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iC3b Arrests Monocytic Cell Differentiation Into CD1c-Expressing Dendritic Cell Precursors: A Mechanism for Transiently Decreased Dendritic Cells *in vivo* After Human Skin Injury by Ultraviolet B

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Our previous data indicated that C3, its bioactive product iC3b, and the iC3b ligand CD11b are critical for ultraviolet-induced immunosuppression. We thus hypothesized that iC3b is an important skin-based factor regulating $CD11b^+$ monocytic cell function in the acute post-ultraviolet period. Although monocytic cell migration peaked at 1–3 d after ultraviolet exposure of skin, dermal CD1c dendritic cells underwent a rapid and prolonged depletion that did not recover until day 7. Because ultraviolet-induced iC3b deposits are reciprocally maximal on day 3, but fade by day 7, we next hypothesized that iC3b can be responsible for the delay in differentiation into dendritic cells of monocytic cells migrating into ultraviolet-exposed skin. Analysis of dermal cells derived from keratome biopsies suggested that iC3b exposure could inhibit the development of $CD1c^+$ dermal cells. To model newly immigrating blood monocytes entering ultraviolet-exposed, iC3b-containing dermis, purified monocytes from human blood were induced with granulocyte-macrophage colony stimulating factor to generate a population of dendritic cell precursors expressing CD1c. Incubation with iC3b markedly inhibited the appearance of $CD1c^+$ cells ($p < 0.05$) and induced $CD1c^-CD14^+$ cells. This inhibition was reversed by coincubation with an anti-CD11b antibody that blocks the iC3b binding site. Other functions associated with dendritic cell maturation were also inhibited by iC3b, such as interleukin-12p70 production as well as CD80 and CD40 expression. Restimulation of monocytes for DC maturation revealed that iC3b induced a temporary inhibition of DC differentiation. Thus, a human skin response in which iC3b is transiently (3–7 d) generated in dermis, such as ultraviolet, can arrest monocytic skin-infiltrating cells from undergoing dendritic cell precursor differentiation. ***Key words: ultraviolet B/monocyte/cell differentiation/complement/cytokine. J Invest Dermatol 120:802–809, 2003***

Ultraviolet B (UVB) induced immunosuppression is an excellent test of skin integrity, requiring the interplay of a number of biologic, structural, and cell migration/differentiation functions. UV has been well known to play a substantial role in modifying contact sensitivity, induction of microbial infections, and development of skin cancer in humans. It has been demonstrated that dermal Langerhans-cell-like dendritic cells (DC) (Meunier *et al*, 1995) as well as epidermal Langerhans cells (Aberer *et al*, 1981; Murphy *et al*, 1993) are depleted in UV-exposed skin, in spite of expansion of a potential precursor population of newly infiltrating monocytic cells, some of which are differentiated to interleukin-10 (IL-10) producing macrophages (Cooper *et al*, 1986; Meunier *et al*, 1995). The mechanism has not been clearly elucidated, however. The changing populations of antigen-presenting cells (APC) caused by UV radiation are involved in UV-induced immunosuppression and tolerance. Our previous studies have shown that infiltrating $CD11b^+$ monocytic/macrophagic cells are critical for induction of tolerance to dinitrofluorobenzene by UV-exposed epidermal cells (Hammerberg *et al*, 1994).

Complement receptor type 3 (CD11b/CD18), a member of the β2 integrin family, is expressed on monocytes and macrophages, polymorphonuclear leukocytes, and natural killer cells, and interacts with a variety of ligands including iC3b (Beller *et al*, 1982), intercellular adhesion molecule 1 (Diamond *et al*, 1990), fibrinogen (Wright *et al*, 1988), and β glucan (Thornton *et al*, 1996). It is known that iC3b can bind the I region of the α subunit of monocytic CD11b.

Complement receptor type 3 is involved in several monocyte and macrophage functions including phagocytosis, transmigration (Shang and Issekutz, 1998), and nitric oxide production (Marth and Kelsall, 1997). In addition, we have reported that iC3b and CD11b are critical for UV-mediated immunosuppression, because blockade of CD11b (Hammerberg *et al*, 1996) and depletion of iC3b via soluble CR1 or C3 gene disruption

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Abbreviations: APC, antigen-presenting cells; DC, dendritic cells; dMFI, delta mean channel fluorescence intensity; EA, IgM-coated sheep erythrocytes; EAiC3b, IgM- plus iC3b-coated sheep erythrocytes.

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(Hammerberg *et al*, 1998) in UV-exposed murine skin reversed UV-induced immunosuppression. Deposition of iC3b can occur in the skin 72 h after UV exposure, in contiguity with monocytes/macrophages that infiltrate the skin at that time point. Furthermore, binding of iC3b to β2 integrin on fresh monocytes induced increased IL-10 production, but downregulated IL-12 production (Yoshida *et al*, 1998), consistent with the IL-10^{high} IL-12^{low} cytokine profile of the monocytic/macrophagic population that infiltrates into UV-exposed skin (Kang *et al*, 1994; 1998).

It has been shown that *in vitro* blood monocytes can develop into DC when incubated with cytokines such as granulocyte-macrophage colony stimulating factor (GM-CSF), IL-4, tumor necrosis factor α, and transforming growth factor β (Akagawa *et al*, 1996; Pickl *et al*, 1996; Zhou and Tedder, 1996; Chapuis *et al*, 1997; Geissmann *et al*, 1998; Palucka *et al*, 1998). In addition, several lines of indirect evidence support the concept that, in human skin, infiltrating monocytes may differentiate into DC. For example, phenotypic transformation of dermal monocytic cells to DC was observed in human skin after bone marrow transplantation (Murphy *et al*, 1986). Our previous work and that of others have shown that CD1$^+$ dermal DC in normal human skin express CD11b (Meunier *et al*, 1993; Gonzalez-Ramos *et al*, 1996), and that some dermal DC are positive for CD14 and CD11b but negative for CD1a (Nestle *et al*, 1993), indicating a transitional state of differentiation toward either CD14$^+$ dermal macrophages or CD1$^+$ DC. Furthermore, *in vitro*, monocyte-derived DC express a CD1c$^+$, CD11b$^+$, factor XIIIa$^+$ phenotype (Grassi *et al*, 1998), as does the dendritic APC population in human dermis (Meunier *et al*, 1993; Nestle *et al*, 1993).

