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Primary Defect in UVB-Induced Systemic Immunomodulation Does Not Relate to Immature or Functionally Impaired APCs in Regional Lymph Nodes1

Shelley Gorman,* Jamie W.-Y. Tan,* Jennifer A. Thomas,* Scott L. Townley,† Philip A. Stumbles,* John J. Finlay-Jones,* and Prue H. Hart2*

UVB irradiation of the shaved dorsal skin of mice can cause both local and systemic suppression of contact hypersensitivity responses; the former demonstrated by administration of the sensitizing Ag/hapten to the irradiated site and the latter by its administration at least 72 h later to distal unirradiated sites. The immunological basis of systemic immunomodulation is not clear. When haptens (trinitrochlorobenzene, FITC) were administered to the shaved ventral skin 4 days after irradiation (8 kJ/m2) to the shaved dorsum of BALB/c mice, CD11c+/FITC+ cells in the skin-draining lymph nodes from control and irradiated mice produced on a per cell basis similar levels of IL-12 and PGE2 were phenotypically mature and efficient at presenting FITC to lymphocytes from FITC-sensitized mice. Ag presentation by FACS-sorted CD11c+ lymph node cells isolated 4 days after UVB irradiation was as efficient as were cells from unirradiated mice at presentation in vitro of an OVA peptide (OVA323–339) to CD4+ cells from OVA-TCR-transgenic DO11.10 mice. Further, IFN-γ levels were increased in the cultures containing CD11c+ cells from UVB-irradiated mice, suggesting that inflammation may precede downstream immunosuppression. These results suggest that the primary cause of reduced contact hypersensitivity responses in mice in which UV irradiation and the sensitizing Ag are applied to different sites several days apart must originate from cells other than CD11c+ APCs that directly or by production of soluble mediators (IL-12, PGE2) affect cellular responses in the nodes of UVB-irradiated mice. The Journal of Immunology, 2005, 174: 6677–6685.

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(15) or lymph node cells that drain from unirradiated sites (16); in one study, lymph node cells were enriched by magnetic bead technology for cells laden with the hapten, FITC (17). Methods and identification of DC enrichment have varied, and no study detailed the yield of lymph node DCs from UVB-irradiated and control mice, or the proportion that were CD11c⁺, a marker used to define DCs (18). Most studies suggested that DC-enriched populations from mice UV-irradiated several days earlier produce reduced levels of IL-12 which polarized subsequent immunological responses with predominant production of Th2 (type 2) cytokines, notably IL-4 and IL-10, rather than IFN-γ (16, 17). It has also been reported that exogenous IL-12 can reverse UVB effects in models of systemic immunomodulation (14, 15), but IL-12 was added at the time of UV administration and was found to reduce keratinocyte apoptosis (19) or cytokine production (14). In a further study, UVB irradiation impaired Th1-mediated immune responses in vivo by suppression of the systemic IL-12 p70 levels; however, upon dissection, splenic APCs from UVB-exposed mice produced levels of LPS-induced IL-12 p70 similar to those produced by spleen cells from unirradiated mice (13). In contrast to these in vitro studies, an earlier in vivo investigation found that when lymph node cells were transferred from FITC-sensitized mice that had been UVB irradiated, or not, at a distant site 3 days previously, a subsequent contact hypersensitivity (CHS) response in all mice was similar (20). Using more advanced immunological tools and multiple approaches, we sought to determine the reason for these different conclusions. We argue that it was not clear in the in vitro studies whether there was a qualitative change or only a quantitative change in the number of DCs harvested from the nodes of UVB-irradiated vs control mice. Due to the potential of UV-induced, DC-derived inhibitory molecules, e.g., IL-12 p40 homodimers (17), it was necessary to investigate the overall functional activity, as well as the phenotype and cytokines produced, of DC populations isolated from lymph nodes of UV-irradiated mice.

Qualitative and quantitative changes in lymph node DCs of UV-irradiated mice were examined in three experimental systems using mice irradiated 4–5 days previously with 8 kJ/m² UVB, a dose consistently equal to 3–4 minimal erythema doses (MED) in BALB/c mice (8, 21). The first involved ventral application of the hapten, trinitrochlorobenzene (TNCB), and 18 h later, the harvest and culture of low density (DC-enriched) populations from the brachial, axillary, and inguinal lymph nodes; similar application of TNCB sensitized for a strong CHS response if reapplied to the ears before sensitization. The limitation of this methodology was that both lymph node-resident CD11c⁺ cells, as well as hapten-loaded CD11c⁺ cells from ventral skin, were harvested. In a second system, FITC was applied as hapten to unirradiated ventral skin, and the FITC⁺ cells draining to the lymph nodes were examined phenotypically and functionally as cells presenting FITC to FITC-sensitized lymphocytes. In the third system, no hapten was used. Instead, CD11c⁺ cells were enriched by FACS sorting from lymph nodes of control mice or mice irradiated with UV light 4 days previously. Their ability to present an OVA peptide to CD4⁺ lymphocytes from OVA-TCR-transgenic DO11.10 mice was examined.

