Impaired Antigen Presentation and Effectiveness of Combined Active/Passive Immunotherapy for Epithelial Tumors

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Background: Although immunization with tumor antigens can eliminate many transplantable tumors in animal models, immune effector mechanisms associated with successful immunotherapy of epithelial cancers remain undefined.

Methods: Skin from transgenic mice expressing the cervical cancer–associated tumor antigen human papillomavirus type 16 (HPV16) E6 or E7 proteins from a keratin 14 promoter was grafted onto syngeneic, non-transgenic mice. Skin graft rejection was measured after active immunization with HPV16 E7 and adoptive transfer of antigen-specific T cells. Cytokine secretion of lymphocytes from mice receiving skin grafts and immunotherapy was detected by enzyme-linked immunosorbent assay, and HPV16 E7–specific memory CD8+ T cells were detected by flow cytometry and ELISPOT.

Results: Skin grafts containing HPV16 E6– or E7–expressing keratinocytes were not rejected spontaneously or following immunization with E7 protein and adjuvant. Adoptive transfer of E7-specific T-cell receptor transgenic CD8+ T cells combined with immunization resulted in induction of antigen-specific interferon gamma–secreting CD8+ T cells and rejection of HPV16 E7–expressing grafts. Specific memory CD8+ T cells were generated by immunotherapy. However, a further HPV16 E7 graft was rejected from animals with memory T cells only after a second E7 immunization. Conclusions: Antigen-specific CD8+ T cells can destroy epithelium expressing HPV16 E7 tumor antigen, but presentation of E7 antigen from skin is insufficient to reactivate memory CD8+ T cells induced by immunotherapy. Thus, effective cancer immunotherapy in humans may need to invoke sufficient effector as well as memory T cells. [J Natl Cancer Inst 2004;96:1611–9]

Many epithelial cancers express tumor-specific antigens (1), and tumor antigen-specific effector T cells can kill human leukocyte antigen–matched tumor cells in vitro. Further, transplantable murine tumors engineered to express model or tumor antigen can be eliminated in vivo by active specific immunotherapy using various protocols (2). However, in patients with cancer, active or passive specific immunotherapy (i.e., cancer vaccines and monoclonal antibody or in vitro expanded T-cell therapy, respectively) targeted to a tumor antigen can induce a tumor antigen–specific T-cell response without necessarily causing tumor regression (3). Many potential mechanisms exist for tumor immunotherapy failure in spite of apparently adequate induced immunity, including mutations in the tumor cell antigen–presenting machinery (4), production of locally immunosuppressive cytokines (5), tumor cell–directed T-cell killing (6), and tumor cell resistance to T-cell effector mechanisms. Without a surrogate marker of effective immunotherapy, it is difficult to determine whether tumor immunotherapy has failed because an inappropriate immune response was induced (7) or because a tumor phenotype prevented an appropriate effector response from functioning. Further examination of the requirements for effective immunotherapy against tumor-specific antigens would be facilitated by a model in which a tumor antigen is presented by cells that have not undergone malignant transformation and therefore have not had the chance to develop resistance to the immunotherapy.

The cells of cervical squamous cell cancer, an epithelial cancer that is common in women worldwide, express two virally encoded nuclear oncoproteins of human papillomavirus type 16 (HPV16), E6 and E7, as do HPV16-infected epithelial cells that display various premalignant phenotypes associated with HPV16 infection (8). These proteins are therefore targets for HPV16-specific immunotherapy. To examine requirements for the elimination of HPV16-infected but non-transformed cells expressing HPV16 proteins, we established a skin graft model. In this model, skin of mice engineered to express HPV16 E6 or E7 as transgenes from the keratin 14 (K14) promoter in keratinocytes but not in professional antigen-presenting cells is grafted onto syngeneic, non-transgenic mice (9). We have previously demonstrated that K14E7 skin grafts are not rejected spontaneously, despite the generation of E7–specific humoral immunity in graft recipients. In addition, K14E7 skin grafts are resistant to active immunotherapy with E7 protein–based immunogens that induce rejection of transgenic E7 tumors transplanted in the same mouse (10). We used this skin graft mouse model to examine the requirements for the eradication of skin cells expressing E7. Specifically, we examined whether the number of and cytokine profile of effector T cells induced following immunization determines whether E7 transgenic skin is rejected.

