Published in final edited form as:

J Immunother. 2005 ; 28(5): 430–437.

Transcutaneous Immunization with Cytotoxic T-Cell Peptide Epitopes Provides Effective Antitumor Immunity in Mice

Tsuyoshi Itoh and Esteban Celis

From the Department of Pediatrics and Stanley S. Scott Cancer Center, Louisiana State University Health Sciences Center, New Orleans, Louisiana

Summary

Much attention has been focused on transcutaneous immunization strategies to stimulate systemic cytotoxic T lymphocyte responses leading to anti-tumor or anti-microbial immunity. Here we report that topical application of vaccines consisting of synthetic peptides formulated with imiquimod, a Toll-like receptor agonist that functions as a potent adjuvant generates strong T cell responses that exhibit effective anti-tumor effects in a murine melanoma model system. These results support the use of peptide-based transcutaneous vaccines as a noninvasive and effective strategy for anti-tumor immunotherapy.

Keywords

cutaneous vaccine; toll-like receptor adjuvant; melanoma; animal model

Successful induction of antitumor cytotoxic T lymphocyte (CTL) responses leading to the prolongation of survival of cancer patients has become the major aim of active cancer immunotherapy. To achieve this goal, many protocols for immunization have been investigated, but few so far have proved effective. Recently, we enumerated what we consider to be some of the major obstacles for the development of effective peptide epitope-based vaccines for cancer. Among these barriers, the inherent poor immunogenicity of pure synthetic peptides, which lack the necessary “danger signals” to awaken the immune system, is a major challenge.

The most common danger signals are derived from components present in various microorganisms that function as ligands to members of the Toll-like receptor (TLR) family, which activate antigen-presenting cells (APCs) to become effective inducers of CTL responses. Because dendritic cells (DCs) are the most potent type of APC, these cells have been used as cell-based therapeutic vaccines against numerous tumor types by loading them with various sources of tumor antigens (peptides, proteins, tumor lysates, genes, and mRNA). Although some encouraging results have been observed, the ex vivo generation of DCs for such therapeutic purposes is time-consuming and labor-intensive and can be quite expensive. Thus, it would be more desirable to deliver the antigen in vivo to DCs and at the same time activate these APCs with TLR ligands to generate the antitumor CTL responses in a more practical manner.

We have previously reported that subcutaneous (s.c.) injections of peptides or proteins formulated in incomplete Freund’s adjuvant (IFA) that were co-administered with a TLR-9 ligand (synthetic deoxyoligonucleotides containing CpG motifs) induced strong and effective antitumor CTL responses.¹ Because Langerhans cells (LCs) are quite abundant in the skin

tissues, direct delivery of antigens to these APCs would certainly be a cost-effective and relatively easy way to stimulate antigen-specific CTLs. LCs are epidermal DCs located at the basal layer of the epidermis, covering nearly 25% of the surface area, and are known to play a sentinel role in the skin. LCs take up antigens and when activated migrate into the draining lymph nodes, where they present antigens to the T cells.²

Although epidermal injections would facilitate the delivery of antigen to LCs, this procedure requires trained personnel to ensure that the injection has been correctly applied into the thin epidermal layer, because immunizations into the dermis or s.c. would not be as effective. In addition, for a vaccine to be successful it must also activate the LCs to induce their migration to the draining lymphoid organs where these APCs are to present antigen to the naive CTLs.

Several vaccine adjuvants have been used for generating the appropriate signals that activate the APCs to become immunogenic. Recently, the use of synthetic ligands that stimulate members of the TLR family expressed by APCs has been investigated as a source of adjuvant. These ligands correspond to pathogen-associated molecular patterns (PAMPS) such as double-stranded RNA, such as poly I:C (TLR-3 ligand), un-methylated CpG motif-containing deoxyoligonucleotides (ODN) that mimic bacterial DNA (TLR-9 ligand), and lipopolysaccharides (TLR-4 ligand), which are present in infectious agents and tend to be absent in higher organisms.³

Transcutaneous immunization (TCI) is a simple and novel vaccination approach that allows the topical administration of antigen and adjuvant and their diffusion into the epidermal layer. TCI has been explored mainly for the induction of humoral immune response⁴⁻⁷ and was shown to provide protective immunity against mucosal challenge with toxins and viruses.⁸⁻¹⁰ TCI has also shown to be effective at inducing cellular immune responses against cholera toxin (CT) and other viral product when peptides were used as immunogens.^{7,11,12} Several types of adjuvants have been tested for their ability to enhance the immune responses by TCI. For example, ADP-ribosylating exotoxins including CT and heat-labile enterotoxin (LT) produced by *Vibrio cholerae* and *Escherichia coli*, respectively, have been reported to augment systemic humoral and cellular immune responses when used as adjuvants in TCI.^{6,13} However, the use of these toxins in humans could pose a significant safety hazard.

