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Intralymphatic Dendritic Cell Vaccination Induces Tumor Antigen–Specific, Skin-Homing T Lymphocytes

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Abstract

Purpose—The identification of tumor antigens recognized by cytotoxic and T helper lymphocytes has led to the development of specific cancer vaccines. Immunization with tumor antigen-pulsed dendritic cells has proved effective at eliciting elevated levels of tumor antigen – specific T cells in patient blood, but objective clinical responses remain rare, suggesting that vaccine-induced T cells are not trafficking optimally to site(s) of tumor burden. Accumulating evidence from animal models suggests that route of immunization can have a substantial influence on the subsequent migration of primed, activated T cells *in vivo*.

Experimental Design—In a clinical trial designed to elicit more effective cytotoxic T-cell mediated antitumor responses, metastatic melanoma patients were immunized directly via a peripheral intralymphatic route with autologous dendritic cells pulsed with HLA-A*0201-restricted melanoma-associated peptide antigens derived from MART-1 and gp100.

Results—Within 10 days of intralymphatic dendritic cell vaccination, four of six patients developed dramatic and diffuse erythematous rashes in sun-exposed areas of skin that showed extensive T-cell infiltration. CTLs grown from rash biopsies were strongly enriched for tumor antigen – specific T cells that had elevated expression of cutaneous lymphocyte antigen and chemokine receptor-6, consistent with a skin-homing phenotype. Of note, the only patient in the study with cutaneously localized disease showed a significant regression of metastatic lesions following the development of a surrounding rash.

Conclusions—The evidence presented here is consistent with immunization studies in animal models and supports the concept that T cells are “imprinted” in peripheral lymph node sites to express specific ligands and chemokine receptors that allow them to migrate to skin. Furthermore, the preferential migration of the T cells to sun-exposed cutaneous sites suggests that inflammation plays a critical role in this migration. These observations suggest that further study of the effects of immunization route and inflammation on T-cell migration in humans is warranted, and could lead

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to vaccination approaches that would more reliably direct trafficking of activated T cells to diverse sites of metastatic disease.

The identification of numerous tumor-associated antigens recognized by specific T lymphocytes has provided opportunities to develop specific and effective immunotherapeutic cancer vaccines for the treatment of solid tumors (1,2). Several clinical trials have attempted to increase tumor-specific T-cell reactivity in cancer patients using a variety of means, including delivery of tumor antigens as peptide- or protein-based vaccines, plasmid DNA, recombinant viruses, and dendritic cell-based approaches (3–8). Although most of these trials have met with success in terms of immunologic end points (often measured by increased frequencies of tumor antigen-reactive T cells in patient peripheral blood), reports of complete tumor regressions have been rare and objective clinical response rates have been disappointing (9–11). The fact that tumor growth often continues unimpeded in the face of large percentages of vaccine-induced, circulating T cells suggests that trafficking of these tumor-reactive T cells out of the vasculature and into tissue sites of metastatic disease is inadequate and may constitute a major rate-limiting step to effective T-cell based immunotherapies.

Accumulating evidence from vaccination studies in animal models has shown that immunization route plays a major role in determining the subsequent migratory capacity of primed, antigen-specific T cells (12). These studies have led to the concept of “imprinting,” whereby priming of T cells within anatomically distinct tissue draining lymph nodes leads to specific expression patterns of ligands and chemokine receptors on the T-cell surface that mediate subsequent migration to the tissue of initial antigen exposure. Thus, T cells initially exposed to antigen in peripheral lymph nodes are imprinted with skin-homing capabilities (13–16), and T cells encountering antigen in mesenteric lymph nodes migrate preferentially to the gut (17–19). Although no studies to date have directly examined the relationship of vaccine route and T-cell imprinting in humans, there is evidence to suggest that human T-cell tissue trafficking patterns are determined similarly by the specific expression of ligands and chemokine receptors homologous to those found in mice (20).

