Immunomonitoring Tumor-Specific T Cells in Delayed-Type Hypersensitivity Skin Biopsies After Dendritic Cell Vaccination Correlates With Clinical Outcome


ABSTRACT

Purpose
Tumor-specific immunomonitoring is essential to evaluate the efficacy of vaccination against cancer. In this study, we investigated the predictive value of the presence or absence of antigen-specific T cells in biopsies from delayed-type hypersensitivity (DTH) sites.

Patients and Methods
In our ongoing clinical trials, HLA-A2.1/melanoma patients were vaccinated with mature dendritic cells (DC) pulsed with melanoma-associated peptides (gp100 and tyrosinase) and keyhole limpet hemocyanin.

Results
After intradermal administration of a DTH challenge with gp100- and tyrosinase peptide–loaded DC, essentially all patients showed a positive induration. In clinically responding patients, T cells specific for the antigen preferentially accumulated in the DTH site, as visualized by in situ tetramer staining. Furthermore, significant numbers of functional gp100 and tyrosinase tetramer–positive T cells could be isolated from these DTH biopsies, in accordance with the applied antigen in the DTH challenge. We observed a direct correlation between the presence of DC vaccine–related T cells in the DTH biopsies of stage IV melanoma patients and a positive clinical outcome (P = .0012).

Conclusion
These findings demonstrate the potency of this novel approach in the monitoring of vaccination studies in cancer patients.

INTRODUCTION

Dendritic cell (DC) vaccines have been successfully used for the induction of antitumor T-cell reactivity in melanoma patients. These early trials have shown that vaccination with DC is feasible, nontoxic, and effective in some patients, provided that the DC are appropriately matured and activated. To exploit the full potential of these immunostimulatory cells, many questions need to be answered. These questions can only be properly addressed in clinical trials. Besides clinical end points, it is of utmost importance to monitor the immune reactivity during therapy.

Most immunologic monitoring assays for antigen-specific cytotoxic T lymphocytes (CTL) in peripheral blood in vaccinated patients depended on their ability to proliferate extensively and acquire lytic activity or to release relatively large amounts of cytokines in vitro. The development of fluorescent major histocompatibility complex (MHC)/peptide tetramers has greatly improved the ability to detect tumor antigen–specific T cells. This method has proven useful for identifying T-cell responses in peripheral
blood to peptide-based vaccines. Additional bioassays, like cytotoxicity assays, or secretion of cytokines, such as interferon gamma (IFN-γ), are essential to measure the functional properties of antigen-specific CTL. Indeed, tetramer-positive T cells in cancer patients have been described that are functionally ineffective in lysing target cells or producing cytokines. Therefore, analysis of both frequency and functionality of natural or vaccine-induced CTL is preferred.

Another major question is the optimal compartment in which vaccine-related immune responses should be monitored. Romero et al described high numbers of MHC/peptide tetramer–positive cells in tumor-infiltrated lymph nodes. Unfortunately, lymph nodes and the tumor site itself are not readily available for monitoring purposes. In this study, we tested the hypothesis that skin biopsies taken from delayed-type hypersensitivity (DTH) reactions are an optimal compartment for immunomonitoring purposes.

**Antibodies, MHC Tetramers, and Immunostaining**

Flow cytometry was performed using fluorescein isothiocyanate–conjugated (anti-HLA class I [W6/32] and anti-HLA DR/DP [Q5/13]) and phycoerythrin–conjugated monoclonal antibodies (anti-CD80, Becton Dickinson, Mountain View, CA; anti-CD14 and anti-CD83, Beckman Coulter, Mijdrecht, the Netherlands; and anti-CD86, Pharmingen, San Diego, CA). Immunohistochemical analysis was performed with monoclonal antibodies purchased from Pharmingen. Tetrameric MHC-peptide complexes (HLA-A2.1-gp100:154-162, HLA-A2.1-gp100:280-288, HLA-A2.1-tyrosinase:369-377, HLA-A2.1-MART-1(ELAGIGILTV), HLA-A2.1-HIVgag (SLYNTVATL), and HLA-A2.1-CMV(GLCTLVAML) were provided by H. Spits (Netherlands Cancer Institute, Amsterdam, the Netherlands) or purchased from Immunomics, Beckman Coulter Inc (San Diego, CA).