Imiquimod is an immune response modifier that functions as a ligand for TLR-7. It has been approved and has been widely used in humans for the treatment of genital warts.¹⁴ Most significantly, TLR-7 is expressed in several DC subsets, including LCs, and is known to activate macrophages and other immune cells inducing the production of local cytokines such as IL-1, IL-6, IL-8, TNF- α , and IFN- α some of which are crucial for maturation of APCs. In the present study we evaluated whether the topical administration of a pharmaceutical-grade imiquimod cream (Aldara) mixed with small synthetic peptides corresponding to CTL epitopes would elicit effective immune responses capable of producing antitumor effects in a mouse melanoma model system. The results show that, indeed, this simple way of immunization results in strong CTL responses that were able to improve the overall survival of mice bearing tumors. These findings bear significance to the design of cost-effective antitumor vaccines that would be easy to manufacture and simple to administer to cancer patients.

METHODS

Animals

Female 6- to 8-week-old C57BL/6 mice were purchased from Frederick Cancer Research Center (Frederick, MD). OT-I T-cell receptor transgenic mice were bred in our facility from mice obtained from Dr. Stephen Schoenberger (La Jolla Institute of Allergy and Immunology). The Institutional Animal Care and Use Committee approved all the protocols for this study.

Cell Lines

The mouse melanoma B16 tumor transfected with the ovalbumin gene was obtained from J. G. Frelinger (University of Rochester, Rochester, NY). The EL-4 thymoma cell line was purchased from the American Type Culture Collection (ATCC).

Immunogens, Peptides, and Adjuvants

The peptide epitope OVA_{257–264} (hereafter referred to simply as OVAp) was synthesized according to standard solid-phase synthesis methods by Applied Biosystems (Foster City, CA) and purified by reverse-phase HPLC. The purity (>95%) and identity of peptides were determined by analytical reverse-phase HPLC and mass spectrometry analysis. A dextran conjugated with Alexa Fluor 488 (MW 10,000) (Molecular Probe, Eugene, OR) was used as an immunogen for antigen-uptake experiments. Aldara, a cream formulation of imiquimod (5%), was purchased from 3M Pharmaceuticals (St. Paul, MN). The previously described immunostimulatory synthetic ODN-1826,¹ containing two CpG motifs (TCCATGACGTTCTGACGTT), was also used as an adjuvant in some experiments.

Immunizations and Tumor Challenge

Most immunizations were performed transcutaneously. Mice were shaved on the dorsal side of the neck under sedation with an intraperitoneal (i.p.) injection of 2,2,2-tribromoethanol to prevent self-grooming for 45 minutes after application of vaccine. The exposed skin surface was hydrated and then blotted with dry paper prior to immunization. Where indicated, in some experiments, exposed skin was stripped with adhesive cellophane tape 10 times to disrupt the epidermal barrier of the stratum corneum. The indicated amount of peptide mixed with 35 μ L Aldara (approximately 1.75 mg of imiquimod) was then applied with a spatula. Where indicated, for comparison some mice were vaccinated via the s.c. route with peptide formulated in IFA and ODN-1826. The antitumor effects of transcutaneous immunizations were evaluated in both the prophylactic and therapeutic models. In the prophylactic protocol, mice were first immunized with vaccine preparations using various protocols indicated. Seven days after the last immunization, mice were challenged with 2×10^5 B16-OVA cells given s.c. in the back hind. In the therapeutic protocol, mice were first inoculated with 2×10^5 B16-OVA cells given i.v. in the tail vein (day 0), followed by corresponding vaccinations starting on day 4. Tumor-bearing mice either died on their own or were killed when tumors surpassed 150 mm² in mean size. Statistical analyses for evaluating the survival advantages were performed using log-rank analysis.

ELISPOT Assays

CTL responses were evaluated in ELISPOT assays. Spleens and draining lymph nodes (bilateral neck and axillary lymph nodes) were aseptically removed 6 days after vaccination and pooled, and single-cell suspensions were made by the passage of the tissue through sterile mesh after red blood cells were lysed with Tris-buffered ammonium chloride. Serial dilutions of cells were incubated with or without 10 μ g/mL peptide at 37°C for 24 hours in 96-well ELISPOT plates. The assays were performed and developed using anti-mouse IFN- γ mAb according to the kit manufacturer's instruction (MABTECH Inc, Mariemont, OH). All experiments were performed in duplicate and the data correspond to the mean (SD) values.

