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Transcutaneous immunization induces mucosal CTLs and protective immunity by migration of primed skin dendritic cells

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Transcutaneous immunization (TCI), the application of vaccines on the skin, induces robust systemic and mucosal antibodies in animal models and in humans. The means by which mucosal immune responses to vaccine antigens are elicited by TCI has not been well characterized. We examined the effect of TCI with an HIV peptide vaccine on the induction of mucosal and systemic CTL responses and protective immunity against mucosal challenge with live virus in mice. Robust HIV-specific CTL responses in the spleen and in the gut mucosa were detected after TCI. The responses were dependent upon the addition of an adjuvant and resulted in protection against mucosal challenge with recombinant vaccinia virus encoding HIV gp160. Although it is clear that adjuvant-activated DCs migrated mainly to draining lymph nodes, coculture with specific T cells and flow cytometry studies with DCs isolated from Peyer’s patches after TCI suggested that activated DCs carrying skin-derived antigen also migrated from the skin to immune-inductive sites in gut mucosa and presented antigen directly to resident lymphocytes. These results and previous clinical trial results support the observation that TCI is a safe and effective strategy for inducing strong mucosal antibody and CTL responses.

Introduction

Major entry points for many infections occur at a mucosal surface: gastrointestinal (e.g., HIV and polio virus), respiratory (e.g., influenza and adenovirus), or genital (e.g., HIV and herpes simplex virus). Thus, the induction of mucosal immune responses and prevention of mucosal transmission are critical goals for many vaccines (1–6). In the case of HIV and other viral infections, control of the viral load is dependent upon potent CD8+ T cell responses, including mucosal CTLs at the site of viral infection (7–11). The best way to induce protective immunity against a mucosal challenge in mice and nonhuman primates is immunization through a mucosal route, while systemic immunization can provide only partial mucosal protection (4, 9, 12).

Transcutaneous immunization (TCI), the application of antigen and adjuvant onto the skin to induce antigen-specific immune responses, is a novel immunization strategy that induces robust mucosal IgG and secretory IgA responses in both mice and humans (13–19) and protective immunity against mucosal challenge with toxins (13, 14) or live virus (19). TCI also induces systemic cell-mediated immunity to peptide (20, 21) and whole protein (16, 21–23), but little information has been generated describing the mucosal cellular responses induced by skin immunization (18).

Adjuvants are required for the induction of potent immune responses to coadministered antigens by TCI; the main adjuvants are ADP-ribosylating enterotoxins that include cholera toxin (CT) and the heat-labile enterotoxin of enterotoxigenic Escherichia coli (LT). TCI is not limited to ADP-ribosylating enterotoxins as the sole source of compounds available with adjuvant properties active in the context of the skin. Many other molecules have adjuvant activity when applied to the skin (24). In their native form, CT and LT cannot be readily administered orally in humans due to their enterotoxicity, but they have been shown to be safe in animal and human skin immunization studies (13, 14, 16–18, 24–26).

Skin immunization utilizes potent bone marrow–derived DCs that are resident in the outer epidermal layers of skin, such as Langerhans cells. These DCs provide immunosurveillance functions, and when they are activated by microorganisms, their products, or inflammatory cytokines, migrate out of the skin to the draining lymph nodes (DLNs) and induce strong effector antigen-specific responses by B and T lymphocytes. In the context of TCI, the addition of immunostimulating agents (microbial toxin or inflammatory signal) at the site of antigen administration provides the necessary activation signal for the DCs to mature, express high levels of costimulatory molecules, secrete cytokines, and become potent antigen-presenting cells (APCs) capable of priming immune responses to the coadministered antigen. DCs are loaded and stimulated in vivo by topical application of vaccines. TCI thereby directly utilizes the most potent of immune activators, DCs, in a manner similar to DC immunotherapy but without the labor-intensive, cumbersome, and individualized ex vivo production and antigen-loading of DCs with subsequent administration back into the patient (27).

Because TCI induces mucosal antibody and can induce robust systemic cell-mediated immune responses, we hypothesized that TCI should also induce cell-mediated immune responses in the mucosa. Therefore, in this study we investigated and characterized the cell-mediated systemic and mucosal immune responses...
induced by a TCI regimen consisting of an HIV peptide construct with CT or LT or CpG oligodeoxynucleotides as adjuvants. These CTL responses were compared with responses induced by intrarectal immunization and by a combined regimen consisting of intrarectal and transcutaneous routes of immunization. We also analyzed the potency of the TCI-induced immune response induced against a live viral challenge and defined a novel mechanism for the induction of the mucosal CTLs induced by skin immunization.