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Skin Graft Models

C57BL/6J (H-2b) mice and C57BL/6J mice transgenic for the HPV16 E7 oncprotein driven from a K14 promoter (designated as K14E7 mice) were obtained from the Animal Resources Centre (Perth, WA, Australia). To generate H-2d K14E7 mice, H-2d K14E7 mice (hK14HPV16E6ttlE7) were backcrossed with C57BL/6J (H-2b) mice for more than 12 generations. Characterization of these mice and of transgene expression has been described (9,11). FVB (H-2b) mice (hK14HPV16E6ttl) transgenic for the HPV16 E6 oncprotein driven from the K14 promoter (designated as K14E6) have been described previously (12). Lines of FVB (H-2b) mice heterozygous for a human growth hormone (hGH) transgene expressed from the K14 promoter (designated as K14hGH mice) were generated by pronuclear injection using an expression construct described by Wang et al. (13) and Zhong et al. (14). The serum hGH level in the 023 K14hGH line used in the current study is approximately 8 mIU/L, and these mice are approximately 10% larger than their sex-matched non-transgenic littermates. First generation C57BL/6J×FVB mice were recipients of first generation C57BL/6J×FVB.K14hGH grafts.

Transgenic donors and non-transgenic syngeneic recipients aged 8–12 weeks were maintained under conventional conditions in specific pathogen-free holding rooms in the Princess Alexandra Hospital Biological Resources Facility (Brisbane, QLD, Australia). All animal protocols were approved by the University of Queensland Animal Ethics Committee.

Skin Grafting

Whole-thickness ear skin grafting is described elsewhere (9,10). Briefly, whole ears from donor mice (C57BL/6J,K14E7, FVB,K14E7, FVB.K14E6, C57BL/6J×FVB.14hGH) were surgically removed, and dorsal and ventral surfaces were separated. Transgenic skin grafts were placed on the flanks of non-transgenic but otherwise syngeneic recipient mice, held in place surgically removed, and dorsal and ventral surfaces were separated. Transgenic skin grafts were placed on the flanks of non-transgenic but otherwise syngeneic recipient mice, held in place with antibiotic-permeated Vaseline gauze (Bactigras; Smith and Nephew, London, U.K.), covered with micropore tape and elastic bandages (CoFlex; Andover, Salisbury, MA) for 7 days, and then with antibiotic-permeated Vaseline gauze and micropore tape for an additional 3 days. Grafted and control mice were immunized as described elsewhere (9,11) with the ovalbumin (OVA) peptide (aa sequence SIINFEKL) intravenously at the tail vein of grafted C57BL/6J mice.

Immunization Protocol

Grafted and control mice were immunized as indicated subcutaneously at the tail base with glutathione S-transferase–HPV16 E7 fusion protein (50 μg per mouse) as previously described (15) or with ovalbumin peptide (Sigma Chemical, St. Louis, MO) (aa sequence = SIINFEKL, 50 μg per mouse). GF001 peptide (H-2Db–restricted minimal CTL epitope of HPV16 E7 protein [aa sequence = RAHYNIVTF], 50 μg per mouse) and MT906 peptide, which includes an H-2Dd–restricted T-helper epitope and a mutant disabled form of the CTL epitope of HPV16 E7 protein (aa sequence = QAEPDRHYNIVTCK CKCD, 50 μg per mouse) were also used for immunization. Antigens were administered with Quil A saponin (Spikoside; Isotec AB, Stockholm, Sweden) with complete Freund’s adjuvant, as indicated.

Cell Culture

To recall E7-specific memory in CTLs, splenocytes collected from C57BL/6J graft recipients and control C57BL/6J mice were restimulated with the minimal E7 CTL peptide (GF001) in vitro. Splenocytes were collected from graft recipients, red blood cells were removed with ACK lysis buffer, and the remaining splenocytes were plated at 7.5 × 10^6 cells per well in a 24-well plate. Syngeneic splenocytes, pulsed for 2–3 hours with GF001 peptide and gamma irradiated (3000 rad), were then added at 3.5 × 10^6 cells per well with 1 ng/mL (approximately 10 U/mL) recombinant mouse interleukin 2 (rIL-2) (BD Pharmingen, San Diego, CA) in EHAA/RPMI complete medium (50% ELAA/50% RPMI supplemented with 0.216 g/L l-glutamine, 60 mg/L benzyl penicillin [CSL, Melbourne, VIC, Australia], 100 mg/L streptomycin [CSL, 50 μmol/L 2-mercaptoethanol, and 10% fetal bovine serum [FBS; Gibco, Gaithersburg, MD]). The splenocytes were then cultured at 37 °C in a 5% CO2 incubator. After 1 week, cultured splenocytes were analyzed by flow cytometry and ELISPOT assay.

