Memory Th1 Cells Augment Tumor-Specific CTL following Transcutaneous Peptide Immunization

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Abstract
Targeting dendritic cells in vivo by transcutaneous peptide immunization (TCI) represents an efficient immunization strategy to induce tumor-specific CTL because it reflects the physiologic conditions occurring during pathogen infection. Here we show that including a Th1 epitope in TCI can activate preexisting memory Th1 (mTh1) responses and thereby enhance the CTL response. For this purpose, peptide-25, a major Th1 epitope of Ag85B from Mycobacterium tuberculosis, was selected. We adoptively transferred peptide-25-specific mTh1 cells and hgp100-specific naive CTL (pmel-1 TCR transgenic) into C57BL/6 mice. Subsequently, mice were transcutaneously immunized with CTL peptide (hgp100) and Th1 peptide (peptide-25). Five days after TCI, the frequency and function of pmel-1 cells was monitored by intracellular IFN-γ staining, ELISPOT, and in vivo cytotoxicity assays. TCI efficiently expanded hgp100-specific, IFN-γ–producing, strongly cytotoxic CD8+ T cells. Concurrent activation of mTh1 cells by peptide-25 induced a 1.5-fold increase in the number of hgp100-specific CTL with enhanced effector functions. Furthermore, TCI elicited not only prophylactic but also therapeutic antitumor responses that were augmented by peptide-25. These results show that TCI facilitates peptide-specific activation of CD4+ T cells, responsible for the augmenting effect of peptide-25 on the hgp100-specific CTL response. Because a significant proportion of the Japanese population has been vaccinated with Bacillus Calmette-Guerin, they are likely to possess Ag85B- or peptide-25–specific mTh1 cells. Therefore, concomitant activation of Ag85B- or peptide-25–specific mTh1 cells together with tumor-specific CTL by TCI might augment antitumor immune responses in a sizeable fraction of patients. [Cancer Res 2008;68(10):3941–9]

Introduction
Successful application of cancer immunotherapy is likely to require the activation, expansion, and differentiation of antigen-specific CD8+ T cells into functional effector cells (1). A current standard approach for generating antigen-specific CTL uses autologous monocyte-derived dendritic cells for the expansion of CTL. The preparation of autologous dendritic cells from the patient has been a limiting step in cancer immunotherapy because underlying disease and pretreatment influences the quantity and quality of dendritic cells obtained (2, 3). Therefore, targeting dendritic cells in vivo would be desirable to increase the efficacy of vaccination against cancer. In this context, transcutaneous immunization (TCI) protocols seem to be particularly promising because they deliver antigen to skin-resident dendritic cells and at the same time activate these dendritic cells to initiate a CTL response (4). Recently, it has been reported that activation of skin-resident cells by TLR7 ligand in the presence of a T-cell epitope is sufficient for CTL priming (5). Topical application of imiquimod to the skin induces a strong inflammatory response, accompanied by activation of dendritic cells, enhancement of their migration to the draining lymph node, and their activation and maturation, together with the release of inflammatory cytokines (e.g., interleukin-6, interleukin-10, IFN-α, IFN-γ, and tumor necrosis factor α; refs. 6, 7). All of these events lead to the enhanced presentation of viral or tumor antigens and facilitate the induction of protective immunity (8). Therefore, this procedure has the potential to replace the ex vivo generation and injection of peptide-pulsed dendritic cells.

However, this approach using imiquimod revealed that the induced immune response is functionally limited and is deficient in potent memory responses (9). It is now clear that activation of dendritic cells requires two phases, first maturation and then licensing to enable the antigen-loaded dendritic cells to migrate to the draining lymph node and induce functional CTL responses (10). The dendritic cell maturation process is closely associated with their capacity to home to lymph node. On reaching the lymph node, antigen-loaded mature dendritic cells require an additional activation step, termed “licensing,” to become potent antigen-presenting cells (APC) that can activate fully functional effector CTL. Licensing stimuli are provided by the CD40 ligand, which is expressed on cognate CD4+ T cells.