These findings raised the question of whether a distinct cell differentiation process affects monocytic infiltrates in the UV-modified micromilieu of the skin, influenced by the induced presence of iC3b (Yoshida *et al*, 1998) and cytokines such as GM-CSF (Takashima and Bergstresser, 1996). We hypothesized that, under the conditions of UV-exposed skin, iC3b and subsequent β2 integrin signaling not only induces IL-10^{high} IL-12^{low} macrophage differentiation, but also inhibits monocytic precursor differentiation toward DC.

In this study, we have investigated the temporal relationship between iC3b deposition and CD1c$^+$ cell depletion in the dermis, which is the primary site of infiltration and activation of monocytic cells (Kang *et al*, 1998). The mechanism for delayed reappearance of CD1c$^+$ DC appears due, at least in part, to the ability of iC3b to inhibit cytokine-induced maturation of monocytes into early DC, including reduced expression of CD1c, CD40, CD80, and IL-12, which are characteristic phenotypic markers on mature DC.

MATERIALS AND METHODS

Subjects Healthy normal human adult volunteers on no systemic medications participated in this study after institutional review board approval of the protocol and informed consent. Punch biopsies and keratomes were taken from normal buttock skin as controls or after a single four minimal erythema dose of UVB irradiation from a bank of Westinghouse FS20 bulbs (PSC Lamps, Pittsford, NY) at different time points. This source has a continuous spectrum from 270 nm to 400 nm, with the predominant UVB emission peaking at 314 nm (Davenport *et al*, 1999).

Immunostaining microscopy studies Six-micron frozen sections of biopsies of normal and UVB-irradiated skin, after thawing and hydration in phosphate-buffered saline (PBS), were first blocked with 10% goat serum/PBS. For human iC3b tissue staining, the slides were treated with primary mouse monoclonal antibody for human iC3b (IgG2b, Quidel, San Diego, CA), or its isotype control IgG2b (Sigma, St. Louis, MO), at a dilution of 2 μg per ml in 10% goat serum/PBS for 1 h, washed with PBS three times, followed by fluorescein isothiocyanate (FITC) conjugated goat antimouse IgG2b secondary antibody (Caltag, Burlingame, CA). Slides were viewed and photographed by fluorescence microscopy. For human CD1c staining, the slides were treated with primary mouse monoclonal antibody for human CD1c (IgG1, Immunotech, Westbrook, ME), or its isotype control mouse IgG1 (Sigma), at a dilution of 1 μg per ml in 10% goat serum/PBS for 1 h, washed with PBS three times, treated with biotin-conjugated goat antimouse IgG1 secondary antibody (Caltag), then with peroxidase-labeled streptavidin (Kirkegaad & Perry, Gaithersburg, MD) for 30 min to 1 h, after washes, and detected with diaminobenzidine (Sigma). Slides were viewed and photographed using a Zeiss Axiophot microscope.

Preparation of dermal cell suspensions In addition to immunostaining, dermal cells were also isolated and analyzed by flow cytometry. Keratomes were taken and placed in Dispase (Collaborative Biomedical Products, Bedford, MA) for 20 min at 37°C. After epidermis was removed, dermal sheets were digested in collagenase, hyaluronidase, and DNase (Sigma) for 3 h, filtered through nylon mesh, and collected in PBS containing 1% fetal bovine serum (FBS), as described previously (Tse and Cooper, 1990; Kang *et al*, 1998). Dermal cell suspensions were placed in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% FBS (HyClone, Logan, UT) and penicillin/streptomycin (Life Technologies) overnight at 37°C before the following experiments.

Isolation of human blood monocytes Peripheral blood mononuclear cells were obtained by centrifugation of heparinized fresh peripheral blood with Ficoll gradients (1.077; Sigma). After incubation of peripheral blood mononuclear cells in tissue culture dishes for 1 h at 37°C, adherent cells were harvested by 0.5 mM ethylenediamine tetraacetic acid in Hanks' balanced salt solution (Life Technologies). Cells were treated with blocking antibodies (FcγRII, anti-CD32, StemCell Technologies, Vancouver, Canada) for 10 min on ice and incubated with antibody mixture (anti-CD2, anti-CD3, anti-CD19, anti-CD56, and glycophorin A, StemCell Technologies) for 15 min and with dextran–iron (StemCell Technologies) for another 15 min at room temperature, followed by adherence to a MACS separation column against a MidiMACS magnet (Miltenyi Biotec, Auburn, CA). The purity of negatively selected monocytes, as determined by flow cytometry, was $>90\%$.

Preparation of IgM- and iC3b-coated sheep erythrocytes IgM-coated sheep erythrocytes (EA) and IgM- plus iC3b-coated sheep erythrocytes (EAiC3b) were prepared as previously described with some modification (Sampson *et al*, 1991; Diamond *et al*, 1993; Yoshida *et al*, 1998). Briefly, EA were generated by incubating erythrocytes with antisheep erythrocyte IgM for 15 min at 30°C. EA were then incubated with C5-deficient human serum (Sigma) for 1 h at 37°C while rotating to convert C3 to C3b and then iC3b, which is unable to cleave C5. Any residual C3b was completely converted to iC3b by incubation with 1% fresh human serum as a fresh source of factor H and I for 1 h at 37°C (Sampson *et al*, 1991). It has been reported that on erythrocytes prepared with IgM and whole serum almost all of the C3b is converted to iC3b, and very little, if any, is degraded to C3d (Ross, 1980), which in any case is not recognized by monocytes because they lack CR2. The presence of iC3b on erythrocytes was verified by flow cytometry using antihuman iC3b or its isotype control IgG2b followed by FITC-conjugated goat antihuman IgG2b. The expression of iC3b on EAiC3b was about 50-fold increased in fluorescence intensity relative to EA.

