Brief Definitive Report

Activated Complement Component 3 (C3) Is Required for Ultraviolet Induction of Immunosuppression and Antigenic Tolerance

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Summary

Complement component 3 (C3), a critical regulator of innate immunity, may also play a role in the regulation of cognate immunity, such as contact sensitivity responses. Because ultraviolet (UV) radiation also activates C3 in the skin, we determined whether the immunosuppressed state that results when a contact sensitizer is applied through UVB-exposed skin requires the presence and activation of C3. This question was addressed through the use of C3-deficient mice, blockade of C3 cleavage to C3b, and accelerated degradation of iC3b by soluble complement receptor 1 (sCR1). Both C3-modulated systems totally reversed the failure to induce a contact sensitivity response to dinitrofluorobenzene (DNFB) upon primary sensitization at the UV-exposed site, as well as immunologic tolerance to a second DNFB immunization through normal skin. Treatment with sCR1 reduced the infiltration of $CD11b^+$ leukocytes into the epidermis and dermis of UV-irradiated skin but did not reverse the UV-induced depletion of epidermal class II MHC^+CD11b^{lo} Langerhans cells. These data, taken together with previous results showing abrogation of locally induced UV immunosuppression by in vivo anti-CD11b treatment, suggest a novel mechanism by which ligation of the leukocyte β2 integrin, CD11b, by iC3b molecules formed from C3 activation in UV-exposed skin, modifies cutaneous $CD11b^+$ cells such that skin antigen-presenting cells are unable to sensitize in a primary immune response, but actively induce antigenic tolerance.

Complement components, especially complement component 3 (C3), have been implicated in the regulation of T cell–mediated responses (1, 2). More specifically, C3 (3), C4 (3, 4), and C5 (5) may be involved in modulating the contact sensitivity response. In addition, C3 fragments have been proposed as an inhibiting factor in the induction of contact sensitivity (3). Thus, C3 activation may also be involved in the UV-induction of an immunosuppressed state to a contact sensitizer that has been applied to UV-irradiated skin. C3, factor B, and the decay-accelerating factor have all been demonstrated to be present in the epidermis of normal skin (6). Cultured keratinocytes have also been demonstrated to be able to produce C3 (7) as well as factor B (8). In addition, the in vitro production of C3 by human keratinocytes is upregulated by the proinflammatory cytokine, TNF-α (9). UVB radiation may be capable of activating the C3 produced by keratinocytes by the alternative pathway (10). Complement activation has also been implicated in UVB-induced local inflammation without affecting the systemic complement system (11). Furthermore, the receptor for the C3 fragment iC3b, CD11b, has recently been demonstrated by in vivo antibody neutralization experiments to be involved in UV-induced immunosuppression (12), as well as in tissue injury. The cells expressing CD11b in UV-exposed epidermis and that have been shown to be critical for inducing immune tolerance are an infiltrating population of monocyte–macrophage cells.

Therefore, we hypothesize that C3 activation is critically required for UV-induction of immunosuppression to a contact sensitizer that is applied to skin exposed to a single UV dose that generates locally inducible immunosuppression. This hypothesis was tested using mice with either a genetic disruption of the C3 gene (C3-deficient mice; reference 13) or of the soluble complement receptor 1 (sCR1) blocking of C3b, and C3b formation/activation (14). Both models conclusively demonstrate that C3 and its activation is required for induction of tolerance in our UVB mouse model.

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Materials and Methods

Mice. C3H/HeN females were purchased from Charles River (Wilmington, MA). (129 × C57BL/6)F1 females were purchased from the Jackson Laboratory (Bar Harbor, ME). C3-deficient females were bred at the Harvard Medical School (Boston, MA). All mice were between the ages of 6 and 12 weeks of age. Groups of four mice were used for each panel of the contact sensitivity experiments.