To this end, here we included a Th1 epitope in TCI to provide dendritic cells with licensing signals. We selected peptide-25, amino acid residues 240 to 254 (FQDAYNAAGGHNAVF) of Ag85B, one of the major proteins secreted by Mycobacterium tuberculosis (11), for this purpose because Ag85B is a potent antigen in both humans and mice (12) and can elicit strong Th1 responses in vitro from purified protein derivative–positive (PPD+) asymptomatic individuals (13-15). Immunization of C57BL/6 mice with peptide-25 induces the differentiation of I-Α2–restricted CD4+ T cells with a Th1 phenotype but does not induce regulatory T cells (16). Coinmunization of mice with peptide-25 and ovalbumin (OVA) or tyrosinase-related protein-2 peptide leads to an increase in antigen-specific CD8+ T cells (17). These results suggest that peptide-25–reactive Th1 cells license dendritic cells and increase their ability to activate antigen-specific CD8+ CTL (17). Therefore, we

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incorporated the TL17 ligand imiquimod into our TCI regimen with the objective of activating dendritic cells, and included peptide-25 to activate memory Th1 (mTh1) cells to license them for antigen presentation to CD8\(^+\) cells.

Here, we show in a murine melanoma model that TCI with peptide-25 stimulates CD4\(^+\) Th1 responses, which augment the CD8\(^+\) T-cell response to tumor-specific antigens by licensing dendritic cells.

Materials and Methods

Mice, tumor cells, and peptides. C57BL/6 mice at the age of 6 to 8 wk were used for vaccination experiments or as hosts in the adoptive transfer experiments. Congenic C57BL/6 (CD45.1\(^+\)) mice and pmel-1 TCR transgenic (Tg) mice recognizing the H-2D\(^{b}\)-restricted epitope EGSRRNQDWL from gp100 (gp100\(^{25–33}\)) were obtained from The Jackson Laboratory (18). All animal procedures were conducted in accordance with the institutional guidelines. B16F10 (H-2b), a gp100\(^+\) spontaneous murine melanoma cell line provided by Dr. N. Restifo (National Cancer Institute, Bethesda, MD), was maintained in culture medium consisting of DMEM (Wako Pure Chemical) with 10% heat-inactivated fetal bovine serum (HyClone), 0.03% L-glutamine, 100 \(\mu\)g/mL streptomycin, and 100 \(\mu\)g/mL penicillin (Invitrogen). The H-2D\(^{b}\)-restricted peptide hgp100 (hgp100\(^{25–33}\), KVPQRNQDWL) and the 1\(^\alpha\)-restricted Th1 epitope peptide-25 (Ag83B\(^{25–25}\)) (QFDAYNAGGHA) were purchased from Invitrogen Japan at purities of >90%, with free N\(_H\)\(_2\) and COOH termini.

Induction of peptide-25–specific mTh1 cells. Mice were immunized by s.c. injection with 50 \(\mu\)g of peptide-25 emulsified in 100 \(\mu\)L of incomplete Freund's adjuvant (Sigma). Four weeks after immunization, peripheral blood mononuclear cells (PBMC) were harvested from 200 \(\mu\)L of peripheral blood. Half of the PBMC were either left unstimulated or stimulated with 1 \(\mu\)g/mL peptide-25 for 16 h. The induction of peptide-25–specific IFN-\(\gamma\)-producing cells was confirmed with a mouse IFN-\(\gamma\) ELISPOT kit according to the manufacturer's instructions (BD Biosciences).

Adoptive transfer and immunizations. Spleen cells from peptide-25–immunized mice and/or pmel-1 TCR Tg mice were prepared and adoptively transferred i.v. into the tail vein of recipient mice in 200 \(\mu\)L of incomplete Freund's adjuvant. Freund's adjuvant (Sigma). Four weeks after immunization, peripheral blood mononuclear cells (PBMC) were harvested from 200 \(\mu\)L of peripheral blood. Half of the PBMC were either left unstimulated or stimulated with 1 \(\mu\)g/mL peptide-25 for 16 h. The induction of peptide-25–specific IFN-\(\gamma\)-producing cells was confirmed with a mouse IFN-\(\gamma\) ELISPOT kit according to the manufacturer's instructions (BD Biosciences).

Harvesting and quantifying Tg T cells. Spleens were harvested from immunized mice at the indicated time points as described in the figure legends. The total numbers of spleen cells were estimated from a fluorescence-activated cell sorting–based cell count of single-cell suspensions. Flowcount beads (Beckman-Coulter) were added to the cell samples and cell counts were calculated by the following equation: (viable cells \(\times\) total beads) / counted beads. The total splenic pmel-1 TCR T-cell population was determined by multiplying the total splenocyte count by the percentage of splenocytes double stained for Thy1.1 and CD8.