Flow Cytometric Analysis

Collected cells were resuspended in PBS/5% FBS and incubated with 1 μg of allopurinol- conjugated rat anti-mouse CD8 + antibody (Clone 53-6.7, BD Pharmingen) and fluorescein isothiocyanate–conjugated mouse anti-mouse TCR Vβ12 + antibody (Clone MR11-1, BD Pharmingen) for 1 hour in the dark at 4 °C. All cells were washed with PBS/5% FBS twice, resuspended in fixative solution (2% formaldehyde in PBS), and analyzed using a FACScan flow cytometer (BD Biosciences, San Jose, CA).

IFN-γ ELISPOT Assay

The IFN-γ ELISPOT assay was performed as previously described (16). Briefly, spleen cell suspensions from C57BL/6J mice were added to membrane-base 96-well plates (Millipore,
Enzyme-Linked Immunosorbent Assay

Splenocytes collected from grafted and control mice and then treated with ACK lysis buffer to remove red blood cells were plated at \(1 \times 10^7\) cells per well in a 24-well plate with \(10^{-8}\) M GF001 peptide and 1 ng/mL rIL-2 (BD Pharmingen) in EHAA/RPMI complete medium. The cells were cultured at \(37^\circ C\) in a 5% CO\(_2\) incubator for 72 hours. Supernatants from the cultures were tested for IFN-\(\gamma\) level by using capture enzyme-linked immunosorbent assay (ELISA). Wells of 96-well ELISA plates (Nalge Nunc, Rochester, NY) were coated with capture monoclonal antibody (100 \(\mu\)L per well) (Clone AN18; MabTech) at 1 \(\mu\)g/mL in 0.05 M Na\(_2\)CO\(_3\) buffer (pH 9.6) and incubated at \(4^\circ C\) overnight. Then the wells were blocked with 200 \(\mu\)L of PBS containing 3% skim milk and 0.05% Tween 20 (blocking buffer) at \(4^\circ C\) overnight. Supernatant sample (100 \(\mu\)L) at a 1:2 dilution in the blocking buffer was reacted with the capture antibody in the well at room temperature for 2 hours. A colorimetric assay using recombinant mouse IFN-\(\gamma\) was used to calculate a standard curve for estimating the IFN-\(\gamma\) concentration of the samples. Wells were washed five times with PBS containing 0.05% Tween 20 and received 100 \(\mu\)L per well of biotinylated detection monoclonal antibody (Clone R4-6A2; MabTech) at a concentration of 0.5 \(\mu\)g/mL in the blocking buffer. The wells were held at room temperature for 1 hour and washed five times with PBS containing 0.05% Tween 20. Then, 75 \(\mu\)L of avidin–horseradish peroxidase solution (Sigma; 1 mg/mL stock solution in PBS diluted at 1:400 in the blocking buffer) was added to each well, and the plates were held at room temperature for 1 hour. The plates were first washed three times with PBS containing 0.05% Tween 20, and then washed twice with PBS. Diaminobenzidine developing reagent was prepared as recommended by the manufacturer, and 200 \(\mu\)L was added to each well. The absorbance at 492 nm was measured after holding the plates for 15 minutes at room temperature. After 3 days of culture in vitro, supernatants were tested for IL-5 and IL-10 by using commercially available capture IL-5 and IL-10 ELISA Kits (R&D Systems, Minneapolis, MN), respectively. Antibodies to HPV16 E7 were measured by ELISA, as described previously (11).

Statistical Methods

Kaplan–Meier plots were used to analyze E7 skin graft survival, and a log rank test was used to examine the statistical significance of differences in the survival curves. The \(t\) test and chi-square test for trend were also used for data analysis, as indicated. All analyses were carried out using a JMP 5.0.1J statistics package (SAS Institute, Cary, NC). Two-tailed \(P\) values were calculated throughout and considered to be statistically significant at less than .05.