**Patients**

Melanoma patients (stage III and IV according to American Joint Committee on Cancer criteria) who are participating in ongoing protocols in which the immune responses of DC vaccines were studied are included. Eligibility criteria included HLA-A2.1 phenotype, HLA-DR4 phenotype (when also MHC class II binding peptides were used), melanoma expressing gp100 and tyrosinase, and WHO performance status of 0 or 1. Additional eligibility criteria for stage IV patients included the following: documented progressive disease within 2 months before study entry, serum lactate dehydrogenase ≤ 2× the upper limit of normal, no prior chemotherapy or immunotherapy within 3 months before study entry, and no residual toxicity from prior treatments. For stage III patients, further eligibility criteria included planned radical lymph node resection and no prior chemotherapy, radiotherapy, or immunotherapy. Patients with brain metastases, serious concomitant disease, or clinical signs of a history of a second malignancy were excluded. The studies were approved by the local regulatory committee, and written informed consent was obtained from all patients.

Patients received the DC vaccine according to different study protocols as listed in Table 1. All patients who remained free of disease progression after the first vaccination cycle were eligible for two maintenance cycles at 6-month intervals, each consisting of three biweekly intranodal vaccinations in a clinically tumor-free lymph node region under ultrasound guidance with mature DC and alternately pulsed with wild-type or modified gp100 and tyrosinase peptides and keyhole limpet hemocyanin (KLH). Nine patients received two maintenance cycles, and 17 patients received one maintenance cycle.

A clinical response was defined as stable disease for more than 4 months or any partial or complete response. Responses were defined according to Response Evaluation Criteria in Solid Tumors. Toxicity was assessed according to National Cancer Institute Common Toxicity Criteria. Progression-free survival was calculated from the day of the first vaccination. Patients were
DTH T Cells Correlate With Clinical Outcome

results were obtained in 10% of patients tested with either CD4 or CD8.


determined by flow cytometry.

Cytotoxicity Assay and Cytokine Production

Cytotoxic activity and production of cytokines by DTH-derived cells in response to T2 cells pulsed with the indicated peptides or BLM (a melanoma cell line expressing HLA-A2.1 and no endogenous expression of gp100 and tyrosinase), transfected with control antigen G250, or with gp100 or an allogenic HLA-A2.1-positive, gp100-positive, and tyrosinase-positive tumor cell line (MEL624) were measured. Cytotoxic activity of DTH-infiltrated lymphocytes (DIL) was measured using the chromium release assay. Cytokine production was measured in supernatants after 16 hours of coculture by the cytokometric bead array (Th1/Th2 Cytokine CBA 1; BD Pharmingen).

Statistics

Statistical significance was evaluated using the log-rank test. Statistical analysis was performed with SPSS 11.0 for Windows (SPSS Inc, Chicago, IL).

DC-Based Vaccination of Melanoma Patients

In total, 39 melanoma patients were treated with tumor peptide and KLH-loaded DC (Table 1). Most patients mounted a potent proliferative (35 of 36 patients tested) and antibody (30 of 37 patients tested) response against KLH (Table 1). The proliferative anti-KLH response was already detected after one DC vaccination (Fig 1). These results demonstrate that the mature DC used were able to mount a de novo immune response.

In Situ Staining With Tetramer

From cryopreserved DTH biopsies, 8-μm thick cryosections were cut. Tissue sections were fixed with 4% paraformaldehyde (3 minutes), and nonspecific protein binding was prevented by using 20% normal goat serum in phosphate-buffered saline (PBS; 20 minutes). Incubation with tetramer (5 μmol/L, 5% normal goat serum in PBS) was performed overnight at 4°C in the absence or presence of CD8 (2 μg/mL, WT82). After washing in PBS, sections were fixed with 4% paraformaldehyde (20 minutes) and incubated with polyclonal rabbit serum against streptavidine (1:800; Rockland Inc, Gilbertsville, PA). The specific binding of tetramer was visualized using a polyclonal goat-antirabbit-Alexa498 (1:500, 30 minutes, RT; Molecular Probes), resulting in a green fluorescent signal. CD8 was visualized using a polyclonal goat-antimouse-Alexa594 (1:500, 30 minutes, RT; Molecular Probes), resulting in a red fluorescent signal. Sections were counterstained with 4',6-diamidino-2-phenylindole, resulting in blue fluorescent nuclei.