### Materials and Methods

**Animals**

Female BALB/c mice were obtained from the Animal Resources Centre, Mouse transgenic for the OVA233-339 (SQVHAAHAEINEAGR)-specific TCR-αβ on a BALB/c background were obtained from The Jackson Laboratory and bred under clean conditions at the Telethon Institute for Child Health Research. Expression of OVA233-339-specific TCR-αβ on T cells was confirmed by staining lymph node cells with biotin-labeled mouse anti-DO11.10 TCR mAb (KJ1–26; Caltag Laboratories) and then CyC-labeled streptavidin (BD Biosciences). Mice were used between the ages of 8 and 12 wk. All experiments were performed according to the ethical guidelines of the National Health and Medical Research Council of Australia.

**Reagents**

Conjugate-labeled mAb from BD Biosciences included PE-labeled hamster anti-CD11c (HL3), PE-labeled hamster IgG1, both biotin-labeled and CyC-labeled rat anti-CD4 (RM4–5), biotin-labeled rat anti-CD8a (53-6.7), biotin-labeled rat anti-CD19 (ID3), biotin-labeled rat IgG2a, biotin-labeled rat anti-CD86 (B7-2), biotin-labeled rat anti-CD40 (3/23), biotin-labeled rat anti-I-A/I-E (2G9), biotin-labeled rat anti-F4/80, FITC-labeled rat anti-I-A/I-E (2G9), and APC-labeled rat anti-CD11b (M1/70). APC-streptavidin was supplied by BD Biosciences, as was the anti-CD16/CD32 that was used to block Fc receptors on cells. mAb used for CD4 purification included rat anti-CD8α mAb (53-6.7; BD Biosciences) and a rat mAb reactive with mouse MHC class II (I-A) molecules (tissue culture supernatant from TIB120 hybridoma). The OVA peptide (OVA233-339) was synthesized by Proteomics International and HPLC, and mass spectrometry analyses confirmed that the purity of the peptide was >95%.

**UV irradiation**

A bank of FS40 sunlamps (Westinghouse) emitting a broad band of UV, 250–360 nm, with 65% of the output in the UVB range (280–320 nm), was used to irradiate mice in individual compartments of Perspex cages on clean-shaven 8-cm² dorsal skin as previously described (8, 21). A new sheet of polyvinyl chloride plastic film (0.22 mm thick) was taped to the top of each Perspex cage immediately before irradiation. By spectroradiometry, we have shown that the polyvinyl chloride film used screens out wavelengths <290 nm and that there is no perceptible change in wave-lengths excluded by the film for the duration of the irradiation (8, 21). The sunlamps were held 20 cm above the cages. In these studies, a UV dose of 8 kJ/m² was delivered which consistently approximates 3–4 MED in BALB/c mice.

**Sensitization of mice by epidermal application of TNCB or FITC**

Freshly prepared 5% TNCB (0.1 ml; Tokyo Kasei Kogyo) in acetone or 0.5% FITC (0.1 ml) in acetone-dibutyl phthalate (1:1) was applied to the shaved ventral surface of mice.

**Isolation of CD11c⁺ cell-enriched and FITC⁺ cell-enriched populations from skin-draining lymph nodes**

The inguinal, axillary, and brachial lymph nodes were harvested into a solution of 11 mM α-glucose, 5.5 mM KCl, 137 mM NaCl, 25 mM Na₂HPO₄, and 5.5 mM NaH₂PO₄·2H₂O (GKN) containing 5% FCS (inactivated 30 min/56°C) before physical and enzymatic disaggregation with collagenase type 4 (1 mg/ml; Worthington Biochemical) and DNase (0.1 mg/ml; Sigma-Aldrich) in GKN with 10% FCS for 30 min at 37°C in a shaking water bath. Cells were filtered through FCS-washed nylon mesh.

### Table I. Recovery of CD11c⁺ cells in lymph nodes draining ventral skin 18 h after 5% TNCB application in acetone

<table>
<thead>
<tr>
<th>Control</th>
<th>UVB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph node cells/mouse</td>
<td>3.21 (±0.32) × 10⁷</td>
</tr>
<tr>
<td>Cells (&lt;1.066 g/ml/mouse)</td>
<td>3.1 (±1.5) × 10⁵</td>
</tr>
<tr>
<td>% CD11c⁺ (of gradient-enriched cells)</td>
<td>200 ± 5.4</td>
</tr>
</tbody>
</table>

* Mice were irradiated with UVB (8 kJ/m²) on their shaved backs 4 days before TNCB application. Mean ± SEM for five experiments; for each experiment, nodes from six mice were pooled per group.
For sorting of CD11c containing 10% FCS, and centrifuged at 1800 rpm for 20 min at 15°C (22). 

A two-step iodixanol gradient (1.068 g/ml solution layered over a 1.094 g/ml solution (Optiprep; Axis-Shield), diluted in RPMI containing (10% FCS) was centrifuged at 1700 rpm for 15 min at room temperature. Cells were harvested from the uppermost interface and washed twice in GKN + 5% FCS for phenotypic analysis and RPMI + 10% FCS for functional analysis. For isolation of FITC+ cells for culture with cells from FITC- sensitized mice, a FITC+ low density (DC-enriched) population was isolated on a 14.5% metrizamide gradient (Sigma-Aldrich) dissolved in RPMI containing 10% FCS, and centrifuged at 1800 rpm for 20 min at 15°C (22). For sorting of CD11c+ cells, single-cell suspensions were stained for CD11c before sorting on an Epics Elite Flow Cytometer (Coulter).

