Imiquimod, a Topical Immune Response Modifier, Induces Migration of Langerhans Cells[1]

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Langerhans cells are bone marrow derived dendritic cells that represent the major antigen-presenting cells in the skin. Langerhans cells take up and process antigen within the epidermis and present processed antigen to T lymphocyte in the regional lymph nodes and thus form an integral part of the cutaneous immune response. The cutaneous immune response can be modified by a number of pharmacologic agents, including corticosteroids, cyclosporine, and retinoids as well as physical agents, such as ultraviolet light. For the most part these agents act by suppressing immune function. A topical immune response modifier, imiquimod has been shown to enhance the cutaneous immune response. Imiquimod has anti-viral and anti-tumor effects in animal models and has been approved for the topical treatment of external genital and perianal warts in humans. The biologic activity of imiquimod in part is due to its effect as a cytokine inducer. Preliminary data suggested that imiquimod could have an effect on Langerhans cells. In order to clarify this effect on Langerhans cells, we examined Langerhans cell morphology and migration in imiquimod-treated skin. The density of Ia^+ cells decreased 2 d after treatment, falling to approximately 43% by day 10. The Ia positive in cells remaining in the skin appeared larger and more dendritic suggesting an activated state. ATPase staining of epidermal sheet confirmed the decreased number of Langerhans cells. To clarify status of Langerhans cells, the activation of B7 was examined. Activation of B7-1 or B7-2 was not detected. Imiquimod, however, did enhance Langerhans cell migration from skin to draining lymph nodes. This enhanced Langerhans cell migration was also associated with an enhanced allergic contact hypersensitivity. These results suggest that the mechanism of modulation of immune response by imiquimod is in part due to effects on Langerhans cells. ***Key words: imiquimod/Langerhans cells. J Invest Dermatol 114:135–141, 2000***

Langerhans cells are bone marrow derived, dendritic cells, which represent the major antigen-presenting cells within the epidermis. Langerhans cells take up antigen, process and present antigen to T lymphocytes in the draining lymph nodes (DLN) resulting in the initiation of the cutaneous immune response (Banchereau and Steinman, 1998). After contact with hapten, morphologic changes in Langerhans cells are seen. Langerhans cells appear to increase in size and change from the characteristic dendritic shape to a less dendritic rounded morphology (Larsen *et al*, 1990; Teunissen *et al*, 1990; Ozawa *et al*, 1996). These changes are thought to facilitate migration of Langerhans cells from skin to DLN and are indicative of activated Langerhans cells (Larsen *et al*, 1990; Kondo and Sauder, 1995) and are thought to involve downregulation of E-cadherin (Borkowski *et al*, 1994; Jakob and Udey, 1998). Following activation, Langerhans cells express a number of cell surface markers including B7, ICAM-1, and ICAM-3 and increase their expression of major histocompatibility complex (MHC) class II antigens (Shimada *et al*, 1987; Aiba and Katz, 1990; Tang and Udey, 1991; Manara *et al*, 1996; Laihira and Jansen, 1997). As Langerhans cells play a crucial part in immunosurveillance there has been considerable interest in agents that modify Langerhans cell function (Simon *et al*, 1992; Bacci *et al*, 1996; Ozawa *et al*, 1996; Qu *et al*, 1997).

Imiquimod [1-(2-methylpropyl)-1H-imidazo[4,5c]quinolin-4-amine] is an immune response modifier, which has been shown to have anti-viral and anti-tumor effects. Previous studies have demonstrated efficiency in infectious and neoplastic diseases including the treatment of primary and recurrent genital herpes simplex virus and cytomegalovirus infection in guinea pigs (Chen *et al*, 1988; Harrison *et al*, 1988, 1994). Imiquimod has been demonstrated to have anti-tumoral properties in a number of murine models, including MC-26 colon carcinoma, Lewis lung carcinoma, FCB bladder tumor (Sidky *et al*, 1992).[2] The biologic activity of imiquimod in part is due to its effect as a cytokine inducer. Imiquimod has been shown to induce interferon (IFN)-α, tumor necrosis factor (TNF)-α, interleukin (IL)-1α, IL-6, and IL-8

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Abbreviations: CHS, contact hypersensitivity; DLN, draining lymph nodes.

[1]The authors have declared a conflict of interest.

[2]Sidky YA, Bryan GT, Weeks JM, Hatcher JM, Borden EC: Effect of treatment with an oral interferon inducer, imidazoquinolinamine (R-837) on the growth of mouse bladder carcinoma FCB. *J Interferon Res* 10:S123, 1990 (abstr.)

(Sidky *et al*, 1992; Weeks and Gibson, 1997; Witt *et al*, 1993; Kono *et al*, 1994; Reiter *et al*, 1994; Gibson *et al*, 1995; Testerman *et al*, 1995) Recent clinical trials demonstrated efficacy of topical imiquimod in the treatment of external genital and perianal warts (Edwards *et al*, 1998 Tyring *et al*, 1998), and in this trial expression of CD1a in the skin was decreased. As CD1a is a marker for Langerhans cells, this decrease suggested that Langerhans cells either migrated out of the skin or were no longer expressing this molecule. We postulated that the mechanism of action of imiquimod may involve enhanced migration of Langerhans cells to the regional lymph node. To confirm this hypothesis, we utilized a murine model of Langerhans cell migration to DLN. The results of these studies demonstrated that imiquimod altered Langerhans cell morphology, enhanced Langerhans cell migration and enhanced allergic contact hypersensitivity.