**Methods**

**Animals.** Female BALB/c or C57BL/6 mice were purchased from Frederick Cancer Research Center (Frederick, Maryland, USA), or Charles River Laboratories Inc. (Wilmington, Massachusetts, USA), maintained in a specific pathogen–free microisolator environment, and used at 6–25 weeks of age. Mice received food and water ad libitum. All procedures with animals were conducted in accordance with the institutionally approved protocols.

**Antigens and adjuvants.** The peptide PCLUS3-18IIIB (KQIINMWQEVKGAMYAPPSQGQRIQRGPRAPVVTIGK) consists of the multideterminant helper segment PCLUS3 (KQINMWWQEVKGAMYAPPSQGQRIQRGPRAPVVTIGK) (28), derived from the CD4 binding domain of HIV-1 IIIB, combined with the immunodominant CTL epitope presented by H-2D	extsuperscript{b} in BALB/c mice, called P18 IIIB (RIQRGPRAPVVTIGK) (29), derived from the V3 loop of the IIIB strain of HIV-1, in a single continuous peptide. The oligodeoxynucleotide 5'-TCCATGACGTTCCTGACGTT-3' contains a CpG motif with immunostimulatory properties in the mouse (30). CT (List Biologica1s, Campbell, California, USA) and LT (Berna Biotech AG, Bern, Switzerland) are ADP-ribosylating enterotoxins with adjuvant activity and no toxic side effects when applied to the skin. Alexa Fluor 488 (Molecular Probes, Eugene, Oregon, USA) was covalently coupled to LT (AF-LT) as described (18).

**Transcutaneous immunization.** Mice were immunized on the skin as described (18, 24). Briefly, each animal was shaved on the dorsum or abdomen and allowed to rest for 48 hours. Mice were anesthetized in the hind thigh intramuscularly or intraperitoneally with a ketamine-xylazine mixture to prevent self-grooming (i.e., oral immunization). For the experiments presented in Figures 1 and 2, the exposed skin surface was hydrated with saline-drenched gauze for 5–10 minutes and then was lightly blotted with dry gauze prior to immunization. For the experiments displayed in Figures 3–8, hydrated skin was buffered with emery paper to disrupt the stratum corneum and to enhance antigen and adjuvant delivery. Disruption of the stratum corneum leaves the epidermis intact, increases the efficiency of antigen and adjuvant delivery, and subsequently elicits stronger immune responses to the vaccine than does simple hydration alone with a similar dosing regimen (26). Each animal received either 25–100 µl of immunizing solution applied onto the shaved skin or the ventral surface of the ear for 1 hour, or a 1-cm	extsuperscript{2} gauze patch containing 25 µl of immunizing solution covered by a semiocclusive backing and placed on the shaved skin for up to 18 hours. The animals were then extensively washed, tails down, under running tap water for approximately 30 seconds and patted dry.

Mice were immunized intrarectally with PCLUS3-18IIIB and LT or CT. A mixture of peptide and adjuvant (150 µl total) was injected intrarectally through an umbilical catheter inserted about 4 cm deep from the anus in mice sedated by inhalation anesthesia (methoxyflurane; Pitman-Moore Inc., Mundelein, Ill) (31).

**Cell isolation.** Splenectomy. Inguinal lymph nodes (LN), superficial cervical LN, and mesenteric LNs were aseptically removed and pooled, and single-cell suspensions were prepared by gentle passage of the tissue through sterile mesh screens (100 µm). For SP cell suspensions, erythrocytes were lysed with Tris-buffered ammonium chloride and the remaining cells were washed extensively in RPMI 1640 (Biowhittaker, Walkersville, Maryland, USA) containing 2% fetal bovine serum (Gemini, Woodland, California, USA) (32).

Peyer’s patches (PPs) were carefully excised from the wall of the small intestine, pooled, and dissociated into single-cell suspensions by enzymatic digestion with collagenase type VIII (300 U/ml; Sigma-Aldrich, St. Louis, Missouri, USA) and DNase I (3 U/ml; Sigma-Aldrich) for 60 min. Cells were collected, washed, and resuspended in complete medium (CM), which is RPMI-1640 supplemented with 10% fetal bovine serum, 10 U penicillin (Invitrogen, Carlsbad, California, USA), 100 µg/ml streptomycin (Invitrogen), 2 mM glutamine (Invitrogen), 50 µM β-mercaptoethanol (Invitrogen), and 20 mM HEPES (Biowhittaker). Cells were then layered onto a discontinuous density gradient containing 75% and 40% chloroform and the remaining cells were washed extensively in RPMI 1640 containing 2% fetal bovine serum. The resulting population was more than 90% viable lymphocytes, with a cell yield of 1 × 10	extsuperscript{6} lymphocytes/mouse. Most PP CD3+ T cells isolated from normal mice were CD4+, while CD3+CD8+ T cells were less frequent. Collagenase VIII digestion does not alter the expression of CD3, CD4, or CD8 on splenic T cells treated with this enzyme (2).