RESULTS

Rejection of Skin Grafts Expressing HPV Nuclear Oncoproteins

The fate of skin from transgenic mice expressing a defined antigen from the K14 promoter and grafted onto syngeneic, non-transgenic mice was antigen-dependent (Fig. 1, A). Skin grafts expressing hGH were spontaneously rejected over 12–20 days in association with a marked inflammatory cell infiltrate (data not shown), as has previously been described for grafts expressing OVA from the keratin 5 promoter (17). In contrast, skin grafts expressing the HPV16 E6 or E7 oncoprotein in a range of genetic backgrounds and demonstrating the skin phenotype of delayed cellular maturation and epithelial thickening associated with transgene expression were not rejected and appeared devoid of inflammatory immune response on histologic analysis. Placement of K14E7 H-2\(^b\) skin grafts on H-2\(^b\) recipients generated an HPV E7 antibody response (Fig. 1, B), as we have previously observed for H-2\(^d\) recipients of K14.E7.H-2\(^d\) skin (9). Thus, HPV E7 was presented by epithelial cells, and it...
induced a different immune response than hGH or OVA presented by the same cells.

Combined Active/Passive Immunotherapy and E7 Skin Graft Rejection

To determine why an immune response to E7 induced by immunization [although able to effect tumor rejection, as previously shown (9)] was inadequate to reject skin grafts expressing the same antigen, we investigated whether adoptive transfer of E7-specific CD8$^+$ T cells to recipients of K14E7 skin grafts could facilitate rejection of the graft by increasing the precursor frequency of E7-responsive T cells. Spleen cells ($5 \times 10^6$ per mouse) from mice expressing a transgenic TCR $\beta$ chain derived from an H-2b-restricted E7 peptide–specific CTL clone (E7TCR mice) were transferred to H-2b recipients of H-2b K14E7 skin grafts (Fig. 2). Consistent with our earlier findings, H-2b K14E7 skin grafts were not rejected by untreated or by E7-immunized H-2b recipients. E7 skin graft recipients that received $5 \times 10^6$ E7TCR tg T cells rejected skin grafts infrequently. However, E7 skin grafts were rejected frequently and rapidly by mice that received E7TCR tg T cells and were immunized with E7 protein ($P = .001$). Although presentation of E7 antigen by keratinocytes did not adequately induce an effector T-cell response, even when considerable numbers of antigen-specific CTL precursors were present, E7 antigen presentation by cells in the graft allowed rejection if sufficient CTL effectors were otherwise induced.

![Fig. 2. Survival of keratin (K)14E7 skin grafts following E7 immunization and/or adoptive transfer of E7-specific T-cell receptor transgenic (TCR tg) T cells. Graft recipients were untreated (group A, open triangles), immunized with E7 protein (group B, solid triangles), adoptively transferred with E7 TCR tg splenocytes (group C, open squares), or immunized with E7 protein and adoptively transferred with E7 TCR tg splenocytes (group D, open circles) at the time of grafting (day 0). Kaplan–Meier survival analysis: group A (survival at day 100 = 100%, 95% confidence interval [CI] = 100% to 100%) versus group D (survival at day 100 = 40.0%, 95% CI = 18.1% to 61.9%) ($P = .02$), group B (survival at day 100 = 100%, 95% CI = 100% to 100%) versus group D ($P = .01$), and group C (survival at day 100 = 85.7%, 95% CI = 70.4% to 100%) versus group D ($P = .001$).](image-url)

Number of E7 TCR tg T Cells Required for E7 Graft Rejection

To evaluate the minimum number of precursor E7-specific T cells required to induce K14E7 graft rejection after immunization, we adoptively transferred increasing numbers of E7 TCR tg splenocytes into E7-immunized recipients of K14E7 skin grafts (Fig. 3, A). Between $5 \times 10^3$ and $5 \times 10^4$ E7 TCR tg splenocytes, including 200–2000 E7/Db$^+$ tetramer–binding T cells, was sufficient to cause skin graft rejection after immunization. Immunization with cell numbers above this threshold was statistically significantly associated with decreased survival of E7 skin grafts ($P_{trend} = .03$) (Fig. 3, A).