In this report, we describe four metastatic melanoma patients who developed an acute, T cell-infiltrated, erythematous rash in sun-exposed areas of skin within 10 days of receiving a dendritic cell-based melanoma vaccine given directly into peripheral lymph nodes by the intralymphatic route. Cytotoxic T lymphocytes (CTLs) grown from skin rash biopsies of two patients were highly reactive against the immunizing tumor antigens and expressed markers indicative of a skin-homing phenotype. Notably, the one patient in the study who showed an objective clinical response to the dendritic cell vaccination presented with cutaneously localized metastatic disease and experienced regression of individual metastatic skin lesions following the development of a surrounding rash.

The observation of T cells migrating to inflamed skin following priming in peripheral lymph nodes is in accordance with data from animal vaccination studies and supports the basic concept of T-cell imprinting in humans. However, these studies also underscore a clear need to better understand the influences of immunization route and tissue inflammation on T-cell migration in humans, principles which could be crucial for the design of improved vaccination strategies that will reliably direct activated T cells to migrate to solid tumors at diverse anatomic locations.

Materials and Methods

Dendritic cell immunization

Under an Institutional Review Board–approved protocol at the National Cancer Institute, HLA-A*0201-positive stage IV melanoma patients were given an autologous peptide-pulsed dendritic cell vaccine via direct intralymphatic injection. Monocyte-derived dendritic cells

were generated from the adherent fraction of patient peripheral blood mononuclear cells (PBMC) by culturing in RPMI 1640 plus 10% human AB serum with recombinant granulocyte-macrophage colony-stimulating factor (1,000 units/mL) and interleukin (IL)-4 (1,000 units/mL) for 6 days. Dendritic cells were activated with trimeric CD40 ligand (100 ng/mL; Immunex, Seattle, WA) for 24 hours before the planned infusion. All dendritic cells for patient treatment were analyzed phenotypically for dendritic cell surface markers by flow cytometry before intralymphatic injection. Table 1 shows the phenotype of monocyte-gated patient dendritic cells, which, depending on the patient, ranged from 76% to 93% of all live cells infused. Before infusion, the dendritic cells were pulsed separately with 10 μ mol/L of the HLA-A*0201-restricted melanoma-associated modified peptide antigens MART-1_{26-35(27L)} or gp100_{209-217(210M)} for 2 hours at 37°C and then washed with PBS. Dendritic cells (2×10^8 total; 1×10^8 per peptide antigen) were infused into the patients via lymphatic vessels, located within the dorsum of the foot, and localized with isosulfan blue dye.

Rash biopsies and T-cell culture

After obtaining appropriate consent, 6-mm punch biopsies of patient rashes and biopsies of noninvolved skin, when possible, were taken at the bedside using sterile technique. A portion of each biopsy was placed in formalin for immunohistochemical analysis, and a portion was placed in culture in complete medium [RPMI 1640 supplemented with HEPES (25 mmol/L), glutamine (1 mmol/L), penicillin (50 units/mL), streptomycin (50 μ g/mL), β -mercaptoethanol (2×10^{-5} mol/L), and 10% human AB serum, supplemented with 6,000 IU/mL recombinant human IL-2]. Culture medium was refreshed every 3 days while monitoring cell growth.

Immunohistochemistry

Immunohistochemical studies were done on deparaffinized formalin-fixed, paraffin-embedded tissue sections. Antigens were localized using the EnVision horseradish peroxidase method with 3,3'-diaminobenzidine as a chromogen and using an automated immunostainer (DakoCytomation, Carpinteria, CA). Primary antibody incubation was done for 30 minutes with antibodies specific for human CD3, E-selectin, or nonspecific isotype-matched control antibodies (DakoCytomation). Positive and negative controls were used in all experiments and stained appropriately.