Flow cytometric analysis, intracellular IFN-\(\gamma\) assay, and ELISPOT. The following monoclonal antibodies (mAb) were used for flow cytometry: FITC–conjugated or biotin–anti-CD90.1, phycoerythrin–conjugated anti-CD4, anti-CD8, allophycocyanin–conjugated anti-CD8 (BD PharMingen), FITC–conjugated anti-IFN-\(\gamma\) ( Immunotech), streptavidin–phycoerythrin/Cy5, and allophycocyanin–conjugated anti-CD45.1 (Biolegend). The cells were stained with antibodies and analyzed on Cytomics FC 500 (Beckman Coulter). Antigen–specific T-cell responses were evaluated by intracellular IFN-\(\gamma\) and ELISPOT assays. Spleen cells (\(1 \times 10^5\)) were stimulated for 5 h at 37°C with 1 \(\mu\)g/mL of the indicated peptide, and intracellular cytokine staining was carried out according to the manufacturer's instructions (Intraprep permeabilization reagent, Immunotech). Serial dilutions of spleen cells were incubated with 2 \(\mu\)g/mL of the indicated peptide at 37°C for 24 h in 96-well ELISPOT plates. The assays were done and developed with antimouse IFN-\(\gamma\) mAb according to the manufacturer’s instructions (BD PharMingen). All experiments were done in duplicate and the data correspond to the mean value.

In vivo cytotoxicity assay. Spleen cells from CD45.1\(^+\) mice were labeled with either 0.5 or 5 \(\mu\)mol/L carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) according to the manufacturer's instructions. The cells labeled with high concentrations of CFSE were incubated with 1 \(\mu\)g/mL hgp100 peptide for 1 h at 37°C, washed thrice with HBSS, and a total of 2 \(\times\) 10\(^5\) cells were adoptively transferred into mice subjected to various immunization protocols. Lymphocytes were isolated from blood and spleens and stained with allophycocyanin-labeled anti-CD45.1mAb at the indicated time points. Target cells were distinguished from recipient cells by gating on CD45.1\(^+\) cells. Peptide-loaded and nonloaded target cells were distinguished by their CFSE fluorescence intensity. The percentage of specific target cell elimination was calculated as follows: 100 – ([% peptide pulsed in immunized recipients / % unpulsed in immunized recipients] / [% peptide pulsed in control recipients / % unpulsed in control recipients]) \(\times\) 100.

Tumor protection. The antitumor effects of TCI with or without peptide-25 were evaluated in both the prophylactic and therapeutic vaccine settings. In the prophylactic model, mice were first immunized according to the vaccine protocols described in the figure legends. Seventeen days after immunization, mice were s.c. injected with 5 \(\times\) 10\(^5\) B16 melanoma cells in 50 \(\mu\)L of HBSS into the right flank. In the therapeutic protocol, mice were first inoculated with 5 \(\times\) 10\(^5\) B16F10 cells s.c. (day 0), followed by corresponding vaccinations starting on day 7. Tumor growth was monitored every 2 or 3 d with calipers in a blinded fashion and was done independently at least twice with similar results. Tumor volume was calculated by the formula 0.5238 \(L_1 L_2 H\), where \(L_1\) is the long diameter, \(L_2\) is the short diameter, and \(H\) is the height of the tumor. The survival of the mice was also monitored periodically. Tumor-bearing mice either died or had to be euthanized when the tumor volume exceeded \(1,500 \text{mm}^3\).

Statistical analysis. Statistical analyses were done with JMP software, version 6.0.3 (SAS Institute, Inc.). Results are shown as mean \(\pm\) SD. Comparison of results obtained for TCI with or without peptide-25 was carried out with two-tailed unpaired t test. Statistical analysis for evaluating survival advantage was done with log-rank analysis. \(P < 0.05\) was considered statistically significant.