Tumor Antigen–Specific T Cells and E7 Graft Rejection

We have previously shown that infection with Listeria monocytogenes facilitates induction of E7-specific immunity by naturally cross-presented antigens from skin grafts and induces E7 graft rejection (9), presumably as a result of activation of innate immune responses locally in the skin. We have also shown that innate immune responses are important for determining the fate of E7-expressing transplanted tumors (18). We therefore wished to establish whether nonspecific antigen–related mechanisms invoked by activation of large numbers of antigen-specific T cells, including release of IFN-$\gamma$ and consequent increased susceptibility of skin cells to T-cell–mediated cytotoxicity (19), might contribute to E7 transgenic skin graft rejection following adoptive transfer of specific T cells and immunization. We therefore examined survival of K14E7 skin grafts following adoptive transfer of OVA-specific TCR tg T cells together with OVA immunization (Fig. 3, B). K14E7 skin grafts were not rejected by recipients of adoptively transferred OVA-specific T cells following OVA immunization, although this treatment facilitated rapid rejection of skin grafts expressing a minimal OVA CTL epitope from the K14 promoter (20) (data not shown). Rejection of skin grafts with tumor antigen–expressing epithelial cells thus appeared to be dependent on antigen-specific CD8$^+$ effector T cells.

Activation of Transferred CD8$^+$ T Cells and E7 Graft Rejection

To address the possibility that spleen cells transferred from HPV E7 TCR tg mice were enhancing E7-specific immunity induced by immunization in the recipient, we immunized graft recipients with E7 protein and Quil-A saponin adjuvant 4 weeks before transferring spleen cells from E7 TCR tg mice and performing skin grafts (Fig. 3, C). Survival of skin grafts placed on previously immunized mice was similar to survival of grafts on nonimmunized animals, suggesting that direct activation of transferred T cells by E7 immunization was necessary for graft rejection. Recipients of skin grafts and E7 TCR tg T cells were then immunized with either a minimal E7 CTL epitope peptide (GF001) (21) or a mutant E7 peptide (MT906) incorporating a B-cell– and H-2b–restricted T helper epitope of E7 in which the phenylalanine anchor residue of the CTL epitope was replaced with lysine because the T helper and CTL epitopes overlap. MT906 peptide, in contrast to GF001, cannot induce E7-specific effector CTLs but induces E7-specific antibody responses in naive C57BL/6J mice (data not shown). Immune recognition with the E7 CTL peptide resulted in rejection of E7 transgenic skin grafts from recipients of E7 TCR tg T cells (Fig. 3, D). Immune recognition...
Fig. 3. Requirements for rejection of human papillomavirus type 16 (HPV16) E7 skin grafts—Kaplan–Meier survival analysis. A) Number of transferred T cells. Various numbers of E7 T-cell receptor transgenic (TCR tg) splenocytes were transferred into the recipient mice together with E7 immunization at the time of grafting (day 0) using \(5 \times 10^5\) (group A, open triangles), \(5 \times 10^4\) (group B, solid triangles), \(5 \times 10^3\) (group C, open squares), or \(5 \times 10^2\) (group D, open circles) cells per mouse. Rejection of keratin (K14-E7) skin grafts was observed in none of the group A mice, in two of the group B mice (survival at day 100 = 71.4%, 95% confidence interval [CI] = 37.3% to 100%), in two of the group C mice (survival at day 100 = 66.7%, 95% CI = 28.2% to 100%), and in five of the group D mice (survival at day 100 = 44.4%, 95% CI = 11.3% to 77.5%) (chi-square test for trend, \(P = .03\)). B) Transfer of antigen-specific T cells. Graft recipients were treated with both adoptive transfer of OT-1 splenocytes (\(5 \times 10^5\) cells) and ovalbumin immunization (OVA; group A, open triangles) or treated with both adoptive transfer of E7 TCR tg T cells (\(5 \times 10^5\) cells) and E7 immunization (E7; group B, solid triangles) at the time of grafting (day 0). Group A (survival at day 100 = 100%, 95% CI = 100% to 100%) versus group B (survival at day 100 = 36.4%, 95% CI = 7.4% to 65.4%) (\(P = .01\)). C) Direct activation of transferred T cells by immunization. All graft recipients were treated by adoptive transfer with E7 TCR tg splenocytes (\(5 \times 10^5\) cells) at the time of grafting (day 0). Mice were either not immunized (Nil; group A, open triangles), immunized with E7 protein at day –28 (i.e., 28 days before grafting and adoptive transfer) (group B, solid triangles), or immunized with E7 protein at day 0 (group C, open squares). Group A (survival at day 100 = 85.7%, 95% CI = 70.4% to 100%) versus group C (survival at day 100 = 40.0%, 95% CI = 18.1% to 61.9%) (\(P = .001\)). Group B (survival at day 100 = 81.8%, 95% CI = 58.5% to 100%) versus group C (\(P = .02\)). D) Activation of specific CD8\(^+\) T cells. Graft recipients were immunized with E7 cytotoxic T lymphocyte (CTL) peptide (TcImm; group A, open triangles) or treated with adoptive transfer of E7 TCR tg splenocytes and immunization with an E7 T-helper peptide (AT+ThImm; group B, solid triangles) or an E7 CTL peptide (AT+TcImm; group C, open squares) at the time of grafting (day 0). Group B (survival at day 100 = 100%, 95% CI = 100% to 100%) versus group C (survival at day 100 = 54.5%, 95% CI = 24.5% to 84.6%) (\(P = .03\)).