T-cell recognition assays

To assess specific peptide recognition by T cells, effector and target cells (1×10^5 each per well) were cocultured in 96-well flat-bottomed plates and supernatants were analyzed for cytokine secretion. Target cells consisted of T2 cells pulsed for 3 hours with 1 μ mol/L of the indicated peptides or HLA-A*0201-positive or HLA-A*0201-negative melanoma tumor lines. Effector cells consisted of T cells cultured from skin rash biopsies. The effector and target cells were cocultured in the absence of IL-2 in 200 μ L of RPMI complete medium for 24 hours and supernatants were collected and analyzed for IFN- γ using commercially available ELISA kits (Endogen, Rockford, IL). T-cell lines known to be reactive against the immunizing MART-1_{26-35(27L)} and gp100_{209-217(210M)} peptides were used for positive controls. T2 cells pulsed with irrelevant HLA-A*0201-restricted control peptides and unpulsed T2 cells were used for negative controls. For T-cell avidity studies, serial dilutions of peptides were pulsed onto T2 cells before coculturing overnight with rash-derived T cells and measuring IFN- γ release.

Flow cytometric immunofluorescence analysis

Before and after treatment, patient PBMC and T-cell cultures were stained with phycoerythrin-labeled MART-1_{26-35(27L)}, gp100_{209-217(210M)}, HIVgag₇₇₋₈₅, and NY-ESO1₁₅₇₋₁₆₅ peptide/HLA-A*0201 tetramer complexes (Beckman Coulter, Inc., Fullerton, CA). For tetramer

staining of PBMC, cells were also stained with lineage markers CD4, CD14, CD16, and CD20, and an exclusion gate was applied so that cells staining positive for these markers were excluded from the analysis. Where indicated, cultured T cells were also stained with fluorescently conjugated monoclonal antibodies against human CD4 and CD8, in addition to monoclonal antibodies against the skin-homing markers chemokine receptor-6 and cutaneous lymphocyte antigen. Cells were fixed with 1% paraformaldehyde and analyzed using FACSCalibur (Becton Dickinson, Franklin Lakes, NJ).

Parallel flow assay

A parallel plate laminar flow chamber (Glycotech, Rockville, MD) connected to an infusion pump was used to simulate the physiologic shear flow stresses present in the microvascular environment as previously described (21). Recombinant E-selectin/immunoglobulin chimeric protein (Glycotech) was coated on the plastic culture plates (Corning, NY) overnight at 1 µg/mL at 4°C in TBS and then rinsed with PBS. T cells were labeled with calcein AM (Molecular Probes, Carlsbad, CA) at 1 µmol/L for 30 minutes at 37°C in PBS, then washed and resuspended in RPMI 1640 at 1×10^6 /mL. A laminar flow of T cells in suspension (5×10^5 /mL– 10×10^5 /mL) was then passed through the flow chamber at 1.5 dynes/cm² shear stress. Cell tethering and adhesion were observed under an inverted phase-contrast microscope (Nikon Eclipse TE300). The number of cells bound within 7 to 11 minutes of flow were counted in a blinded fashion from 8 to 10 fields and analyzed with IPLab software (Scanalytics, Fairfax, VA).

Results

Development of T cell-infiltrated skin rash following intralymphatic dendritic cell vaccination

Dendritic cells normally sample antigens in tissues and, on receiving appropriate activation and maturation signals, migrate to lymph nodes to present these acquired antigens to prime naïve T lymphocytes. In an effort to enhance priming of tumor-reactive T cells in patients with stage IV metastatic melanoma, patients were immunized via an intralymphatic route with CD40 ligand-activated, autologous monocyte-derived dendritic cells pulsed with the HLA-A*0201-restricted, melanocyte-specific tumor antigens gp100₂₀₉₋₂₁₇(210M) and MART-1₂₆₋₃₅(27L). The infused dendritic cells displayed an intermediate to mature phenotype, as determined by flow cytometric analysis of dendritic cell maturation markers CD80, CD83, and CD86 (Table 1).

Unexpectedly, four of the six patients receiving the dendritic cell vaccination developed a dramatic and diffuse erythematous macular papular pruritic rash 7 to 10 days following infusion, which was largely confined to sun-exposed areas of skin (Fig. 1). For patients 1 and 2, the rash was serious enough to require hospitalization and steroid treatment. The rash resolved within a week for patient 1 whereas it took 3 weeks to completely resolve the skin rash for patient 2. Aside from the rashes, there were no other toxicities reported following intralymphatic dendritic cell immunization of the six patients.