Results

TCI is as efficient as dendritic cells for priming naive TCR Tg CTL. To compare the efficacy of TCI and dendritic cell immunization for priming naive antigen-specific CD8\(^+\) T cells, we adoptively transferred naive spleen cells from pmel-1 TCR Tg mice into C57BL/6 hosts and subsequently immunized them by TCI or with dendritic cells. ELISPOT assays were used to monitor the frequency of hgp100-specific T cells in the spleen by their ability to produce IFN-\(\gamma\) on hgp100 peptide restimulation in vitro. Whereas as many as 460 \(\pm\) 79 spots per 10\(^5\) spleen cells were detected in the mice that underwent TCI with imiquimod and hgp100, treatment with either component alone, or no treatment at all, resulted in a lack of any detectable IFN-\(\gamma\) spots (Fig. 1A). The efficacy of TCI seemed to be \(\sim 50\%\) of that of immunization with hgp100-pulsed dendritic cells [460 versus 861 (\(\pm\) 149) spots per 10\(^5\) spleen cells; see Fig. 1A]. However, it must be noted that imiquimod-treated mice had splenomegaly and that there were twice as many spleen cells in TCI mice as in the dendritic cell–immunized mice (Fig. 1B). Therefore, the actual numbers of IFN-\(\gamma\)-producing cells were
comparable between TCI and dendritic cell–immunized mice (Fig. 1C). These results show that priming is required for transferred naive TCR Tg CTL to carry out their effector functions and that neither the peptide nor imiquimod alone is sufficient for priming. Moreover, TCI is comparable to dendritic cell vaccination for the priming of antigen-specific CTL.

Antigen-specific priming of naive CTL by TCI with imiquimod and CTL epitope peptide. The frequency of pmel-1 TCR Tg T cells was monitored in the spleen with the congenic marker CD90.1 expressed only by these cells (Fig. 2A). Consistent with the results from ELISPOT analysis, treatment with imiquimod ointment alone or hgp100 peptide alone did not induce the expansion of CD90.1+/CD8+ cells, indicating that the proliferation of pmel-1-specific cells after TCI is dependent on hgp100 antigen and not on nonspecific stimulation by imiquimod (Fig. 2A). CD90.1+/CD8+ cells comprise 2.4 ± 0.4% of spleen cells in mice immunized by TCI combined with imiquimod and hgp100 peptide, whereas 5.2 ± 1.5% of spleen cells are positive for CD90.1 and CD8 in hgp100 peptide–pulsed dendritic cell–immunized mice (Fig. 2A). Again, the absolute numbers of CD90.1+/CD8+ cells are comparable in TCI mice and dendritic cell–immunized mice, suggesting that TCI with imiquimod represents an effective and practical way to induce CTL responses to a peptide antigen in vivo.

To investigate whether TCI activated and expanded the antigen-specific CTL, intracellular IFN-γ production was analyzed by flow cytometry. Five days after TCI with imiquimod and hgp100 peptide, spleen cells were stimulated ex vivo with hgp100 peptide (Fig. 2C, R2 gate). IFN-γ production was not detected in CD8− cells, indicating that the CTL response elicited by TCI is completely restricted to the topically applied epitope in TCI (Fig. 2C, R1 gate). As shown in (Fig. 2C, R3 gate), IFN-γ production by CD90.1−CD8+ cells was negligible, suggesting the ability of only the transferred naive pmel-1 TCR Tg T cells to manifest antigen-specific responses. These results are consistent with those of the ELISPOT analysis and show that transcutaneous peptide uptake is very efficient and that the activation of CTL to carry out their effector functions was specific for the epitope used for immunization.

Concomitant activation of mTh1 cells by TCI augments the tumor-specific CTL response. We included a Th1 epitope with the TCI to stimulate helper T cells that can provide licensing signals to dendritic cells. We also targeted the activation of mTh1 cells rather than naive Th cells because it is known that there is a high frequency of memory T cells in individuals vaccinated even a long time ago. The continued presence of such cells could be
exploited for immunization with novel antigens. Therefore, we hypothesized that concomitant activation of preexisting Ag85B-specific mTh1 cells by TCI would augment the generation of tumor-specific CTL in PPD+ asymptomatic individuals possessing Ag85B-specific mTh1 cells and tumor antigen–specific naive CTL.

