
<td>IL-8</td>
<td>1,305</td>
<td>1,500</td>
<td>6,586</td>
<td>10,395</td>
<td>7,266</td>
<td>39,156</td>
</tr>
<tr>
<td>IL-12</td>
<td>&lt;32</td>
<td>&lt;32</td>
<td>&lt;32</td>
<td>&lt;32</td>
<td>&lt;32</td>
<td>&lt;32</td>
</tr>
<tr>
<td>IL-18</td>
<td>169</td>
<td>164</td>
<td>152</td>
<td>74.8</td>
<td>&lt;40</td>
<td>114</td>
</tr>
<tr>
<td>TNF-α</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>40</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>64</td>
</tr>
<tr>
<td>Expt. 2</td>
<td>IL-1β</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>10.2</td>
<td>8.9</td>
</tr>
<tr>
<td>IL-6</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>611</td>
<td>38</td>
<td>44</td>
<td>894</td>
</tr>
<tr>
<td>IL-8</td>
<td>1,748</td>
<td>1,254</td>
<td>11,262</td>
<td>4,673</td>
<td>3,974</td>
<td>17,614</td>
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<tr>
<td>IL-12</td>
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<td>&lt;32</td>
<td>&lt;32</td>
<td>&lt;32</td>
<td>&lt;32</td>
<td>&lt;32</td>
</tr>
<tr>
<td>IL-18</td>
<td>68.0</td>
<td>89.8</td>
<td>85.8</td>
<td>73.5</td>
<td>73.5</td>
<td>114</td>
</tr>
<tr>
<td>TNF-α</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>212</td>
<td>44</td>
<td>31</td>
<td>284</td>
</tr>
<tr>
<td>Expt. 3</td>
<td>IL-12 (18 h)</td>
<td>&lt;32</td>
<td>&lt;32</td>
<td>&lt;32</td>
<td>&lt;32</td>
<td>&lt;32</td>
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<td>IL-12 (72 h)</td>
<td>&lt;32</td>
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<td>&lt;32</td>
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<td>IL-18</td>
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<td>107</td>
<td>101</td>
<td>40</td>
<td>89.6</td>
<td>68.0</td>
</tr>
<tr>
<td>IL-18 (72 h)</td>
<td>89.6</td>
<td>52</td>
<td>86.5</td>
<td>86.6</td>
<td>71.2</td>
<td>86.6</td>
</tr>
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</table>

Table II. Viability of the HLA-DR-low and -high subpopulations of LC after ex vivo irradiation of the skin

<table>
<thead>
<tr>
<th>Viability (%) at Time After Irradiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>UVB dose (J/m²)</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>18 h</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>200</td>
</tr>
<tr>
<td>400</td>
</tr>
<tr>
<td>800</td>
</tr>
<tr>
<td>200</td>
</tr>
<tr>
<td>400</td>
</tr>
<tr>
<td>800</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>200</td>
</tr>
<tr>
<td>400</td>
</tr>
<tr>
<td>800</td>
</tr>
<tr>
<td>Range</td>
</tr>
<tr>
<td>Mean</td>
</tr>
</tbody>
</table>
of IL-18 was not affected after 72 h of culture. In clear contrast, a marginal level of IL-8 and TNF-α was measured in the LC-depleted fraction, consisting mainly of KC. IL-18 was detectable in some unirradiated samples, but mostly after irradiation. IL-12 p70 protein could not be detected in supernatants from LC or KC, even after stimulation by CD40L plus IFN-γ and after prolonged culture, although mRNA for both IL-12 p35 and IL-12 p40 was clearly present in LC-enriched fractions and both seemed to be up-regulated by stimulation with CD40L or UVB (Fig. 5). Similar results were obtained when LC were stimulated with SAC instead of using J558-CD40L (data not shown).

To assess which of the two LC subpopulations was responsible for the enhanced secretion of IL-6 and IL-8, we performed intracellular cytokine staining for IL-6 and IL-8 in LC that were stimulated as above. Fig. 6 shows the profiles of electronically gated HLA-DR<sup>+</sup> LC using the vertical line of the quadrant-setting to separate the HLA-DR-low (left two quadrants) and HLA-DR-high (right two quadrants) LC subsets. Some of the unstimulated LC from unirradiated skin already had an intracellular expression of IL-8. Stimulation by CD40L plus IFN-γ enhanced the percentage as well as the MFI of IL-8<sup>+</sup> cells, especially in the HLA-DR-high LC population (Fig. 6a; bottom row; left vs right dot plot, MFI: 41 vs 52 in LC of the upper right quadrant). UVB irradiation enhanced the MFI of the IL-8<sup>+</sup>-HLA-DR-high LC (Fig. 6b; bottom row; left vs right dot plot, MFI: 90 vs 138 in LC of the upper right quadrant), although the percentage of IL-8<sup>+</sup> cells in that LC subset remained unchanged as compared with CD40L-stimulated LC from unirradiated skin. IL-6-positive LC were hardly detectable in any culture condition. These results suggest that HLA-DR-high LC are mainly responsible for the secretion of the proinflammatory cytokines.