To more closely evaluate the nature of the rashes, biopsies of involved and noninvolved skin were obtained from patients 1 and 2 before the onset of steroid treatment. Immunohistochemical staining revealed a large number of inflammatory cells infiltrating the epidermal and dermal junction of the skin involved in the rashes that were not evident in biopsies taken from noninvolved areas of skin (Fig. 2A, B, E, and F). Staining with an anti-CD3 monoclonal antibody confirmed that the majority of the infiltrating cells in the rash were T lymphocytes (Fig. 2C, D, G, and H).

T cells from skin rash biopsies recognize immunizing tumor antigens

To characterize the rash-infiltrating T cells, skin biopsies were cultured in medium containing the T-cell growth factor IL-2 (6,000 IU/mL), resulting in the nonspecific expansion of large

numbers of T cells from the rash biopsies, but no significant outgrowth of T cells from biopsies of noninvolved skin (not shown). After 2 weeks in culture, >90% of the rash-derived T cells were CD8⁺, as measured by flow cytometry (Fig. 3A). T cells cultured from rash biopsies of both patients strongly recognized both of the immunizing gp100 and MART-1 peptide antigens, as determined by high levels of specific IFN- γ secretion in response to stimulation with peptide-pulsed target cells or HLA-matched allogeneic melanoma lines expressing both gp100 and MART-1 (Fig. 3B). Cultured tumor antigen-specific T cells from both patients were highly avid, secreting IFN- γ in response to peptide concentrations as low as 100 pmol/L (Fig. 3C). These T cells were also specific for the immunizing antigens because they failed to recognize HLA-mismatched melanoma cell lines or irrelevant HLA-A*0201-restricted peptides derived from several other tumor or viral antigens (not shown). Rash-derived T cells secreted IFN- γ and granulocyte-macrophage colony-stimulating factor, but not IL-4, in response to recognition of the immunizing melanoma antigens, demonstrating a type 1 cytokine secretion profile (Table 2).

Tumor antigen – specific T cells preferentially migrate to inflamed skin

Tetramer analysis showed that CD8⁺ T cells cultured from the skin rash of patient 1 contained strikingly high percentages of MART-1 and gp100 tumor antigen-specific cells (39% and 54%, respectively; Fig. 4A). Likewise, cultured, rash-derived T cells from patient 2 contained 93% gp100-reactive T cells (Fig. 4A). Although detectable in the initial culture from patient 2 (Fig. 3B), MART-1-reactive T cells decreased in number after further culture and were no longer detectable at the time of tetramer analysis.

By contrast, peripheral blood drawn from patients 1 and 2 contained relatively few tumor antigen-specific cells at all time points tested, for up to 4 weeks following dendritic cell vaccination (Fig. 4A and B). The peak response was measured 10 days following vaccination in patient 2, at which time only 0.3% of CD8⁺ T cells stained positively with the gp100 tetramer (Fig. 4B). Culture of these PBMC in IL-2 (6,000 IU/mL) failed to significantly increase the percentage of tumor antigen-reactive T cells (data not shown). Collectively, these results suggested that the tumor antigen-specific CD8⁺ T cells induced by the intralymphatic dendritic cell vaccination had migrated preferentially to skin.

Rash-derived T cells express functional skin-homing markers

Because T-cell homing to cutaneous sites is mediated through surface expression of specific ligands and chemokine receptors, we next examined whether the IL-2-cultured, patient rash-derived T cells expressed surface markers consistent with a skin-homing phenotype. As shown in Fig. 5A, rash-derived T cells from patients 1 and 2 showed elevated expression of both cutaneous leukocyte antigen and chemokine receptor-6. Cutaneous leukocyte antigen mediates lymphocyte adherence to E-selectin expressed on inflamed cutaneous endothelium as one of the first steps in transmigration of T cells out of the bloodstream and into skin tissue. Chemokine receptor-6 is also known to mediate T-cell trafficking to sites of inflammation through binding to the soluble inflammatory mediators CCL20 and HBD2 (22–24). Although chemokine receptor-4 has also been reported as a marker for skin-homing CD8⁺ T cells, expression of this chemokine receptor was not detectable in rash-derived T cells from either patient (not shown). This may reflect a culture-induced down-regulation of chemokine receptor-4 or might indicate the usage of alternative chemokine receptors, like chemokine receptor-6, to mediate homing of these T cells to inflamed skin (15,25). Coculture of patient PBMC with autologous MART-1 peptide-pulsed dendritic cells did not result in a significant up-regulation of skin-homing markers in MART-1-specific T cells, implying that factors present *in vivo* within the lymph node environment were essential for inducing the skin-homing phenotype observed in the rash-derived T cells (Fig. 5B).