To this end, a model for PPD+ asymptomatic individuals was constructed by transferring peptide-25–specific mTh1 cells and naive pmel-1 TCR Tg T cells into naive C57BL/6 mice (designated mTh1+nCTL+ mice). Five days after adoptive transfer of $3.5 \times 10^7$ peptide-25–specific mTh1 cells, $1 \times 10^7$ hgp100-specific naive CTL (pmel-1 TCR Tg) were transferred into the same mice. Subsequently, animals were divided into three groups and were untreated (group 1) or transcutaneously immunized with hgp100 (group 2) or hgp100 and peptide-25 (group 3). Five days after TCI, spleen cells were harvested and stimulated with peptide-25 or hgp100 as indicated. As expected, peptide-25–specific IFN-γ spots were detected in group 3 mice, suggesting that TCI efficiently activated preexisting mTh1 cells (Fig. 3A). The frequency of CD90.1+CD8+ T cells in the spleen was 0.3 ± 0.1%, 1.6 ± 0.1%, and 1.8 ± 0.1% in groups 1, 2, and 3, respectively (Fig. 3B). The percentages of hgp100-specific IFN-γ–producing cells among the CD90.1+CD8+ T cells were 0.5 ± 0.4 (group 1), 36.8 ± 5.1 (group 2), and 49.6 ± 4.9 (group 3; Fig. 3C). Note that the total number of IFN-γ+CD90.1+CD8+ T cells in the whole spleen induced by TCI with hgp100 and peptide-25 ($9.4 \times 10^7$) was 1.5 times that of mice receiving TCI with hgp100 alone ($6.2 \times 10^7$; $P = 0.01$; data not shown). These results suggest that concomitant activation of preexisting peptide-25–specific mTh1 cells would augment the activation and expansion of tumor-specific CTL following TCI.

**In vivo detection of cytotoxic activity induced by TCI.** To show that the CTL induced by TCI mediated cytolytic activity in vivo, CFSE-labeled target cells were transferred into mTh1+nCTL+ mice that had been immunized according to the TCI protocols indicated. At the indicated time points after transfer, specific elimination of hgp100-loaded target cells was assessed in the blood and spleen. By 6 hours, this was 43.2% and 53.9% in the blood of mice receiving TCI with hgp100 alone or immunized with hgp100 and peptide-25, respectively. These results suggest that TCI with a CTL epitope is sufficient to induce CTL effector function. Moreover, immunizing with peptide-25 together with hgp100 enhanced the TCI-induced cytotoxicity. Specific target cell elimination from the spleen was 44.1%, 81.8%, and 91.2% following TCI with hgp100 peptide at 6, 12, and 18 hours after transfer, respectively (Fig. 3D). In mice receiving TCI including both hgp100 and peptide-25, these values were increased to 53.7%, 88.5%, and 95.4%, respectively (Fig. 3D; $P < 0.01$, at 18 hours, compared with
These results suggest that concomitant activation of preexisting mTh1 cells by TCI further enhanced the tumor-specific CTL response.

Tumor growth is suppressed in mice prophylactically immunized by TCI. To assess whether TCI can prevent tumor growth, mTh1+nCTL+ mice were immunized using the indicated TCI protocols. Naïve C57BL/6 mice (n = 6) were left untreated as controls. Seventeen days after TCI, mice were challenged s.c. with 5 \times 10^5 B16F10 cells. Two days before challenge, the presence of hgp100-specific CTL within PBMC was confirmed by IFN-γ ELISPOT assay. On average, 325 ± 207 IFN-γ spots were detected in 120 μL of peripheral blood from mice immunized by TCI. This assay revealed that hgp100-specific CTL were primed in mice immunized with hgp100 with or without peptide-25 (data not shown). By day 6, all mice in the control groups had developed tumors (Fig. 4A and C). This was correlated with an absence of hgp100 alone). These results suggest that concomitant activation of preexisting mTh1 cells by TCI further enhanced the tumor-specific CTL response.