Antibodies and Reagents. 2,4-dinitro-1-fluorobenzene (DNFB) was purchased from Sigma Chemical Co. (St. Louis, MO). Soluble CR1 was a gift from T Cell Sciences, Inc. (Needham, MA). Ethidium monoazide and Cascade blue–conjugated avidin were obtained from Molecular Probes (Eugene, OR). PE-conjugated rat anti-CD11b (rat IgG2b, clone M1/70) was purchased from Boehringer Mannheim (Indianapolis, IN). Biotin-conjugated mouse anti-$I\alpha^k$ (mouse IgG2b, clone 11-5.2) and FITC-conjugated rat anti–mouse Gr-1 (rat IgG2b, clone R86-8C5) were obtained from PharMingen (San Diego, CA).

UV Induction of Tolerance. Razor-shaved and chemically depilated mouse skin was exposed as previously described (15) to UVB irradiation from a band of six FS-40 fluorescent lamps from which UVC and UVB wavelengths not present in natural solar radiation have been filtered by Kodacel cellulose film (Eastman Kodak Co., Rochester, NY) (16). A single UV exposure was administered to each strain at a dose such that tolerance was induced only if DNFB was applied to the UV-exposed skin and not a non–UV-exposed distant site (low-dose, locally inducible tolerance). The UVB emission was measured with an IL-443 phototherapy radiometer (International Light, Newburyport, MA) equipped with an IL SED 240 detector fitted with a W side angle quartz diffuser and a SC5 280 filter. 48 h after UV exposure, mice were sensitized with DNFB by administering 25 ml of 0.5% DNFB in acetone/olive oil (4:1) onto the UV-exposed skin. 5 d later, the dorsal surface of the ear was challenged with 20 ml of 0.2% DNFB in acetone/olive oil (4:1). 24 h later, ear thickness was measured using an engineer's micrometer (Mitutoyo, Tokyo, Japan) and compared with ear thickness just before the challenge. Tolerance was determined by resensitizing mice on razor-shaved non–UV-exposed skin with DNFB 48 h after the primary challenge. 5 d after the secondary sensitization, mice were rechallenged with DNFB on the dorsal skin of the left ear. Ear swelling was measured immediately before and 24 h after secondary challenge. In these experiments four animals were used in each group.

Microosmotic pumps (Alzet model 1007D; Alza Corp., Palo Alto, CA) containing sCR1 or PBS were inserted into the peritoneal cavity of C3H/HeN mice 3 d before irradiation with a single UVB dose of 72 mJ/cm^2. C3-deficient mice and their (129 × C57Bl/6)F1 controls received a single UVB dose of 140 mJ/cm^2. Contact sensitizer was applied and tolerance was determined as outlined above.

Flow Cytometric Analysis. Epidermal cell suspensions were prepared in 0.25% trypsin as previously described (12). In preparation of epidermal cell suspensions 6–8 mice were used in each group of the experiment.

As previously described (15), 10^6 FcIgG receptor–blocked (anti-CD16/32, clone 2.4G2; PharMingen) epidermal cells or dermal cells obtained from skin 48 h after UV exposure were incubated with the following set of mAbs: sCR1-treated C3H/HeN mice; FITC-conjugated anti-Gr-1; PE-conjugated anti-CD11b; and biotin-conjugated anti-$I\alpha^k$. After washing, cells were incubated with Cascade blue–conjugated avidin. Control tubes were stained with matched conjugated isotype control immunoglobulins. Dead cells were identified by uptake of ethidium monoazide (15). Stained cells were fixed in 1% paraformaldehyde before flow cytometric analysis by an Epics Elite Cytometer (Coulter Electronics, Hialeah, FL). Positive cell percentages were determined by calculating the percentage of the positive cell population in terms of total viable epidermal or dermal cells and subtracting the percentage of cells found within the same positive gate in the isotype control–stained samples.