MATERIALS AND METHODS

Animals C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were housed in the animal facility of Sunnybrook Health Science Center, University of Toronto. Healthy 8–12 wk old mice were used for this study. Each experimental group contained five mice. All animal protocols were approved by the Institutional Animal Care and Use Committee. Each experiment was replicated three times.

Reagents Five percent imiquimod cream and vehicle alone were prepared and supplied by 3M pharmaceuticals (St Paul, MN). Fluorescein isothiocyanate (FITC, isomer), ammonium thiocyanate, collagenase type IV, adenosine 5′-triphosphate disodium salt (ATP), ammonium sulfide, croton oil, and dibutylphthalate were purchased from Sigma (St Louis, MO). DNFB was purchased from INC Biomedicals (Aurora, OH). Murine Ia antibody (IgG1 from OX-6) was purchased from Serotec Canada (Toronto, Ontario). Biotin-conjugated rat anti-mouse IgG antibody, streptavidin-conjugated FITC, FITC-conjugated mouse anti-mouse Ia^b, phycoerythrin (PE)-conjugated rat anti-mouse CD80 (B7-1), PE-conjugated rat anti-mouse CD86 (B7-2), FITC-conjugated mouse IgM and PE-conjugated rat IgG2a were purchased from Cederlane Laboratories (Hornby, Ontario, Canada). DNAse I was purchased from Boehringer Mannheim (Lavel, Quebec, Canada). Anti-CD32/CD16 (FcγRII/III) and anti-Ia/PE were purchased from Pharmigen (San Diego, CA).

Immunolabeling of Langerhans cells To investigate whether imiquimod induces Langerhans cell migration, we needed to first examine Langerhans cell density and morphology after the topical application of imiquimod. Anti-Ia *in situ* immunolabeling was performed as previously described (Wang *et al*, 1992). Mice were divided in three groups. Six microliters of either imiquimod cream or vehicle alone was applied to the dorsum of each ear once a day for 1–7 d. Vehicle alone without imiquimod was used as a negative control. As a further control we utilized mice who did not receive either imiquimod or vehicle. Epidermal sheets were separated from the dermis using 0.5 M ammonium thiocyanate. Acetone-fixed epidermal sheets were labeled with anti-Ia in a three-step immunolabeling procedure as previously described (Wang *et al*, 1992). Briefly, the epidermal sheets were incubated with a murine anti-Ia antibody overnight at room temperature, washed with phosphate-buffered saline (PBS), and then incubated with biotin-conjugated rat anti-mouse IgG antibody IgG at 37°C for 1 h. After washing with PBS, the sheets were incubated with streptavidin-conjugated FITC 37°C for 1 h. Positively labeled cells were counted and morphology was examined under a fluorescent microscope. Ten randomly selected fields were examined, and Ia^+ cells were expressed as cells per mm^2 (mean ± SEM). All data were examined by an independent observer who was blinded to the specific experimental treatment.

Preparation of single cell suspension and flow cytometric analysis To confirm the decrease of MHC class II positive cells in epidermis and to investigate further, the effect of imiquimod on Langerhans cells by means of flow cytometric analysis was performed utilizing single cell suspension of mouse epidermis. Mice were divided in five groups. Sixty microliters of imiquimod cream or vehicle alone was applied to the shaved trunk once a day for 2 or 7 d. Vehicle alone without imiquimod was used as a negative control. As a further control we utilized mice who did not receive either imiquimod or vehicle. Single cell suspension was prepared as previously described with some modification (Wang *et al*, 1996). Briefly, epidermal sheets were separated from the dermis using 0.5% dispase incubating for 60 min at 37°C. Single cell suspension was obtained by trypsinization (0.25% trypsin/PBS) for 20 min at 37°C, then filtered through nylon mesh. Cells were immunolabeled with anti-Ia and anti-B7 (CD80 or CD86) as follows: Cells were incubated for 5 min on ice with anti-CD32/CD16 to block FcγRII/III, and then reacted with FITC-conjugated anti-Ia for 30 min on ice, then washed with 1% fetal bovine serum, 0.1% NaN_3/PBS twice. Next, cells were incubated with PE-conjugated anti-CD80 or PE-conjugated anti-CD86 for30 min on ice. For isotype control, sample stained with FITC-conjugated mouse IgM and PE-conjugated rat IgG2a was prepared. Two-color immunofluorescence of FITC and PE was analyzed by means of Coulter EPICS 75L flow cytometer (Hialeath, FL). At least 10,000 cells were analyzed.

ATPase staining of Langerhans cells The result of immunolabeling suggested either a decrease in Langerhans cell number or loss of MHC class II antigens. To determine whether or not the number of Langerhans cells decreased, ATPase staining of epidermal sheets was performed. Briefly, mice were divided in three groups. Six microliters of either imiquimod cream or vehicle alone was applied to the dorsum of each ear once a day for 7 d. Vehicle alone without imiquimod was used as a negative control. Epidermal sheets were separated from the dermis by incubation for 3 h at 37°C in 20 mM ethylenediamine tetraacetic acid in PBS. The epidermal sheets were rinsed in Tris/maleate with 8.3% sucrose buffer (pH 7.3) and fixed for 20 min at 4°C in 4% formaldehyde in PBS. Subsequently, sheets were incubated for 30 min at 37°C in 15 mg of ATP-disodium salt, 42 ml of Tris/maleate with 8.5% sucrose buffer, 5 ml of 5% $MgSO_4$, and 3 ml of 2% $Pb(NO_3)_2$. Samples were rinsed with Tris/maleate with 8.3% buffer, incubated for 10 min at room temperature in a 1% solution of ammonium polysulfide in PBS, rinsed, and mounted in glycerol. The number of $ATPase^+$ Langerhans cells per mm^2 was counted. Ten randomly selected fields were examined, and $ATPase^+$ cells were expressed as cells per mm^2 (mean ± SEM).