Figure 3. Concomitant activation of mTh1 cells by TCI augments the tumor-specific CTL response. Spleen cells from peptide-25–immunized mice were used as a source of peptide-25–specific mTh1 cells. First, 3.5 \times 10^7 spleen cells from peptide-25–immunized mice were transferred into naïve C57BL/6 mice by i.v. injection. Five days later, these mice were injected with 1 \times 10^8 naïve spleen cells from pmel-1 TCR Tg mice to produce mTh1+nCTL+ mice. Subsequently, these animals (four per group) were transcutaneously immunized with imiquimod mixed with hgp100 peptide alone or hgp100 and peptide-25. Control mTh1+nCTL+ mice were left untreated. Five days after TCI, spleens were harvested and assayed. A, spleen cells were incubated with 1 μg/mL peptide-25 for 16 h and IFN-γ–producing cells were detected by ELISPOT. Images show representative wells in each group with peptide stimulation. No, or negligibly few, spots were observed in wells without peptide. Numbers on the top of the images show the numbers of spots per 10^6 spleen cells (mean ± SD). B, CD90.1^+CD8^+ pmel-1 TCR Tg cells in the spleen were detected by flow cytometry. C, spleen cells (1 \times 10^7) were stimulated for 5 h at 37°C with 1 μg/mL hgp100 peptide and intracellular cytokine staining was carried out. Cells represented in the figure were gated on CD90.1^+CD8^+ cells and their IFN-γ production was evaluated. Dot plots are representative data of spleen cells stimulated by hgp100 peptide. Production of IFN-γ was not observed without peptide stimulation. D, in vivo cytotoxicity assay. mTh1+nCTL+ mice (five per group) that received 2.5 \times 10^7 spleen cells from peptide-25–immunized mice and 5 \times 10^5 from pmel-1 TCR Tg mice were left untreated or transcutaneously immunized by imiquimod mixed with hgp100 peptide alone or hgp100 and peptide-25. Five days later, target cells consisting of equal amounts of CD45.1^+ spleen cells, unpulsed and pulsed with hgp100 peptide and stained with CFSE at low (0.5 μmol/L) and high (5 μmol/L) concentrations, respectively, were injected i.v. PBMC and spleen cells were harvested and analyzed by flow cytometry for the detection of CFSE^+ cells 6, 12, and 18 h after injection. Histograms for cells gated on CD45.1^+ populations are shown. Numbers on the right corner of the images show the percentage of specific target cell elimination calculated as described in Materials and Methods. Experiments were repeated twice with similar results.
hgp100-specific CTL in PBMC in naive C57BL/6 mice and untreated mTh1+nCTL+ mice. In contrast, a significant delay of tumor growth was observed in mice that had received TCI (Fig. 4A, middle and right). The sizes of the tumors in mice after TCI with or without peptide-25 were significantly smaller compared with controls (Fig. 4B). Furthermore, two of six mice immunized with hgp100 and four of six mice immunized with hgp100 together with peptide-25 were tumor-free on day 13 (Fig. 4C).

However, there were no significant differences between the sizes of tumors in mice immunized with or without peptide-25 (P = 0.09). These results suggest that TCI is highly effective for the induction of functional hgp100-specific CTL and for the generation of antitumor activity in vivo. Although TCI without Th1 peptide did have some effect on tumor suppression in a prophylactic setting, it is clear that concomitant activation of preexisting mTh1 cells benefits the induction of antitumor CTL responses following TCI.

**TCI results in antitumor activity in a therapeutic tumor model.** Efficacy of TCI was evaluated not only in the prophylactic model but, more clinically relevant, also in a therapeutic model. Groups of naive C57BL/6 and mTh1+nCTL+ mice first received an inoculation of B16F10 melanoma cells, and 7 days later, after the tumor had become established, they were treated with various therapeutic immunization protocols as indicated. Tumor growth was measured every 2 or 3 days. Up to 5 days after treatment, there was no difference in tumor growth among the groups of animals, but thereafter, it was gradually delayed in the animals receiving TCI (Fig. 5A). Furthermore, the suppression of tumor growth was more prominent in mice given TCI containing hgp100 and peptide-25 than those immunized with hgp100 alone (Fig. 5A, bottom right; P = 0.03). Control mice bearing large tumors were euthanized on day 17 according to the institutional guidelines for animal care. Survival times of the remaining mice were recorded (Fig. 5B). TCI with peptide-25 significantly increased median survival by 10 days (35 versus 25 days) compared with TCI with hgp100 alone (P < 0.01). This suggests that addition of a Th1 peptide to the CTL peptide TCI enhanced antitumor activity in this therapeutic setting.

Together, these results show that TCI induces strong CTL responses to the immunizing peptide and that these responses are sufficiently potent to generate not only prophylactic but also therapeutic antitumor effects. Concomitant activation of mTh1 cells together with tumor-specific CTL by TCI enhances the activation and expansion of tumor-specific CTL, leading to better control of tumor growth.