Cell Isolation and Detection of APCs by Fluorescent Antibody Staining

Lymph nodes were harvested from mice that underwent the application of 35 μ L imiquimod with or without dextran conjugated with Alexa Fluor 488 (10,000 MW), pooled, and dissociated into single-cell suspensions by enzymatic digestion with collagenase D (Roche Diagnostics, Mannheim, Germany) and DNase (Sigma-Aldrich, St. Louis, Missouri) for 45 minutes. Cells were collected, washed, and then stained with anti-CD11c mAb-PE, anti-I-A^b mAb-PerCP for

30 minutes at 4°C with blocking for Fc receptors using anti-CD16/CD32 mAb (BD Biosciences, San Diego, CA) prior to staining. After being washed, the cells were analyzed by FACSscan (Becton Dickinson, San Jose, CA) using CellQuest software with the expression of CD11c, I-Ab, and Alexa Fluor 488.

Cytotoxicity Assays

In vivo cytotoxicity assay was performed as previously described.¹⁵ Splenocytes from naive B6.PL mice (expressing the Thy1.1 allele) were stained with either 5 or 0.5 μ M carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) according to the manufacturer's instructions. The cells labeled with high concentrations of CFSE were incubated with antigen peptide for 1 hour at 37°C. Cells were washed and mixed at equal amounts, and a total of 10^7 cells were adoptively transferred into mice treated with various immunization protocols. Thirty-six hours later, lymphocytes were isolated from the spleen and target cells distinguished from host cells by the expression of the Thy1.1 marker were analyzed for CFSE fluorescence intensity by FACS. The percent cytotoxicity was calculated as follows: $([\%CFSE^{high} \text{ in untreated mice}] - [\%CFSE^{high} \text{ in treated mice}]) / [\%CFSE^{high} \text{ in untreated mice}] \times 100$. In vitro cytotoxic activity of the cells was determined by 4-hour ^{51}Cr release assays. Spleen and lymph nodes were harvested and pooled from mice that underwent vaccination with various protocols and restimulated with peptide (5 $\mu\text{g/mL}$) in the presence of IL-2 (25 U/mL) and IL-15 (10 ng/mL) for 7 days. Peptide-pulsed target cells were prepared by overnight culture of EL-4 cells with 10 $\mu\text{g/mL}$ peptide. Various numbers of effector cells from each culture were mixed with the target cells that had been incubated with or without peptide overnight and labeled with 200 μCi Na ^{51}Cr (Amersham Pharmacia Biotech) in 96-well round-bottomed plates. After a 4-hour incubation period at 37°C, the radioactivity in the supernatants was determined using a TopCount scintillation counter (Packard Instruments, Meriden, CT), and the percentage of specific lysis was defined by the formula $([\text{experimental } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}] / [\text{maximum } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}])$, where spontaneous release was the radioactivity of target cells released into the supernatant in the absence of effector cells, and maximum release was the radioactivity released by the targets incubated with 0.1% Triton X-100. All experimental determinations were performed in duplicate, and the averages and SD were consistently below 15% of the value of the mean.

Cell Proliferation Assays

Cell proliferation assays were performed to measure the response of CD8⁺ T cells derived from OT-1 mice upon stimulation with CD11c⁺ cells purified from B6 mice given topical applications of OVA_p mixed with imiquimod. CD8⁺ T cells were isolated from spleens of OT-1 mice and used as responder cells. CD11c⁺ APCs were isolated from either draining lymph nodes or non-draining lymph nodes of mice given the immunizations with OVA_p. Anti-mouse CD8 and CD11c microbeads (Miltenyi Biotec, Germany) were used for cell purifications. CD8⁺ T cells (3×10^4 /well) were co-cultured with the indicated numbers of APCs in 96-well U-bottom culture plates and incubated at 37°C for 48 hours, and during the final 16 hours each well was pulsed with 0.5 mCi [^3H] thymidine (Amersham Pharmacia Biotech, Piscataway, NJ). The radioactivity incorporated into DNA, which correlated with cell proliferation, was measured using the TopCount scintillation counter (Packard Instruments). All experiments were performed in triplicate, and results correspond to the mean (SD) values.