Assay for hapten-induced Langerhans cell migration To examine whether imiquimod application altered Langerhans cell migration from the skin to the regional lymph nodes, we utilized the fluorescent hapten FITC. Previous investigations have shown that FITC can be used both as a contact sensitizer and as an immunofluorescent marker for cells (Kurimoto and Streilein, 1992; Wang *et al*, 1996). In this study, mice were pretreated with 60 μl of imiquimod cream or vehicle alone for 3 d once a day, then painted on the shaved trunk, foot pads, and ears with 400 μl of 0.5% FITC in a 50:50 (vol/vol) acetone/dibutylphthalate mixture 12 h after last application of pretreatment. As a further control the mice receiving neither drug nor vehicle were also painted with FITC. Twenty-four hours later inguinal and axillary lymph nodes were obtained. Lymph nodes were gently disrupted and cell suspensions were prepared by filtering the disrupted tissues through nylon mesh. The stromal fragments were digested with a solution of collagenase IV (0.5 mg per ml)/DNAase I (0.02 mg per ml) at room temperature for 25 min, and again filtered through nylon mesh. LN cells were immunolabeled with anti-Ia as follows: LN cells were incubated for 5 min on ice with anti-CD32/CD16 to block FcγRII/III, and then reacted with anti-Ia/PE for 45 min on ice. Two-color immunofluorescence of FITC and PE was analyzed by means of Coulter EPICS 75 L flow cytometer. At least 10,000 cells were analyzed.

Allergic contact hypersensitivity To investigate whether Langerhans cell migration was associated with an alteration of contact hypersensitivity (CHS), we examined the effect of imiquimod on DNFB-induced contact hypersensitivity. After 3 successive days of 18 μl imiquimod or vehicle treatment (placebo), CHS was induced as described previously (Kondo *et al*, 1994a). Briefly, the mice were painted with 20 μl of 0.5% DNFB in 4:1 acetone:olive oil. As a further control, some mice that did not receive either drug or vehicle were sensitized as above. Five days later, the mice were challenged on the dorsal surface of the ears with 10 μl of 0.2% DNFB. Non-sensitized mice were challenged with 0.2% DNFB as a control for the irritation. Ear thickness was measured with a Peacock spring-loaded micrometer (Ozaki, Tokyo Japan) immediately before challenge and at 24 and 48 h after challenge.

To determine whether imiquimod enhanced irritant contact dermatitis, studies with croton oil as an irritant were preformed. Mice were divided in three groups. Six microliters of either imiquimod cream or vehicle alone was applied to dorsum of each ear once a day for 3 d. Vehicle alone without imiquimod was used as a negative control. For further control mice without chemical treatment were utilized. Contact sensitization was induced as described previously with modification (Kondo *et al*, 1996). Briefly, 10 μl of 1% croton oil in 4:1 acetone/olive oil was applied on both sides of their right ears. Vehicle solution was applied on their left ears. Ear thickness was measured with a Peacock spring-loaded micrometer in 24 h after croton oil application.

Table I. Sequences for each primer

	Upstream primers	Downstream primers
IL-6	5′-ATG AAG TTC CTC TCT GCA AGA GAC T-3′	5′-CAC TAG GTT TGC CGA GTA GAT CTC-3′
IL-10	5′-CTC TTA CTG ACT GGC ATG AGG ATC-3′	5′-TTA AAC TGT AGA AGT AGT TGA CGT ATC-3′
TNF-α	5′-ATG AGC ACA GAA AGC ATG ATC CGC-3′	5′-CCA AAG TAG ACC TGC CCG GAC TC-3′
IFN-α	5′-GAC TCA TCT GCT GCT TGG AAT GCA ACC CTC C-3′	5′-GAC TCA CTC CTT CTC CTC ACT CAG TCT TGC C-3′
IFN-γ	5′-TGC ATC TTG GCT TTG CAG CTC TTC CTC ATG GC-3′	5′-TGG ACT TGT GGG TTG TTG ACC TCA AAC TTG GC-3′
β-actin	5′-GTG GGC CGC TCT AGG CAC CAA-3′	5′-CTC TTT GAT GTC ACG CAC GAT TTC-3′