E-selectin, known to be up-regulated on skin endothelial cells in response to skin inflammation, was expressed at significantly higher levels in rash-involved skin compared with noninvolved skin (Fig. 6A and B). To test whether the cutaneous lymphocyte antigen expressed by the rash-derived T cells could mediate binding to E-selectin under physiologic conditions, a recombinant E-selectin/immunoglobulin chimeric protein was coated onto plastic and a laminar flow of T cells in suspension was passed over the coated layer using an infusion pump to simulate physiologic levels of shear stress. T-cell tethering and adhesion were then observed and photographed in real time using phase-contrast microscopy. In these experiments, rash-derived T cells from patient 2 failed to bind to control protein bovine serum albumin but showed significant levels of tethering and adhesion to E-selectin/immunoglobulin-coated plastic (Fig. 6C-E). The addition of EDTA, which disrupts the binding of cutaneous lymphocyte antigen to E-selectin, reduced the number of bound T cells to background levels. Similar results were obtained with rash-derived T cells from patient 1 (not shown).

These results collectively suggest that a significant proportion of T cells induced by intralymphatic dendritic cell vaccination migrated to sun-exposed cutaneous sites. These T cells were highly reactive against the immunizing tumor antigens, expressed characteristic markers of skin homing, and interacted functionally with E-selectin, suggesting a plausible mechanism for their migration into inflamed skin.

Regression of cutaneous lesions following induction of rash

Of the six metastatic melanoma patients receiving intralymphatic dendritic cell vaccination, five had noncutaneous disease, such as lung metastases, and one had cutaneously localized disease. Whereas none of the patients with visceral or subcutaneous disease showed significant objective clinical responses to the dendritic cell vaccine, the single patient with cutaneous disease showed a significant partial response following immunization (Table 3). As shown in Fig. 7, a metastatic cutaneous lesion on the thigh of patient 2 developed a surrounding erythematous rash ~12 days following vaccination and exposure to sunlight. By day 29 posttreatment, the lesion had almost completely regressed (Fig. 7), suggesting that tumor antigen-specific T cells associated with the rash may have mediated destruction of the tumor. No apparent disease was observed for 5 months, at which time the lesion recurred. A second lesion near the first one showed a 70% reduction in total area following dendritic cell vaccination, but had begun progressing again by 3 months postimmunization (Table 4).

Discussion

The goal of all cancer vaccination strategies is to induce the generation of endogenous tumor-reactive T cells that can subsequently mediate the destruction of solid tumors. Several of these strategies have realized the first part of the equation, but the successful generation of large numbers of circulating tumor antigen-specific T cells has not, to date, correlated well with clinical responses (11). This seemingly paradoxical observation can be explained, at least in part, by the fact that vaccine-induced T cells may be largely incapable of successfully migrating out of the bloodstream and into tissue sites of metastatic disease where they are needed. Overcoming this substantial hurdle to T cell-based immunotherapies will require a better understanding of, and consideration for, the principles governing T-cell trafficking *in vivo*.

This report highlights two variables known to have a major influence on T-cell migration: site of T-cell priming and presence of inflammation at the target tissue. T-cell imprinting proposes that the site of initial antigen exposure dictates the future migration patterns of primed effector and memory T cells, essentially limiting trafficking to those anatomic compartments where specific antigen encounters are most likely to recur (12). This process is mediated by the differential expression of specific ligands, integrins, and chemokine receptors expressed at the T-cell plasma membrane. Such receptors recognize molecular “addressins” in the form of other

tissue-specific molecules, including selectins and chemokines expressed on endothelial cells (26). These molecular interactions collectively mediate rolling and adherence to vascular endothelium and transmigration of lymphocytes into peripheral tissue sites (27). This imprinting mechanism is thought to have evolved as a means to efficiently direct lymphocyte migration from tissue draining lymph nodes back to the initial sites of pathogen exposure, which most frequently are skin, gut, and bronchus (20,28).