Culture conditions Purified monocytes (2×10^6 per ml) or isolated dermal cell suspensions were incubated with RPMI 1640 supplemented with 10% FBS and penicillin/streptomycin. EA or EAiC3b (erythrocyte:monocyte or dermal cell ratio 25:1) were added in round-bottomed tubes (Becton Dickinson Labware, Lincoln Park, NJ) and incubated in a 5% CO_2 incubator at 37°C for 2 h. For monocytes, the addition of human recombinant GM-CSF (Endogen, Woburn, MA) was performed in some tubes and incubated for 40 h for CD40 staining, and for 3 d for CD1c, CD14, CD80, CD86 staining. For anti-CD11b blocking studies, cells were preincubated with purified heat-aggregated (for 30 min at 65°C) human IgG (2.5 mg per ml, Sigma) for 30 min at 4°C, and then with anti-CD11b (clone 44, BD Pharmingen, San Diego, CA, 100 μg per ml) or isotype control (mouse IgG1, BD Pharmingen) for 30 min at 4°C before addition of sheep erythrocytes. For dermal cells, GM-CSF (500 U per ml) was added and cells were incubated for 3 d. Prior to flow cytometry, sheep red blood cells were lyzed with lysis buffer (NH_4Cl, $KHCO_3$) for 10 min on ice. For double color flow cytometry, cells were incubated with GM-CSF (500 U per ml) with or without IL-4 (50 U per ml, Endogen) for 3 d. After sheep red blood cells were lyzed, cells were further cultured with GM-CSF with or without IL-4 for an additional 3 d.

Flow cytometry The cells either from dermis or after culturing were first incubated with purified heat-aggregated human IgG (2.5 mg per ml, Sigma) for 30 min at 4°C. Cells were then directly stained with FITC-conjugated anti-CD14 (Caltag), FITC-conjugated anti-CD40

(BD Pharmingen), phycoerythrin (PE) conjugated anti-CD80 (BD Pharmingen), PE-conjugated anti-CD86 (Ancell Corporation, Bayport, MN), or antigen-presenting-cell-conjugated anti-HLA-DR (Caltag), and their appropriate isotypes were used as controls. For CD1c staining, the cells were treated with primary mouse monoclonal antibody for CD1c followed by FITC/PE-conjugated goat antimouse IgG1 secondary antibody (Boehringer Mannheim, Indianapolis, IN; Caltag). For anti-CD11b blocking study, the cells were stained with anti-CD1c antibody (ICN Biomedicals, Aurora, OH) and PE-conjugated goat antimouse IgG2a secondary antibody (Caltag). The stained cells were analyzed by flow cytometry using WinList software (Verity Software House, Topsham, ME). Data were expressed as percentage positive staining cells after subtraction of cells in the same gate stained with isotype controls, or as delta mean channel fluorescence intensity (dMFI). dMFI was calculated as mean fluorescence of isotype staining subtracted from staining with specific antibody.

Cytokine enzyme-linked immunosorbent assay (ELISA) Cytokine proteins in cell supernatant were quantitated by ELISA with antibody pairs for either IL-12 or IL-10 (Endogen). The sensitivity of all ELISAs was >30 pg per ml.

Statistics Results were expressed as mean $\pm$ standard error for n repeat experiments. Statistical significance was determined by Student's t test. A p-value of <0.05 was considered significant.

RESULTS

The time course of CD1c$^+$ dermal DC disappearance/reappearance after UV reveals an inverse correlation with iC3b deposition The skin of normal human subjects was exposed *in vivo* to UVB at various time points (0–10 d) prior to biopsy, and frozen sections derived from this extended time course were stained for both iC3b and CD1c.

We first examined iC3b deposition after UV, which we hypothesized to be a regulator of CD1c$^+$ dermal cells. The results showed that iC3b deposits are increased in human skin in the papillary dermis and along the dermal–epidermal junction on day 3, and further demonstrated that iC3b deposits are faded on day 7 and are back to normal by day 10 following UV exposure (**Fig 1*A***). By contrast, non-UV control skin lacked significant iC3b deposits and isotype control staining was negative.

Meanwhile, we also examined whether the time course of iC3b deposition correlated with the time course of reappearance of CD1c$^+$ dermal DC following UV exposure. As shown in **Fig 1(*B*)**, CD1c$^+$ cells are prominent in the papillary dermis of normal control skin. Even though this UV exposure protocol induces a marked infiltration of monocytic cells between 6 h and 3 d after exposure (Kang *et al*, 1998), 3 d after UV exposure CD1c$^+$ cells are exceedingly difficult to visualize. It is not until day 7 that CD1c$^+$ cells are replenished in the papillary dermis, concordant with the time course of disappearance of iC3b in this microanatomic area.

To quantitate these findings, flow cytometry was performed on dermal cells isolated from keratomes from non-UV skin, and skin at days 3, 7, and 10 following UV exposure. Consistent with the immunostaining results, the percentage of CD1c$^+$ cells in a representative subject's dermal cell suspensions was reduced from 5.5% in normal control skin to 2.1% on day 3, and recovered to 4.7% on day 7 and 8.2% on day 10 following UV exposure (**Fig 2*A***), after subtracting the matching isotype controls. Aggregated data from four separate individuals (**Fig 2*B***) demonstrated that the reduction of dermal CD1c$^+$ cells on day 3 ($1.6\% \pm 0.9\%$) was statistically significant compared with non-UV skin ($4.3\% \pm 0.9\%$, $p = 0.01$). The reappearance of CD1c$^+$ dermal cells at day 7 ($5.2\% \pm 1.6\%$, $p = 0.036$) and day 10 ($7.6\% \pm 1.2\%$, $p = 0.008$) was also statistically significant relative to day 3. In addition, when gated on HLA-DR$^+$ cells, the percentage of CD1c$^+$ cells showed the same pattern of the time course with a higher percentage (non-UV, $23.1\% \pm 4.9\%$; day 3, $6.7\% \pm 1.8\%$; day 7, $23.1\% \pm 8.8\%$; day 10, $35.5\% \pm 3.4\%$).

Thus, despite the presence of numerous monocytic cells in the dermis after UV, CD1c$^+$ dermal DC recovery from depletion in the papillary dermal–epidermal junction area is spatially and temporally correlated to the reciprocally regulated iC3b deposition in this microanatomic location following UV exposure.