RESULTS

Topical Application of Peptide with Imiquimod Induces Efficient Antigen-Specific CTL Responses

We first tested the ability of imiquimod to serve as an adjuvant for TCI by measuring the systemic CTL response induced by a single application of peptide mixed with either a cream

formation of imiquimod or in vehicle alone (PBS with 15% DMSO). For these experiments C57BL/6 mice were immunized with OVA_p, a synthetic peptide corresponding to a well-characterized CTL epitope, and 7 days later, ELISPOT assays were carried out to assess the overall CTL response to the immunogen by determining the number of IFN- γ -producing T cells upon peptide stimulation. Although noticeable CTL responses were detected in the mice that underwent TCI with OVA_p in vehicle alone, peptide immunization by TCI with imiquimod increased by approximately fourfold the level of the CTL response (Fig. 1A). When we compared the effectiveness of TCI against our conventional vaccine protocol via an s.c. injection of OVA_p emulsified in IFA and CpG-ODN, another TLR adjuvant, we observed that the responses to TCI were approximately 50% of those obtained by the s.c. vaccine (see Fig. 1B). Although we cannot determine whether these differences were due to either the administration route of the vaccine or to the TLR adjuvant (or to both), it is clear that TCI using imiquimod constitutes an effective and practical way to induce CTL responses to a peptide antigen.

CTLs Generated by TCI are Capable of Killing Target Cells

To assess the functional effector properties of CTLs generated by TCI, we performed in vitro cytotoxicity assays of lymphocytes isolated from mice that underwent immunization by topical application of peptide with or without imiquimod. First, cells from the spleens and the lymph nodes from three mice were pooled and stimulated in vitro with OVA_p (5 μ g/mL) in the presence of IL-2 (25 U/mL) and IL-15 (10 ng/mL) for 7 days. The cytolytic activity of the effector cells was then measured using a standard 51 Cr release assay. As shown in Figure 2, target cells (EL-4) pulsed with relevant peptide were killed to some extent by CTLs generated in mice immunized with OVA_p without imiquimod (see Fig. 2B). However, mice immunized with OVA_p plus imiquimod produced a significantly higher CTL response than the immunized mice that did not receive the imiquimod (see Fig. 2A), consistent with the results observed in the ELISPOT assay shown in Figure 1A.

Because culturing cells for several days in vitro could give rise to an artificial expansion of CTLs and thus may not reflect the existing conditions prior to in vitro stimulation, in vivo cytotoxic assays were carried out in the following experiment. CTL responses were assessed in vivo in mice that had undergone either TCI or s.c. vaccination using various OVA_p doses (50, 150, and 300 μ g for TCI; 50 and 150 μ g for s.c.). Comparable high levels of specific cytotoxic activity (measured by the elimination of adoptively transferred CFSE^{high} OVA_p-pulsed target cells, compared with the CFSE^{low} antigen-negative target cells) were observed in the two corresponding groups of mice immunized with the highest OVA_p dose (see Fig. 2C). The percentages in Figure 2C correspond to the specific cytotoxicity obtained for each group. Thus, TCI using this approach required roughly twice the amount of peptide used for an s.c. vaccine to obtain an equivalent response. Nevertheless, these results indicate that CTLs induced by TCI are functionally active and capable of killing antigen-bearing cells both in vitro and in vivo.

Topical Imiquimod Application Promotes Antigen Uptake by LCs and Their Migration into Draining Lymph Nodes

Resident epidermal LCs are thought to act as the main APC to elicit systemic immune responses induced by TCI. To address the role of these cells for inducing CTL response to TCI with peptide and imiquimod, we performed a cell tracking study of a topically applied fluorescent low-molecular-weight compound. Alexa Fluor 488-conjugated dextran (approximately 10,000 Da) was administered by topical application with imiquimod into the mice, and the presence of fluorescent cells in lymphoid organs was studied by flow cytometry. Draining and non-draining lymph nodes were harvested from the mice 24 hours after receiving the topical antigen preparation. Notably, a significant number of Alexa Fluor 488-positive cells were found in the

lymph nodes draining the area of topical application, whereas no fluorescent cells were observed in the distal lymph nodes (Fig. 3A). As shown, the fluorescent cells expressed high levels of MHC class II (I-A^b) and CD11c (data not shown), indicating that these cells are typical DCs.

We further investigated whether CD11c⁺ cells taking up peptide applied onto the skin and subsequently migrating into the draining lymph nodes would function as effective APCs for activating and inducing the proliferation of CD8⁺ T cells. OVAp-specific CD8⁺ T cells that were purified from OT-1 T-cell receptor transgenic mice were co-cultured with purified CD11c⁺ cells derived from either the draining or the distal lymph nodes obtained from mice that had received TCI using OVAp. As shown in Figure 3B, the CD11c⁺ cells derived from draining lymph nodes of mice given OVAp plus imiquimod effectively stimulated the proliferation of the OT-1 CD8⁺ T cells, whereas neither the APCs from distal lymph nodes of mice immunized with OVAp plus imiquimod nor the cells from mice immunized with imiquimod alone were able to display this activity. Control experiments, pulsing all of the CD11c⁺ populations with OVAp, showed that the APCs in all cases were equally potent for stimulation of T cells (see Fig. 3C). These results confirm that skin-resident APCs can pick up peptides administered topically with imiquimod and take this antigen to the draining lymph nodes, where they can be presented to antigen-reactive CTLs.