In this study, we found that four of six patients receiving a peptide-pulsed dendritic cell vaccine administered by an intralymphatic route developed an acute rash in sun-exposed areas of skin within 10 days of immunization. A detailed characterization of skin rash biopsies from two of the patients revealed large numbers of infiltrating, highly avid T cells that specifically recognized the immunizing tumor antigens. These T cells expressed elevated levels of functional cutaneous lymphocyte antigen, known to mediate tethering and rolling on the walls of postcapillary venules through interactions with E-selectin, an early step in the transmigration of T cells into the dermis. The observation of much higher frequencies of tumor antigen-specific CD8⁺ T cells in rash-involved skin compared with peripheral blood suggests that the intralymphatic immunization endowed the primed T cells with the ability to preferentially migrate to cutaneous sites, consistent with the concept of T-cell imprinting. In previous clinical trials using an i.v. dendritic cell vaccination route, no such rash development or T-cell skin-homing was observed following immunization,⁴ implying that intralymphatic administration was crucial for imparting skin-homing potential.

It should be noted that both melanoma patients and normal donors have a relatively high frequency of MART-1-reactive T cells compared with other melanoma antigens. T cells with specificity for gp100, however, are not normally detectable in normal and nonimmunized patient donors, underscoring the remarkable effectiveness of intralymphatic dendritic cell vaccination for expanding these tumor antigen-specific cells.

Notably, the rashes appeared primarily on sun-exposed areas of skin in all patients described. This is likely due to T-cell chemotaxis triggered by local inflammation stimulated by UV light (29). T cells are known to infiltrate the dermis following UVB exposure, which is thought to be mediated by an inflammatory process involving initial vasodilation through prostaglandin E2 and nitric oxide, followed by up-regulation of CCL20, E-selectin and P-selectin, and intercellular adhesion molecule-1 on dermal endothelium (30). Inflammation, through the up-regulation of adhesion molecules, selectins, and chemokines recognized by lymphocyte receptors, seems to be a requirement for optimal migration of T cells into target tissues, as has been shown by numerous elegant studies in animal models (31,32). In this context, chemokine receptor-6, expressed by rash-derived T cells and known to bind to CCL20, may have contributed to the migration of vaccine-induced T cells into inflamed skin.

Whereas this report illuminates two important principles that influence T-cell migration *in vivo*, the challenge ahead will be to incorporate these concepts into future vaccination strategies for cancer patients. The observed skin homing of T cells following vaccination via peripheral lymph nodes suggests that this immunization route may be particularly effective for treatment of cutaneously localized metastases, as we observed in patient 2. However, consideration of these principles also begs the question: What types of T cells are we generating with our current cancer vaccines? Imprinting would suggest that current vaccines, the majority of which are given in peripheral extremities, generate T cells that preferentially home to the skin, providing a potential explanation for the ineffectiveness of cancer vaccines against visceral metastases. Whereas addressins have been identified for the most common sites of pathogen exposure

⁴Unpublished observations.

(skin, gut, and bronchus), it is unknown whether such addressins have evolved to mediate lymphocyte trafficking into other organ sites or to tumor.

Finally, whereas imprinting may provide the potential means for T cells to migrate into target tissues, only in the context of local inflammation can this potential be fully realized. It seems that the majority of malignancies emerge within a noninflammatory immune environment that is fostered largely by immune inhibitory factors secreted by or induced by the tumors themselves. The induction of local inflammation at tumor sites through exogenous means may therefore be critical for attaining enhanced clinical responses to T cell–based immunotherapy. Incorporating the natural principles of T-cell migration illustrated here into future immunization strategies may be an important key to improving the efficacy of cancer vaccines.

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Fig. 1.