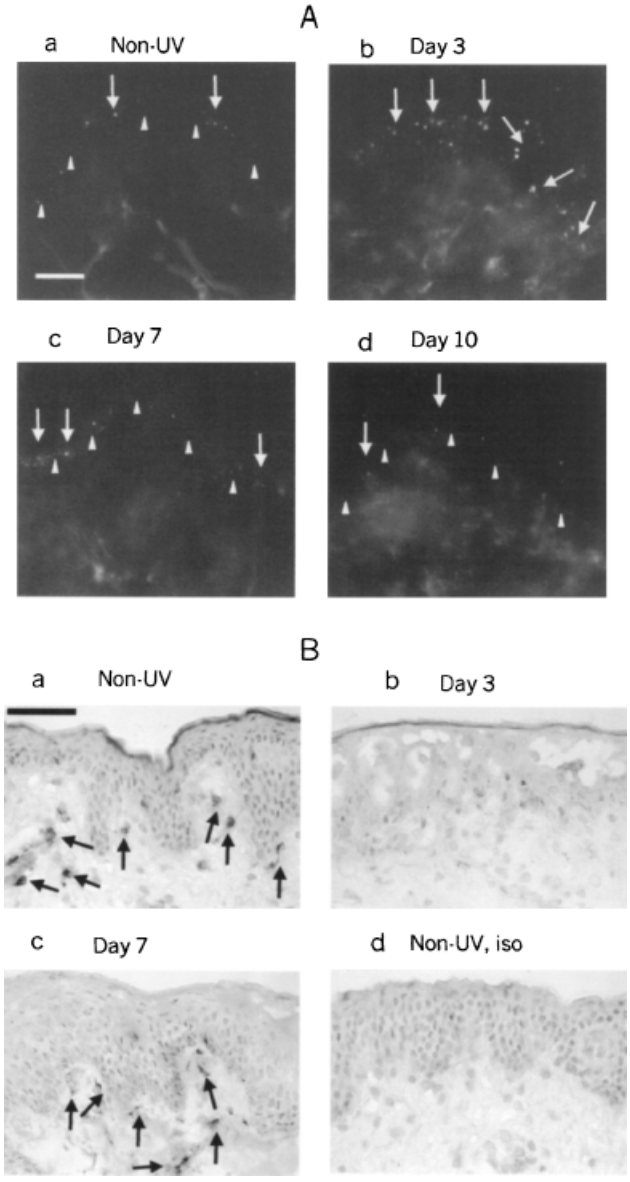


Figure 1. Time course of iC3b deposition and CD1c$^+$ dermal dendritic APC disappearance/reappearance reveals an inverse correlation. (*A*) Fluorescence immunostaining of skin biopsies. Non-UV skin (*a*), and skin taken at day 3 (*b*), day 7 (*c*), and day 10 (*d*) following UV exposure. *White arrowheads* show the dermal–epidermal junction (*a*, *c*, *d*). *White arrows* show iC3b deposits at the dermal–epidermal junction. *Scale bar*: 10 μm. $n = 2$. (*B*) Non-UV skin (*a*), and skin taken at day 3 (*b*) and day 7 (*c*) following UV exposure were stained with anti-CD1c monoclonal antibody or its isotype control (day 3, *d*). *Black arrows* show CD1c$^+$ cells. Dermal CD1c$^+$ cells were depleted on day 3, and recovered to normal levels by day 7 following UV exposure. *Scale bar*: 40 μm.

Dermal cells from a keratome biopsy showed that iC3b inhibits CD1c-expressing HLA-DR$^+$ cells We hypothesized that iC3b in the papillary dermis was modifying the monocytic immigrants in the dermis from differentiating into CD1c$^+$ DC. Dermal cell suspensions were isolated from keratomes 6 h after UVB exposure, when infiltration of monocytic precursors has begun but iC3b deposits have barely appeared (Kang *et al*, 1998; Yoshida *et al*, 1998). The cells were incubated with EA or EAiC3b

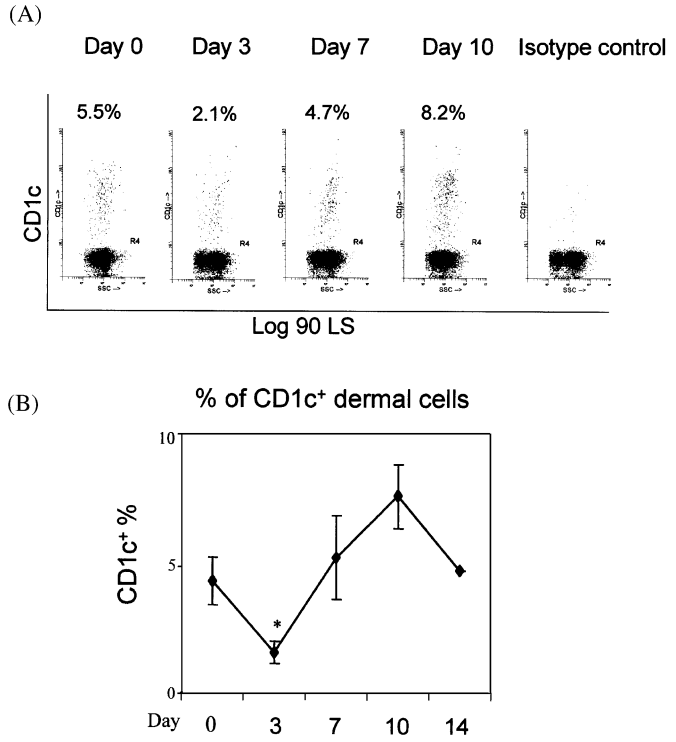


Figure 2. Time course of the percentage of dermal CD1c$^+$ cells following UV exposure determined by flow cytometry. (*A*) Representative flow diagrams of CD1c expression on dermal cells. Dermal cell suspensions were isolated from keratomes taken at 3, 7, and 10 d after UV exposure or from keratomes of non-UV-exposed skin. Cells were stained with anti-CD1c monoclonal antibody or with the isotype-specific controls and analyzed by flow cytometry. Percentages of stained cells after subtracting the isotype background are given for the circles. (*B*) Results are expressed as the mean percentage of CD1c$^+$ cells $\pm$SEM. *p-values are 0.01, 0.036, 0.008, respectively, for day 3 compared with non-UV, day 7, and day 10 (paired *t* test) ($n = 4$ subjects).

in the presence of the CD1c inducer GM-CSF for 3 d, and analyzed by flow cytometry. Incubation with EAiC3b diminished the percentage of CD1c$^+$ cells on HLA-DR$^+$ cells relative to the EA control in two out of two subjects. A representative experiment is shown in **Fig 3**. The mean change of CD1c$^+$ cells in EAiC3b relative to EA was 17%. These data suggested that iC3b can affect dermal monocytic cell differentiation into CD1c$^+$ precursor cells.