Enhancement of TCI-Induced CTL Responses by Booster Immunizations

In the TCI protocol described so far, the intensity of the immune responses elicited by a single topical application of antigen seemed to be somewhat weak compared with that generated by s.c. immunization using the same amount of peptide (see Fig. 1B, and data not shown). Thus, we proceeded to study whether TCI booster applications could augment the CTL response before we evaluated this approach for its antitumor effect. As shown in Figure 4, when TCI was applied twice with a week's interval between the applications (condition 2), no significant increase in the response was observed compared with a single TCI application (condition 1). However, when TCI was applied for three consecutive times at weekly intervals (condition 3), a small but significant increase was observed ($P < 0.03$). In this experiment we also evaluated the application of booster TCI at shorter time intervals. Interestingly, a substantial increase in the CTL response was obtained when the vaccine was applied twice a week at a 3-day interval (condition 4; $P < 0.01$), but this effect could not be further augmented if the vaccine was applied three times a week (at a 2-day interval, condition 5). TCI application two or three times a week gave responses close to those obtained with the conventional s.c. injection (condition 6).

Enhancement of CTL Responses by Adhesive Tape-Stripping Followed by TCI

Previous studies have shown that disruption of the epidermal barrier by adhesive tape-stripping (ATS) leads to an enhanced immune response.¹⁶ Therefore, we studied whether ATS could enhance the CTL response induced by TCI using imiquimod as an adjuvant. The results (Fig. 5) show that TCI plus imiquimod after ATS increased the CTL response approximately twofold over that obtained with TCI alone, to a level close to that obtained with the s.c. injection. The above results indicate that either booster immunization or ATS is a viable approach to augment the effect of TCI, and either one could be applicable to experiments designed to evaluate anti-tumor effects.

Prophylactic Antitumor Effect of TCI and Imiquimod

In view of the results so far presented, we proceeded to investigate the antitumor effects elicited by TCI in a mouse melanoma model system. For the first experiment, groups of five mice received TCI-2X (twice in a week) with either OVAp plus imiquimod or with imiquimod alone; 7 days after the second topical application, the animals were challenged with an intravenous injection of 1×10^5 B16-OVA melanoma cells; 2 weeks later, the lung metastases were counted.

As shown in Fig. 6 (specific example presented in Fig. 6B), the mice that received TCI-2X with OVA plus imiquimod had significantly ($P < 0.01$) fewer metastases (approximately 10 nodules) than those that received imiquimod alone (>150 nodules). The effect of prophylactic TCI was also evaluated in mice challenged s.c. with tumor cells 7 days after the second immunization. As shown in Figure 6c, significant delay of tumor growth was observed in the mice that had received peptide vaccination with either TCI-2X with OVA plus imiquimod or the s.c. vaccine consisting of OVA plus CpG-ODN, compared with the nonvaccinated controls. In contrast, no substantial antitumor effect was observed in the mice that received imiquimod alone.

Therapeutic Antitumor Effect of TCI with Peptide Plus Imiquimod

The TCI-2X protocol was also evaluated in a therapeutic tumor model. Here, mice first received an intravenous inoculation of B16-OVA melanoma cells, and 4 days later they were treated with various therapeutic vaccination protocols. Two weeks later the lung tumors (nodules) were enumerated. As shown in Figure 7A, treatment with either TCI-2X with OVA/ imiquimod or the s.c. vaccine (OVA/CpG-ODN) reduced significantly the number of tumor nodules compared with non-treated mice or the animals treated with imiquimod alone ($P < 0.01$). In this experiment we also evaluated the effects of TCI-2X in providing a survival benefit in mice bearing 4-day established lung tumors. The results (see Fig. 7B) indicate that TCI-2X with OVA/imiquimod significantly increased the median survival time by approximately 20 days compared with the control mice. On the other hand, topical administration of imiquimod alone had little effect in prolonging the survival of the mice. Overall, these results show not only that TCI induces strong CTL responses to the immunizing peptide but also that these responses are sufficiently strong to generate both prophylactic and therapeutic antitumor effects.

