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Tumor Immunotherapy by Epicutaneous Immunization Requires Langerhans Cells

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A role for Langerhans cells (LC) in the induction of immune responses in the skin has yet to be conclusively demonstrated. We used skin immunization with OVA protein to induce immune responses against OVA-expressing melanoma cells. Mice injected with OVA-specific CD8+ T cells and immunized with OVA onto barrier-disrupted skin had increased numbers of CD8+ T cells in the blood that produced IFN-γ and killed target cells. These mice generated accelerated cytotoxic responses after secondary immunization with OVA. Prophylactic or therapeutic immunization with OVA onto barrier-disrupted skin inhibited the growth of B16.OVA tumors. LC played a critical role in the immunization process because depletion of LC at the time of skin immunization dramatically reduced the tumor-protective effect. The topically applied Ag was presented by skin-derived LC in draining lymph nodes to CD8+ T cells. Thus, targeting of tumor Ags to LC in vivo is an effective strategy for tumor immunotherapy. The Journal of Immunology, 2008, 180: 1991–1998.

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Materials and Methods

Mice

Breeding pairs of the inbred strains C57BL/6 (CD45.2+) and the congenic strain B6-SJ ptprca (CD45.1+) were obtained from The Jackson Laboratory, and from the Animal Resource Centre, respectively. OT-I and OT-II mice express transgenic Vα2 Vβ5.1/5.2 TCR specific for K b + OVA257–264 and I-Ab + OVA223–239, respectively (15, 16). Relevant breeding pairs were provided by S. Hook (School of Pharmacy, Dunedin, NZ), with the permission of F. Carbone (Melbourne University, Melbourne, Australia). Langerin-DTR-GFP and Langerin-EGFP knockout mice expressing a DTR and/or EGFP in LC were used (10). All mice were bred at the animal facility of the Malaghan Institute of Medical Research, and used for experiments at 7–16 wk of age. All experimental protocols were approved by the Victoria University Animal Ethics Committee and performed according to institutional guidelines.
Medium and reagents
Culture medium was IMDM supplemented with 5% FBS, 2 mM glutamax, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µM 2-ME (all Invitrogen Life Technologies). OVA was purchased from Sigma-Aldrich. OVA257–264 (SIINFEKL) and OVA323–339 (ISQAVHAAHAEINEAGR) were purchased from Mimotopes. The B16.OVA cell line, generated by E. Lord and J. G. Frelinger (University of Rochester, Rochester, NY) (17), was provided by R. A. Kemp and R. W. Dutton (Trudeau Institute, Saranac Lake, NY).

FACS analysis
Anti-CD4 (clone GK1.5) mAb was affinity purified from hybridoma culture supernatants using protein G-Sepharose (Pharmacia Biotech) and conjugated to allophycocyanin. The following Abs were purchased from BD PharMingen: anti-CD107a PE and anti-CD107a PerCP-Cy5.5 (both clone Ly-2), anti-CD45.2 bioi and anti-CD45.2 PE (both clone 104), and anti-IFN-γ allophycocyanin (clone XM1G1.2). Propidium iodide (BD PharMingen) was added to samples to exclude or determine percentage of dead cells. All Ab incubations were conducted on ice for 10 min. All samples were analyzed on a FACS Excalibur using CellQuest software (BD Biosciences).

Detection of Ag-specific CD8+ T cells
SIINFEKL/2Kb pentamers conjugated to PE were obtained from ProImmune. Mice were bled through the tail vein on days 3, 5, 7, and 21 after immunization. After RBC lysis, the leukocytes were stained with pentamer on ice. All samples were analyzed on a FACS Excalibur using CellQuest software (BD Biosciences).

Epicutaneous immunization
For barrier disruption, anesthetized mice were tape stripped on ear skin by application of adhesive tape for 12 times onto both ears. Twenty-four hours later, 0.5 mg of OVA (in 20 µl of an aqueous cream, i.e., hydrophilic oil in water emulsion, Ultrasiss, Hecht Pharma) was applied onto either untreated or tape-stripped skin on each ear, for a total of 1 mg of OVA per mouse. As a control, PBS in 20 µl of cream was applied onto the epidermis of untreated skin. TLR ligand imiquimod was used in its pharmaceutical form, Aldara cream (5% imiquimod; 3M Pharmaceuticals), and PBS or OVA was mixed 1:1 with Aldara cream.

Isolation of T cells
Spleens and lymph nodes were pressed through a cell strainer, and RBC were lysed with ammonium chloride buffer. Cells were then incubated with anti-CD8a allophycocyanin mAb (clone Ly-2; BD PharMingen) for 10 min on ice. All samples were analyzed on a FACS Excalibur using CellQuest software (BD Biosciences).