In the profiles of LC enriched from the migrated epidermal cell population of unirradiated skin, significant numbers of HLA-DR-low LC (50 ± 1% of the total LC) were present after 18 h of in vitro culture (Fig. 6a). These HLA-DR-low LC were not present in the migrated epidermal cell population from unirradiated skin (Fig. 1), thus they appeared during the 18-h incubation of purified LC. In the culture of UVB-irradiated LC, the percentage of HLA-DR-low LC increased (69 ± 2%) (Fig. 6b). Stimulation by CD40L had little effect on the ratio of the two LC subpopulations irrespective of UVB irradiation.

**FIGURE 5.** Expression of IL-12 p35 and IL-12 p40 mRNA in LC. Cells migrated from epidermal sheets were collected, and LC were enriched using anti-HLA-DR-coated paramagnetic microbeads as described in Materials and Methods. The LC-enriched fraction as well as LC-depleted fraction was stimulated with control- or CD40L-transfected J558 plus 1000 U/ml of IFN-γ for 4 h. RNA was extracted and analyzed by RT-PCR. The PCR products were subjected to electrophoresis and were visualized by ethidium bromide staining. The expression of housekeeping gene GAPDH served as a control for the quality and the quantity of each sample. The data shown are representative of four experiments.

**FIGURE 6.** IL-8 production is mainly confined to the HLA-DR-high LC subpopulation. Flow cytometric analysis of intracellular cytokine for IL-6 and IL-8 is shown. LC derived from unirradiated (a) or UVB-irradiated (b) skin were enriched and stimulated with CD40L-transfected or control J558 cells plus IFN-γ (1000 U/ml) in the presence of brefeldin A (3 μg/ml) for 18 h. Cells were collected, and intracellular cytokine staining of IL-6 and IL-8 was performed. In the profiles shown, LC were electronically selected as described in Materials and Methods before analysis. The setting of the horizontal line of the quadrant was based on the negative staining control, using isotype-matched Ab, and the vertical line of the quadrant discriminates between HLA-DR-low and -high LC. The percentages given in the upper left or upper right quadrant represent the cytokine-positive cells within the HLA-DR-low or -high LC subset, respectively, which was calculated as follows: % = [(number of IL-8<sup>+</sup> HLA-DR-low (or -high) LC)/(number of HLA-DR-low (or -high) LC)] × 100. One representative of three independent experiments is shown.

**FIGURE 7.** Separation of the HLA-DR-low and -high subpopulation by FACS. LC migrated from unirradiated (a) or 400 J/m² UVB-irradiated (b) skin after 42 h of culture were stained with FITC-conjugated anti-HLA-DR mAb, and sorted using FACS Vantage SE. The sorted cells were reanalyzed for their purity and viability (Fig.
After sorting, the viability of the sorted LC decreased rapidly in HLA-DR-low LC (2–14% in HLA-DR-low LC, 65–81% in HLA-DR-high LC, in contrast to 82–91% in LC from unirradiated skin, data from four independent experiments) as compared with the viability in LC before sorting (Table II). After the calculation of the viability, graded numbers of viable LC from either of the two LC subsets as well as LC from unirradiated skin were cultured with purified allogeneic T cells. As demonstrated in Fig. 8, the HLA-DR-low LC subset was significantly less able to stimulate T cells as compared with HLA-DR-high LC or LC from unirradiated skin. The stimulatory function of HLA-DR-high LC seemed to be less than LC from unirradiated skin, but this was not significant at some points in the compared range. These findings suggest that HLA-DR-high LC have a better functional capacity than the HLA-DR-low LC subset.

Discussion

UVB irradiation of the skin depletes LC from the epidermis and alters the phenotype of LC. These phenomena are considered to be involved in the UVB-induced inhibition of contact hypersensitivity and generation of Ag-specific tolerance (24). To elucidate the alterations in the number and phenotype of migrated LC after UVB, we have introduced an approach, as an attempt to mimic natural conditions as much as possible, in which skin was irradiated ex vivo. Subsequently, the irradiated epidermal sheets were cultured, enabling us to collect and analyze the LC that had migrated from the sheets into culture media (21).