**Phenotypic analysis of lymph node cells**

The phenotype of cells were analyzed at 4°C using cells (5 x 10^5) in GKN supplemented with 0.2% BSA. Cells were first incubated with 0.05 ml of anti-mouse CD16/CD32 (to block Fc receptors). After 5 min, conjugated or unconjugated Abs were added at optimized concentrations and incubated for 30 min on ice. If a biotin-labeled Ab was used, cells were washed (PBS with 0.5% BSA, 0.1% azide) and then incubated with optimized APC-labeled streptavidin for 30 min at 4°C. Cells were washed and resuspended in 0.3 ml of PBS supplemented with 1% formaldehyde (fixative buffer) until flow cytometric analysis (FACSCalibur flow cytometer; BD Biosciences), and data were analyzed using FlowJo software (version 4.6.1; TreeStar).

**Assay of IL-12 and PGE₂ production in vitro by DC-enriched populations**

CD11c-enriched populations from skin-draining lymph nodes were isolated and prepared as described. After two washings, cells were resuspended in complete RPMI (RPMI supplemented with 2 mM L-glutamine, 50 μM 2-ME, 5 μg/ml gentamicin, 1 mM sodium pyruvate (Sigma-Aldrich), 10 mM MEM nonessential amino acid solution (Sigma-Aldrich), 25 mM HEPES, and 10% FCS). Cells (5 x 10^5 in 1 ml) were aliquoted into Immuno Minisorp tubes (Nunc) and cultured with 100 ng/ml recombinant murine GM-CSF (Peprotech), with or without 10 μg/ml LPS (Sigma-Aldrich) and 100 ng/ml recombinant murine IFN-γ (BD Biosciences) at 37°C in 5% CO₂. Culture supernatants were harvested after 24 and 48 h, and IL-12 levels were determined using the IL-12 p70 OPTEIA set (BD Biosciences; sensitivity, 30 pg/ml) in a dissociation-enhanced time-resolved fluorescence immunoassay (PerkinElmer Life Sciences) with europium (Eu³⁺) as label. PGE₂ levels in culture supernatants were measured using a PGE₂ DIA kit, Monoclonal (Cayman Chemical; sensitivity, 10 pg/ml) according to the manufacturer’s instructions.

**Assay of intracellular IL-12**

Intracellular IL-12 was assayed using a BD Cytofix/Cytoperm Plus Kit (BD Biosciences). GolgiStop was added 4 h before harvesting after 24 h of culture. After washing and pelleting, cells were resuspended in 0.05 ml of rat anti-mouse Fc block and incubated for 5 min on ice before staining with PE-labeled hamster anti-mouse CD11c in GKN supplemented with 0.2% BSA for 30 min on ice. After two washes, cells were incubated in 0.25 ml of buffer with 0.25 ml of BD Cytofix/Cytoperm Plus solution for 20 min on ice. Cells were washed twice in cold 1 x Perm/Wash solution before incubation with 0.05 ml of intracellular block (PBS with 2% BSA, 0.1% saponin; Sigma-Aldrich) for 30 min, followed by APC-labeled rat anti-mouse IL-12 (C15.6; BD Biosciences) for 30 min on ice. Cells were then washed twice with 1 x Perm/Wash solution, fixed in 0.3 ml of fixative buffer before flow cytometric analysis as described above.

**Table II. Phenotype of gradient-enriched cells (<1.068 g/ml) for two representative experiments**

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Control (%)</th>
<th>UVB (%)</th>
<th>Control (%)</th>
<th>UVB (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+</td>
<td>52.4</td>
<td>58.3</td>
<td>54.1</td>
<td>60.4</td>
</tr>
<tr>
<td>CD8α+</td>
<td>20.4</td>
<td>21.2</td>
<td>18.2</td>
<td>20.3</td>
</tr>
<tr>
<td>CD19+</td>
<td>14.9</td>
<td>13.3</td>
<td>18.8</td>
<td>13.5</td>
</tr>
<tr>
<td>CD11c+</td>
<td>18.3</td>
<td>13.2</td>
<td>17.2</td>
<td>15.7</td>
</tr>
</tbody>
</table>

| Of CD11c+ | 1-A/I-E+ | 82 | 83 | 79 | 79 |
| CD11b+    | 66        | 60 | 53 | 48 |
| CD86+     | 69        | 67 | 69 | 71 |
| CD40+     | 50        | 49 | 50 | 50 |
| F4/80+    | 28        | 21 | 28 | 21 |

*Percent of total cells expressing marker (staining by labeled isotype control mAb subtracted).