In vivo proliferation assays
T cells were isolated from TCR transgenic OT-I and OT-II mice and labeled with 0.2 µM CFSE (Molecular Probes). Groups of three CD45.1+ congenic mice were injected i.v. with a mix of 2 × 10^6 CFSE-labeled OT-I and OT-II T cells, respectively, and tape stripped on the same day. Twenty-four hours later, mice were immunized with Ag in cream, as described above. Mice were sacrificed 72 h later, and the auricular lymph nodes were harvested for FACS analysis. The proliferation of Ag-specific CFSE+ T cells was detected by the decrease of CFSE fluorescence intensity, as described (18).

Detection of intracellular cytokines (IFN-γ)
Groups of three congenic CD45.1+ mice were injected i.v. with 10^6 CD45.2+ OT-I T cells and immunized through the skin, as described above. Auricular lymph node and spleen cells were prepared 1 wk later and restimulated for 4 h with 1 µM OVA257–264 in the presence of Golgi Stop (BD PharMingen). Intracellular IFN-γ staining was conducted using the Cytofix/Cytoperm kit (BD PharMingen), according to manufacturer’s instructions.

In vivo killing assay
Groups of three mice were injected with 10^6 OT-I T cells i.v. and immunized through the skin. On day 7 after immunization, mice were injected i.v. with mixtures of differentially CFSE-labeled spleen cells (20 or 200 nM) loaded with 10 or 100 nM OVA257–264 and unloaded spleen cells labeled with 10 µM chromomethyl-benzoyl-aminotetramethyl-rhodamine (CMTMR; Molecular Probes). Twenty-four hours after injection of target cells, mice were bled through the tail vein and blood was analyzed for presence of viable injected spleen cells, as determined by propidium iodide staining. Ingual lymph node cells were harvested at 48 h, and cell suspensions were analyzed as well. Percentage of killing was calculated using the formula, as described before (19): 100% − [(number of CFSE+ cells/number of CMTMR+ cells) × 100%]. For memory responses, mice were challenged on the neck skin 2–3 mo after the first immunization, and injected with target cells 1 day later.

Tumor challenge
Mice were injected s.c. into the flank with 10^6 B16.OVA tumor cells. Tumor size was assessed three times per week by measuring the short and long tumor diameters using calipers, and is expressed as mean product of tumor diameters ± SEM. Four to five mice were used in each group. Measurements were stopped when first mouse in the cage reached maximum tumor size (150 mm^2) and had to be euthanized. The tumor sizes from all mice in each group were used to calculate the mean tumor size. This means that mice with no tumor were counted as zeros, and we were able to draw lines for groups in which none of the mice developed tumors during the observation time. Tumor challenge was 7 days after skin immunization (prophylactic setting), or 7 days before immunization (therapeutic setting). For rechallenge experiments, mice were injected with 10^6 B16.F1 melanoma cells (American Type Culture Collection), or 10^5 B16.OVA on the contralateral flank 1 mo after the first tumor challenge.

In therapeutic experiments, we injected one group of mice i.v. with 200 ng of α-galactosylceramide (α-GalCer; Industrial Research) (20) and 400 µg of OVA as a positive control because previous experiments have shown that this treatment inhibits tumor growth (21).

Depletion of LC
A quantity amounting to 1 µg of DT (Sigma-Aldrich) in PBS was injected i.p. 2 days prior, on the day and 5 days after immunization to ensure the complete and persistent depletion of Langerin-positive cells.

In vitro proliferation assays with FACS-sorted LC
Langerin-EGFP mice were tape stripped 12 times and immunized, as described above. Two days later, auricular lymph node cells were digested with 0.5 mg/ml collagenase P (Roche Diagnostics) for 30 min at 37°C, pressed through cell strainers (Falcon; BD PharMingen), and enriched on a Nyco-denz gradient, as described (22). The cells in the interface were stained with anti-CD8 PerCP-Cy5.5 (clone Ly-2), anti-MHC class II PE (clone M5/114b), and anti-CD11c allophycocyanin (clone HL-3; all from BD PharMingen), and sorted on a FACSVantage-SE (BD Biosciences) for skin-derived EGFP+ CD8+ LC, blood-derived EGFP+ CD8+ DC, and remaining EGFP− DC (purity was routinely above 97%). Graded numbers of DC/LC were incubated with 2 × 10^5 OVA-specific OT-I T cells for 48 h, and proliferation of T cells was measured by incorporation of [3H]thymidine (CMTMR; Molecular Probes). Twenty-four hours after injection of target cells, mice were bled through the tail vein and blood was analyzed for presence of viable injected spleen cells, as determined by propidium iodide staining. Inguinal lymph node cells were harvested at 48 h, and cell suspensions were analyzed as well. Percentage of killing was calculated using the formula, as described before (19): 100% − [(number of CFSE+ cells/number of CMTMR+ cells) × 100%]. For memory responses, mice were challenged on the neck skin 2–3 mo after the first immunization, and injected with target cells 1 day later.