iC3b inhibits upregulation of CD1c on monocytic precursors of DC and macrophage via a CD11b-dependent mechanism The complexity of the mixture of cells in human dermis and yield limitations prompted us to model monocyte immigrant responses using blood monocytes. Because GM-CSF is effective in inducing CD1c and downregulating CD14 on monocytes (Kasinrerk *et al*, 1993), we have used iC3b along with short-term exposure to GM-CSF to explore the effect of iC3b on monocytes undergoing differentiation to DC precursors as measured by the CD1c differentiation marker. As shown in **Fig 4(*a*)**, the addition of GM-CSF resulted in upregulation of CD1c expression (**Fig 4*a***, *top panel vs second panel*) whether expressed as dMFI (51.0 ± 6.4) or as percentage of CD1c$^+$ cells ($63.3\% \pm 9.3\%$; **Fig 4*b***) ($n = 6$ subjects). In contrast, monocytes cultured without GM-CSF minimally expressed CD1c, either as dMFI (6.6 ± 2.4; **Fig 4*a***, *top panel*), or as a percentage of CD1c$^+$ cells ($4.6\% \pm 3.1\%$; **Fig 4*b***). This upregulation of CD1c was comparable to the results when the cells were incubated with EA (**Fig 4*a***, *third panel*) (dMFI $= 42.7 \pm 5.1$; $59.3\% \pm 8.8\%$ positive; **Fig 4*b***). In contrast, when cultured with EAiC3b in the presence of GM-CSF, CD1c expression and the percentage of

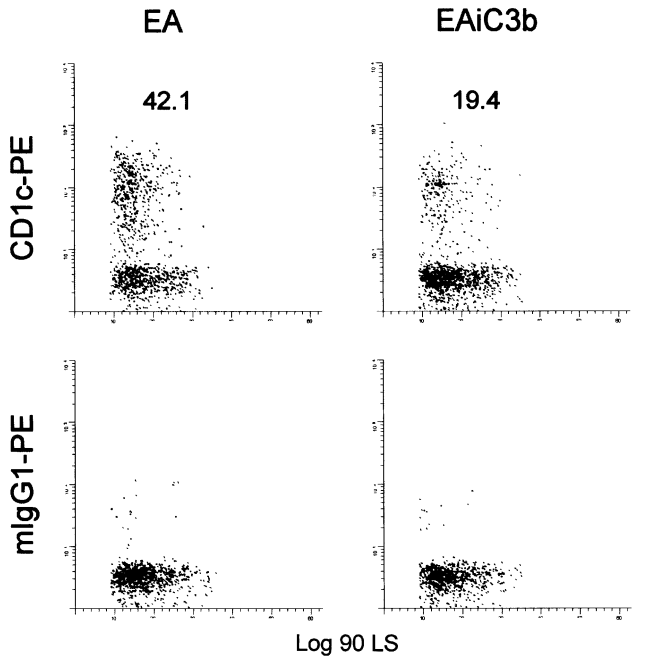


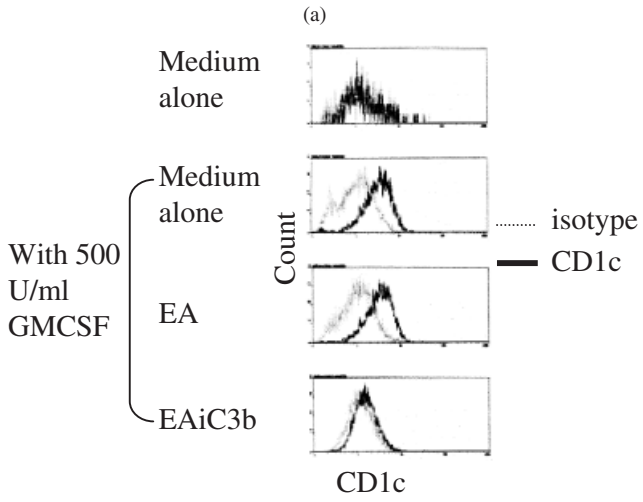
Figure 3. iC3b inhibits CD1c expression on HLA-DR$^+$ dermal cells. Isolated dermal cell suspensions from skin, 6 h post-UV exposure to stimulate an early influx of monocytes *in vivo*, were incubated with EA or EAiC3b (erythrocyte:dermal cell ratio 25:1) for 2 h, followed by incubation with GM-CSF (500 U per ml) for 3 d. Cells were analyzed by flow cytometry. A representative experiment demonstrating the percentage of CD1c$^+$ cells on HLA-DR$^+$ cells after subtracting the isotype background is shown within the gated areas.

CD1c$^+$ cells were markedly downregulated (**Fig 4*a***, *bottom panel*) (dMFI $= 17.6 \pm 3.8$; $28.4\% \pm 8.5\%$; **Fig 4*b***). There was no difference in yield of cells between EA and EAiC3b.

To verify whether this effect is iC3b–complement receptor type 3 ligation specific, we performed antibody blocking studies using anti-CD11b antibody, clone 44, in which the antibody binding site overlaps with that of iC3b (Zhang and Plow, 1999). A preliminary experiment confirmed that clone 44 blocked rosette formation of monocytes with EAiC3b (80% inhibition, data not shown). Monocytes were preincubated with clone 44 or isotype control antibody, followed by incubation for 3 d with EA or EAiC3b in the presence of GM-CSF. As shown in **Fig 4(*c*)**, pretreatment with clone 44 restored CD1c expression inhibited by iC3b, indicating that the effect of iC3b on DC differentiation is specifically inhibited by antibody to its receptor on monocytes, CD11b.

iC3b differentiates monocytes *in vitro* to a CD1c$^-$CD14$^{+/-}$ non-DC monocytic population, which retains the capacity to mature into CD1c$^+$ DC upon IL-4 stimulation Double color flow cytometry has been employed to further analyze the transitional differentiation of monocytes under the influence of cytokines and iC3b. The results revealed that incubation of monocytes (93.1% CD1c$^-$CD14$^+$; **Fig 5**, *left panel*) with GM-CSF induced maturation of cells expressing the CD1c DC marker and lacking the CD14 macrophage marker (59.8% CD1c$^+$CD14$^-$; **Fig 5**, *lower middle panel*). Exposure to iC3b blocked the maturation of CD1c$^+$CD14$^-$ cells to 39.9% (**Fig 5**, *upper middle panel*). Exposure to iC3b also blocked the appearance of CD1c$^+$CD14$^+$ cells, another dermal dendritic APC phenotype, from 26.3% to 16.2% (**Fig 5**, *middle panels*). Conversely, iC3b induced an almost 4-fold increase in cells expressing a monocytic/macrophagic non-DC phenotype (CD1c$^-$CD14$^+$) from 3.3% to 14.1% (**Fig 5**, *middle panels*).