Single-cell suspensions of lung lymphocytes were isolated by DNase (3,500 U/ml) and collagenase VIII (75 U/ml) digestion of pooled perfused lung tissue at 37°C for 2 hours. Cells were subjected to centrifugation on Ficol-Paque Plus solution at 400 g for 30 min (33).

CD11c+ APC populations were isolated from inguinal LN and P18 single-cell suspensions using magnetic cell sorting (MACS) anti-mouse CD11c MicroBeads (Miltenyi Biotec, Bergish-Gladbach, Germany). Single-cell suspensions were incubated with mouse immunoglobulin (1 µg per 500 µl) to block Fc receptor binding sites before anti–mouse CD11c MicroBeads were added. CD11c+ cells were positively selected by magnetic retention, eluted by removal of the magnetic field, and washed with CM.

**Cytotoxic T lymphocyte assay.** Single-cell suspensions of pooled SP, lung or PP cells were activated and expanded by culture with antigen for 6 or 7 days and were used as the effector cell population in the assay. Cells were cultured at a density of 5 × 10	extsuperscript{6} per ml in 12-well culture plates with 1 µM of the synthetic CTL epitope peptide P18-110 (I10; RGPGRAPVTI), the minimal epitope of P18 IIIB, in CM (31). Three days after the culture was initiated, a medium containing 10% concanavalan A supernatant (T-stim; Collaborative Biomedical Products, Bedford, Massachusetts, USA) was added as a source of IL-2. Cytolytic activity of the CTL cultures was measured in a 4-hour ³¹Cr-release assay using labeled P815 targets cells that had been cultured in the presence or absence of P18-110 peptide (1 µM). The percent specific ³¹Cr release was calculated as 100 × (experimental release – spontaneous release) / (maximum release – spontaneous release). Maximum release was determined from supernatants of cells that were lysed by the addition of 5% Triton-X 100. Spontaneous release was determined from target cells incubated without added effector cells (34).

**Fluorescent antibody staining.** Single-cell suspensions of inguinal LNs, cervical LNs, mesenteric LNs, SP, and PPs in PBS plus 1% bovine serum albumin (BSA) (Sigma-Aldrich) were blocked for 20
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Figure 1
Induction of CTL responses in SP and PPs after TCI with HIV peptide and adjuvant. BALB/c mice (n = 5) were immunized twice 3 weeks apart with 50 μg of PCLUS3-18IIIB peptide alone (triangles), with 50 μg CT as an adjuvant (circles), or with 50 μg CT and 50 μg CpG as adjuvants (squares). SP (A) and PP (B) cells from immunized mice were incubated in vitro with 1 μM P18-I10 peptide for 7 days before cytolytic activity was measured in a 4-hour 51Cr-release assay against P815 target cells alone (open symbols) or pulsed with P18-I10 peptide (filled symbols). E/T, effector-to-target ratio.

Results
TCI induces systemic and mucosal CTLs that are protective against mucosal live virus challenge. To study the induction of mucosal and systemic CTL responses after TCI, we immunized BALB/c mice twice with an HIV peptide construct (PCLUS3-P18IIIB; 50 μg/mouse) in the presence or absence of CT and CpG oligodeoxynucleotide as adjuvants. CTL responses were assayed 2 weeks after the last immunization (day 35) (Figure 1). We found that TCI with HIV peptide with CT and CpG as adjuvants can induce P18IIIB-110–specific CD8+ CTLs in PPs of the intestine (Figure 1B) as well as in the SP (Figure 1A). In multiple previously published studies, we found that subcutaneous immunization with peptide did not elicit a significant CTL response in the PPs (2, 12). An HIV peptide construct with CT as an adjuvant but without CpG was able to induce CTLs both in the SP (Figure 1A) and in PPs (Figure 1B). However, the level of CTL responses in the gut and in the SP was greater in magnitude in the groups with CpG and CT as adjuvants (Figure 1). TCI with PCLUS3-P18IIIB alone did not induce mucosal (data not shown) or systemic (Figure 1A) CTLs. Because the addition of CpG oligodeoxynucleotide directs a more Th1-like response (i.e., CTLs) (30, 35, 36) and the addition of the oligodeoxynucleotide showed some potential enhancing effects on the cellular response after TCI, the CpG oligodeoxynucleotide was included in subsequent immunization regimens unless otherwise noted.