Statistical analysis
Unpaired Student’s t test was used (data were approximate Gaussian) to determine the statistical significance of differences between OVA treatment on tape-stripped vs untreated skin in Figs. 2, 3, and 4, as well as in Fig. 7 to compare T cell proliferation in response to PBS and OVA immunization. For the tumor experiments, we performed Kaplan-Meier analyses to determine percentage of survival. Probability values are expressed as the following: ***, p < 0.001; **, p < 0.01; and *, p < 0.05.

Results
OVA-specific T cells proliferate in mice immunized onto barrier-disrupted skin
We investigated the effect of epicutaneous immunization with OVA on the expansion of T cells in vivo. CD45 congenic mice were injected with 2 × 10^6 CFSE-labeled OVA-specific CD4+ and CD8+ T cells, and immunized with OVA onto either undisturbed or tape-stripped skin. In lymph nodes draining the immunization site, CD4+ and CD8+ T cells did not divide after immunization with PBS. In mice immunized with OVA onto undisturbed skin, both CD4+ and CD8+ T cells proliferated (28–94% of CD4+ T cells
CD4+ and CD8+ T cells proliferate after immunization on barrier-disrupted skin. A, Congenic CD45.1+ mice were injected i.v. with 2 × 106 CFSE-labeled OVA-specific CD4+ and CD8+ T cells (CD45.2+), and on the same day tape stripped 12 times on the dorsal surface of both ears, or left untreated. Next day, 0.5 mg of OVA or PBS in 20 μl of cream was applied to each tape-stripped or untreated ear. Proliferation of T cells was measured 3 days later in the auricular lymph nodes. Histograms show CFSE proliferation for cells gated on CD45.2+CD4+ or CD45.2+CD8+ T cells. Similar results were obtained in three independent experiments using three mice/group. B and C, Mice were injected with 106 OT-I T cells and immunized through the skin, as described in A. On days 3, 5, 7, and 21, mice were bled and the percentages of OVA-specific CD8+ T cells in blood were determined by OVA257–264/H-2Kb pentamer staining. Representative FACS dot plots of pentamer staining on gated CD8+ T cells on day 3 after immunization are shown in B. Results from two separate experiments are shown in C (three mice per group, mean ± SEM). In both experiments, the tape-stripped + OVA groups were significantly higher than the group receiving OVA on untreated skin, but peak responses were on different days. Experiments were done seven times. t str, Tape stripped.

FIGURE 1. CD4+ and CD8+ T cells proliferate after immunization on barrier-disrupted skin. A, Congenic CD45.1+ mice were injected i.v. with 106 OVA-specific OT-I T cells, and on the same day tape stripped 12 times on the dorsal surface of both ears, or left untreated. Next day, 0.5 mg of OVA or PBS in 20 μl of cream was applied to each tape-stripped or untreated ear. Proliferation of T cells was measured 3 days later in the auricular lymph nodes. Histograms show CFSE proliferation for cells gated on CD45.2+CD4+ or CD45.2+CD8+ T cells. Similar results were obtained in three independent experiments using three mice/group. B and C, Mice were injected with 106 OT-I T cells and immunized through the skin, as described in A. On days 3, 5, 7, and 21, mice were bled and the percentages of OVA-specific CD8+ T cells in blood were determined by OVA257–264/H-2Kb pentamer staining. Representative FACS dot plots of pentamer staining on gated CD8+ T cells on day 3 after immunization are shown in B. Results from two separate experiments are shown in C (three mice per group, mean ± SEM). In both experiments, the tape-stripped + OVA groups were significantly higher than the group receiving OVA on untreated skin, but peak responses were on different days. Experiments were done seven times. t str, Tape stripped.

FIGURE 2. Ag-specific CD8+ T cells produce IFN-γ after immunization on barrier-disrupted skin. Congenic CD45.1+ mice were injected i.v. with 106 OVA-specific OT-I T cells, and on the same day tape stripped 12 times on the dorsal surface of both ears, or left untreated. Next day, 0.5 mg of OVA or PBS in 20 μl of cream was applied to each tape-stripped or untreated ear. On day 7 after immunization, auricular lymph node and spleen cell suspensions were prepared and restimulated in vitro with 1 μM OVA257–264 for intracellular IFN-γ staining. A, Shows representative FACS dot plots of gated CD8+ T cells from auricular lymph nodes for individual mice; percentage of positive cells in each quadrant is shown. B, Shows data from three separate experiments giving similar results. ***p < 0.001; *p < 0.05. t str, Tape stripped.