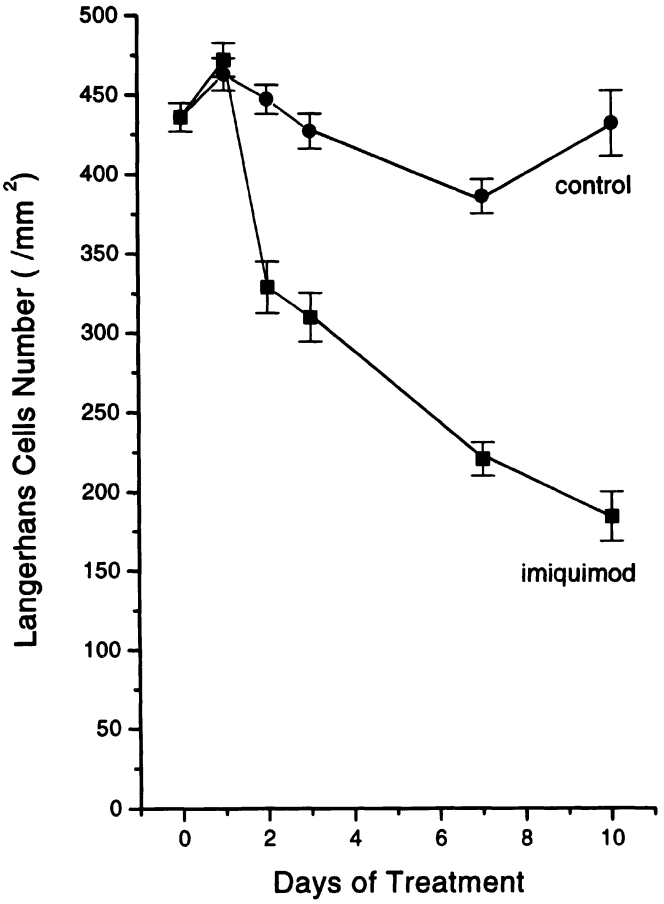

Figure 1. Effect of imiquimod on Langerhans cell density with immunolabeling. Topical imiquimod or vehicle was applied to the skin of mice and skin was obtained from 0 to 10 d post-treatment. Epidermis was separated from dermis and stained for Ia^+ cells as described in *Materials and Methods*. A decrease in Langerhans cell density was noted 2 d after treatment ($p < 0.05$) falling to approximately 43% of control by day 10 (184 ± 16 *vs* 432 ± 21 cells per mm^2, $p < 0.05$).

RNA extraction and reverse transcription In the course of the challenge phase of allergic contact sensitization, a number of cytokines are known to be induced (Enk and Katz, 1992; Kondo and Sauder, 1995). In addition, imiquimod is a known cytokine inducer (Harrison *et al*, 1988; Witt *et al*, 1993; Kono *et al*, 1994; Testerman *et al*, 1995). To determine whether imiquimod further enhanced expression of cytokines during contact sensitization, reverse transcriptase–polymerase chain reaction (reverse transcriptase–PCR) was performed. The cytokines evaluated were TNF-α, IFN-γ, IL-6, IL-8, and IL-10. Twenty-four hours after challenge, samples from the ears were collected from the mice and placed in an aluminum mortar containing a small amount of liquid nitrogen. Then samples were carefully ground to a fine powder and lysed in RNA Stat-60 (TEL-TEST "B", Friendswood, TX). Total RNA was extracted according to manufacturer's instructions. Reverse transcription was performed by standard methods with some modifications (Kono *et al*, 1994). One microgram of total RNA dissolved in 10 μl of water containing 10 U of RNasin (Pharmacia, Lavel, Quebec, Canada) and 1 μg of oligo-dT-primers (Pharmacia) was heated to 65°C for 5 min, then chilled on ice. This solution was mixed with reverse transcriptase buffer [final concentration, 60 mM KCl, 50 mM Tris (pH 8.3), 3 mM $MgCl_2$] (Gibco BRL, Burlington, Ontario, Canada), 10 mM dithiothreitol (Gibco BRL), and 10 mM of each dNTP (Boehringer Mannheim, Dorval, Canada) and 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL) to total volume 20 μl. After 1 h incubation at 37°C, the reaction was stopped by heating at 95°C for 5 min.

PCR This was performed as described previously (Kondo *et al*, 1996). Primer sets for β-actin and IFN-α were purchased from Clontech Laboratories (Palo Alto, CA). Primers for murine IL-6, IL-10, TNF-α, and IFN-γ were synthesized at Dalton Chemical Laboratories INC. (Toronto, Ontario, Canada). The sequences for each primer sets were as shown in **Table I**.

Specific cDNA obtained from the reverse transcriptase mixture were amplified in a total volume of 10 μl containing PCR buffer [final concentration on 50 mM KCl, 10 mM Tris–HCl (pH-8.3), 1.5 mM $MgCl_2$] (Pharmacia) 200 mM each of all four dNTPs, 10^{-6} M tetramethylammonium chloride (TMAC), 20 pmol each primer, and 0.25 U of Taq DNA polymerase (Pharmacia). The mixture was overlaid with 15 μl of mineral oil. PCR cycles were performed in a Perkin-Elmer Cetus Thermal Cycler 480 (Perkin-Elmer/Cetus, Norwalk, CT) with denaturation for 1 min at 95°C, annealing at 60°C for 45 s and extension for 30 s at 72°C. Optimal PCR cycle numbers were determined for each gene product, respectively.

Analysis of PCR products An aliquot (5 μl) of each PCR product was loaded on to 1.5% agarose gels containing ethidium bromide and electrophoresed in Tris-acetate buffer for 10–20 min at 100 V. The gels were photographed under ultraviolet light. Negative films were scanned by means of a laser densitometer (KLB 2222–020, ULTRASCAN XL, Pharmatica) for semiquantitation of c-DNA. As β-actin was unaffected by imiquimod treatment, densitometric values of each cytokine were normalized to that of β-actin.

Statistical analysis All data were expressed as mean ± SEM. The statistical significance of differences between the means was determined by applying the Student's t test (two tails). A difference was considered to be statistically significant if $p < 0.05$.