Effectiveness of the Specific Memory CD8\(^+\) T Cells That Develop Following Combined Active/Passive Immunotherapy

We have previously shown that animals primed to reject E7 skin grafts by grafting and administration of L. monocytogenes develop E7-specific memory such that subsequent grafts are rejected without further immune manipulation (9). To establish whether functional E7-specific memory was similarly induced by immunization in mice that received E7 TCR tg T cells, second E7 skin grafts were placed 100 days after the first graft onto immunized recipients of adoptively transferred E7 TCR tg T cells. Of 11 TCR tg T-cell recipients that were also immunized with HPV E7, seven mice rejected the first E7 skin graft and only one rejected the second E7 skin graft. To confirm that memory CTLs were established following adoptive transfer of CTLs and immunization in these mice, spleen cells from immunized recipients of V812\(^+\) TCR tg T cells, and control mice were assayed after 200 days of E7 peptide–specific IFN-\(\gamma\)–secreting T cells (Fig. 5, A) and for V812\(^+\) CD8\(^+\) T cells (Fig. 5, B) after restimulation in vitro, and the results were compared with those obtained from cells of mice immunized 6 days previously and similarly restimulated. E7-specific IFN-\(\gamma\) secretion by splenocytes after in vitro culture with antigen was observed in the recipients of grafts, E7 immunization, and E7
TCR tg T cells, confirming that a substantial persistent memory CTL population was present in grafted recipients.

**DISCUSSION**

In this study, we used a mouse skin graft model to simulate the immunobiology of HPV-associated cervical precancer in the clinical setting. In the model system (9), as in the clinical setting (22), epithelial cells expressed the nuclear oncoprotein antigens of the relevant oncogenic virus, HPV16, and induced a specific antibody response, but the host did not reject the HPV16-expressing cells. Thus, HPV16 E7 was presented to the adaptive immune system without inducing an effective CD8<sup>+</sup>/H11001 (MHC Class I) restricted E7-specific T effector response. This finding is somewhat at variance with the concept that low levels of antigen are more effectively cross-presented for CTL induction than for induction of antibody response (23), and it demonstrates that functional tolerance can be a consequence of tumor or viral antigen presentation restricted to somatic cells (24). In this study, we show further that a CD8<sup>+</sup> T-cell response sufficient to effect epithelial rejection can be induced only by a combination of active immunization with the relevant antigen and passive immunotherapy with specific T-cell precursors and that neither immunization nor passive immunotherapy (i.e., adoptive transfer) alone is sufficient. Thus, in a model system in which naive CD8<sup>+</sup> precursors are not limiting, immunization activated E7-specific CD8<sup>+</sup>/H11001 T cells to a functional state more effectively than native E7 antigen presented from recently grafted skin.

In clinical trials, specific immunotherapies for cervical cancer and precancer have induced a range of E7-specific immune responses, including E7-specific CD8<sup>+</sup>/H11001 T-cell responses (8), but evidence for therapeutic efficacy of these responses is limited. Although transfer of effector CD8<sup>+</sup> T cells has been effective for some patients with advanced melanoma (25,26), passive immunotherapy with CD8<sup>+</sup> T cells has not been attempted for cervical cancer. Our current data, when taken together with the
Findings in the murine transplant model used in this study replicate findings in cervical cancer and chronic cervical HPV infection when the E7 protein of high-risk oncogenic HPV types is presented by epithelial cells over many years to immunocompetent hosts. Immune responses to the E7 protein of HPV16 are observed in invasive malignancy (22) and in association with cervical intraepithelial neoplasia. Whether persistent infection reflects failure of an effective immune response is not yet known. Immune suppression increases the risk of persistent HPV infection and its progression to malignancy during HIV infection (28) and following transplantation (29); cell-mediated responses to HPV E7 are associated with clearance of premalignant cells (30). However, in general, persistent infection also occurs in immunocompetent individuals and, as with the mouse skin graft model, does not lead to effective presentation of E7 protein, at least as measured by antibody induction (31).