CD8+ T cells activated by epicutaneous immunization mediate effector function

Immunization through the skin induces extensive proliferation of injected CD4+ and CD8+ T cells; thus, we wished to determine whether CD8+ T cells also acquired effector function. CD45-congenic mice were injected with 106 CD8+ T cells from OT-I mice, and immunized with OVA onto either untreated tape-stripped skin. On day 7 after treatment, lymph node and spleen cells were harvested and restimulated in vitro with 1 μM OVA257–264 to determine IFN-γ production. CD8+ T cells from mice treated with PBS or OVA onto undisturbed skin failed to produce IFN-γ. In contrast, roughly half of the injected CD45.2+CD8+ T cells from mice immunized with OVA onto tape-stripped skin produced IFN-γ (Fig. 2). No significant IFN-γ production was observed in cultures stimulated with an irrelevant peptide, or no peptide (data not shown). Similar results were obtained when OVA-specific CD8+ T cells were identified by OVA257–264/H-2Kb pentamer staining, although some of the CD8+ IFN-γ+ cells appeared negative for pentamer staining, indicating that the restimulation of OT-I T cells in vitro induced a down-regulation of the TCR. This is further underlined by the observation that lymph node cells restimulated for 6 h in vitro were no longer able to bind pentamers (data not shown).

Effector function of CD8+ T cells is not just measured by their IFN-γ production, but also, and more importantly, by their cytotoxic activity. We tested whether Ag-specific CD8+ T cells activated by epicutaneous immunization are able to kill target cells in vivo. For this purpose, skin-immunized mice were injected with...
spleen cells loaded with different amounts of OVA257–264 and CFSE, together with spleen cells labeled with CMTMR and not loaded with Ag, and killing was determined at 24 h in the blood and at 48 h in the lymph nodes. We observed low cytotoxic activity in mice treated with PBS or OVA onto undisturbed skin. In contrast, by 24 h most of the target cells were killed in mice immunized with OVA on tape-stripped skin (Fig. 3A). This shows that CD8⁺ T cells activated by epicutaneous immunization develop effector function in vivo within the first week after immunization.

To test whether skin immunization also induced the development of memory CD8⁺ T cells, we treated mice with PBS or OVA on untreated or tape-stripped ear skin, and rested them for 2–3 mo. During this time, cytotoxic activity declined to very low levels in all mice (data not shown), thus allowing us to determine the effect of a secondary boost on cytotoxic activity. All mice were immunized again with 1 mg of OVA on tape-stripped skin, and injected with CFSE-labeled spleen cells. Cytotoxic activity was high in mice that had received OVA onto barrier-disrupted skin in the first immunization, whereas cytotoxicity in the other groups remained low (Fig. 3B). When OVA257–264-loaded target cells were injected 2–7 days after the secondary immunization, killing was similarly high in all the treatment groups (data not shown). Thus, epicutaneous immunization leads to the activation of CD8⁺ T cells with effector activity, and the development of memory CD8⁺ T cells in vivo.

**Mice immunized onto barrier-disrupted skin are protected from tumor growth**

The CD8⁺ T cells activated by epicutaneous immunization were able to produce IFN-γ in vitro, kill target cells in vivo, and home to the skin, as detected by increased infiltration of CD8⁺ T cells into immunized skin (data not shown). We asked whether these CD8⁺ T cells could also mediate effector function in vivo, and prevent the growth of B16 melanoma tumor cells expressing OVA (B16.OVA). We injected mice with 10⁶ OVA-specific CD8⁺ T cells, immunized them through the skin, and 7 days later challenged them with 10⁶ B16.OVA tumor cells injected s.c. into the flank. Tumors became palpable after 8–10 days in the mice treated with PBS or OVA on untreated skin. Mice treated with OVA on undisturbed skin occasionally remained tumor free (∼1 of 5 mice in each experiment), indicating partial protection by this treatment. However, nearly all of the mice treated with OVA on tape-stripped skin remained tumor free over the time of the experiment (Fig. 4A). When mice were challenged with tumor on day 21, instead of day 7, after immunization, they again remained tumor free, indicating that the induced response was long-lived (data not shown).

Tape-stripped and immunized mice that remained tumor free after the first injection of B16.OVA cells were rechallenged 1 mo later with the same number of tumor cells on the contralateral flank. All mice were protected against a second challenge with the same tumor, suggesting the development of long-lasting immunity (Fig. 4B). To investigate whether this protection was OVA specific, we rechallenged mice with B16.F1 melanoma cells not expressing OVA. Some of these mice developed tumors at the same time as the control mice (nonimmunized mice), some developed tumors later, and some remained tumor free, suggesting that some immunity to melanoma Ags had developed in these mice (Fig. 4B).