To determine the optimal skin immunization site for inducing maximal systemic and mucosal CD8+ CTL responses, we compared the effects of TCI in mice immunized on the back, abdomen, or ear. Systemic (SP) and mucosal (PP) CTL responses were measured for all three anatomical sites of skin immunization (Figure 2). The highest CTL response in both mucosal and systemic sites was observed after TCI on the back, although TCI on the abdomen gave a comparable level of PP CTL response, albeit with a higher background.

To determine the protective efficacy of the mucosal immune response induced by TCI using the HIV peptide with CT or LT as an adjuvant, we challenged immunized mice intrarectally with live vaccinia virus carrying the gene encoding HIV-1 IIIB gp160 (37). Six days after the mucosal challenge with virus, mice were sacrificed and their ovaries were removed and assayed for virus titer (12). TCI using the HIV peptide with CT or LT as an adjuvant partially protected mice against an intrarectal challenge with HIV–recombinant vaccinia virus (Figure 3), as shown by a 2-log reduction in viral PFU in the ovaries of TCI mice compared with those of mice immunized with adjuvant alone. TCI using the HIV peptide with CT or LT as an adjuvant was equally effective for protection against mucosal challenge (Figure 3). The efficacy of the TCI results contrasts with sub-

Figure 2
(A and B) TCI at multiple anatomic skin sites elicits systemic (A) and mucosal (B) CTL responses. BALB/c mice (n = 5) were skin-immunized on the back (squares), abdomen (diamonds), or ear (triangles) with 100 μg PCLUS3-18IIIB peptide with 50 μg CT and 50 μg CpG as adjuvants. Immunizations occurred on weeks 0, 1, 3, and 5. SP (A) and PP (B) cytolytic activity was measured in a 51Cr-release assay against P815 target cells alone (open symbols) or pulsed with P18-I10 peptide (filled symbols).
cutaneous immunization with peptide and incomplete Freund’s adjuvant, which does not protect against intrarectal challenge (12). Transcutaneous and intrarectal immunization regimens are effective strategies for priming systemic and mucosal CTL responses. To compare priming and boosting regimens with various transcutaneous and intrarectal immunization sequences, we analyzed the CTL response in mucosal and systemic compartments after four immunizations with an interval of 2 weeks between each immunization (Figure 4). HIV-specific CTL responses in the SP and PPs were studied 2 weeks after the last immunization (Figure 4). Mice were immunized four times transcutaneously; three times transcutaneously followed by once intrarectally; or once intrarectally followed by three times transcutaneously. We found that all three regimens induced a highly comparable level of CTL activity in the SP (Figure 4A), although the HIV-specific CTL response in the SP after one intrarectal followed by three transcutaneous immunizations may have been marginally weaker than that after four transcutaneous immunizations or three transcutaneous plus one intrarectal immunization (Figure 4A). In contrast, the rank order of HIV-specific CTL responses in the PPs of the three groups of immunized animals differed (Figure 4B). The highest CTL response in the PPs was found in mice that received three transcutaneous immunizations and one intrarectal booster immunization. In the groups of animals treated with four transcutaneous immunizations or one intrarectal followed by three transcutaneous immunizations, HIV-specific CTL responses were observed at the highest effector/target ratio only and were significantly lower than in mice with three transcutaneous immunizations followed by one intrarectal booster.

In view of this better response when transcutaneous immunization was given first, we investigated several prime and boost strategies in which transcutaneous immunization was used to prime or boost immune responses. Mice were immunized with HIV peptide, CT, and CpG oligodeoxynucleotide twice at 2-week intervals and were sacrificed 2 weeks after the last immunization to analyze their CTL responses to HIV peptide in PPs (Figure 5A), SP (Figure 5B), and lung (Figure 5C). The regimens that the mice received included transcutaneous plus intrarectal immunization (group 1); two transcutaneous immunizations (group 2); subcutaneous plus intrarectal immunization (group 3); intraperitoneal plus intrarectal immunization (group 4); two intrarectal immunizations (group 5); and a single intrarectal immunization without boosting (group 6). We found the highest HIV-specific CTL response in the PPs after intrarectal prime and intrarectal boost immunizations (Figure 5A). Animals in group 1 (transcutaneous prime and intrarectal boost) and group 2 (transcutaneous prime and transcutaneous boost) also had a significant CTL response in the PPs. The other prime and boost strategies did not induce a mucosal CTL response. The HIV-specific CTL response in the systemic circulation (SP) was optimal after intraperitoneal priming immunization and intrarectal boost (Figure 5B). However, transcutaneous immunization as a priming immunization with transcutaneous or intrarectal boost also induced a significant CTL response in the SP.