Development of rash in patients following intralymphatic dendritic cell infusion. HLA-A*0201-positive stage IV melanoma patients were infused intralymphatically with autologous, CD40 ligand – activated, monocyte-derived dendritic cells pulsed with the melanoma-associated peptides MART-1₂₆₋₃₅(27L) and gp100₂₀₉₋₂₁₇(210M). A and B, the majority of treated patients developed a diffuse, erythematous rash in sun-exposed areas of skin 7 to 10 days following dendritic cell infusion. Representative photographs of patients 1 and 2.

Fig. 2.

Skin rash biopsies contain extensive T-cell infiltrates. Histologic analysis of two representative patient biopsies of noninvolved (A–D) and rash-involved (E–H) skin. A, B, E, and F, H&E staining of skin tissue reveals dramatically increased lymphocytic infiltrates in rash sections as compared with normal skin. C, D, G, and H, immunohistochemical staining with anti-CD3 antibody shows that T cells constitute the majority of infiltrating lymphocytes in rash sections.

Fig. 3.

Rash-derived CD8⁺ T cells recognize immunizing tumor antigens with high avidity. *A*, T cells cultured from skin rash biopsies of patients 1 and 2 contained 93% and 98% CD8⁺ cells, respectively, after 14 days of expansion in 6,000 IU/mL IL-2. *B*, rash-derived T cells show immune reactivity against HLA-A2-restricted melanoma tumor antigens MART-1₂₆₋₃₅(27L), gp100₂₀₉₋₂₁₇(210M), and HLA-matched allogeneic melanoma lines, as measured by IFN-γ release. *C*, cultured T cells were incubated overnight with T2 cells pulsed with titrated doses of gp100₂₀₉₋₂₁₇(210M) peptide and IFN-γ production was measured by ELISA on cultured supernatants. Peptide doses as low as 100 pmol/L were recognized by CD8⁺ T cells derived from both patients. These experiments were done twice with similar results.

Fig. 4.

Tumor antigen – specific T cells show preferential migration to inflamed skin. HLA-A*0201 tetramer analysis of pretreatment PBMC, patient PBMC collected 10 to 12 days post-dendritic cell infusion, and skin rash – derived T cells cultured in 6,000 IU/mL IL-2. Pretreatment and posttreatment PBMC from patient 1 (A) and patient 2 (B) show very low frequencies of CD8⁺ T cells that stain specifically with HIV-1gag₇₇₋₈₅ (negative control), gp100_{209-217(210M)}, or MART-1_{26-35(27L)}/HLA-A*0201 fluorescently conjugated tetramers. In contrast, cultured T cells derived from rash skin biopsies contained significantly higher percentages of T cells specific for the immunizing gp100 and/or MART-1 peptides. These experiments were done twice with representative data shown.

Fig. 5.

Rash-derived T cells express the skin-homing markers chemokine receptor-6 and cutaneous lymphocyte antigen. **A**, flow cytometric analysis of rash-derived T cells cultured in 6,000 IU/mL IL-2 from patients 1 and 2 stained with monoclonal antibodies against skin-homing markers chemokine receptor-6 (*CCR6*) and cutaneous lymphocyte antigen (*CLA*). **B**, PBMC from patient 1 were stimulated *in vitro* with MART-1 peptide – pulsed dendritic cells for 21 days before staining for chemokine receptor-6 and cutaneous lymphocyte antigen and analyzing by flow cytometry. In contrast to antigen-specific rash-derived T cells generated *in vivo* by dendritic cell vaccination, *in vitro* dendritic cell – stimulated CD8⁺ T cells failed to show significant up-regulation of skin-homing markers. These experiments were done twice with representative results shown.

Fig. 6.