RESULTS

Imiquimod alters the density and morphology of Ia^+ Langerhans cells Immunolabeling of the epidermal sheets demonstrated a decrease in Ia^+ dendritic cells (Langerhans cells) at 48 h after imiquimod treatment (imiquimod-treated group, 329 ± 16 per mm^2; control, 427 ± 11 per mm^2, mean ± SEM). This decrease in density persisted throughout treatment, decreasing to approximately 43% of control by day 10 (**Fig 1**). The Langerhans cells remaining in the skin following imiquimod treatment of mice appeared larger and more dendritic compared with Langerhans cells from control mice (**Fig 2**). Moreover the expression of Ia antigen on Langerhans cells from imiquimod-treated mice appeared stronger than in control mice at day 2 and these changes persisted throughout the experiment until day 10 (**Fig 1**).

Imiquimod altered the population of Langerhans cells in epidermal cells, but did not activate costimulatory factor To confirm the decrease of MHC class II/Ia positive cell in epidermis flow cytometric analysis was performed utilizing single cell suspension of mouse epidermis. We also examined whether imiquimod altered expression of the costimulatory molecules B7-1 (CD80) and B7-2 (CD86). The decrease of

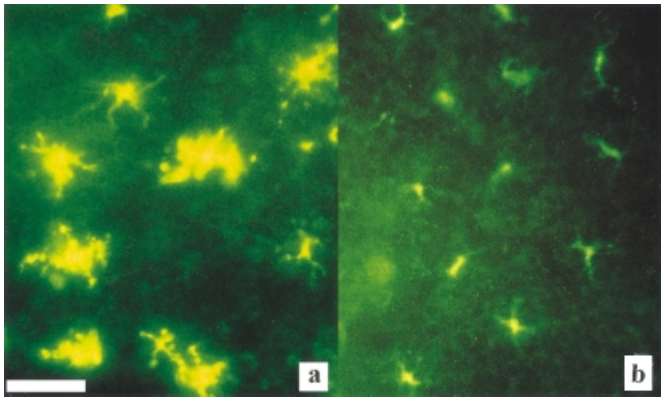

Figure 2. Effect of imiquimod on Langerhans cell morphology. Topical imiquimod or vehicle was applied to the skin of mice, then skin was obtained from 0 to 10 d post-treatment. Epidermis was separated from dermis and stained for Ia^+ cells as described in the *Materials and Methods*. A representative photomicrograph from day 10 is shown. Langerhans cells in the imiquimod-treated group appeared larger with brighter Ia antigen staining (*a*) compared with control (*b*); magnification × 400. These changes were seen from day 2 to day 10. *Scale bar*: 50 μm.

MHC class II/Ia positive cells in epidermal cells and the expression of B7-1 and B7-2 were examined by means of two-color flow cytometry. In the imiquimod-treated group 1.8% of epidermal cells were Ia positive after 7 d of treatment compared with 3.2% in the placebo-treated group. Neither imiquimod treatment nor placebo treatment increased expression of costimulatory factors, B7-1 and B7-2 (**Fig 3**).

Imiquimod alters the density of $ATPase^+$ Langerhans cells To confirm the data from immunolabeling of a decrease in Langerhans cell number, ATPase staining of epidermal sheets was performed. $ATPase^+$ dendritic cells (Langerhans cells) decreased by 61% after imiquimod treatment (imiquimod-treated group, 150 ± 7 per mm^2; control, 247 ± 10 per mm^2, mean ± SEM) (**Fig 4**).

Imiquimod enhances Langerhans cell migration from skin to DLN After demonstrating a decrease in Langerhans cells in imiquimod-treated skin, we hypothesized that this decrease was due to enhanced Langerhans cell migration from skin to DLN. To test this hypothesis, we assessed FITC-induced Langerhans cell migration following imiquimod treatment (Macatonia *et al*, 1987; Kurimoto and Streilein, 1992; Wang *et al*, 1992). The uptake and transport of FITC by Langerhans cells was traced by fluorescence. Twenty-four hours after FITC painting (following imiquimod treatment), the draining LN were collected and cell suspensions were prepared. LN cells were immunolabeled with anti-Ia/PE, then analyzed by two-color FACS analysis. In the imiquimod-treated groups, the frequency of Ia^+, FITC bearing cells was significantly higher than the vehicle alone treated group (**Fig 5**) ($4.2 \pm 0.628\%$ *vs* $2.3 \pm 0.691\%$, mean ± SEM, $p < 0.05$) thus demonstrating that imiquimod significantly enhanced Langerhans cell migration from skin to DLN.

Imiquimod enhances contact hypersensitivity but did not alter the response of irritant contact hypersensitivity To determine if the enhanced Langerhans cell migration was translated into an enhanced CHS response, we examined the effect of imiquimod on DNCB-induced allergic contact hypersensitivity. Ear swelling was significantly enhanced at 24 h after challenge in mice treated with imiquimod 24 h before sensitization compared with the control group ($p < 0.01$) (**Fig 6*A***). CHS was 61% higher in imiquimod-treated animals than the control group.

To determine the role of imiquimod on irritant contact dermatitis response, mice were treated with 0.1% croton oil after imiquimod or placebo treatment. Unlike our results demonstrating enhanced allergic contact dermatitis, imiquimod did not enhance irritant contact dermatitis (**Fig 6*B***).