We observed that the number of migrating LC increased by UVB irradiation, although regressed after high doses of UVB. After low doses of UVB, as well as in unirradiated control cultures, the number of viable LC increased 42 h after the irradiation in comparison with that after 18 h, probably because of the continuous migration of LC during the prolonged culture period. However, high doses of irradiation rendered viable LC numbers to be lower after 42 h of culture than those after 18 h despite the prolonged culture, probably due to the cytotoxic effect of UVB on LC as indicated by less viability (Table I). These results are consistent with those of Tang and Udey demonstrating that in vitro exposure of LC to UVB was ultimately cytotoxic (25).

FACS analysis of the LC migrated from irradiated epidermal sheets demonstrated bidirectional changes in the profiles of LC. One subpopulation of LC had become small in size with low HLA-DR expression, and was unable to up-regulate costimulatory molecules and surrendered to apoptotic cell death. The other LC was larger in size and HLA-DR-high with enhanced up-regulation of costimulatory molecules. Corresponding with our observations, the occurrence of the subpopulation of large LC in situ was previously reported in two independent studies in mice (8, 9). In addition, Laihia and Jansen (26) recently reported the up-regulation of costimulatory molecules on human LC remaining within the epidermis after solar-simulating irradiation. The mechanisms for the dual effects of UVB are not certain, but the anatomical location of LC within the epidermis might be responsible, i.e., some LC being located in the upper portion of epidermis receiving more UV damage vs others settled in the deeper suprabasal layers covered with more layers of KC receiving less damage. Another explanation might be the intrinsic difference in the state of maturation among the LC in situ. Analysis by flow cytometry revealed that different subpopulations of LC exist within the freshly prepared LC with respect to HLA-DR intensity, as well as signal transduction properties (27), and to the phases of the cell cycle (28).

UVB also affects the cytokine levels in the epidermal microenvironment in which LC reside. Surrounding irradiated KC can produce various kinds of cytokines: GM-CSF, TNF-α, as well as IL-1β are reported to promote the up-regulation of B7-1 and B7-2 on murine LC (29, 30), and these cytokines are also involved in inducing or enhancing migration of LC from epidermis (31–33). The production of the immunosuppressive factor IL-10 is also strongly up-regulated in UVB-exposed skin due to the influx of macrophages (34). IL-10 is able to inhibit the up-regulation of costimulatory molecules on murine (30) as well as human (35) LC after in vitro culture. However, the addition of the culture supernatant from UVB-irradiated epidermal sheets (50% v/v) as well as the addition of recombinant IL-1β and TNF-α (up to 10 ng/ml), GM-CSF (up to 500 ng/ml), or a mixture of these cytokines to the culture media of unirradiated epidermal sheets did not induce the two LC subpopulations (data not shown). IL-10 was not detectable in the supernatant of epidermal sheet cultures derived from both UVB-irradiated and unirradiated skin (by ELISA, detection limit: 50 pg/ml, data not shown). Taken together, we conclude that UVB is responsible for the induction of two LC subpopulations. The up-regulation of costimulatory molecule on HLA-DR-high LC might be caused by a direct effect of mild UVB irradiation in a dose below the threshold causing cytostatic/cytotoxic effects, or by indirect effects via cytokines secreted by KC or LC themselves.

We found that the HLA-DR-low LC subpopulation was more susceptible to UVB-induced apoptotic cell death than HLA-DR-high LC as determined by higher annexin V binding and low viability. It is reported that the CD95 system is involved in UVB-induced apoptosis (36–38) and immune tolerance (39). However, apoptosis observed in HLA-DR-low LC did not seem to be caused via CD95 pathway because there was little up-regulation of CD95 in the HLA-DR-low LC subset as compared with the HLA-DR-high counterpart. Other mechanisms, such as p53/bcl-2 (40) or TNF-α/TNF receptor p55 pathway (41), might also be involved in UVB-induced apoptosis of LC, which should be studied in forthcoming experiments.

Although the secretion of cytokines such as IL-1β, IL-6, IL-8, IL-10, IL-12, IL-13, and TNF-α by human blood-derived DC were well investigated (42–46), there has been few reports concerning cytokine production by epidermal LC, mainly due to the difficulty of the isolation and the very low yield of LC. Murine LC can produce IL-1β, IL-6, IL-12, IL-18, GM-CSF, TNF-α, macrophage inflammatory protein-1α, and macrophage inflammatory protein-2 (47–50), but so far there were few reports concerning cytokine production by human LC. It has been demonstrated that IL-1α (51), IL-1β (51, 52), IL-8 (51), TNF-α (53), and a very low

**FIGURE 8.** The HLA-DR-high LC subset retains accessory function. Each of the LC subpopulation from UVB-irradiated as well as from unirradiated skin was purified by FACS. Graded numbers (300–10,000) of viable purified LC were cultured with CD3+ T cells (1.5 × 10^5) for 5 days. The incorporation of [3H]thymidine, which was present during the final 6 h of culture, was measured using liquid scintillation counter. Data are shown as the mean ± SD from triplicate cultures. The cpm from the culture of T cell or LC alone did not exceed 100 in all experiments (data not shown).