**Table III. IL-12 and PGE₂ production by iodixanol gradient-enriched lymph node cells isolated from mice treated with ventral TNCB 18 h previously and incubated for 24 and 48 h**

<table>
<thead>
<tr>
<th></th>
<th>After 24 h</th>
<th>After 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>UVB</td>
</tr>
<tr>
<td>IL-12 (pg/10⁶ CD11c+ cells)</td>
<td>43.8 ± 11.3</td>
<td>43.6 ± 15.0</td>
</tr>
<tr>
<td>PGE₂ (pg/10⁶ CD11c+ cells)</td>
<td>5.2 ± 1.3</td>
<td>4.0 ± 0.8</td>
</tr>
</tbody>
</table>

* Mice were UVB irradiated (8 kJ/m²), or not, 4 days before TNCB administration. Mean ± SEM for the five experiments detailed in Table I.
FITC-specific Ag presentation to FITC-sensitized lymph node cells

The responder cells were isolated from the inguinal, axillary, and brachial lymph nodes of mice sensitized 7 days previously to 0.5% FITC administered to the shaved ventral surface. Nodes were physically disaggregated in RPMI containing 10% FCS and filtered through FCS-washed nylon mesh filters. After two washings, 5 × 10^5 cells in 0.1 ml were aliquoted into round-bottom wells of 96-well plates.

For use as presenters, FITC-laden DCs were harvested from the inguinal, axillary, and brachial lymph nodes of mice exposed to 0.5% FITC (shaved ventral surface) 18 h previously. The nodes were physically disaggregated and filtered, and the larger, less dense cells were enriched on a 14.5% metrizamide gradient (see above). These cells were added to the responder cells at ratios of 1:20, 1:40, and 1:80 in 0.1 ml of RPMI containing 10% FCS (four replicates for each ratio). After culture for 48 h at 37°C in 5% CO₂, [³H]thymidine (0.25 μCi; Amersham TRA-306) was added for a further 18 h before harvest. Incorporation of label by responders alone and by presenters alone was subtracted from the [³H]thymidine counts for the mixed culture.

OVA-specific Ag presentation to CD4 cells from OVA-TCR-transgenic mice

CD4+ T cells were isolated from lymph nodes of naive DO11.10 mice. Lymph nodes were disaggregated and filtered through FCS-washed nylon mesh filters as previously described. Erythrocytes were removed from cell preparations by ammonium chloride lysis. MHC class II+ and CD8α+ cells were removed using a rat anti-CD8α mAb (2.5 μg/ml) and supernatant from the TIB-120 hybridoma (1/200 dilution) and M-450 sheep anti-rat IgG Dynabeads (Dynal Biotech). In some experiments, the purified CD4+ T cells (≥95%) were then labeled with 5 μM CFSE (Molecular Probes) for 10 min at room temperature. After three washings, cells were resuspended in RPMI with 10% FCS and aliquoted into round-bottom 96-well plates at 10^5 cells/0.1 ml/well. OVA323–339 peptide was added to reach a final concentration of 10 μg/ml. CD11c+ cells were added to CD4+ T cells at decreasing ratios. For cultures in which the CD4 cells were not labeled with CSFE, [³H]thymidine was added for the last 18 h of a 66-h culture; for equivalent plates, [³H]thymidine was not added, and supernatants were harvested after 66 h for cytokine measurements. Cultures with CSFE-labeled CD4 cells were incubated for 96 h, supernatants were harvested for cytokine analysis, whereas the cells were washed and stained with CyC-labeled rat anti-CD4 mAb for 30 min at 4°C. Cells were fixed in 0.3 ml of fixative buffer before FACS.

Assay of IFN-γ and IL-10 in culture supernatants from DC-T cell cultures

IFN-γ and IL-10 levels were determined using the IFN-γ and IL-10 OPTIAEA sets (BD Biosciences; sensitivity of both assays, 25 pg/ml) in a dissociation-enhanced time-resolved fluorescence immunonanassy with europium (Eu³⁺) as label.

Results

Effect of UVB on sensitization to the hapten TNCB applied to a nonirradiated site

As previously published by us (8, 21) and many others (23), UVB (8 kJ/m²) administered to the shaved dorsal skin of BALB/c mice significantly reduces a CHS response to the hapten, TNCB, when applied to the ventral skin 4 days after irradiation. In this model, the ear swelling when challenged a further 5 days later with TNCB (10 days after irradiation) is reduced by ~50%. To dissect the effect of UVB on the sensitization phase of a CHS response, we examined the cells that will present TNCB to naive lymphocytes in these mice. First, the cellular content in the nodes draining the site of ventral TNCB sensitization (inguinal, brachial, axillary nodes) was examined after 18 h. Density gradient centrifugation was used and enriched for cells with densities of <1.068 g/ml. These cells were larger and included all CD11c+ cells (data of absence of CD11c+ cells among cells of higher density not shown). A summary of the harvest of CD11c+ cells in the draining nodes of mice from five experiments is shown in Table I. The phenotype of the cells harvested from the iodixanol gradient for two representative experiments is shown in Table II. Other than CD11c+ cells, approximately one-half of the isolated cells were CD4+, together with lesser numbers of CD8+ and CD19+ cells. The percentage decrease in CD11c+ cells was balanced in both experiments by an increased percentage of CD4+ cells (Table II).

Effect of UVB irradiation on IL-12 and PGE₂ production by DC-enriched lymph node cells

The iodixanol gradient-enriched CD11c+ cell populations as described in Table I were incubated for 24 or 48 h in complete RPMI supplemented with GM-CSF (100 ng/ml), with or without LPS (10 μg/ml) + IFN-γ (100 ng/ml). IL-12 and PGE₂ were not detectable in the supernatants of cells incubated without LPS + IFN-γ. There was also no detectable IL-12 or PGE₂ in the supernatants of unfraccionated lymph node cells or cells of higher density that were isolated from a lower band of the iodixanol gradient and incubated for 24 or 48 h with LPS + IFN-γ. Allowing for the reduced percentage of CD11c+ cells in the gradient-enriched populations from UV-irradiated mice, and assuming that only CD11c+ cells produced IL-12 and PGE₂, there was no significant difference in the levels of IL-12 or PGE₂ produced on a per cell basis between the cells harvested from control and UVB-irradiated mice (Table III).