We next asked whether the CD1c$^-$CD14$^{+/-}$ population, which was expanded by iC3b exposure, is differentiated to the point where DC differentiation is no longer possible, or whether these



(b)

	% of CD1c$^+$ cells	dMFI
M	4.6± 3.1	6.6± 2.4
M+ GMCSF	63.3± 9.3	51.0± 6.4
M+EA+GMCSF	59.3± 8.8	42.7± 5.1
M+EAiC3b	28.4± 8.5*	17.6± 3.8*

(c)

	% of CD1c$^+$ cells	dMFI
EA	32	13.4
EAiC3b	19.1	7.6
EAiC3b+isotype	21.2	8.4
EAiC3b+anti-CD11bAb	30.1	11.9

Figure 4. Inhibition of CD1c expression on monocytes by iC3b; reversal by anti-CD11b I region antibody. (*a*) Purified monocytes were incubated with medium alone (M), EA, or EAiC3b (erythrocyte:monocyte ratio 25:1) for 2 h, followed by incubation with or without GM-CSF (500 U per ml) for 3 d. Cells were analyzed by flow cytometry. *Dark lines*: cells were stained with anti-CD1c monoclonal antibody and with secondary antibody. *Light lines*: cells were stained with mouse IgG1 isotype controls and with secondary antibody. The *y* axis represents the number of cells at a given fluorescence intensity (*x* axis). (*b*) Purified monocytes were incubated as described in (*a*); data are expressed as the mean of the percentage of CD1$^+$ cells ± SEM and dMFI ± SEM of six separate experiments from six individuals. *$p < 0.05$ *vs* M + GM-CSF or EA + GM-CSF. (*c*) EA: GM-CSF-stimulated monocytes exposed to EA only. EAiC3b: GM-CSF-stimulated monocytes exposed to iC3b on EA. EAiC3b + isotype: Incubation with human IgG for 30 min, then addition of mIgG1 isotype control antibody for 30 min, preceded by GM-CSF and iC3b exposure. EAiC3b + anti-CD11b Ab: Incubation with human IgG for 30 min, then addition of anti-CD11b antibody (clone 44), preceded by GM-CSF and iC3b exposure. Cells were analyzed by flow cytometry using mIgG2a anti-CD1c and PE-conjugated secondary antibody. Data for this subject are expressed as both the percentage of CD1c$^+$ cells in total cells and dMFI.

cells are only temporarily blocked and still contain DC precursor capabilities. Because IL-4 has been shown to differentiate monocytes more effectively into DC together with GM-CSF (Pickl *et al*, 1996; Palucka *et al*, 1998), we used IL-4 as a DC differentiation signal that could model intracutaneous signals *in vivo* (i.e., mast cell IL-4) that result in DC recovery after iC3b deposition wanes after UV. Indeed, IL-4 + GM-CSF differentiated almost the entire population of iC3b-induced CD1c$^-$ cells into CD1c$^+$CD14$^-$ cells (91.7%; **Fig 5**, *right upper panel*). These cells morphologically expressed dendrites under microscopy. By contrast, iC3b-pre-exposed cells cultured

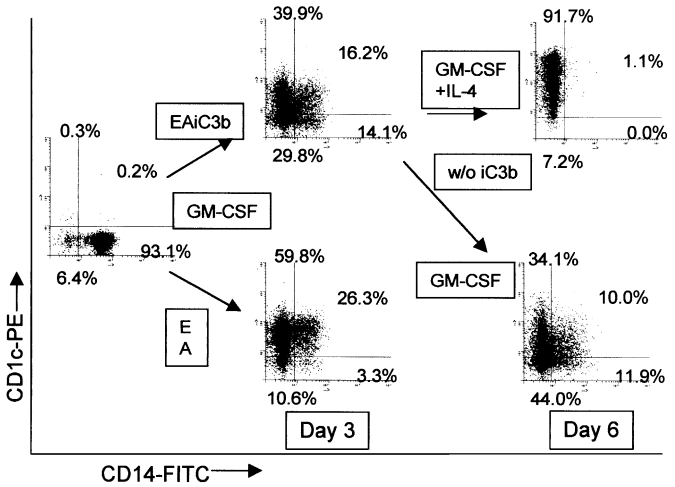


Figure 5. iC3b differentiates monocytes to a CD1c$^-$CD14$^{+/-}$ non-DC myeloid population that retains capacity to mature into CD1c$^+$ DC upon IL-4 stimulation. Purified monocytes were incubated with EA or EAiC3b (erythrocyte:monocyte ratio 25:1) for 2 h, followed by incubation with GM-CSF (500 U per ml) for 3 d. In some tubes, cells were further incubated with GM-CSF (500 U per ml) with or without IL-4 (50 U per ml) for a further 3 d. Cells were analyzed by flow cytometry. Data are displayed as two-parameter dot-blots (PE-CD1c, FITC-CD14). Markers were set according to the isotype controls. $n = 2$.

without the IL-4 DC signal, but only continued in GM-CSF, maintained large numbers of CD1c$^-$ cells, both CD14$^+$ and CD14$^-$ (11.9%, 44.0%, respectively; **Fig 5**, *right lower panel*), and did not recover the level of CD1c$^+$ cells that were induced by GM-CSF without iC3b at the onset of culture (**Fig 5**, *lower middle panel*).