We also studied the HIV-specific CTL response in the lung after immunizing mice twice transcutaneously, priming with transcutaneous immunization and boosting intrarectally, or immunizing twice intrarectally (Figure 5C). Two weeks later we studied the HIV-
specific CTL response in the lung. We found the strongest CTL response in the lung after transcutaneous priming and transcutaneous boosting, while transcutaneous priming and intrarectal boosting also induced a CTL response in the lung (Figure 5c). Taken together, these results suggested that TCI was an effective means of antigen delivery to induce CTL responses systemically (i.e., SP) as well as in the gut (i.e., PP) and the lower respiratory tract (i.e., lung).

After TCI, APCs traffic to the gut mucosa, present skin-derived antigen to CTLs, and have upregulated activation markers. Although TCI clearly induced strong mucosal immune responses, the mechanism by which these responses were initiated was unknown. Three models were hypothesized based on known paradigms to explain these observations: after TCI, (a) APCs traffic to the regional DLNs and activate resident T cells, and then the T cells migrate to mucosal sites; (b) APCs traffic directly to the inductive sites of the mucosa and activate resident T cells in situ; or (c) a combination of models a and b occurs. To evaluate the role of APC trafficking to inductive sites, we conducted three studies: coculture of APCs isolated from DLNs or PPs with antigen-specific T cells as an assay of antigen presentation; analysis of the effects of LT upon activation markers on PP APCs; and monitoring of the migration of APCs to inductive sites of the peripheral and mucosal immune system.

To directly demonstrate that 24 hours after TCI, APCs in the PPs possess sufficient skin-derived antigen to activate antigen-specific T cells, we conducted an ex vivo coculture experiment in which a T cell line was cultured with PP APCs after TCI. Mice were skin-immunized with LT plus CpG or with LT, CpG, and an HIV peptide (PCLUS3-18IIIB). Twenty-four hours after TCI, all mice were sacrificed and the

**Figure 5**

TCI generated CTL responses in the PPs, SP, and lung. BALB/c mice (n = 5) were immunized twice 2 weeks apart with 50 µg PCLUS3-1IIIB peptide, 10 µg CT, and 50 µg CpG by the indicated route of administration for each group as follows: group 1, transcutaneous prime and intrarectal boost; group 2, transcutaneous prime and boost; group 3, subcutaneous (SC) prime and intrarectal boost; group 4, intraperitoneal (IP) prime (peptide and CpG given intraperitoneally and CT administered intraperitoneally because CT is toxic given intraperitoneally) and intrarectal boost; group 5, intraperitoneal prime and intrarectal boost; and group 6, no treatment during the first immunization and an intrarectal immunization at the second immunization interval (Single IR). Four weeks after the final immunization, PPs (A), SP (B), and lung (C) were removed, processed, stimulated in vitro with P18-I10 peptide (1 µM) for 7 days, and assayed for cytolytic activity in a 51Cr-release assay.

**Figure 6**

CD11c-enriched cells isolated from inguinal LN and PPs of skin-immunized mice have acquired antigen that activates high- or low-avidity CTLs ex vivo. (A–D) Mice were immunized transcutaneously with 50 µg LT or with 50 µg LT and 50 µg PCLUS3-1IIIB peptide and were sacrificed 24 hours after immunization. Pooled inguinal LN and PP CD11c+ cells (APCs in figure) were enriched by MACS (enrichment of CD11c+ cells to about 70% by FACScan analysis). APCs (5 × 10⁴, 1 × 10⁴, or 1 × 10⁵) were cocultured in 96-well U-bottomed plates with 2 × 10⁴ cells/well high-avidity (0.001 µM; A and C) or low-avidity (10 µM; B and D) P18-I10-specific CTLs. Negative controls: CTL + Contr APCs, 5 × 10⁴ CD11c+ APCs isolated from the inguinal LN or PPs of mice immunized with LT alone were cocultured with 2 × 10⁴ high-avidity (A and C) or low-avidity (B and D) P18-I10–specific CTLs; CTL alone, 2 × 10⁴ high-avidity (A and C) or low-avidity (B and D) P18-I10–specific CTLs were cultured alone; ING APC alone, 5 × 10⁴ CD11c-enriched cells isolated from the inguinal LN of mice immunized with LT and peptide were cultured alone; 89.6 CTL + APC, 5 × 10⁴ CD11c+ APCs isolated from the inguinal LN of LT- and peptide-immunized mice were cocultured with 2 × 10⁴ high-avidity (0.001 µM) P18-89.6A9–specific CTLs (89.6 strain of HIV), which do not cross-react with P18-I10–specific CTLs (IIIB strain of HIV). The level of IFN-γ produced by the CTL lines in the supernatant after a 48-hour coculture with CD11c-enriched APCs was measured by semiquantitative ELISA.
mononuclear cells from the inguinal LNs and PPs were purified, and then the cell populations were enriched for CD11c+ cells using magnetic beads. CD11c-enriched APCs (5 × 10⁴, 1 × 10⁵, or 1 × 10⁶ APCs) from animals immunized with PCLUS3-18IIIB by TCI were cocultured in vitro with 1 × 10⁶ T cells of a high-avidity (0.001 M) LT and were sacrificed 24 hours later. Immunofluorescence microscopy was used to identify CD11c+ APCs in the PPs. The addition of CpG oligodeoxynucleotide with LT in the immunization regimen did not alter the population compared with LT alone (data not shown). These results demonstrated that skin-applied LT has a direct effect upon APCs resident in the PPs and suggested that APCs can migrate from the skin to the PP.