To validate the assumption that IL-12 was produced predominately by CD11c+ cells, those cells producing IL-12 were characterized by intracellular staining. Fig. 1, A and B, demonstrates increased intracellular IL-12 in response to LPS + IFN-γ by cells from control and UVB-irradiated mice, respectively, for a representative experiment. Fig. 1, C and D, show that 78 and 80% of the IL-12-positive cells were CD11c+ for cells from control and UVB-irradiated mice, respectively. The intracellular staining of IL-12 also suggests that the amount of IL-12 produced in response to LPS + IFN-γ for cells from UVB-irradiated mice is not less than that detected for unirradiated mice.

Table IV. Recovery of FITC+ cells in lymph nodes draining ventral skin 18 h after FITC application (0.5% in 0.1 ml of acetone-dibutyl phthalate, 1:1)⁎

<table>
<thead>
<tr>
<th>Yield/mouse from 14.5% metrizamide gradient</th>
<th>Control</th>
<th>UVB</th>
</tr>
</thead>
<tbody>
<tr>
<td>105 (±0.27) × 10⁴</td>
<td>1.03</td>
<td>3.03</td>
</tr>
<tr>
<td>FITC+ (% cell yield)</td>
<td>17.7 ± 4.8</td>
<td>7.0 ± 2.3</td>
</tr>
<tr>
<td>Total FITC+ mouse</td>
<td>1.82 (±0.48) × 10⁴</td>
<td>2.12 (±0.46) × 10⁴</td>
</tr>
</tbody>
</table>

⁎ Mice were UVB irradiated (8 kJ/m²), or not, 4 days before FITC administration. Mean ± SEM for three experiments.
(data not shown), and comprised approximately one-half of the harvested CD11c\textsuperscript{+} cells (Fig. 2 for cells from a representative experiment). For cells harvested from both control and UVB-irradiated mice, \(>94\%\) of FITC\textsuperscript{+} cells expressed high levels of the costimulatory molecules (CD40 and CD86) and MHC class II (Fig. 2).

**Effect of UVB on Ag presentation by FITC\textsuperscript{+} cells that have drained to nodes from nonirradiated skin administered FITC**

The gradient-enriched FITC\textsuperscript{+} cell populations as described in Table IV were incubated at different ratios with lymphocyte responders isolated from the nodes of FITC-sensitized, unirradiated mice. There was reduced \([^{3}\text{H}]\)thymidine incorporation when cells from UVB-irradiated mice were added (Fig. 3A). However, when the results were plotted according to the number of FITC\textsuperscript{+} cells incubated with FITC-sensitized lymphocytes, levels of \([^{3}\text{H}]\)thymidine incorporated were not significantly different between cells from control and UVB-irradiated mice (Fig. 3B). At presenter-responder cell ratios of 1:40, 1:80, and 1:200, the mean cpm\textsuperscript{S} for cells incubated with FITC\textsuperscript{+} cells from UV-irradiated mice were 50, 58, and 36\%, respectively, of that measured with FITC\textsuperscript{+} cells from control mice, reflecting the 60\% reduced concentration of FITC\textsuperscript{+} cells in the nodes of UVB-irradiated mice.

**Effect of UVB on Ag presentation by lymph node CD11c\textsuperscript{+} cells**

The inguinal, axillary, and brachial lymph node cells were harvested from BALB/c mice irradiated with 8 kJ/m\textsuperscript{2} UVB 4 days previously. These cells were representative of 1) those APCs at distant sites that might have been bathed by soluble mediators generated at UV-irradiated skin and 2) APCs that have drained from irradiated skin during the past 4 days. Cells were isolated on an iodixanol density gradient and then sorted according to levels of expression of CD11c (Fig. 4, A and B; Fig. 5, A and B; Fig. 6, A–D, for profiles from control and UVB-irradiated cells, respectively, for three experiments). The CD11c\textsuperscript{+} cells were incubated for 66 or 96 h with purified CD4\textsuperscript{+} cells from DO11.10 mice, together with the OVA\textsubscript{323–339} peptide. For the first experiment when CD11c\textsuperscript{+} cells were added at CD11c:CD4 T cell ratios of 1:10–1:1280, levels of \([^{3}\text{H}]\)thymidine incorporated by the cultures were not significantly different (Fig. 4C). In contrast, the cultures with CD11c\textsuperscript{+} cells from UVB-irradiated mice contained significantly increased levels of IFN-\(\gamma\) (Fig. 4D); IL-10 was not detectable. In the second experiment, sorted CD11c\textsuperscript{+} cells from both control and UVB-irradiated mice were incubated with CSFE-labeled CD4\textsuperscript{+} cells. After 96 h, at CD11c:CD4 T cell ratios of 1:50–1:5000, there were equal percentages of divided cells (CSFE\textsuperscript{low}) in the cultures.