**Figure 4.** Tumor growth is suppressed following TCI in the prophylactic model. Protective immunity against B16F10 melanoma was examined. As described in the Fig. 3 legend with slight modification, mTh1+nCTL+ mice were prepared and immunized. Spleen cells (2.0 ¥ 10^7) from peptide-25-immunized mice were transferred into naive C57BL/6 mice, followed by i.v. injection with 1 ¥ 10^7 naive spleen cells from pmel-1 TCR Tg mice. Subsequently, mTh1+nCTL+ mice (6 per group) were transcutaneously immunized by imiquimod mixed with hgp100 peptide alone or hgp100 and peptide-25. Control mTh1+naiveCTL+ mice were left untreated. Seventeen days after immunization, all mice were challenged s.c. with 5 ¥ 10^5 B16F10 cells. A, the size of the tumors was measured every 2 or 3 d with tissue calipers. Tumor volume was calculated as described in Materials and Methods. Each line represents an individual mouse. B, columns, mean tumor volume of each group of mice at day 13; bars, SD. There were significant differences between the volumes of untreated and TCI mice (P < 0.01). The volumes of the tumors among the TCI groups were not different whether or not peptide-25 had been included (P = 0.09). C, Kaplan-Meier plots of the tumor-free mice after tumor challenge. Experiments were repeated twice with similar results.
Discussion

In this report, we have used a well-established transplantable B16 melanoma model in C57BL/6 mice with naive hgp100-specific pmel-1 TCR Tg spleen cell transfer (18). We showed that TCI efficiently expanded hgp100-specific CD8+ T cells, which produced IFN-γ on peptide stimulation and exerted cytolytic activity in vivo. Importantly, in a model for the concurrent activation of preexisting mTh1 cells, the generation of hgp100-specific CTL with effector function was enhanced. TCI facilitated direct peptide-specific activation of CD8+ memory T cells, resulting in augmentation of the hgp100-specific CTL response by the mycobacterial antigen Ag85B-derived peptide-25. These responses are sufficient to generate antitumor effects, which can suppress the growth of established tumor. In the present study, we used the TLR7 ligand imiquimod and targeted antigens to dendritic cells in situ, while

Figure 5. Tumor growth is suppressed following TCI in the therapeutic model. A, groups of naive C57BL/6 and mTh1+nCTL+ mice (5–7 per group) were s.c. injected with 5 × 10^5 B16F10 cells. C57BL/6 mice (top left) and one group of mTh1+nCTL+ mice (top middle) were left untreated. Other groups of mice received transcutaneous application of imiquimod alone (top right), TCI with imiquimod and hgp100 (bottom left), or TCI with imiquimod, hgp100, and peptide-25 (bottom middle). Tumor size was measured and reported as described in Fig. 4 legend. Each line represents one mouse. Columns, mean tumor volume of each group of mice at day 17; bars, SD. Although tumor growth was not suppressed in control mice (top), it was markedly delayed in TCI mice (P < 0.01 in TCI groups; bottom). Importantly, tumor size was smaller in TCI with hgp100 and peptide-25 mice than in TCI with hgp100 alone mice (P = 0.03; bottom). B, Kaplan-Meier plots of the survival of mice after tumor challenge. Surviving mice of control groups were euthanized on day 17 because tumor volume surpassed 1,000 to 1,500 mm^3. Experiments were repeated twice with similar results.
activating and maturing the targeted dendritic cells. By using TCI, the need for complex patient-specific dendritic cell preparations to induce effective antitumor immune responses can be circumvented.

Dendritic cells constitute a heterogeneous population of antigen-presenting cells (20, 21). TCI targets dendritic cells in the skin by topical application of antigenic peptides. Of the different subsets of dendritic cells, only epidermal Langerhans cells and dermal dendritic cells are resident in normal skin. These cells are activated at the site of application, take up and process antigen, and migrate to the draining lymph node where they initiate antigen-specific immune responses. Similarly, topical application of the TLR7 ligand induces Langerhans cell migration (6), although these cells might not express TLR7 (22, 23). If so, these findings would imply that the Langerhans cells do not respond directly to TLR7 ligand. Although plasmacytoid dendritic cells do express TLR7, they might not be able to initiate these events. In addition to the dendritic cells of the skin in the steady state, rapid infiltration of plasmacytoid dendritic cells into the skin is induced by imiquimod (24). Recently, Heib et al. (25) reported that mast cells are responsible for the TLR7 ligand–induced activation of Langerhans cells and that plasmacytoid dendritic cells accumulate in the dermis and spleens of mice topically treated with imiquimod. Therefore, imiquimod might rather induce the activation of other skin-resident cells resulting in the secretion of soluble mediators that induce the activation and migration of Langerhans cells and/or plasmacytoid dendritic cells. Although it remains to be elucidated which cells capture the antigenic peptides and present them to the antigen-specific T cells in the lymph nodes, targeting the skin with TLR7 ligand and peptide is an efficient strategy to initiate the antigen-specific immune response.