We have shown that APCs migrate directly to the DLNs and do not traffic to other peripheral LNs within 72 hours after application of an LT patch (18). Our functional data here (Figure 6) showed that APCs carrying skin-applied antigen migrated from the skin to the inductive sites of the gut mucosa. We sought to visually confirm this migration from the skin to mucosal sites (i.e., PPs) by looking for PP APCs containing skin-derived and fluorescently labeled LT after TCI. Mice were left untreated (naive) or were immunized with fluorescent LT (AF-LT). Then, 24 (data not shown) and 48 (Figure 8) hours later, inguinal, superficial cervical, and mesenteric LNs, SPs, and PPs were analyzed for the presence of resident APCs containing fluorescent LT. As we have shown previously (18), CD11c+ MHC class II+ APCs in the inguinal DLNs contained LT after TCI with the fluorescent antigen (Figure 8, B and C). LNs (superficial cervical and mesenteric) that did not drain the site of immunization and the SP were negative for labeled LT.

APCs in the PPs contain skin-derived fluorescent LT after TCI. (A–F) BALB/c mice were left untreated (A and D) or were immunized with 50 µg of Alexa Fluor 488–conjugated LT (AF-LT; B, C, E, and F). Forty-eight hours later, inguinal LNs and PPs were isolated and analyzed for APC populations containing AF-LT (A, B, D, and E). The CD11c, MHC class II+ (H-2 I-A^d) surface expression of AF-LT–positive cells was determined for both inguinal LN (C) and PP (F) cells. Similar results were observed 24 hours after application of AF-LT. The percent of AF-LT–positive cells are listed in A, B, D, and E. The percent of cells present in each quadrant are listed in C and F.
response, it is seems reasonable to consider the skin as behaving component of IgA (49), and can prime and boost a mucosal immune coat with immunoglobulins including IgA and secretory com-

tions. The current results suggest that the migration of DCs from alcations in the cytoplasm of the APCs and not on the surface of the cells (data not shown). The PPs also contained a small population of non-B (CD19-), non-T (CD3-), non-
macrophage (F4/80-), large granular cells of an unknown pheno-
type that contained LT (Figure 8F). Taken together, these results suggested that APCs have a direct migration route from the skin to the gut mucosa that can be initiated by the application of LT on the skin and in which skin-derived antigen is presented directly in the gut mucosa to induce mucosal immune responses.

Discussion
In this study, we demonstrated that the application of antigen on the skin is an effective means for inducing robust systemic and mucosal CD8+ CTL responses. Mucosal immune responses are considered desirable for their ability to stop pathogens at the point of entry at mucosal surfaces and, in the case of influenza infections, for CTLs to mediate in viral clearance (39). Parenteral immunization with nonreplicating HIV agents often fail to contribute to specific mucosal immune responses, and only immunization by mucosal routes can induce optimal protection against mucosal challenge (4, 12, 40), although most licensed vaccines can protect against mucosal pathogens after intramuscular or subcutaneous injection of antigen (41-43). Because skin immunization generates mucosal responses in the gut, lung, saliva (15), and female repro-
ductive tract (15), it appears to be a unique route by which to deliver antigens to multiple mucosal sites and differs from traditional immunization routes that induce compartmentalized mucosal responses. Examples of compartmentalized responses include those to intrarectal and oral immunizations, which induce mucosal responses mainly in the gut, and to intranasal immunizations, which produce mucosal responses located mainly in the lung and nasal-associated lymphoid tissues. The mechanism by which TCI induces immune responses in both systemic and multiple mucosal compartments, each having very different lymphocyte homing markers (44-47), is not known and remains a subject of investiga-
tion. The current results suggest that the migration of DCs from skin to both systemic and mucosal compartments may provide such a mechanism. Furthermore, because the mucosa and skin share similar elements such as DCs, secretory organs, the presence of IgA such as that found in sweat glands (48), and microorganisms coated with immunoglobulins including IgA and secretory component of IgA (49), and can prime and boost a mucosal immune response, it is seems reasonable to consider the skin as behaving like a “distant relative” of a mucosal surface.