Conversely, addition of IL-4 + GM-CSF at the onset of culture differentiated most of the monocytic cells into a CD1c$^+$CD14$^-$ population, whether exposed to either EA or EAiC3b (EA *vs* EAiC3b, 92.1% *vs* 90.7%; data not shown). Thus, iC3b interaction with monocytic precursors at an early stage of DC differentiation can result in an arrest of such differentiation, which can subsequently be rescued upon provision of a strong DC-inducing signal. These data are extremely consistent with the *in vivo* sequence of events in human dermis.

iC3b inhibits upregulation of the costimulatory molecules CD40 and CD80 as well on monocytes cultured with GM-CSF Because CD40 and B7 are important costimulatory molecules involved in APC functioning (Linsley *et al*, 1990; Cayabyab *et al*, 1994), and are upregulated during DC differentiation, we next extended our examination to the effect of iC3b on the expression of these costimulatory molecules by monocytes. GM-CSF was used to upregulate expression of monocyte CD40, CD80 (B7.1), and CD86 (B7.2), compared to the freshly isolated monocytes (data not shown). In each individual tested, monocytes incubated with EAiC3b in the presence of GM-CSF consistently expressed lower levels of CD80 relative to monocytes incubated with EA + GM-CSF (percentage change in dMFI relative to GM-CSF alone for EAiC3b *versus* EA was −66.9% ± 6.2% *vs* −2.7% ± 10.9%). For CD40, percentage change in dMFI relative to GM-CSF alone for EAiC3b *versus* EA was −28.1% ± 8.4% *vs* −9% ± 3.6%. By contrast, CD86 did not exhibit consistent regulation by EAiC3b (**Fig 6**). Thus CD80 and CD86 are differentially regulated by iC3b.

GM-CSF-induced monocytic cells treated with iC3b produce decreased levels of IL-12 but increased levels of IL-10 To explore if the iC3b-arrested GM-CSF-treated cells are coordinately modified in their cytokine production, we further investigated IL-12p70 and IL-10 by iC3b-treated monocytes cultured with GM-CSF. Freshly purified monocytes

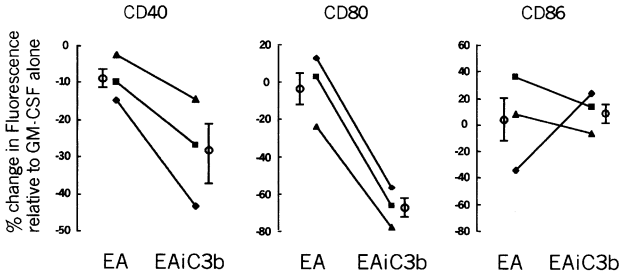


Figure 6. iC3b differentially regulates costimulatory molecules on monocytes. Purified monocytes were incubated with medium alone (M), EA, or EAiC3b (erythrocyte:monocyte ratio 25:1) for 2 h, followed by incubation with GM-CSF (10 U per ml for CD40 staining, 500 U per ml for CD80, CD86 staining). The cells were stained with anti-CD40 monoclonal antibody, anti-CD80 monoclonal antibody, anti-CD86 monoclonal antibody, or the matching isotype controls. Results are expressed as percentage change in fluorescence intensity relative to M, after subtracting the isotype controls. Each line represents different individuals.

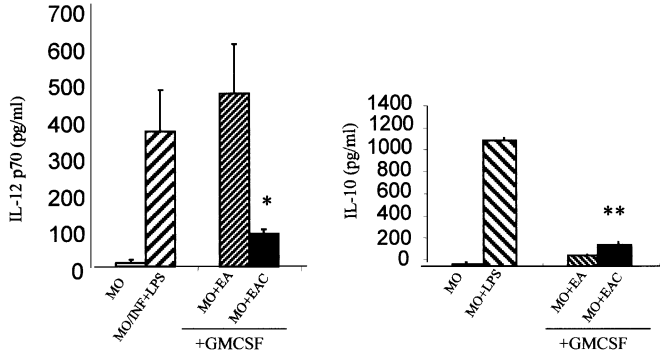


Figure 7. GM-CSF-induced monocytic cells treated with iC3b produce decreased levels of IL-12 but increased levels of IL-10. Purified monocytes were cultured as described for **Fig 4** for 72 h. Freshly purified monocytes stimulated with interferon-γ 1 ng per ml + lipopolysaccharide 1 ng per ml for IL-12 production served as controls. Supernatants were then harvested for ELISA analysis. Results are expressed as picogram per ml and as the mean ± SE. $n = 4$. *$p = 0.033$. **$p = 0.034$.

and EA- or EAiC3b-treated monocytes were cultured with GM-CSF for 3 d. ELISA analysis showed that monocytes cultured with GM-CSF (CD1c upregulated) and EA (control for EAiC3b) produced significantly higher amounts of IL-12 (471 ± 133 pg per ml, $n = 4$) relative to monocytic cells cultured with EAiC3b (iC3b-regulated, CD1c-inhibited cells, 89 ± 13 pg per ml) ($p = 0.033$) (**Fig 7**). Fresh monocytes stimulated with interferon-γ + lipopolysaccharide served as a control (366 ± 113 pg per ml). In contrast, although monocytes cultured with GM-CSF produced IL-10, the amount was low; however, EAiC3b still stimulated significantly higher IL-10 levels (187 ± 84 pg per ml) relative to EA control (100 ± 32 pg per ml) ($n = 4$, $p = 0.034$). Fresh monocytes stimulated with lipopolysaccharide served as a positive control (1141 ± 39 pg per ml). These data indicate that IL-12, a potent cytokine produced by human epidermal Langerhans cells (Kang *et al*, 1996) and dermal CD1c$^+$ DC (Kang *et al*, 1998), is downregulated in monocytic precursor cells by iC3b, in conjunction with arrest of the monocytic precursors' ability to differentiate into CD1c$^+$CD14$^-$ cells. In addition, even in this precursor condition, iC3b still plays a role in inducing IL-10, a reciprocal regulatory cytokine to IL-12.

DISCUSSION

UVB irradiation of human skin causes a shift in the dominant APC population in the dermis from CD1$^+$CD11b$^+$ DC to CD1$^-$CD11b$^+$ macrophages. This change has been largely accounted for by both a rapid disappearance of CD1c$^+$ DC and new blood monocytes entering the dermis (Meunier *et al*, 1995). The milieu encountered by the freshly immigrating monocytic population probably plays a critical role in determining whether precursor cells differentiate into DC replacements for Langerhans cells or into tissue macrophages. This study indicates that the delay between infiltration beginning 6 h after UV exposure and the day 7 reconstitution of CD1c$^+$ DC in the dermis may be due to the deposition of iC3b in the dermis at this time and its ability to arrest DC differentiation and promote CD1c$^-$CD14$^+$ macrophage phenotype expression.