To determine whether epicutaneous immunization could induce therapeutic antitumor immunity, mice were injected with 10⁷ OT-I T cells i.v., and injected with 10⁵ B16.OVA tumor cells on the next day. Adoptive transfer of OT-I T cells increased the percentage of Ag-specific CD8⁺ T cells in blood to ∼0.5%, and this percentage did not change after tumor cell injection (data not shown). On day 7 after tumor inoculation, mice were immunized on the skin, as described above. As a positive control, one group of mice was injected i.v. with α-GalCer and OVA, because this immunization protocol has been shown to inhibit tumor growth (21). Immunization with OVA on tape-stripped skin did not block tumor growth, but tumors remained small until day 22/24 when they started to progress again. The difference in tumor size between mice immunized with PBS or OVA on untreated skin vs mice immunized with OVA on tape-stripped skin was statistically significant on day 13 (p < 0.05) and day 15 (p < 0.005) after immunization (Fig. 4C). In contrast, tumors grew to maximum size in the mice immunized with PBS or OVA on untreated skin. Treatment with α-GalCer and OVA i.v. caused a decrease in tumor size, so that tumors were barely palpable by day 13, but started to progress again by day 20 (Fig. 4C). Results similar to those shown in Fig. 4C were obtained when mice were immunized epicutaneously on day 10 after tumor administration, when tumors were already palpable; however, inhibition of tumor growth was less marked in this case (data not shown).

Immunization with OVA on tape-stripped skin induced expansion of Ag-specific CD8⁺ T cells in tumor-bearing mice, peaking at ∼2.5% of CD8⁺ T cells during week 1. Mice immunized with α-GalCer plus OVA i.v. also had increased frequencies of OVA-specific CD8⁺ T cells in peripheral blood, which peaked at ∼25% of CD8⁺ T cells during the first week after immunization (data not shown). Altogether, these results indicate that CD8⁺ T cells activated by epicutaneous immunization are able to control tumor growth in both a prophylactic and therapeutic setting.

**FIGURE 3.** Immunization on barrier-disrupted skin leads to generation of specific cytotoxic activity in vivo. Mice were injected i.v. with 10⁶ OVA-specific OT-1 T cells, and on the same day tape stripped 12 times on the dorsal surface of both ears, or left untreated. Next day, 0.5 mg of OVA or PBS in 20 μl of cream was applied to each tape-stripped or untreated ear. A. On day 7 after immunization, peptide-loaded CFSE-labeled spleen cells and unloaded CMTMR-labeled spleen cells were injected i.v. into immunized mice. Percent specific in vivo killing was determined 24 h later on blood cells. Results are from one experiment of four that gave similar results. B. 2.5 mo after epicutaneous immunization, all mice were immunized again on tape-stripped neck skin with 1 mg of OVA in cream. One day after immunization, peptide-loaded CFSE-labeled spleen cells and unloaded CMTMR-labeled spleen cells were injected i.v. into immunized mice. Percent specific in vivo killing was determined 48 h later in lymph node cells. Results are from one experiment of four that gave similar results. Mean ± SEM for three mice are shown. **p < 0.001; *p < 0.01. t str, Tape stripped.**
Tumor protection after epicutaneous immunization does not require the presence of TCR transgenic CD8\(^+\)/H\(_{11}\) T cells

In the experiments described to date, the number of Ag-specific CD8\(^+\)/H\(_{11}\) T cells was artificially increased by i.v. injection of OVA-specific OT-I T cells. We wanted to know whether epicutaneous immunization was also effective in mice that have a normal frequency of OVA-specific T cells. Ear skin of C57BL/6 mice was tape stripped 12 times to disturb the skin barrier. Next day, we applied either PBS or 0.5 mg of OVA in a cream onto the ear skin of untreated or tape-stripped mice, respectively. Seven days after immunization, mice were challenged with B16.OVA tumor cells. Tumor growth was delayed in mice immunized with OVA on tape-stripped skin, but eventually all mice developed tumors (Fig. 5). Similar results were observed when mice were challenged with tumors 21 days after immunization, indicating that the induced response was long lasting (data not shown).

We tested the ability of imiquimod, a TLR-7 ligand, to increase the efficiency of epicutaneous immunization. OVA was mixed with cream containing 5% imiquimod (Aldara cream) and compared with OVA in the standard cream. Mice immunized with OVA in Aldara cream were better able to delay the progression of B16.OVA tumors than mice immunized with the same amount of OVA in standard cream, although this difference was not statistically significant (Fig. 5). Again, even in the presence of TLR ligands, immunization delayed, but did not prevent tumor growth.