As with the skin graft model, induction of immunity following immunization produces both humoral and cell-mediated immunity in patients with vulval and cervical HPV infection (8) without, however, causing disease resolution in the majority of patients. Thus the available evidence suggests that the immunobiology of cervical cancer and premalignancy more closely mimics that of E7 skin grafts than that of E7-expressing transplantable tumors, although both express E7 mRNA and protein in approximately equal amounts (10). Why is the combination of immunization and adoptive transfer of specific CD8+ T cells necessary for effective immunotherapy of skin expressing E7 but not for that of transplantable tumors that express the same amount of E7 protein (10)? The C57BL/6J mouse mounts an effective CD8+ T-cell response to the major dominant H-2Db epitope (aa sequence = RAHYINIVTF) of HPV16 E7 protein. However, larger numbers of effector T cells may be required to kill skin cells than tumor cells, perhaps because trafficking of effector cells to skin is less effective than to tumors (32–34). Certainly, more effector cells are induced by combined therapy than by immunization alone. This explanation is supported by the ability of immunization to result in tumor but not graft rejection in the same animal (10).

Alternatively or additionally, E7-specific CD8+ T-cell precursors may not respond optimally to immunization in an E7 graft recipient as a consequence of presentation of E7 from the graft. However, immunization of a graft recipient before, rather than after, grafting does not render the active immunotherapy more effective (10). The results presented here thus indicate a role for activated antigen-specific CD8+ T cells in skin immunotherapy. Adoptive transfer of immunocytes might also increase capacity to deliver an innate immune response, and innate immunity can, under restricted circumstances, be sufficient to invoke rejection of HPV16 E7-expressing transplantable tumors (18). However, our current data show that E7-expressing grafts were not rejected following transfer and specific activation of OT-1 T cells, which recognize an OVA peptide. Thus, transferred innate immune response effectors, even when activated by co-administered antigen-activated T cells, are insufficient to induce graft rejection—that is, T cells specific for an antigen expressed by graft keratinocytes are required.

The fate of grafts expressing HPV16 E6 and E7 in keratinocytes was markedly different than that of grafts similarly expressing two secreted proteins—OVA from the keratin 5 promoter (17) and hGH, from the K14 promoter; these were promptly rejected, though rejection of hGH grafts is not ob-