We have shown that iC3b deposits are present in contiguity with infiltrating monocytic/macrophagic cells in the dermal–epidermal junction area shortly after UV exposure of human skin (Yoshida *et al*, 1998). In this time course study, we show that CD1c$^+$ dermal DC remain downregulated whereas iC3b deposition is prominent in the skin following UV exposure (resolution by day 7). Notably, upon full clearance of iC3b the percentage of dermal CD1c$^+$ cells rebounded at day 10 following UV exposure relative to that of non-UV skin, followed by stabilization to normal levels at day 14 (**Fig 2**).

Because UV preferentially induces keratinocytes to produce GM-CSF (Gallo *et al*, 1991), it is highly likely that blood monocytes entering UV-damaged skin will be exposed to GM-CSF. GM-CSF has been shown to expand DC APC in human dermis *in vivo* (Kremer *et al* 2000) and to enhance myeloid-derived APC function (Witmer-Pack *et al*, 1987; Fischer *et al*, 1988; Heufler *et al*, 1988; Chantry *et al*, 1990). Furthermore, GM-CSF alone is able to induce CD1 molecules, a specific marker for DC, and can downregulate CD14 on monocytes (Kasinrerk *et al*, 1993). The addition of iC3b to the dermal cell suspensions with GM-CSF supplement resulted in a lower percentage of CD1c$^+$HLA-DR$^+$ cells, compared to the EA control (**Fig 3**). Although human dermal cells contain a variety of monocytic cells and DC, even in this complex mixture we were able to detect an effect of iC3b in decreasing the number of CD1c$^+$ cells. This suggested that the use of fresh peripheral blood monocytes to model newly immigrated cells into UV-exposed skin would be a better model for this study and is valid for the linking of *in vitro* studies and *in vivo* dermal monocytic cells.

Indeed, in concordance with the dermal cell data, our *in vitro* results with fresh human monocytes showed that addition of GM-CSF to monocytes resulted in enhancement of CD1$^+$CD14$^-$CD40$^+$ cells (**Figs 5, 6**), which is comparable to the phenotype of dermal DC/dermal APC *in vivo* (Nestle *et al*, 1993; Meunier *et al*, 1995). Moreover, these CD1c$^+$ cells also demonstrated enhanced IL-12p70 production consistent with early DC differentiation and Langerhans cells (Kang *et al*, 1998). By contrast, incubation with iC3b in the presence of GM-CSF inhibited CD1c expression and produced significantly decreased levels of IL-12p70 production (**Fig 7**). Interestingly, in terms of IL-10, a reciprocal regulatory cytokine to IL-12, iC3b still functions to significantly induce IL-10 production by the GM-CSF-treated, CD1c-upregulated monocytic precursors, implying that the dichotomous phases of precursor cells are strongly affected by the iC3b molecule.

These findings are consistent with the decreased IL-12 production by macrophage-colony-stimulating-factor-differentiated macrophages (Smith *et al*, 1998), and with *in vivo* data demonstrating that human skin macrophages present in UV-exposed skin exhibit low IL-12 production (Kang *et al*, 1994; 1998).

DC maturation is also associated with upregulation of accessory molecules such as CD40 and B7 on DC that are critically involved in T cell activation and tolerance through the B7:CD28 and CD40:CD40L pathways (Howland *et al* 2000). Under the conditions of our study, addition of GM-CSF to monocytic cells clearly induced expression of CD40, CD80, and CD86 (Alderson *et al*, 1993; Liu *et al*, 1999). Consistent with our hypothesis, incubation of monocytic cells with EAiC3b in the presence of GM-CSF demonstrated the novel finding that iC3b not only

inhibited CD1c but CD40 and CD80 expression as well (**Fig 6**), again suggesting that iC3b binding to β2 integrin on monocytes arrests DC differentiation, and involving molecules critical for initiating immune responses. Because *in vivo* UV-induced macrophages harvested from the skin at the peak of iC3b deposition are also low expressors of costimulatory molecules (Kremer *et al*, 1998), and inhibition of iC3b after UVB reverses immune suppression and tolerance (Hammerberg *et al*, 1998), these processes may be an important mechanism of UV immunoregulation. These findings are also consistent with the *in vivo* data (**Figs 1, 2**) showing a temporal and spatial correlation between UV-induced iC3b and the shifting APC populations in the dermis after UVB irradiation.

Of interest is that iC3b differentially regulated B7 molecules on monocytic cells, in which B7.1, but not B7.2, expression was blocked. These data are consistent with our *in vivo* observation that UV macrophages are minimal for B7.1, but obviously positive for B7.2 (Kremer *et al*, 1998). Several studies have demonstrated that B7.1 and B7.2 differ in their capacity to generate Th1 *versus* Th2 responses (Freeman *et al*, 1995; Kuchroo *et al*, 1995), suggesting that an iC3b effect on monocytes may favor a Th2 response or at least diminish a Th1 response.

In summary, this study provides insight into the mechanism behind the finding that blockade of iC3b formation reverses the UV-induced inhibition of contact hypersensitivity and delayed-type hypersensitivity (Hammerberg *et al*, 1998). Thus, iC3b deposited in UV-exposed skin may actively participate in the prolonged but transient depletion of dendritic APC populations in the dermis after UV exposure, and simultaneously promote macrophage differentiation of precursors in the newly immigrating monocytic populations. These findings may be relevant to other conditions associated with complement activation, such as occur in microbial infections. Thus, we propose that UV exposure generates iC3b deposition and infiltration of monocytic cells into the dermis, including $CD1c^{-}CD11b^{+}$ monocytic precursor cells. CD11b recognition of iC3b then causes the precursor cells to be transiently arrested in their ability to differentiate into $CD1c^{+}$ DC, but to be promoted into $CD1c^{-}$ macrophage differentiation. Such macrophage differentiation at this time may be adaptively advantageous, allowing phagocytosis of UV-damaged or infected cells and generation of antimicrobial reactive oxygen species and nitric oxide. On the other hand, preexisting tissue DC already expressing CD1c would be able, at least early on before depletion, to uptake and process tumor-initiated cell neoantigens or microbial antigens, migrate to lymph nodes, and activate antigen-specific T cells. Following disappearance of iC3b, monocytic precursor cells are hypothesized to be able to begin to differentiate into $CD1c^{+}$ DC again, based upon *in vivo* observations (**Figs 1, 2**) and our *in vitro* observations (**Fig 5**). Taken together, the critical nature of iC3b and CD11b interactions in UV-induced immune suppression may be due to the ability of iC3b to transiently arrest monocytic DC precursor differentiation while simultaneously allowing or promoting macrophage differentiation in the skin.

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