•, LC from unirradiated skin; ■, HLA-DR-high LC; and ▲, HLA-DR-low LC from UVB (400 J/m^2)-irradiated skin. Two representative data of three independent experiments with different donors are shown.
amount of IL-12 p40 (54) protein are produced by human LC. We demonstrated that human LC can also secrete IL-6 and IL-18 and that the production of IL-1β, IL-6, IL-8, and TNF-α was enhanced by UVB. Although KC, which contaminate the enriched LC, can also secrete these cytokines (55–57), we may conclude that they were indeed produced by LC by comparing the cytokine level in the LC-enriched and LC-depleted fractions.

We could argue that HLA-DR-high LC are the major source of the proinflammatory cytokines as judged by a higher percentage, as well as MFI, of IL-8^+ LC (Fig. 6), though IL-6 in LC was not detectable by the intracellular cytokine-staining method, probably due to a lower detection limit in comparison to the ELISA. Although very few numbers of HLA-DR-low LC were found in the cells from unirradiated skin just after migration from epidermis (Fig. 1), significant numbers of these cells were observed in this population after enrichment for LC and subsequent culture for 18 h (Fig. 6). This is considered as the spontaneous apoptotic change of the purified LC following in vitro culture as reported (58). However, this apoptotic process itself did not induce the secretion of the proinflammatory cytokines, except for low amount of IL-8, without additional stimulation by CD40L (Table III).

Unexpectedly, we could not measure any IL-12 p70 protein in the supernatant from the cultured LC, even upon stimulation by bacterial Ags, or CD40L and IFN-γ, which is considered to be the most potent stimuli for DC to produce IL-12 (46, 59). Interestingly, we could detect mRNA of both p35 and p40 of IL-12 in LC by RT-PCR (Fig. 5), indicating that LC might be able to produce IL-12 p70. Prolonged culture up to 72 h, which was suitable to detect IL-12 p70 secretion in a murine LC-like immature DC line (60), did not result in detectable IL-12 release in our case. There may be several explanations: 1) LC need additional stimuli to secrete IL-12 p70 protein-like primary blood DC, which require preincubation with GM-CSF and IL-4 (59); 2) our LC number (5 × 10^7/well) was too small to reach the detection limit of the ELISA (32 pg/ml); 3) during the preparation and the culture of epidermal sheets, LC were already affected by inhibitory signals, like PGE_2, which was reported to prohibit DC from secreting IL-12 (61). However, a recent study has demonstrated no presence of IL-12 in the suction blister fluid up to 72 h after 3 MED (minimal erythemal dose) of solar-simulated irradiation, suggesting that little or no synthesis of IL-12 in human skin occurs in vivo as well (62). IL-18 secretion by LC was not influenced by UVB, bacterial Ag-stimulation, or CD40 ligation.

These results imply that the selective suppression of Th1 cells by UVB-irradiated LC (17, 18) may not be ascribed to their inability to secrete IL-12 or IL-18. Other factors such as IL-6, reported to polarize naive CD4^+ T cells to effector Th2 cells (63), might be responsible as we observed up-regulation of IL-6 secretion by UVB-irradiated LC.

HLA-DR-high LC from UVB-irradiated skin exhibited significantly higher T cell stimulatory function than the HLA-DR-low LC (Fig. 8). This is probably owing to the low expression of co-stimulatory molecules in the latter LC subset (Fig. 2) as well as to a more rapid apoptotic change (Fig. 4). The accessory function of these HLA-DR-high LC was slightly less than LC from unirradiated epidermis in spite of the enhanced costimulatory molecules expression (Fig. 3). The enhanced secretion of proinflammatory cytokines (Table III). The reason was unclear, but we speculate that this decrease could be due to the lower capability of the HLA-DR-high LC to survive after cell sorting or during longer culture period following UVB (25).

In summary, we found that irradiation of skin specimens ex vivo with low doses of UVB caused the development of clearly distinguishable HLA-DR-low and HLA-DR-high LC subpopulations. The former LC subset was prohibited to mature with their phenotypic resembling freshly prepared LC and more susceptible to apoptotic cell death, whereas the latter LC subset underwent enhanced maturation with further up-regulation of costimulatory molecules such as B7-1, B7-2, ICAM-1, and CD40 than unirradiated controls. Furthermore, UVB enhanced the production of proinflammatory cytokines by the latter LC, which retained more accessory function than the former LC. We speculate that the UV exposure in vivo causes various effects on LC, which could not merely be extrapolated from the data obtained in vitro. Comparative studies between in vitro- and ex vivo-irradiated LC are in progress in our laboratory.

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