Results

Absence of C3 Prevents UV Induction of Tolerance to a Contact Sensitizer. Mice deficient in C3 due to the disruption of the C3 gene, which resulted in a mouse strain with undetectable levels of serum C3 (13), were assayed for the effect of UV upon contact sensitivity induction in a C3-deficient environment. As done previously (17), a single UVB dose was determined (data not shown) that induced tolerance only when the DNFB was applied 48 h after UV irradiation to the UV-exposed skin (locally induced tolerance) and not at a distant non–UV-exposed site (systemically induced tolerance). Based upon these criteria, a single UVB dose of 140 mJ/cm^2 for the C3-deficient mice and their (129 × C57BL/6)F1 controls was used. The absence of C3 did not affect the ability of C3-deficient mice to mount a contact sensitivity response to DNFB (Fig. 1, bar *2* from the top) that was comparable to that observed in (129 × C57BL/6)F1 controls (Fig. 1, bar *5*). By contrast, C3-deficient mice differed markedly from their (129 × C57BL/6)F1 controls; absence of C3 conferred protection from UV's ability to inhibit contact sensitization (Fig. 1, *left*, bar 6 [UV inhibition] versus 5 [no UV sensitization in C3 sufficient controls], as compared to no reduction in sensitization after UV in C3-deficient mice [bar *3* versus *2*]). Similarly, C3-deficient mice were protected against the development of tolerance to DNFB; UV-exposed C3-deficient mice were not blocked in their ability to be resensitized through normal skin (Fig. 1, *right*, bars *2* and *3*), as were normal

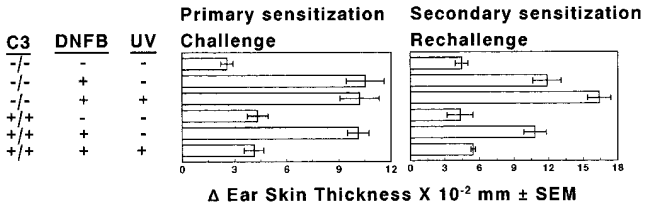

Figure 1. Failure of a single low UVB dose (140 mJ/cm^2) to induce an immunosuppressed state to a contact sensitizer applied through the UV-irradiated skin of C3-deficient mice. The contact sensitivity responses of C3-deficient mice (−/−) and their +/+ controls (129 × C57BL/6)F1 to DNFB in the absence of UV exposure are shown in the second and fifth bars from the top, respectively. The effect of a single UVB dose given 48 h before application of DNFB to the UV-exposed site on the response to DNFB by C3-deficient mice and their controls is given in the third and sixth bars from the top, respectively. The left panel shows the ear swelling response to primary sensitization through dorsal skin and challenge. The right panel indicates the response to a second sensitization through normal back skin and rechallenge. Data are expressed as the mean ± SEM difference in ear thickness between pre- and postchallenge measurements. Four mice were used in each group.

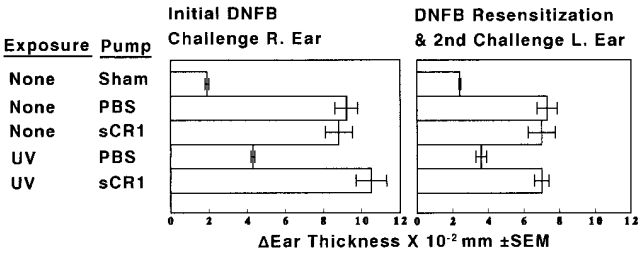

Figure 2. Reversal by sCR1 of induction of tolerance to a contact sensitizer immunized through skin of C3H/HeN mice exposed to a single UVB dose (72 mJ/cm^2). The contact sensitivity response to DNFB of mice receiving no UV with and without sCR1 treatment is given in the third and second bars from the top, respectively. The response of mice receiving UV with no sCR1 is shown in the fourth bar from the top, and with sCR1 in the fifth bar from the top. The left panel shows the ear swelling response to primary sensitization through dorsal skin and challenge. The right panel indicates the response to secondary sensitization through normal back skin and rechallenge. Data are expressed as described in Fig. 1. Four mice were used in each group.