**FIGURE 2.** The phenotypic profile of low density lymph node cells (inguinal, axillary, brachial) harvested from BALB/c mice 18 h after administration of 0.5\% FITC to shaved ventral skin. Mice were irradiated, or not, with UVB (8 kJ/m\textsuperscript{2}) 5 days previously on shaved dorsal skin. FITC\textsuperscript{+} cells were enriched on an iodixanol gradient to \(29\) and 20\%, for control and UVB-irradiated mice, respectively. In A, staining of FITC\textsuperscript{+} cells with a PE-hamster isotype control Ab or a PE-labeled CD11c Ab is shown. For control and UV-irradiated mice, 39 and 35\%, respectively, of cells were CD11c\textsuperscript{+}, and 52 and 42\%, respectively, of CD11c\textsuperscript{+} cells were FITC\textsuperscript{+}. In B, the FITC\textsuperscript{+} cells were stained for I-A/I-E, CD40, and CD86, respectively, for cells from control (---) and UVB-irradiated (-----) mice. Little to no staining with an APC-labeled rat IgG2a control Ab is also shown. In C, the gradient-purified cells from both control and UV-irradiated mice were stained for I-A/I-E and CD11c, with a population of CD11c\textsuperscript{dim} cells expressing intermediate levels of I-A/I-E. FL, Fluorescence.
containing CD11c⁺ cells from control and UVB-irradiated mice (Fig. 5C); however, again, levels of IFN-γ were increased in cultures with CD11c⁺ cells from UVB-irradiated mice (Fig. 5D). IL-10 levels were not significantly different for cultures containing CD11c⁺ cells from control and UVB-irradiated mice (Fig. 5E).

In a third experiment, CD11clow and CD11chigh cells from both control and UVB-irradiated mice (Fig. 6, A and B) were incubated at CD11c:CD4 T cell ratios of 1:20–1:1280. CD11clow cells from all mice were more efficient than CD11chigh cells at Ag presentation, which supports the data in Fig. 2C showing a population of CD11clow cells with intermediate levels of I-A/E. CD11chigh cells from UVB-irradiated mice compared with CD11clow cells from control mice stimulated significantly enhanced CD4⁺ T cell proliferation (measured by [³H]thymidine incorporation) at a CD11c:T cell ratio of 1:40 and 1:80 (Fig. 6E). Levels of IFN-γ (Fig. 6, G and H) and IL-10 (Fig. 6, I and J) in the culture supernatants at 66 h were significantly greater for cultures with CD11chigh cells, but not CD11clow cells, isolated from UVB-irradiated mice. Levels of IFN-γ produced were significantly greater than the levels of IL-10 detected.

Discussion

All three lines of experimental investigation suggest that there is no primary phenotypic or functional Ag presenting defect in CD11c⁺ cells isolated from the brachial, axillary, and inguinal lymph nodes of mice 4 or 5 days after UVB irradiation to shaved dorsal skin (Tables I–IV and Figs. 1–6). Instead, stimulation of an inflammatory environment, characterized by enhanced IFN-γ production (Figs. 4–6) and a high IFN-γ:IL-10 ratio, was detected when CD11c cells from UVB-irradiated mice were cocultured with CD4⁺ T cells. This inflammatory response complemented the increased cellularity, and in turn the reduced concentration of CD11c⁺ or FITC⁺ cells, in the skin-draining lymph nodes (with or without hapten) 4 or 5 days post-UV irradiation.

In many of these experiments, CD11c⁺ cells were investigated ex vivo 5 days after UVB irradiation and 18 h after hapten exposure; this scenario addresses most models of UVB-induced systemic suppression of CHS responses in which experimental hapten is applied to nonirradiated skin at least 3 days postirradiation (1). Initially, TNCB⁺ or FITC⁺ cells in lymph nodes draining ventral skin treated with TNCB or FITC, respectively, were enriched by density gradient centrifugation and examined for their 1) phenotype, 2) IL-12 and PGE₂ production in response to activation/differentiation with LPS + IFN-γ, or 3) efficiency at Ag (FITC) presentation to sensitized lymphocytes. In the final line of experimentation, hapten was not administered to mice and CD11c⁺ cells purified from the brachial, axillary, and inguinal nodes 4 days after UVB irradiation to shaved dorsal skin. These experiments used lymph node CD11c⁺ cells as a surrogate for CD11c⁺ cells potentially located at any nonirradiated distant site; i.e., they represented those APCs potentially altered by soluble mediators produced at the irradiated site and that would subsequently handle Ag. Furthermore, there is recent evidence for significant Ag presentation by DCs that permanently reside within lymph nodes with Ag transfer documented between migrating and lymphoid-resident DCs in cases of T cell priming and tolerance induction (24). This final line of investigation also confirmed that if there were any functionally altered CD11c⁺ DCs in the same nodes that had drained from the UVB-irradiated site, such altered function was not detectable 4 days after UVB irradiation.