DISCUSSION

We have shown here that TCI with peptide and imiquimod resulted in the generation of strong CTL responses leading to efficient antitumor immunity. TCI is a simple and relatively new method of vaccination that uses a topical application of an antigen and adjuvant onto intact skin. Our results indicate that topically applied CTL peptide antigens appear to be small enough (MW of approximately 1,000 Da) to traverse the epidermal layer to generate immune responses. Because our stock solutions of synthetic peptide (at 10 mg/mL) are prepared in 100% DMSO and we estimate that the final cream formulation of the vaccine could contain approximately 15% (vol/vol) DMSO, it is likely that this extremely potent solvent could affect the adsorption of the antigen through the skin. Likewise, we cannot rule out that some of the excipient ingredients of the commercial imiquimod cream Aldara used here (isostearic acid, acetyl alcohol, stearyl alcohol, white petrolatum, polysorbate 60, sorbitan monostearate, glycerin, xanthan gum, benzyl alcohol, methylparaben, and propylparaben) could influence the transport of the peptide from the skin outer layers into a site where it becomes accessible to APCs. Experiments are underway to determine whether DMSO or the other compounds mentioned above play any role in the effectiveness of TCI to peptide antigens. Nonetheless, the data presented in Figure 3A indicate that DMSO may not be required for the internalization of molecules of relatively low molecular weight because the fluorescent dextran (10,000 Da) formulation used did not contain DMSO. The same experiment also suggests that larger synthetic peptides than those tested here, containing more than one CTL epitope, could be used in TCI. One attractive possibility for a multiple-epitope TCI-based vaccine would be to use the Trojan peptide constructs (of approximately 6,000 Da), which we recently reported as being effective for inducing CTL responses to at least three distinct CTL epitopes when injected s.c.¹⁷ This appealing possibility is under investigation.

We have presented a simple and practical immunization procedure that could revolutionize the use of vaccines for the treatment and prevention of both malignant and infectious diseases. Among the obvious advantages of TCI over conventional injected vaccines is the likely increase in compliance on the part of the patient due to the lack of syringes and needles, but also the possibility that patients could apply their own vaccines with the frequency needed to maintain immunity and prevent disease recurrence.

Acknowledgements

This work was supported by the National Institutes of Health Grants R01CA103921 and P50CA91956.

References

1. Davila E, Celis E. Repeated administration of cytosine-phosphorothiolated guanine-containing oligonucleotides together with peptide/protein immunization results in enhanced CTL responses with anti-tumor activity. *J Immunol* 2000;165:539–547. [PubMed: 10861094]
2. DeBenedictis C, Joubert S, Zhang G, et al. Immune functions of the skin. *Clin Dermatol* 2001;19:573–585. [PubMed: 11604304]
3. Takeda K, Akira S. Toll-like receptors in innate immunity. *Int Immunol* 2005;17:1–14. [PubMed: 15585605]
4. Glenn GM, Taylor DN, Li X, et al. Transcutaneous immunization: a human vaccine delivery strategy using a patch. *Nat Med* 2000;6:1403–1406. [PubMed: 11100128]
5. Zuber AK, Brave A, Engstrom G, et al. Topical delivery of imiquimod to a mouse model as a novel adjuvant for human immunodeficiency virus (HIV) DNA. *Vaccine* 2004;22:1791–1798. [PubMed: 15068863]
6. Kersten TS, Yu JM, Vassell R, et al. Transcutaneous immunization with bacterial ADP-ribosylating exotoxins, subunits, and unrelated adjuvants. *Infect Immun* 2000;68:5306–5313. [PubMed: 10948159]
7. Partidos CD, Beignon AS, Mawas F, et al. Immunity under the skin: potential application for topical delivery of vaccines. *Vaccine* 2003;21:776–780. [PubMed: 12531358]
8. Glenn GM, Scharton-Kersten T, Vassell R, et al. Transcutaneous immunization with cholera toxin protects mice against lethal mucosal toxin challenge. *J Immunol* 1998;161:3211–3214. [PubMed: 9759833]
9. Beignon AS, Briand JP, Muller S, et al. Immunization onto bare skin with heat-labile enterotoxin of *Escherichia coli* enhances immune responses to coadministered protein and peptide antigens and protects mice against lethal toxin challenge. *Immunology* 2001;102:344–351. [PubMed: 11298834]
10. Beignon AS, Briand JP, Tappuoli R, et al. The LTR72 mutant of heat-labile enterotoxin of *Escherichia coli* enhances the ability of peptide antigens to elicit CD4⁺ T cells and secrete gamma interferon after co-application onto bare skin. *Infect Immun* 2002;70:3012–3019. [PubMed: 12010992]
11. Kahlon R, Dutz JP. Skin immune responses to peptide and protein antigen are LTR4 independent. *Cell Immunol* 2003;226:116–123. [PubMed: 14962499]
12. Kahlon R, Hu Y, Orteu CH, et al. Optimization of epicutaneous immunization for the induction of CTL. *Vaccine* 2003;21:2890–2899. [PubMed: 12798632]
13. Xabier MG, Hammond SA, Epperson DE, et al. Immunostimulant patch containing heat-labile enterotoxin from *Escherichia coli* enhances immune responses to injected influenza virus vaccine through activation of skin dendritic cells. *J Virol* 2003;77:5218–5225. [PubMed: 12692224]
14. Saunderson DN. Imiquimod: modes of action. *Br J Dermatol* 2003;149:5–8.
15. Wolint P, Betts MR, Koup RA, et al. Immediate cytotoxicity but not degranulation distinguishes effector and memory subsets of CD8⁺ T cells. *J Exp Med* 2004;199:925–936. [PubMed: 15051762]
16. Nishijima T, Tokura Y, Imokawa G, et al. Altered permeability and disordered cutaneous immunoregulatory function in mice with acute barrier disruption. *J Invest Dermatol* 1997;109:175–182. [PubMed: 9242504]
17. Lu J, Higashimoto Y, Appella E, et al. Multiepitope Trojan antigen peptide vaccines for the induction of antitumor CTL and Th immune responses. *J Immunol* 2004;172:4575–4582. [PubMed: 15034075]