E-selectin is up-regulated on rash endothelium and mediates adhesion to cutaneous lymphocyte antigen on IL-2-cultured, rash-derived T cells. *A* and *B*, immunohistochemical analysis of a representative patient biopsy showing E-selectin expression in noninvolved (*A*) and rash-involved (*B*) skin. Arrows in *B* indicate E-selectin expression. *C* to *E*, simulating the physiologic shear flow stresses present in the microvascular environment, a laminar flow of suspended, rash-derived T cells from patient 2 was passed through a flow chamber coated with bovine serum albumin control protein (*C*) or recombinant E-selectin/immunoglobulin chimeric protein (Glycotech; *D*). Cell tethering and adhesion were observed under an inverted phase-contrast microscope. *E*, numbers of T cells bound within 7 to 11 minutes of flow with either

bovine serum albumin or E-selectin/immunoglobulin chimeric protein coated on the flow chamber. EDTA, which interferes with E-selectin/cutaneous lymphocyte antigen binding, was used as a negative control in this experiment. Representative data from three experiments.

Fig. 7. Regression of cutaneous lesion following development of rash. A to C, photographs document the regression of a cutaneous metastatic melanoma nodule in patient 2 following intralymphatic infusion of peptide-pulsed dendritic cells. By day 12 posttreatment, a rash developed surrounding the cutaneous tumor nodule, which showed a complete regression over the following 3 weeks.

Table 1

Phenotype of infused, CD40 ligand-activated dendritic cells

Patient	CD11c ⁺	HLA-DR ⁺	CD80 ⁺	CD83 ⁺	CD86 ⁺	ICAM-1 ⁺
1	99	99	38	42	99	98
2	99	98	43	25	96	93
3	99	99	49	85	99	99
4	99	98	39	22	99	ND
5	99	99	25	2	93	84
6	99	99	65	73	99	99

NOTE: Table indicates percent of DCs expressing each phenotypic marker.
Abbreviations: ICAM-1, intercellular adhesion molecule 1; ND, not determined.

Rash-derived T cells recognize and secrete Tc1 cytokines in response to melanoma antigens

Table 2

Cytokine	Target cells	Rash-derived T cells	Control T cells	
			gp100 specific	MART-1 specific
Granulocyte-macrophage colony-stimulating factor (pg/mL)	T2 cells pulsed with:	No peptide	<30	<30
		gp100 ₂₀₉₋₂₁₇ (210M)		
		gp100 ₂₀₉₋₂₁₇	8,252	191
		MART-1 ₂₆₋₃₅ (27L)	9,516	227
		MART-1 ₂₆₋₃₅	117	13,329
IFN-γ (pg/mL)	T2 cells pulsed with:	No peptide	204	2,855
		gp100 ₂₀₉₋₂₁₇ (210M)	<30	<30
		gp100 ₂₀₉₋₂₁₇	>10,000	<30
		MART-1 ₂₆₋₃₅ (27L)	>10,000	<30
		MART-1 ₂₆₋₃₅	<30	>10,000
IL-4 (pg/mL)	T2 cells pulsed with:	No peptide	380	7,320
		MART-1 ₂₆₋₃₅	3,635	8,880
		gp100 ₂₀₉₋₂₁₇ (210M)	<30	<30
		gp100 ₂₀₉₋₂₁₇	<11	<11
		MART-1 ₂₆₋₃₅ (27L)	174	<11
	Melanoma cell lines	No peptide	159	<11
		gp100 ₂₀₉₋₂₁₇	<11	61
		MART-1 ₂₆₋₃₅ (27L)	<11	18
		MART-1 ₂₆₋₃₅	18	18
		gp100 ₂₀₉₋₂₁₇ (210M)	<11	<11

Table 3

Summary of patient characteristics, treatment-related toxicities, and clinical response

Patient	Age/sex	Sites of metastatic disease	Treatment side effects	Clinical response
1	54/M	Lung	Rash	None
2	39/F	Cutaneous skin	Rash	Partial
3	59/M	Lymph node, s.c. skin	Rash	None
4	29/M	Lymph node, s.c. skin	Rash	None
5	52/M	S.c. skin	None	None
6	54/F	Lymph node, s.c. skin	None	None

Table 4

Documented objective clinical response for patient 2

Cutaneous lesion	Anatomic location	Lesional area (mm ²)			
		8/27/02*	10/18/02	11/27/02	1/25/03
1	Anterior superior tibia	42	12	20	25
2	Inferior lateral thigh	16	2	0	0
Total	N/A	58	14	20	25
					42
					20
					62

* Pretreatment measurement.