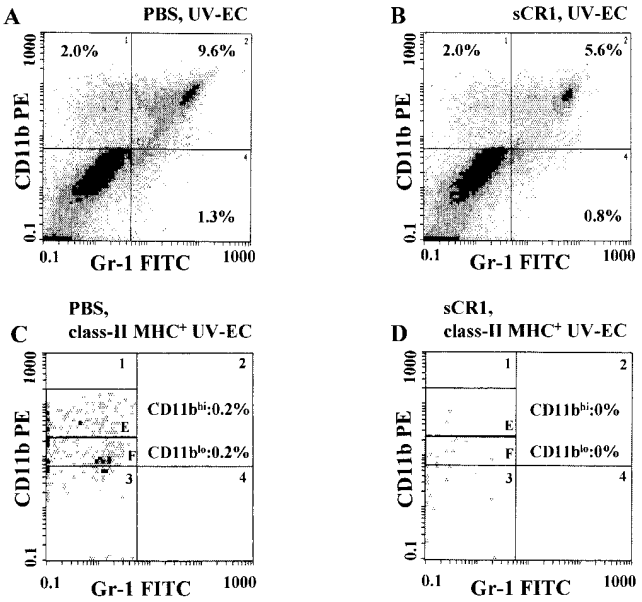

Figure 3. sCR1 treatment of UV-irradiated C3H/HeN mice partially blocks infiltration of CD11b$^+$ leukocytes but does not prevent UV-induced depletion of class II MHC$^+$CD11b^{lo}Gr-1$^-$ LC. Three-color flow cytometric analysis was used to determine the expression of CD11b and Gr-1 by epidermal cells obtained from the skin of C3H/HeN mice (*A* and *B*), 48 h after UV exposure, that had been treated with either PBS (*A*) or sCR1 (*B*). Cells were first selected based upon their viability (EMA exclusion), then analyzed for CD11b expression (x-axis represents PE intensity) and Gr-1 expression (y-axis represents FITC intensity). Cells stained with the matching antibody isotype were confined to quadrant 3. Percentage of stained cells after subtracting the isotype background are given for each quadrant. Four-color flow cytometric analysis was used to identify class II MHC$^+$CD11b^{lo}Gr-1$^-$ LC from UV-exposed epidermis of C3H/HeN mice with (*D*) or without (*C*) sCR1 treatment. Determination of the expression of CD11b (y-axis represents PE intensity) and Gr-1 (x-axis represents FITC intensity) on class II MHC$^+$ epidermal cells electronically selected by their reactivity to biotin-conjugated anti-Ia^k and avidin-Cascade blue was determined by flow cytometric analysis. For the preparation of epidermal cell suspensions six to eight mice were used in each group.

mice (Fig. 1, *right*, bars *5* and *6*). Thus, although C3 is not needed for positive immune response development in this model, C3 does appear to be critical for UV-induced downregulatory responses.

C3 Activation Required for UV Induction of Immunosuppression. We next determined whether C3 activation was required for the locally induced effects of UV upon suppression of contact sensitivity responses. C3 activation can be blocked in vivo by sCR1, which both inactivates C3 convertase (and C5 convertase) and accelerates degradation of iC3b (14, 18) and in the mouse inhibits the alternative pathway of C3 activation (19). Relative to non-sCR1–treated C3H/HeN mice (Fig. 2, bar *2* from top), sCR1 treatment did not affect the contact sensitivity response to DNFB after either the primary or secondary challenge (Fig. 2, bar *3*). We have previously demonstrated that for C3H/HeN, 72 mJ/cm^2 is sufficient to create the local skin milieu that can induce tolerance, but is not high enough to also generate systemically acting factors that can induce tolerance even upon immunization through normal skin (17). UV irradiation with 72 mJ/cm^2 did inhibit both the primary and secondary contact sensitivity response in non-sCR1–treated mice (Fig. 2, bar *4*). However, sCR1 treatment of UV-exposed C3H/HeN mice completely prevented UV injury of the skin's ability to induce a primary contact sensitivity response (Fig. 2, *left*, bar *5*). Furthermore, sCR1 fully reversed UV induction of tolerance (active suppression of ability to sensitize) (Fig. 2, *right*, bar *5*).