**LC are required for antitumor responses induced by epicutaneous immunization**

It is unknown to what extent LC contribute to immunization strategies through the skin. We used Langerin-DTREGFP mice

**FIGURE 4.** Ag application on barrier-disrupted skin induces prophylactic and therapeutic antitumor responses. Mice were injected i.v. with 10\(^6\) OT-I T cells, and on the same day tape stripped 12 times on both ears, or left untreated. Next day, 0.5 mg of OVA or PBS in 20 \(\mu\)l of cream was applied to each tape-stripped or untreated ear. On day 7 after immunization, mice were injected with 10\(^8\) B16.OVA melanoma cells s.c. into the flank. A, Tumor size (upper panel) and percentage of survival (lower panel) are shown from one representative experiment of three (five mice per group). B, Tumor-free mice were rechallenged with either B16.OVA or B16.F1, melanoma cells in the contralateral flank 1 mo after the first tumor challenge. Control mice received tumor cells for the first time. Percentage of survival is shown for one representative experiment of three (four mice per group). C, Mice were injected with 10\(^8\) OT-I T cells i.v. on day 0 and with 10\(^8\) B16.OVA melanoma cells on day 1. Seven days later, mice were tape stripped 12 times on both ears, or left untreated. Next day, 0.5 mg of OVA or PBS in 20 \(\mu\)l of cream was applied to each tape-stripped or untreated ear. As a positive control, one group of mice was injected with 200 ng of a-GalCer and 400 \(\mu\)g of OVA i.v. on day 7. The differences in tumor size between mice immunized with OVA on tape-stripped skin and OVA or PBS on untreated skin were statistically significant on day 13 (\(p > 0.05\)) and day 15 (\(p < 0.01\)). Results from two experiments are pooled together (10 mice each group). Tumor size is expressed as mean ± SEM. ***, \(p < 0.01\); *, \(p < 0.05\). t str, Tape stripped.

**FIGURE 5.** The antitumor effect of epicutaneous immunization does not require the presence of TCR transgenic CD8\(^+\)/H\(_{11}\) T cells and is amplified by simultaneous administration of TLR7 ligands. Mice were tape stripped 12 times on the dorsal surface of both ears, or left untreated. Next day, 0.5 mg of OVA or PBS was applied to each tape-stripped or untreated ear, in 20 \(\mu\)l of standard cream or 20 \(\mu\)l of cream containing the TLR7 ligand imiquimod. Seven days after immunization, mice were injected with 10\(^5\) B16.OVA melanoma cells s.c. into the flank. Results from two experiments (9–10 mice each group) are pooled, and tumor sizes are expressed as mean ± SEM. Tumor sizes (upper panel) and percentage of survival (lower panel) are shown. ***, \(p < 0.01\); *, \(p < 0.05\). t str, Tape stripped.
expressing the DTR in LC to answer this question (10). Mice were injected i.v. with OT-I T cells, and LC were depleted by three i.p. injections of 1 μg of DT 2 days prior, on the day and 5 days after epicutaneous immunization. Two days after the first injection of DT, the skin was depleted of LC, and no LC were detected for up to 2 wk after DT injection, as confirmed by immunofluorescence staining of the epidermal sheets (data not shown). In addition, in the skin-draining lymph nodes, the percentage of Langerin+ cells was decreased, as described earlier (10). One week after skin immunization, mice were challenged with 10^6 B16.OVA tumor cells s.c. into the flank, and tumor growth was monitored thereafter. We used two different doses of OVA for skin immunization, because application of large amounts of Ag might cause diffusion into the dermal layer of the skin, or even to the draining lymph node. Langerin-DTREGFP mice not treated with DT (control mice) and immunized with a high dose of OVA were protected against tumor challenge, whereas about half of the mice immunized with the low dose of OVA developed tumors that appeared to grow at a slower rate compared with control mice. When LC were depleted with DT, immunized Langerin-DTREGFP mice became highly susceptible to tumor development. More than half of the mice immunized with the high dose of OVA, and all the mice immunized with the low dose, developed tumors (Fig. 6). No delay in tumor growth was observed in either of the DT-treated groups.