FIGURE 1.

Imiquimod induces efficient antigen-specific CTL responses when co-applied with OVA_{257–264} peptide (OVAp) onto mouse skin. A, Naive C57BL/6 mice (3 mice per group) were immunized with the application of OVAp (300 μ mol) mixed with either 35 μ L of the cream formation of imiquimod (OVAp/Iq) or the same amount of vehicle alone (OVAp/vehicle). As a negative control, mice were immunized with imiquimod alone. Six days later, spleens and draining lymph nodes were harvested, pooled, and subjected to 24-hour IFN- γ release ELISPOT assay to determine peptide-specific CTL responses. A series of 1:3 dilutions of total spleen and lymph node cells starting from 2×10^6 cells were incubated with OVAp (10 μ g/mL) in each well of an antibody-coated ELISPOT 96-well plate for 24 hours under tissue culture conditions. Pictures show one of the duplicate wells in each group with (+) or without (–) peptide stimulation. Each bar represents the average of the numbers of spots indicated in duplicate samples. B, Naive C57BL/6 mice (3 per group) were immunized transcutaneously with OVAp (100 μ mol) mixed with imiquimod (OVAp/Iq). Subcutaneous injection of OVAp (100 μ mol) emulsified with IFA and CpG (100 μ g) was also set up as a standard vaccine method. Seven days later, spleens and draining lymph nodes were harvested, and the antigen-specific CTL responses were analyzed by a 24-hour IFN- γ release ELISPOT. Although the differences in the intensity of the immune response to OVAp/Iq observed between A and B are likely due to differences in the peptide dose, we also observed substantial variations from experiment to experiment.

FIGURE 2.

Efficient induction of CTL in mice vaccinated by TCI with OVAp and imiquimod. A and B, Naive C57BL/6 mice (3 mice per group) were immunized by TCI using OVAp (150 μ mol) with vehicle alone (OVAp/vehicle) or with imiquimod (OVAp/Iq). Six days after vaccination, spleens and draining lymph nodes were harvested, pooled, and stimulated with peptide (5 μ g/mL) in the presence of IL-2 (25 U/mL) and IL-15 (10 ng/mL) for 7 days. T-cell responses were detected with or without peptide pulsing in a 4-hour chromium assay. All experimental determinations were performed in duplicate, and the averages and SD were consistently below 15% of the value of the mean. Each data point represents the average of duplicate samples. C, In vivo cytotoxic assay in mice that underwent immunization with various amount of peptide in comparison between s.c. injection and transcutaneous application of peptide. Mice were transcutaneously immunized with imiquimod alone (Iq) or 50 μ mol (Iq50), 150 μ mol (Iq150), or 300 μ mol (Iq300) of OVAp with imiquimod. As a standard immunization protocol by injection, mice were immunized s.c. with either 50 μ mol (SC50) or 150 μ mol (SC150) of OVAp emulsified with IFA and CpG (100 μ g). 7 days later, target cells consisting of equal amounts of syngeneic CD45.1⁺ spleen cells nonpulsed and pulsed with OVAp and stained with CFSE at low and high concentrations, respectively, were administered intravenously. Spleen cells were harvested and analyzed by flow cytometry for the detection of CFSE 36 hours after injection. Histograms for cells gated on PI-negative and CD45.1-positive populations are shown. Numbers in the bottom of the panel show the % specific cytotoxicity calculated as indicated in text.

FIGURE 3.