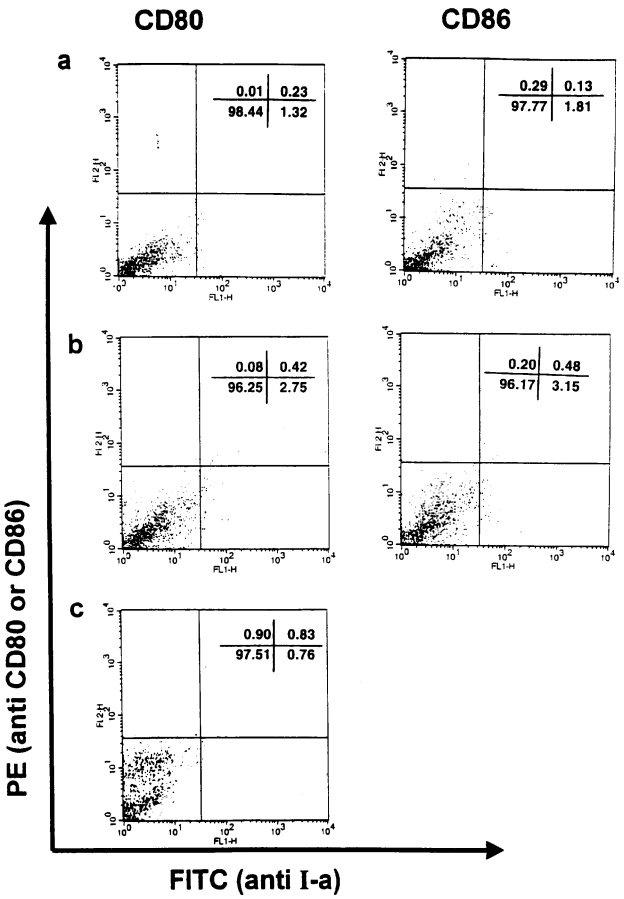

Figure 3. Imiquimod decreased the number of Langerhans cells in epidermis but did not activate expression of B7. Mice were treated with imiquimod or vehicle alone for 7 d then single cell suspension was prepared. Cells were immunolabeled with anti-Ia and anti-B7 (B7-1/CD80 or B7-2/CD86) using a two-color immunofluorescence of FITC and PE. A decreased number of Langerhans cells was observed in the 7 d imiquimod-treated group (*a*) compared with the placebo-treated group (*b*). Flow cytometric gate was chosen so that the isotype control had ⩽ 0.1% positive cells 9c). No change was seen in B7-1 and B7-2 expression with imiquimod treatment.

Imiquimod upregulates cytokines mRNA expression To determine if the above enhanced CHS was associated with enhanced cytokine expression, reverse transcriptase–PCR was performed. For relative quantitation, the densitometry values for each cytokine were normalized to that of the β-actin, which was not affected by DNFB or imiquimod treatment. Twenty-four hours after challenge, mRNA expression of IL-6, IL-10, IFN-α, IFN-γ, and TNF-α of challenge site were upregulated in both groups. In the mice treated with imiquimod, however, cytokine levels were significantly higher than vehicle-treated group (**Fig 7*A*, *B***).

DISCUSSION

The immune response modifier, imiquimod, has anti-viral and anti-tumor effects. Anti-viral activity has been shown in several animal models including herpes simplex virus and cytomegalovirus in guinea pigs (Chen *et al*, 1988; Harrison *et al*, 1988, 1994). Anti-tumor effects of imiquimod have also been demonstrated. Imiquimod is able to inhibit the growth of murine colon carcinoma and bladder carcinoma (Sidky *et al*, 1992).[2] Whereas the exact mechanism of action of imiquimod is unclear, it is known that imiquimod induces a number of cytokines including IL-1, IL-2, TNF-α, IFN-α, and PGE_2 (Kono *et al*, 1994; Testerman *et al*, 1995). Imiquimod upregulates the production of TNF-α, IFN-α,