FIGURE 3. [³H]Thymidine incorporation by lymph node cells isolated from FITC-sensitized mice (responders) and incubated with density gradient-enriched FITC⁺ cells (presenters) from the skin-draining lymph nodes of mice treated with 0.5% FITC to shaved ventral skin 18 h previously. For presenters from control, unirradiated mice, FITC⁺ cells were enriched to a mean of 17.7%; for those from UVB-irradiated mice, the mean was 7.0%. [³H]Thymidine was added for the last 18 h of a 72-h culture. A, [³H]Thymidine incorporated for each culture. B, Results expressed according to the number of FITC⁺ cells in the cultures. Results are shown as cpm, mean ± SEM, n = 12 replicates from three pooled experiments. ∗, [³H]Thymidine incorporated by responder cells in the absence of presenters.

FIGURE 4. Proliferation and IFN-γ production by CD4⁺ T cells incubated for 66 h with sorted CD11c cells isolated from lymph nodes (inguinal, axillary, brachial) harvested from BALB/c mice irradiated with UVB (8 kJ/m²) four days previously on shaved dorsal skin. A, CD11c purity of 95% for sorted cells from control mice. B, CD11c purity of 95% for sorted cells from UVB-irradiated mice. C, Incorporation of [³H]thymidine after 66 h for cultures of CD11c⁺ cells from control (——) and UVB-irradiated (––––) mice with CD4⁺ T cells at ratios of 1:10 to 1:1280. ∗, [³H]Thymidine incorporated by CD4⁺ cells in the absence of CD11c⁺ cells. D, IFN-γ levels in the culture supernatants at the termination of culture. Mean ± SEM for triplicate cultures. ∗, Significant difference, p < 0.05.
This study was modeled on two previous findings: 1) that UVB alters the Ag-presenting function of Langerhans cells at irradiated sites and converts them via perturbation of membrane-bound co-stimulatory molecules from immunogenic to tolerogenic APCs (25, 26); and 2) that epidermal cells from the ears of mice that have received chronic systemic treatment with cis-urocamic acid were unable to suppress the mixed skin lymphocyte reaction response (27). The hypothesis behind this study was that soluble products from UVB-irradiated skin altered the Ag-presenting function of DCs at nonirradiated sites. This hypothesis reflected published findings that keratinocyte-derived IL-10 or mast cell-derived histamine at low concentrations could subvert the differentiation of DCs into mature, immunocompetent cells (7, 12, 28, 29). Instead, by reduced IL-12 production, IL-10- or histamine-treated DCs could signal less T cell proliferation and a less inflammatory T cell response driven by reduced levels of type 1 cytokines. Another UV-induced soluble mediator that may polarize DCs for reduced IL-12 production and the skewing of T cell differentiation toward a Th2 phenotype is PGE2 (7, 21, 30, 31). In our studies, in which 10^6 cells from nodes of mice were incubated with LPS with IFN-γ, IL-12 levels were lower in the culture supernatants for cells from UVB-irradiated mice (Table III). However, if calculated according

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Proliferation and IFN-γ production by CD4+ T cells incubated for 96 h with sorted CD11c cells isolated from lymph nodes (inguinal, axillary, brachial) harvested from BALB/c mice irradiated with UVB (8 kJ/m²) 4 days previously on shaved dorsal skin. A, CD11c purity of 91% for sorted cells from control mice. B, CD11c purity of 91% for UVB-irradiated mice. C, percent of cells that were CSFElow (% divided) after 96 h for CD11c+ cells incubated with CD4+ cells at ratios of 1:50 to 1:5000. D, IFN-γ levels in the culture supernatants at the termination of culture; E, IL-10 levels in the culture supernatants at the termination of culture. C–E, cultures with CD11c+ cells from control mice; --, cells from UVB-irradiated mice. Mean ± SEM for triplicate cultures. *, Significant difference, p < 0.05.

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** Proliferation and IFN-γ and IL-10 production by CD4+ T cells incubated for 66 h with sorted CD11chigh and CD11clow cells isolated from lymph nodes (inguinal, axillary, brachial) harvested from BALB/c mice irradiated with UVB (8 kJ/m²) 4 days previously on shaved dorsal skin. A, CD11chigh staining for cells sorted from control mice. B, CD11chigh staining for cells sorted from control mice. C, CD11clow staining for cells sorted from UVB-irradiated mice. D, CD11chigh staining for cells sorted from UVB-irradiated mice. The purity of the sorted cells is indicated. E, Incorporation of [3H]thymidine after 66 h for cultures of CD11chigh cells from control (-----) and UVB-irradiated mice (---) with CD4 T cells at ratios of 1:20 to 1:1280. F, Incorporation of [3H]thymidine after 66 h for cultures of CD11clow cells from control (-----) and UVB-irradiated mice (---) with CD4 T cells at ratios of 1:20 to 1:1280. G and H, IFN-γ levels in the culture supernatants at the termination of culture. I and J, IL-10 levels. Mean ± SEM for triplicate cultures. *, Significant difference, p < 0.05.
to the number of CD11c<sup>+</sup> cells in the starting population, IL-12 production, and similarly PGE<sub>2</sub> production, were similar on a per cell basis. We propose that this is the reason for previous reports of less competent APCs in the secondary lymphoid organs of UVB-irradiated mice. The assay for IL-12 used in our study would not have detected UV-induced IL-12 p40 homodimers as reported in studies using unfiltered and higher doses of UVB radiation (17). Furthermore, if IL-12 p40 homodimers were produced in sufficient amount by APCs from the UV-irradiated mice, the Ag-presenting function of the CD11c<sup>+</sup> cells should have been reduced. This study supports a previous in vivo finding using FITC-sensitized lymph node cells from control and UVB-irradiated mice (20). This study, however, provides a more extensive investigation of the mechanisms of systemic UVB-induced immunomodulation to debate the contrasting conclusions of many other laboratories (12–17).