Effect of sCR1 Treatment on UV Modulation of Skin Leukocyte Populations. We have previously observed that treating mice with blocking antibodies to CD11b, the receptor for iC3b, blocked CD11b$^+$ leukocyte infiltration into both the dermis and epidermis of UV-exposed skin (12). Since sCR1 treatments block the UV-induced formation or stable accumulation of the CD11b ligand iC3b (data not shown), it was determined whether sCR1 treatment also affects leukocyte movement into UV exposed skin. Because skin-infiltrating leukocytes express both CD11b and Gr-1 (17), and the tolerogenic cells in UV-exposed epidermis express CD11b, class II MHC (20), and GR-1 (not shown), these markers were used to quantitate by flow cytometry the effect of sCR1 treatment on UV-induced leukocyte infiltration. The degree of UV-induced infiltrating CD11b$^+$Gr-1$^+$ leukocytes was decreased in sCR1-treated C3H/HeN in both epidermal (Fig. 3, *A* and *B*, quadrant *2*) and dermal experiments (data not shown). Changes in the other two cell populations, CD11b$^+$Gr-1$^-$ and CD11b$^-$ Gr-1$^+$ in sCR1-treated UV-exposed mice did not consistently increase or decrease. Essentially identical results were obtained in a duplicate experiment.

Four-color flow cytometry was performed to determine whether restoration of class II MHC$^+$ antigen-presenting cells is occurring in the UV-irradiated epidermis as a result of sCR1 treatment (Fig. 3, *C* and *D*). Class II MHC$^+$ epidermal Langerhans cells (LC) can be distinguished by their low expression of CD11b compared to a high expression of

CD11b by monocytes/macrophages (17). Normal levels of class II MHC$^+$CD11b^{lo} LC are ~4% (17); as a result of UV irradiation, this population drops to 0.2% in C3H/HeN (Fig. 3 *C*) mice. However, sCR1 pretreatment of UV-irradiated C3H/HeN mice did not affect the UV-induced class II MHC$^+$CD11b^{lo} LC depletion (Fig. 3 *D*, no restoration of LC in quadrant *F*). Shown is the experiment that resulted in the median value from three separate experiments of six to eight mice in each group. Similar results were observed by staining for LC with anti–class II MHC antibodies in tissue sections of skin from UV-irradiated and sCR1-pretreated plus UV-irradiated C3H/HeN mice (data not shown).

Discussion

Using two different in vivo mouse model systems in which either C3 is deficient (C3 gene disruption) or C3 is present and activation of C3 is inhibited (sCR1), it was demonstrated that C3 is essential for UV induction of an immunosuppressed state to a contact sensitizer. The UV dose used for each mouse system was such that only when the contact sensitizer was applied to the UV-irradiated skin, and not at a distant site, was tolerance induced. Using such UV doses in both C3 mouse models, reversal of UV induction of an immunosuppressed state was complete, indicating not only that C3 is required but that activation of C3 (sCR1 model) is essential. Treatment of normal mice with sCR1 did not alter their ability to generate a contact sensitivity response, indicating that sCR1 is not affecting the normal antigen-presenting cell–T cell interaction that results in contact sensitivity. Because the UV dose used was local in its effect on contact sensitization induction, this would imply that the influence of UV-induced C3 activation upon the generation of a contact sensitization response is also restricted to the skin at the site of UV irradiation.