A major goal of tumor immunotherapy is the generation of functional CTL that efficiently kill tumor cells. Because many targets for tumor immunotherapy are nonmutated self-antigens, and due to the deletion of high-avidity CD4+ and CD8+ T cells recognizing self antigens, the remaining tumor antigen–specific T-cell precursors are ignorant of or unresponsive to tumor antigens. Therefore, to overcome the immunologic tolerance of tumor-specific CTL, T-cell help may be crucial for activating these CTL precursors (26). However, because CD4+ T cells are also tolerant to self-antigens, here we have used a foreign antigen to help the induction of tumor-specific CTL. It has been reported that linked foreign T-cell help for tetanus toxoid could activate otherwise tolerant OVA-specific CTL precursors from RIP-mOVA mice (27). The same antigen-presenting cell needs to present both the helper epitope and the CTL peptide to overcome unresponsiveness to self-antigens by providing cognate T-cell help. In the present study, we used foreign peptide-25 rather than a T-cell epitope derived from B16 melanoma cells to stimulate CD4+ helper T cells. Topical application of hgp100 peptide and peptide-25 allowed dendritic cells to capture and present both the CTL and Th epitope, so that these two peptides behaved as if they were derived from the same cognate antigen. In this way, dendritic cells could be successfully licensed by peptide-25–specific Th1 cells and then efficiently primed gp100-specific CTL.

Bacillus Calmette-Guerin (BCG), the only available vaccine against M. tuberculosis, is a live attenuated strain of Mycobacterium bovis that was first used in 1921 (28). Since then, it has become a well-established part of the WHO Expanded Program on immunization and is widely administered at birth throughout the developing world (29). Japan has had a policy of universal BCG vaccination of infants against tuberculosis since 1951 (30). Therefore, a significant proportion of the Japanese population who received BCG vaccination is expected to possess Ag85B- or peptide-25–specific mTh1 cells. Ag85B and some synthetic peptides representing parts of its sequence are recognized by IFN-γ–secreting Th1 cells in association with multiple HLA-DR molecules (31). Our results suggest that concomitant activation of Ag85B- or peptide-25–specific mTh1 cells together with tumor-specific CTL might be clinically applicable to augment antitumor immune responses. Hamaoka et al. (31) reported the augmentation of tumor-specific immunity by BCG cross-reactive T cells in 1985, long before the identification of T-cell epitopes or understanding of dendritic cell biology. Since then, a great deal of data has accumulated, such that current understanding of the molecular immunobiology of T-cell responses to BCG and tumor antigens allows us to increase the potential for clinical application of this strategy.

Here, we transferred spleen cells from pmel-1 TCR Tg mice into C57BL/6 mice to increase the naive hgp100-specific CTL precursor frequency before immunization. These well-established procedures permit ex vivo analysis of functional CTL; however, they may also affect antitumor activity induced by TCI. Accordingly, experiments are under way in different tumor models in our laboratory to reevaluate antitumor activity induced by TCI without using TCR Tg mice.

One of the other advantages of TCI is treatment compliance. The lack of syringes and needles makes it possible for patients to apply their own vaccines at the frequency required to maintain antitumor immunity. However, the most important point is that targeting dendritic cells in situ by TCI can initiate optimal immune responses by facilitating interactions between the relevant cells and can result in the efficient induction of antitumor CTL responses with full effector functions. Incorporation of a Th1 epitope peptide into TCI schedules to activate preexisting mTh1 cells should contribute to enhancing the induction of functional CTL responses against tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Memory Th1 Cells Augment Tumor-Specific CTL following Transcutaneous Peptide Immunization

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