The effectiveness of any prophylactic immunization strategy in the infectious disease setting is its ability to protect against challenge by a live organism. Several TCI studies have demonstrated the potency of this route of administration against infectious disease agents such as bacterial toxins (13, 14, 16, 24, 50), live bacteria (51), and live viruses (19, 52). Protective immunity mediated by antibody against live influenza virus challenge was also demonstrated using a similar but slightly different skin immunization strategy in which dry powder containing antigen and adjuvant were driven into the skin by a gas-
powered gun (53, 54). We have extended these findings by demonstr-
ating increased protection in skin immunized mice against a mucosal (i.e., intrarectal) challenge with a live vaccinia virus vector. As the recombinant vaccinia does not incorporate HIV-1 gp160 into the virus particle and thus is not sensitive to neutralization by anti-
gp160, the reduction in viral load was solely due to cell-mediated effector mechanisms, in contrast to the other TCI challenge studies in which protective responses were mediated mainly through humoral immune components. Although the protection seen in these experiments was a 2-log reduction in viral PFU, slightly less than the 3- to 4-log reduction we have seen with four doses of peptide and LT given intrarectally (12), the strong dependence on the number of immunizations makes these results difficult to compare directly. Although the recombinant vaccinia expressing HIV-1 gp160 is less virulent than wild-type vaccinia, because the insert replaces the tyrosine kinase gene, T cells have been shown to protect against death from lethal vaccinia virus challenge (55).

Mucosal and systemic CTL responses were produced after TCI with antigen administered at several anatomical sites. Responses were greater in magnitude when antigen was applied to the abdomen or back compared with the ear, although the epidermis of the ear, abdomen, and back has a similar density and distribution of DCs (56, 57). After TCI, these sites also have equivalent “immunopotential” to elicit similar levels of antibody (58). These results suggest that TCI targets mucosal responses regardless of the site of administration and that immunogens can be applied any-
where on the skin where DCs are present to induce a systemic or mucosal response. However, local responses in the DNLNs proximal to the site of TCI may be greater in magnitude than those of distal immunization sites (data not shown), and this may be an impor-
tant consideration in determining the location of antigen place-
ment on the skin when immune responses need to be directed to a localized anatomical site (e.g., for cancer treatment).

Unmethylated oligodeoxynucleotides containing CpG motifs are effective immunomodulating agents and act as adjuvants to elicit a predominantly Th1 CD4+ T cell response to coadministered antigens in mice (30). CpGs were combined with CT in our initial immunization regimen, as it was believed that CT by itself would be unable to elicit a robust Th1-directed CTL response (59-61). CT and LT were able to act as adjuvants in eliciting systemic and mucosal CTL responses with or without the addition of CpG oligodeoxynucleotides, but CT levels were increased by the inclusions of CpG oligodeoxynucleotides. CT and LT also induced functionally similar levels of protective immunity against virus challenge. The Th2 bias reported for immunizations using CT as an adjuvant (59, 61, 62) may be related to a number of factors in addi-
tion to the known inhibition of IL-12 production (63), including the route of delivery, the coadministered antigen, and the microen-
vironment and its effects on the cytokines and chemokines pro-
duced by APCs after encountering the adjuvant and antigen. Inter-
estingly, CT as an adjuvant on the skin primes a CTL response in the absence of IL-12 (21), a known promoter of Th1 CD4+ T cells and CD8+ CTL development (64) and an enhancer of the activation of naive CD8+ T cells (65). However, IL-12 produced by DCs is not required for the in vivo priming of CTLs (66). Although still contro-
versial, a number of the DC parameters (receptors, cytokines, chemokines, etc.) that direct Th1 and Th2 lymphocyte develop-
ment have been identified (67–71). However, the in vivo effects of CT and LT on skin-derived APC and their effect upon Th1 and Th2 lymphocyte development are unknown and are under investigation. From our study and results reported elsewhere (24, 14, 18, 21), it would appear that the skin contains and provides the appropriate microenvironment for both CT and LT to act as adjuvants in eliciting an effective Th1 response as well as a Th2 response directed against protein-based antigens. For application to humans, the CpG-containing sequence motifs effective in humans are different from those active in mice, but most of the same effects can be elicited with the appropriate sequence (30, 72). However, there may still be differences in outcome in view of the fact that there are differences in TLR9 expression in mice and humans. In mice, both plasmacytoid DCs and myeloid DCs express TLR9, while in humans only plasmacytoid DCs, not myeloid DCs, express this receptor for CpG oligodeoxynucleotides (71, 73–76).