The complement components required for the activation of C3 by the alternative pathway are all present in the skin. C3 and its activating protease, factor B, have both been detected in normal epidermis (6). In addition, production of C3 and factor B by cultured keratinocytes has been demonstrated (6–8). Cleavage fragments of C3 have also been observed in the normal epidermal basement membrane zone (21, 22). Moreover, complement deposits of C3b and C3d have been detected in and on epidermal cells within 24 h of exposure of the skin to UVB, indicating activation of C3 by UVB (10). Treatment with sCR1 eliminated the presence of C3 deposits (data not shown), indicating that the activation of C3 in the skin by UV was blocked by sCR1 (inhibiting C3 cleavage to C3b).

sCR1 treatment of UV-irradiated C3H/HeN mice resulted in a decreased, but not total inhibition of, leukocyte infiltration into the UV-irradiated skin and little restoration of class II MHC$^+$ cells. Specifically, a recovery of class II MHC$^+$CD11b^{lo} LC was not observed in sCR1-treated, UV-exposed C3H/HeN mice where sCR1 treatment also restored a primary contact sensitivity response as well as blocked tolerance induction. The lack of retention of LC after UV when C3 degradation is blocked indicates the effect is not due to iC3b triggering of tolerogenic UV–LC emigration. This finding implicates the involvement of products of UV-induced C3 activation in the stimulation of the development of a tolerance-inducing antigen-presenting cell. C3a, a product of the initial cleavage of C3, is capable of activating leukocytes as measured by increased reactive oxygen species production (23). More direct evidence for the involvement of C3 fragments in modifying the development of antigen-presenting cells derives from findings that C3b, the second fragment resulting from the initial cleavage of C3, cross-links the C3b receptor (CD46), resulting in inhibition of induction of IL-12 production (24). IL-12 is critical for the successful induction of contact sensitivity and reverses UV-induced immune suppression (25–28). We have found that a deficiency of IL-12–producing cells within the draining lymph nodes of UV-exposed, contact-sensitized mice is associated with the induction of tolerance in these animals (29).

However, C3b is highly unstable and rapidly undergoes further cleavage by factor I, in the presence of factor H, resulting in the generation of the more stable product, iC3b, that binds to its receptor, CR3 (CD11b/CD18). Stimulation of leukocytes through CD11b/CD18 by iC3b results in activation of leukocytes (30). Cross-linking of CD11b integrin on leukocytes results in increased tyrosine phosphorylation (31, 32), changes in intracellular calcium levels (33–35), and increased reactive oxygen species production (33, 34, 36). Thus, C3 fragments generated in UV-irradiated skin may, through binding to their receptors, be providing a critical signal necessary for the development of an antigen-presenting cell capable of inducing T suppressor cells. Our previous data demonstrating complete blockade of UV-induced tolerance by in vivo anti-CD11b treatment and only partial blockade of CD11b$^+$ leukocyte infiltration (12) suggests the additional involvement of the CD11b molecule (iC3b receptor) in tolerance induction. This hypothesis is supported by recent publications that demonstrate cross-linking the CD11b/CD18 molecule downregulates induction of interleukin-12 by monocytes (37, 38). Furthermore, we have recently found that iC3b is capable of inducing IL-10 in human monocytes (39).

Therefore, in our model both infiltrating and indigenous CD11b-expressing antigen-presenting cells would be triggered by UV-activated C3 into a state of differentiation/activation that is insufficient to support initiation of a primary immune response, but in fact induces antigenic tolerance. However, in the absence of the iC3b/CD11b signaling the infiltrating and indigenous (at least those remaining after UV) antigen-presenting cells would acquire functionalities (IL-12, costimulatory molecules) that support initiation of a primary immune response. These results implicate a novel mechanism of immunoregulation that can be operative or manipulated in UV injury, UV tumor immunity, photosensitivity, and other cutaneous immune-mediated diseases.

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