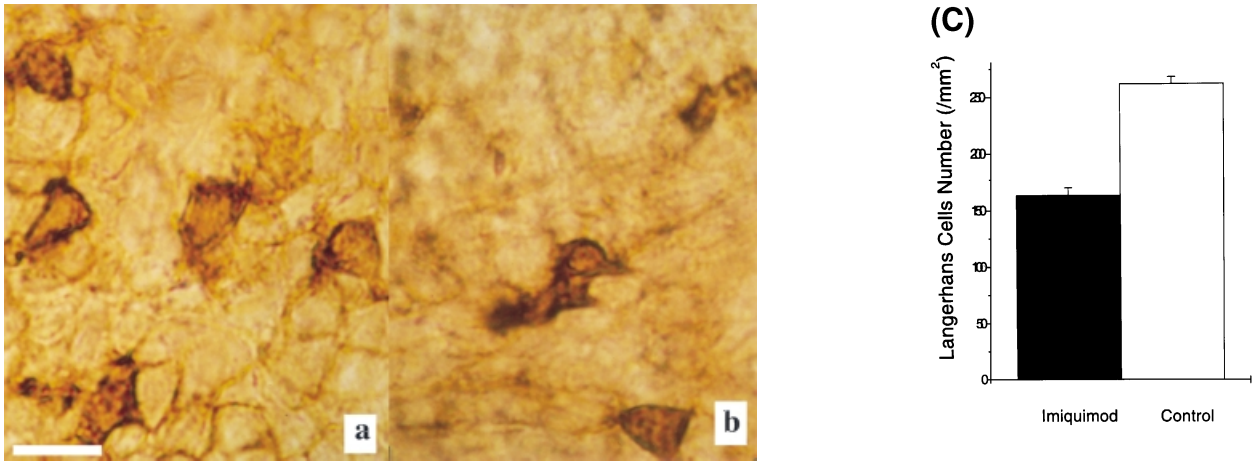

Figure 4. Imiquimod reduces Langerhans cell density. (*a*, *b*) Effect of imiquimod on Langerhans cell density by ATPase staining. Imiquimod or vehicle alone was applied to dorsum of each ear once a day for 7 d. Epidermis was separated from dermis and stained for ATPase$^+$ cells (Langerhans cells) as described in the *Materials and Methods*. A significant decrease in the density of Langerhans cells was observed in the imiquimod-treated group (*a*) compared with control (*b*); magnification $\times 400$. *Scale bar*: 50 μm. (*c*) Langerhans cells were enumerated by examining at least 10 random fields in each group. Langerhans cell density decreased by approximately 61% compared with control (150 ± 7 *vs* 247 ± 10 cells per mm^2, $p < 0.05$).

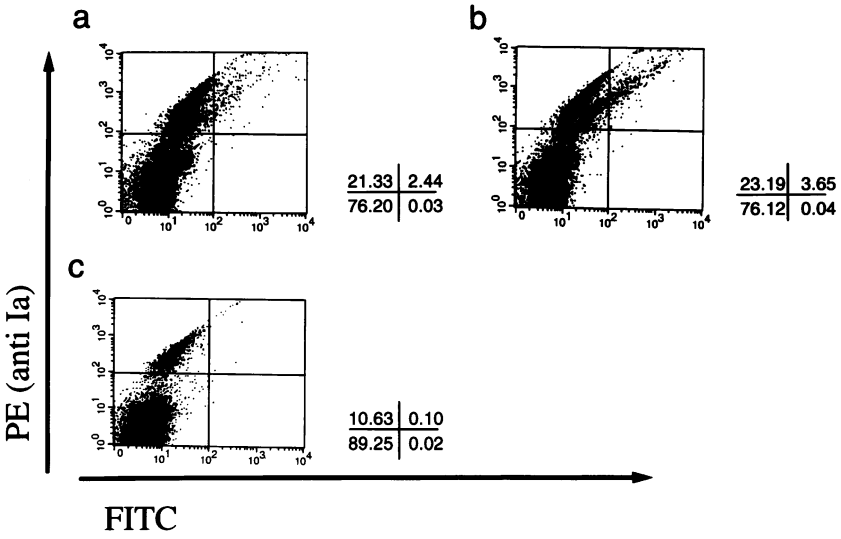

Figure 5. Effect of imiquimod on Langerhans cell migration. Mice were treated with topical imiquimod or vehicle alone, then FITC was applied epicutaneously. Twenty-four hours later DLN were obtained and single cell suspensions were prepared, immunolabeled with anti-Ia/PE and then analyzed for PE/FITC-labeled cells as described in *Materials and Methods*. Enhanced Langerhans cell migration was seen in the imiquimod-treated group (*b*) compared with the vehicle-treated control group (*a*). Flow cytometric gate was chosen so that the negative control (no FITC) had $\leq 0.1\%$ positive cells (*c*).

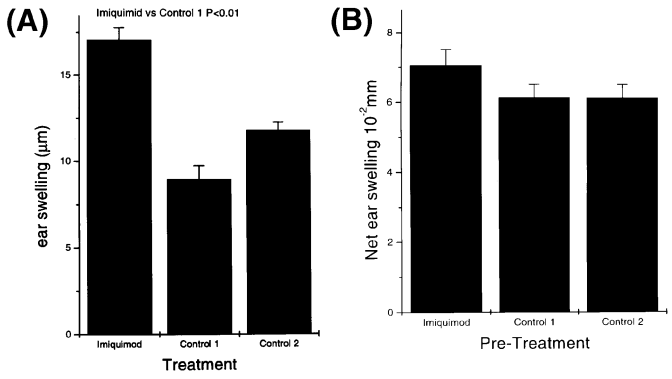

Figure 6. Imiquimod enhances allergic contact hypersensitivity. (*A*) Mice were treated with topical imiquimod or vehicle alone (control 1), and then CHS was induced by DNFB. For further control CHS was induced on the mice without chemical treatment (control 2). Mice were challenged on the dorsal surface of ears and ear thickness was measured as a manifestation of CHS (mean ± SEM). (*B*) Imiquimod did not alter irritant contact hypersensitivity. Mice were treated with topical imiquimod or vehicle alone (control 1), and then irritation was induced by croton oil. As a further negative control, irritation was induced with croton oil in mice receiving no pretreatment (control 2). Ear thickness was measured as a manifestation of irritation after 24 h (mean ± SEM).

IL-6, and IL-10 in human blood cell and monocyte-enriched culture, and induces IL-6 and IL-8 production of keratinocyte and human epidermal carcinoma cells (Gibson *et al*, 1995; Testerman *et al*, 1995). The anti-viral and anti-tumor effects of imiquimod are thought to be mediated in part by the effect on inducing these cytokines (Sidky *et al*, 1992; Reiter *et al*, 1994).

Both anti-viral and anti-tumor effects are thought to involve the presentation of viral or tumor antigens by antigen-presenting cells which in turn present processed antigen to T lymphocytes in DLN (Vestey *et al*, 1990; Cavanagh *et al*, 1996). This process leads to the activation and subsequent proliferation of antigen-specific T lymphocytes, which give rise to the specific immune response (Reinherz *et al*, 1983). Langerhans cells represent the major antigen-presenting cells in skin and are capable of presenting viral and tumor antigens (Knight and Macatonia, 1988; Grabbe *et al*, 1991; Kondo and Sauder, 1995; Memar *et al*, 1995; Cavanagh *et al*, 1996).