This study had multiple approaches that were designed to account for, or balance, the deficiencies of any one system. The study of FITC<sup>+</sup> cells allowed us to specifically study cells migrating from the FITC-treated site and to test whether they have reduced expression of costimulatory molecules, and thus reduced ability to present FITC to FITC-sensitized lymphocytes. However, FITC, unlike TNCB, does not induce a strong CHS response (32) and instead has been characterized to induce a Th2-driven immune response. Thus, studies of TNCB administration to nonirradiated sites were included as UVB consistently reduces a TNCB-induced CHS response (8, 21). Lymphocytes from DO11.10 mice allowed us to study potential UV effects on CD11c<sup>+</sup> APCs in a more defined system, but the Ag, the OVA peptide (OVA<sub>233–339</sub>), was handled in vitro, not in vivo. Using sorted CD11c<sup>+</sup> cells, the influence of coisolated inflammatory lymph node cells (previously FITC<sup>+</sup> cells were enriched to <20%) was removed and allowed a clear analysis of the ability of mediators or cells from irradiated sites to influence DCs and DC function several days later.

In all experiments (with or without hapten administration), there was an increased cellularity of the brachial, axillary and inguinal lymph nodes of UVB-irradiated mice. A dose of UVB equivalent to 3–4 ¥ MED was administered to mice and represented a dose of UVB necessary for significant suppression of systemic CHS responses in BALB/c mice (21). The increased cellularity reflected a general inflammatory response with parallel increases in T and B lymphocytes. In contrast, the concentration of CD11c<sup>+</sup> cells was reduced in nodes from irradiated mice and as discussed above could explain the reduced responses by lymph node cells from irradiated mice reported by others. This is to our knowledge also the first study to measure the CD11c<sup>+</sup> cell concentration in nodes after UVB; others have characterized DCs by morphology (16) or I-A<sub>1</sub>-surface expression (33). Taking into consideration this reduced concentration of CD11c<sup>+</sup> cells, our studies did not detect any altered properties of the CD11c<sup>+</sup>FITC<sup>+</sup> cells from UVB-irradiated mice. One could argue that the increased number of inflammatory cells in the nodes of UV-irradiated mice may alter qualitatively, as well as quantitatively, DC behavior.

Our FITC studies suggest that in the nodes of UV-irradiated mice, the ratio of Ag-presenting DCs to responding T cells could be altered by a factor of 2 or 3. There are a number of published in vitro studies in which the ratio of APCs to responder cells was titrated, with a low ratio favoring Th1 development (34, 35). In these studies, the ratio of type 1 (IFN-γ) to type 2 cytokines (IL-10) produced vary with different ratios. This was not found in our studies of purified CD11c<sup>+</sup> cells incubated with CD4<sup>+</sup> T lymphocytes (Figs. 4–6). With the dynamic interactions occurring in a lymph node, we do not propose that an altered ratio of 2 to 3 would significantly alter the immunological outcome. Instead, with the absence of a primary defect in APCs at sites distant to the UVB-irradiated site, we propose that the primary defect resides in a different cell population. We propose that UV-induced soluble mediators drive a different outcome in vivo for APCs that are not defective in vitro. It has also been recently proposed that UVB irradiation does not affect the sensitizing phase of CHS (36); however, the many reports of UVB-induced Ag-specific regulatory T cells cannot be ignored (37, 38). In one published study, the Ag presenting ability of DCs enriched from spleens of irradiated mice was altered 7 but not 3 days after UVB irradiation (33). However, this model could not explain the UV-immunomodulatory effects in the CHS studies from our laboratory and that of others (14, 16). This study suggests that 4 to 5 days after a dose of UVB that causes a systemic suppression of CHS responses, skin-draining lymph nodes are inflamed. Further, CD11c<sup>+</sup> cells that have drained to the nodes with the sensitizing hapten applied to a non-irradiated site are phenotypically and functionally mature. So why are CHS responses significantly suppressed? In the inflamed environment, is there increased destruction or apoptosis of potential APCs? Numbers of CD11c<sup>+</sup> cells are enhanced in nodes of UV-irradiated mice but their concentration is reduced. Alternatively, with functionally mature CD11c<sup>+</sup> APCs, are there UV-induced regulatory cells or molecules in the nodes that regulate the function of CD11c<sup>+</sup> cells? These questions are the subject of ongoing experiments.

It will be important that the mechanism(s) of UVB-induced systemic immunomodulation are understood, particularly as they will provide insight into the effect of sunlight on immune responses in infectious and autoimmune diseases, and vaccination. Nonmelanoma skin cancer is the most common cancer in Caucasians in the world. There is also an increased cancer mortality after a history of skin cancers (39); this highlights the importance of studying the systemic immunomodulation that follows the release of keratinocyte/mast cell products from UV-irradiated skin (8).

**Disclosures**

The authors have no financial conflict of interest.

**References**