Skin-derived LC present OVA after epicutaneous immunization

We wished to determine which of the DC populations found in lymph node was responsible for presenting the OVA Ag applied to the skin. Mice expressing EGFP under the Langerin promoter, which allows to sort Langerin+EGFP+ LC from skin-draining lymph nodes, were tape stripped and immunized on ear skin with either PBS or high dose OVA in cream. Two days later, lymph nodes draining the immunization site were harvested, and DC populations were purified by electronic sorting for use as APCs in vitro. Three CD11c+MHC class II+ cell populations were compared, as follows: the skin-derived EGFP+CD8− LC, the blood-derived EGFP+CD8+ DC (23), and the remaining EGFP− DC population, which includes dermal DC and lymph node resident DC. As shown in Fig. 7, the skin-derived EGFP+CD8− LC and the EGFP− DC subpopulations had both captured OVA Ag in vivo, and could present the OVA epitope to specific CD8+ T cells in vitro. In contrast, the blood-derived EGFP+CD8+ DC were unable to induce any proliferation. None of the DC populations from mock-immunized mice could elicit T cell proliferation in vitro. We conclude that DT treatment of Langerin-DTREGFP mice prevents the effect of epicutaneous immunization by depleting skin Langerin+ cells, whereas the depletion of blood-derived CD8+ Langerin+ cells is irrelevant because these cells do not present OVA in vivo after epicutaneous immunization.

Discussion

Immunization strategies through the skin have the potential to mobilize large numbers of DC, are noninvasive, and easy to perform. Therefore, they offer an attractive alternative to conventional immunotherapies. Previous studies have shown that mice immunized...
with epidermal cells loaded with tumor material generated protective antitumor responses (24), suggesting that LC have the capacity to initiate productive antitumor immune responses. In this study, we extend those findings to show that antitumor immune responses could also be generated by loading LC with Ag in situ. Whole protein Ag applied onto the epidermis induced proliferation of CD4⁺ and CD8⁺ T cells, development of long-lasting effector function in CD8⁺ T cells, and inhibition of tumor growth in prophylactic and therapeutic settings. Although this method of immunization was especially effective when the frequency of tumor-specific CD8⁺ T cells was increased by adoptive transfer of transgenic CD8⁺ T cells, it was also active in mice with normal T cell repertoires, and its efficacy could be increased by including TLR ligands such as imiquimod in the vaccine formulation. LC played a critical role in the induction of this response, because mice in which LC were depleted by DT treatment were unable to resist tumor challenge after epicutaneous immunization. Moreover, LC in lymph nodes draining the immunization site were able to present ex vivo Ag to CD8⁺ T cells in vitro. Therefore, targeting of LC via skin immunization could be an innovative strategy to treat cancers.

Studies of skin immunization strategies have shown that optimal T cell responses require an adjuvant (6, 7, 25), which is necessary to induce skin inflammation and activation and migration of skin DC. Disturbing the skin barrier by tape stripping (7), or by simultaneous application of cholera toxin (6) or TLR ligands such as CpG oligonucleotides (6) or imiquimod (25) have all been shown to provide an adjuvant effect. We chose to use skin barrier disruption because this method might be applicable in clinical trials, and because we had observed that it effectively induced migration of LC from the skin to the draining lymph nodes (26). Throughout our study, we compared groups of mice that received OVA on untreated or on tape-stripped skin. Barrier disruption proved to be essential to achieve optimal and long-lasting CD8⁺ T cell activation and antitumor responses. Clinical studies have also shown that barrier disruption in combination with peptide immunization resulted in activation of specific T cell responses and reduction of lesion size in melanoma patients (8). In contrast, the clinical applicability of TLR ligands and cholera toxin as adjuvants remains to be proven. The TLR-7 ligand imiquimod, commercially available as Aldara cream, is clinically approved for treatment of genital warts and basal cell carcinoma. We used imiquimod cream as a vehicle for the application of OVA on tape-stripped skin, and observed improved antitumor effects compared with application of OVA in a cream not containing TLR ligands. Therefore, our results support further exploration of this immunization strategy in tumor immunotherapy.

We show in this study that epicutaneous immunization on barrier-disrupted skin induced expansion of CD8⁺ T cells, and their differentiation into effector cells able to secrete IFN-γ and express cytotoxic activity in vivo. IFN-γ has been shown to play a critical role in tumor immunity (27). In particular, B16 melanoma cells treated with IFN-γ up-regulate expression of MHC class I and MHC class II, express increased levels of fas ligand, and become more susceptible to lysis mediated by non-Ag-specific mechanisms (28). In addition, IFN-γ mediates antitumor activity through its antiangiogenic effects (27). It is likely that IFN-γ was also a critical mediator of the antitumor effects observed in this study. Pretreatment with IFN-γ was necessary to induce up-regulation of MHC class I molecules on B16.OVA tumor cells, and render them susceptible to OVA-specific CTL-mediated killing in vitro (data not shown). Rejection of B16.OVA cells in vivo may have been mediated by a similar mechanism because we measured increased IFN-γ production after skin immunization. Although OVA-specific T cells were probably playing a major role in rejection, a contribution of melanoma-specific T cells is also likely, because immunized mice were partly protected from rechallenge with parental tumors, reminiscent of observations in human models in which vaccination against one melanoma Ag can trigger responses against other tumor Ags (29).