In this study, we sought to determine the effect of imiquimod on Langerhans cells. The density of Langerhans cells in the skin decreased 2 d after treatment with imiquimod and fell to 43% by day 10. In addition, remaining Langerhans cells from imiquimod-treated skin appeared larger and stained stronger for Ia antigen. Imiquimod itself does not act as a hapten as during the clinical trials no evidence of allergic contact hypersensitivity was observed.

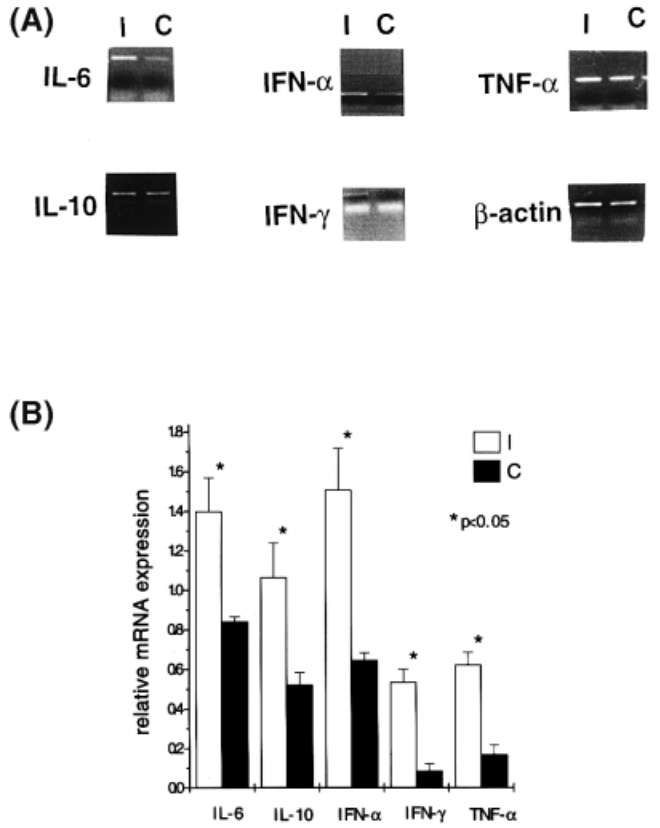

Figure 7. Imiquimod upregulates cytokines mRNA expression. (*A*) Twenty-four hours after challenge, total RNA from the challenge skin site was obtained, and mRNA expression for IL-6, 10, IFN-α, IFN-γ, TNF-α, and β-actin was evaluated by reverse transcriptase–PCR. PCR products were resolved an agarose gel and photographed under ultraviolet light. Imiquimod-treated group (I) and vehicle-treated group (C). (*B*) The densitometry values for each cytokine were quantitated by determining the ratio of the cytokine mRNA to that of β-actin. Imiquimod-treated group (I) demonstrated enhanced cytokine expression compared with the vehicle-treated group (C).

These observations suggest that imiquimod activates Langerhans cells and induces Langerhans cell migration. The activation, however, was not associated with the induction of the costimulatory factor B7. As a decreased number of Langerhans cells in the skin could be the result of enhanced migration of Langerhans cells from skin to DLN, we next evaluated the imiquimod effect on Langerhans cell migration.

To examine Langerhans cell migration we utilized FITC induced Langerhans cell migration as previously described (Wang *et al*, 1992). Flow cytometric analysis of DLN cells demonstrated a higher frequency of FITC bearing cells present in imiquimod-treated mice compared with the controls (1.8–3.8% in imiquimod). It is possible the Ia^+, FITC bearing population include both epidermal and dermal dendritic cells. Dermal dendritic cells have a role as antigen-presenting cells as well as epidermal Langerhans cells; however, 50–80% of the dendritic cells in the lymphatic vessels are thought to be Langerhans cells (Lukas *et al*, 1996; Wang *et al*, 1997), hence our results support the hypothesis that imiquimod treatment enhanced Langerhans cell migration from skin to DLN.

To examine whether the enhanced Langerhans cell migration was translated into enhanced contact hypersensitivity, we evaluated the effect of imiquimod in DNFB-induced CHS. We demonstrated a 61% enhanced CHS response in imiquimod-treated animals compared with vehicle alone ($p < 0.01$). To confirm further the immune enhancement and cytokine inducing effect of imiquimod, we examined cytokine expression in skin. Enhanced mRNA expression of IL-6, IL-10, IFN-α, IFN-γ, TNF-α were seen in imiquimod-treated skin compared with vehicle-treated skin. As previous studies have shown that these cytokines are seen in the challenge phase of contact dermatitis (Enk and Katz, 1992; Kondo *et al*, 1994b), these data suggest that imiquimod enhances contact hypersensitivity in part by upregulating cytokine expression.

These results suggest that the mechanism by which imiquimod enhances immune function is in part due to effects on Langerhans cells. In particular, imiquimod increases Langerhans cell migration to the regional lymph node, which in turn leads to enhanced Langerhans cell antigen presentation exclusively for allergic hypersensitivity. The results of this study also suggest other disease targets where imiquimod treatment may be beneficial, for example enhanced Langerhans cell antigen presentation could be important in the treatment of cutaneous neoplasms.

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