Effect of imiquimod on antigen uptake by LCs. A, Antigen uptake was determined by measuring the presence of fluorescent antigen on I-A^b positive, CD11c⁺ cells in draining and non-draining lymph nodes. As an antigen, Alexa Fluor Dextran 488 (FL1) was applied onto the skin of C57BL/6 mice, and 24 hours later, draining or non-draining lymph nodes were harvested and stained with anti-CD11c⁺, anti-I-A^b mAbs. Cells represented in the figure were gated on CD11c⁺ population. The numbers represent the percentage of I-A^b positive cells that contain fluorescent antigen. B, Detection of antigen in LCs from draining lymph nodes. Draining lymph nodes (DLN) and non-draining lymph nodes (NDLN) were harvested from mice that underwent topical applications of OVAp either with or without imiquimod and CD11c⁺ cells were purified using magnetic beads. CD8⁺ T cells from OT-1 mice were used as responder cells for a cell proliferation assay measuring the incorporation of ³H-thymidine into DNA. C, Cell proliferation assays were also performed using APCs exogenously pulsed with OVAp (10 µg/mL) 1 hour before the assay. Values shown are triplicates. Experiments were repeated twice with nearly identical results.

FIGURE 4.

Evaluation of booster immunization effect on CTL responses induced by TCI. Peptide-specific CTL responses were determined by ELISPOT assay. C57BL/6 mice (3 mice per group) were immunized either with the transcutaneous application of OVAp (100 μ mol) with imiquimod (OVAp/Iq) once (condition 1), twice (condition 2), or three times (condition 3) on a weekly basis. Booster immunizations with TCI were also done two times in the same week (3 days apart, condition 4) or 3 times in the same week (2 days apart, condition 5). Seven days after the last immunization, spleens and draining lymph nodes were harvested, pooled, and subjected to a 24-hour IFN- γ ELISPOT assay. Subcutaneous injections of OVAp with CpG were included for comparison as a standard immunization protocol.

FIGURE 5.

Evaluation of the effect of the adhesive tape-stripping (ATS) procedure on CTL responses induced by TCI. C57BL/6 mice (3 mice per group) were immunized with TCI using OVAp (100 μ mol) and imiquimod with (OVAp/Iq + ATS) or without ATS (OVAp/Iq) pretreatment. Six days later, pooled cells of spleens and draining lymph nodes from immunized mice were subjected to IFN- γ ELISPOT assay. TCI using OVAp without imiquimod with ATS (OVAp/ATS) and s.c. vaccine (OVAp/IFA, CpG) were included as controls.

FIGURE 6.

Co-application of OVAp with imiquimod induces protective immunity against a tumor challenge. A, Protective immunity against the B16-OVA melanoma was examined. C57BL/6 mice (5 mice per group) were first immunized twice (3 days apart) by TCI either with the application of OVAp (150 mmol) and imiquimod (TCI-OVAp/Iq) or imiquimod alone (TCI-Iq alone). Untreated mice were included as controls (No Tx). Seven days after the last TCI all mice were challenged intravenously with 2×10^5 B16-OVA cells; 2 weeks later, the mice were killed and lungs were harvested to enumerate the tumor nodules. Data represent the average numbers of lung metastases in each group. B, Graphic example of one mouse in each group. C, Effect of prophylactic TCI with peptide and imiquimod to a subcutaneous tumor challenge. C57BL/6 mice (10 mice per group) were first immunized by TCI either with the application of OVAp (150 μ mol) and imiquimod (TCI-OVAp/Iq) or imiquimod alone (TCI-Iq alone) 3 days apart. A group of mice given subcutaneous injection of OVAp (150 μ mol) emulsified with IFA and CpG (SC-OVAp/IFA, CpG) and another group of mice left untreated (No Tx) were included as controls. One week after the last vaccination, the mice were challenged with subcutaneous injection of 2×10^5 B16-OVA cells. Tumor sizes (in mm²) were scored by measuring perpendicular by longitudinal diameters and averaged for all tumor-bearing mice within each group. By day 20 the mice from the No Tx and TCI-Iq alone groups had to be killed due to their large tumors. The experiment was terminated on day 30. These results are representative of two experiments.

FIGURE 7.

Evaluation of therapeutic antitumor effect of TCI using OVAp and imiquimod. A, 2×10^5 B16-OVA were first injected intravenously into C57BL/6 mice (15 animals per group). Four days later, mice were given the application of OVAp (150 μ mol) with imiquimod (TCI-OVAp/Iq) or imiquimod alone (TCI-Iq alone) twice a week for 2 weeks. One group of mice was given s.c. injection of OVAp (100 μ mol) emulsified with IFA and CpG (SC-OVAp/IFA, CpG), and another group of mice was left untreated (No Tx). Twenty days later, 5 mice from each group were killed and the lungs were harvested to count the tumor nodules. Data represent the average numbers of nodules in each group. B, The remaining 10 mice in each group were observed for their survival.