It has been proposed and subsequently demonstrated that priming an immune response in a specific inductive site by one method of immunization (e.g., transcutaneously) should further modulate or enhance a subsequent immune response developing through the same inductive site by an alternative route of immunization (e.g., intrarectally) (77). We utilized this concept to ascertain the potential of priming by one mucosal route of immunization (transcutaneously or intrarectally), boosting with a second (intrarectally or transcutaneously), and then measuring the effects by the level of mucosal CTLs elicited using the various combinations. Overall, the responses with the greatest magnitude found in the PPs, lung, and SP were all primarily dependent upon the priming route (intrarectal for PPs, transcutaneous for lung, and intraperitoneal for SP) and secondarily on the boosting route. Transcutaneous immunization was able to boost an intrarectally primed response and vice versa. These results further support the concept that transcutaneous administration is an excellent route for initiating a mucosal immune response, but raised the question of how intrarectal and transcutaneous immunization can target similar inductive sites and modulate each other’s responses when the two anatomical sites of antigen application are far removed from each other and the sites of immune induction (i.e., PPs for intrarectal and DLNs for transcutaneous) are not known to be the same.

As presented above, three models based on known paradigms were put forth to explain how TCI primes and amplifies mucosal CTL responses: (a) APCs migrate from the skin to the regional DLNs into T cell–rich areas and prime and activate resident T cells, and then the T cells migrate from the regional DLNs to peripheral and mucosal effector sites; (b) APCs migrate from the skin to the PPs (or other mucosal inductive sites) and activate T cells in situ; and (c) a combination of (a) and (b). We focused on developing models to determine if APCs can migrate from the skin to the PPs by assessing the migratory properties of skin-resident APCs after TCI. By using an APC/T cell coculture study and tracking migrating APCs by flow cytometry, we demonstrated that CD11c+ APCs carrying skin-derived antigens can be isolated and identified in the PPs within 24 hours of topical application of antigen. Our data are consistent with those of Enioutina et al. (78), who demonstrated that an increase in injected bone marrow–derived CD11c+ DC numbers could be found in PPs after the cells had been treated ex vivo with vitamin D3, CT, or forskolin and then injected intravenously or intradermally. Our in vivo DC tracking results are also consistent with recent reports suggesting that DCs located in the mucosa inductive sites are mainly responsible for imprinting gut-homing potential to T cells (79–81). In addition, a small population of CD11c+ cells was identified that contained skin-derived antigen, were located in the PPs, and were not B cells (CD19+), T cells (CD3+), or macrophages. The identity and functional significance of this population in the PPs is unknown and is under investigation. In view of the recent study by Allan et al. (82) distinguishing between Langerhans cells and CD8α+ DC in viral immunity, it would be of interest to know which of these populations is involved in the transport of antigen to the PPs. Our current data do not allow us to distinguish these subpopulations, but future studies should allow us to resolve these subsets.

Although we did not address the hypothesis of T cell migration from regional DLNs to mucosal effector sites for the induction of mucosal responses after TCI, it was clear from literature on contact sensitization (83) and from our own studies (18) that after they are activated, skin-derived APCs migrate to the regional DLNs and activate T cells to migrate to peripheral sites that may include the gut mucosa. T cell activation in the DLNs is mediated by migratory APCs that through direct cell-to-cell contact, cytokine secretion, and chemokine secretion alter the phenotype of lymphocytes to exit the DLNs through the efferent lymph and home to a variety of anatomical sites as determined by their surface marker expression and the different chemokine and adhesion molecule expression of peripheral lymphoid and nonlymphoid tissue (84–88). Although recent reports suggest that the gut-homing tropism of T cells may be directed mainly by DCs resident in the PPs or mesenteric LN and not in peripheral LNs (79–81), we have observed that topical application of LT, a majority of T cells present in the skin DLNs express the peripheral lymphocyte homing markers B1 integrin and CD62L, while a small population of T cells express the mucosal homing marker α4β7 (data not shown). In the context of topical delivery, LT may act as a mucosal adjuvant, providing a specific signal for DCs in the DLNs to activate the expression of mucosal homing receptors on resident naïve T cells. Thus, the APC, microenvironmental signals, and/or adjuvant may promote T cells to modulate homing receptor expression with a specificity for a wide range of anatomical migratory destinations. However, from our studies and published reports, it appears that APC migration to mucosal inductive sites is the main mechanism in vivo by which robust mucosal immune responses are produced after TCI.

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