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**Fig. 5.** Long-term survival of transferred E7 T-cell receptor transgenic (TCR tg) T cells in skin graft recipients. Splenocytes were tested for E7 cytotoxic T lymphocyte (CTL) peptide–specific interferon gamma–secreting T cells by ELISPOT assay (A) and Vβ12+ CD8+ T cells by flow cytometry (B) after 1-week culture with E7 CTL peptide (aa sequence = RAHYINIVTF). Mice were immunized as follows: group A (n = 3), Naive; group B (n = 3), immunized with E7 protein 200 days prior to testing (E7 Imm, day 200); group C (n = 3), adoptive transfer of E7 TCRtg T cells and E7 immunization 200 days prior to testing (AT+E7, day 200); and group D (n = 3), E7 minimal CTL peptide 6 days prior to testing (E7 Imm, day 6). P values for comparisons between groups in panel A were assessed with the Mann–Whitney U test. Horizontal bars indicate mean number of spots. Outcomes of these clinical studies, suggest that effective immunotherapy for HPV-associated skin cancer in humans may require a combination of active and passive immunotherapy. This observation conflicts with the finding that a range of specific immunotherapies eliminates established E7-expressing transplantable murine tumors [reviewed in (8)]; thus, skin expressing E7 may be a more demanding target for immunotherapy than a transplantable tumor expressing E7. However, B16 melanoma, a transplantable tumor that presents only self-antigen, requires not only active/passive immunotherapy but also exogenous IL-2 for successful immunotherapy (27). Thus, the requirements for effective immunotherapy of epithelial tumors expressing only self-antigen would be predicted to be even more stringent.
served on all genetic backgrounds (14). Presentation of antigen by professional antigen-presenting cells is necessary (35,36) to induce an effective CD8\(^+\)-restricted immune response but can result in functional tolerance (36,37). The amount of cross-presented antigen can determine whether immunity or tolerance is induced (38), and E6 and E7 are expressed at relatively lower levels than hGH in our model (data not shown). However, a secreted antigen is better at inducing tolerance than a non-secreted antigen (39), which is somewhat inconsistent with our findings. Systemic *L. monocytogenes* infection (9), induced at the time of grafting E7 skin, allows induction of specific effector responses that eliminate transgenic skin grafts, presumably by modulating cross-presentation through the induction of a wide range of proinflammatory cytokines (40,41). This result implies that the consequence of presentation of antigen is determined less by the amount of cross-presented antigen than by the environment and site. Chronic viral antigen exposure results in preferential induction of CD8\(^+\) T cells with a Tc2 phenotype (42,43). Use of an expanded antigen-specific CD8\(^+\) T-cell population in this model demonstrates that specific CD8\(^+\) T cells have a Tc2 phenotype before antigen challenge by immunization, secreting IL-10 and IL-5 but not IFN-\(\gamma\), whereas after immunization, the phenotype is dominated by IFN-\(\gamma\) secretion and is permissive for rejection. Thus, successful active and passive immunotherapy may be a consequence of a change of polarity of the specific immune response from Tc2 to Tc1, and the need for large numbers of precursors may reflect less efficient trafficking of effectors to skin than to other sites. The absence of second graft rejection in recipients of active and passive immunotherapy demonstrates that the newly placed grafts did not adequately cross-present E7 antigen to the circulating memory CTL population. However, mice that initially received a graft, TCR tg T cells, and immunization, when given further E7 immunization at the time of a second graft, rejected the second graft faster than they rejected the first graft (Fig. 6, B), demonstrating that the grafts themselves present antigen directly to effector T cells, once they have been induced.

The target cell in our model for CD8\(^+\) effector function is likely to be the keratinocyte, although cross-presentation of antigen by endothelial cells within skin grafts has been described (37). *In vitro*, treatment of E7-expressing keratinocytes with IFN-\(\gamma\) is necessary for their recognition and lysis by specific T cells (19) and, *in vivo*, well-healed grafts are not rejected, even in animals primed to reject a recently placed graft by co-administration of *L. monocytogenes* (9) or by combined immunization and adoptive T-cell immunotherapy (data not shown). Thus, a pro-inflammatory environment may be necessary not only for induction of effector function but also for effective direct presentation of antigen.

Overall, our data support a model for the consequences of peripheral antigen presentation in which a critical determinant of outcome is the amount of antigen cross-presented by professional antigen-presenting cells, as has been observed for induction of tolerance when antigen is cross-presented at different levels (44). Non–self-antigens sequestered within cells are not effectively cross-presented to naive or memory T cells, even when presented in the relatively pro-inflammatory environment of a skin graft, although stronger inflammatory signals, including infection with live *L. monocytogenes*, can overcome this limitation to antigen cross-presentation. Ignorance of or functional tolerance to antigens presented by skin cells, even in the face of moderate local inflammation, will thus persist. However, if antigen cross-presentation in an appropriately inflammatory environment elsewhere can induce an adequate population of effector T cells, then direct presentation of antigen, even in a less inflammatory environment, will allow epithelial cell destruction.

Interestingly, the memory effector T-cell population induced by grafting and co-administration of *L. monocytogenes* (9) differs substantially from that induced by adoptive transfer and immunization described in the current experiments, in that the *L. monocytogenes*–induced memory T-cell response could induce rejection of a second graft without further immune manipulation of the recipient mouse, whereas the memory response induced by adoptive transfer and immunization required further activation by immunization at the time of second grafting to induce graft rejection. One difference between the models is that the *L. monocytogenes*–invoked response is more likely to include a CD4\(^+\)-restricted memory component than adoptive transfer and priming of a CD8\(^+\) TCR tg T-cell population. This difference is important because CD4\(^+\) memory is important for long-term immune effector function against tumors (45). Ongoing studies using the skin graft model may thus further clarify requirements for effective immunotherapy for human skin tumors.

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Notes

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