Conventional vaccinations are routinely delivered by s.c. injection and might bypass skin DC. Thus, the potential of skin DC as Ag carriers in immunotherapy remains largely uncharacterized. A number of experimental and clinical studies have demonstrated that immunization through the skin induces activation of CD4⁺ T cells, Ab production, and systemic and mucosal immunity (4, 30). Protection from subsequent challenge with a lethal dose of cholera toxin was also observed (31). In a very recent study, CD8⁺ T cell responses were induced in melanoma patients by application of peptides on barrier-disrupted skin (8). Although minimal CD8 peptide responses have been successfully used in clinical tumor immunotherapy studies, they have some limitations, as follows: they fail to activate CD4⁺ T cells, and their use is restricted to patients of the appropriate HLA phenotype. Both of these limitations could be overcome by using whole protein Ags instead of peptides. In this manuscript, we build on our previous observation that LC are able to cross-present present Ag to CD8⁺ T cells (14) to show that a protein Ag applied onto barrier-disrupted skin can induce potent effector CD8⁺ T cell responses with cytokine production, cytotoxic activity, and inhibition of tumor growth in prophylactic and therapeutic tumor settings. Considerably delayed tumor growth was observed in unmanipulated mice that had received one single epicutaneous immunization, and this delay was amplified by addition of the TLR-7 ligand imiquimod in the Ag formulation. Topically applied imiquimod can induce emigration of LC from the epidermis (own observation and Ref. 32). However, TLR-7 is not expressed by murine or human LC, but by surrounding keratinocytes, suggesting an indirect effect through induction of cytokine production in keratinocytes (33, 34). Further improvements might be obtained by using combinations of different TLR ligands that may act directly on LC (35) and repeated immunizations to boost the T cell responses.

Over 20 years ago, Schuler and Steinman (36) demonstrated for the first time that LC are very potent APCs in vitro. However, the contribution of LC to in vivo immune responses has recently been questioned. Allan et al. (37) observed that in mice infected with HSV, LC were not involved in the presentation of viral Ag in the skin-draining lymph nodes. Three other studies used mouse models in which LC were ablated by DT in a conditional (9, 10) or constitutive way (11). In one of these studies, LC were found to be dispensable for contact sensitivity (10), whereas the second study reported that LC made a small, but detectable contribution to the contact sensitivity reaction (9). In the third mouse model, in which LC are absent at all times, contact sensitivity reactions were increased (11). There is evidence now that the discrepancy in the conditional knockout mouse models might be due to the use of different doses and types of contact allergen. When low doses of contact allergen are used, LC are essential for the contact sensitivity response, whereas high doses override the need for LC (B. Clausen, unpublished observation). We observed that tumor resistance induced by immunization with 0.025 mg of OVA was completely ablated by the depletion of LC in the skin. In contrast, immunization with 0.5 mg of OVA through the skin was sufficient to induce some tumor resistance in LC-depleted mice, although this was clearly decreased compared to intact mice. One possible explanation for this finding is that high Ag doses can diffuse to the dermis or even to the lymph nodes, and be taken up by local DC, whereas low Ag doses remain confined to the epidermal layer of the skin. Thus, our data suggest that, in conditions in which Ag loading of other populations of DC, such as dermal or lymph node...
DC, is minimal, LC are indeed effective APCs that can induce productive antitumor immune responses. As further evidence for the importance of LC in epicutaneous immunization, LC sorted from lymph nodes draining the immunization site were shown to activate naive, OVA-specific CD8+ T cells in vitro, suggesting that LC may also directly prime specific CD8+ T cells in vivo. In contrast, blood-derived EGFP+CD8+ DC (23) were unable to activate cells in vitro, indicating that they are unlikely to be involved in the response to epicutaneous immunization. In addition to skin-derived LC, EGFP+DC from lymph nodes draining the immunization site were also able to present OVA to naive CD8+ T cells in vivo. These cells may represent DC that have taken up Ag cross from the epidermis into the dermis or directly to the lymph node. However, the observation that depletion of Langerin+ cells in vivo is sufficient to ablate the effects of epicutaneous immunization suggests that these cells have a secondary role in the response to skin application of Ag.

In conclusion, our findings reveal a critical and nonredundant role of LC in the induction of immune responses to Ags in the epidermis, and support the view that epicutaneous immunization may have important applications in tumor immunotherapy.

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Disclosures
The authors have no financial conflict of interest.

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