MHC Tetramer Staining

DTH-derived cells (1 × 10⁸ cells in 10 μL) or PBMC (1 × 10⁶ cells in 10 μL) were incubated with phycoerythrin–labeled tetrameric–MHC complexes for 1 hour at RT. In the last 20 minutes of this incubation, fluorescein isothiocyanate–conjugated monoclonal antibodies directed against either CD4 or CD8 (both Becton Dickinson) were added. After washing, the samples were analyzed by flow cytometry.

RESULTS

DC Preparations and Characterization

KLH-loaded DC were generated from peripheral-blood mononuclear cells (PBMC) and matured with autologous monocyte-conditioned medium with prostaglandin E2 (10 μg/mL; Pharmacia & Upjohn, Puurs, Belgium) and 10 ng/mL of recombinant tumor necrosis factor alpha (provided by Dr G. Adolf, Bender Wien, Vienna, Austria), as described previously. This procedure gave rise to mature DC meeting the release criteria described previously.9

Peptide Pulsing

DC were pulsed with the HLA class I gp100-derived peptides gp100:154-167 (wild type or modified Q→A), and gp100:280-288 (wild type or modified A→V) and the tyrosinase-derived peptide tyrosinase:369-376. DC from HLA-DR4–positive patients were also pulsed with HLA-DR4–binding peptides of both gp100 and tyrosinase (gp100:44-59 and tyro:448-462 analog; both provided by D. Schadendorf, Department of Dermatology, University Hospital Mannheim, Mannheim, Germany).26,27 Peptide pulsing was performed as described previously, and cells were resuspended in 0.1 mL for intradermal (ID) or intranodal injection.6

DTH

One to 2 weeks after a vaccination cycle consisting of three or four DC vaccinations, a DTH skin test was performed. Briefly, unpulsed DC, DC pulsed with the indicated peptides, DC pulsed with KLH, and DC pulsed with the indicated peptides plus KLH (2 to 10 × 10⁶ DC each) were injected ID in the skin of the back of the patients at four different sites. The maximum diameter of induration was measured after 48 hours.

From positive DTH sites (> 2 mm), punch biopsies (6 mm) were obtained and cut in half; one part was cryopreserved, and the other part was cut in pieces. Leukocytes emigrating from these tissue pieces were cultured in RPMI 1640/7% human serum supplemented with interleukin (IL)-2 (100 U/mL). Every 7 days, half of the medium was replaced by fresh IL-2–containing RPMI 1640/7% HS. After 2 to 4 weeks of culturing, T cells were tested.

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Cytotoxicity Assay and Cytokine Production

Cytotoxic activity and production of cytokines by DTH-derived cells in response to T2 cells pulsed with the indicated peptides or BLM (a melanoma cell line expressing HLA-A2.1 and no endogenous expression of gp100 and tyrosinase), transfected with control antigen G250, or with gp100 or an allogenic HLA-A2.1-positive, gp100-positive, and tyrosinase-positive tumor cell line (MEL624) were measured. Cytotoxic activity of DTH-infiltrated lymphocytes (DIL) was measured using the chromium release assay. Cytokine production was measured in supernatants after 16 hours of coculture by the cytokometric bead array (Th1/Th2 Cytokine CBA 1; BD Pharmingen).

Statistics

Statistical significance was evaluated using the log-rank test. Statistical analysis was performed with SPSS 11.0 for Windows (SPSS Inc, Chicago, IL).
Induration at the DTH Is Not Predictive of a Vaccine-Induced Immune Response

To investigate the immune response generated in the vaccinated patients, DTH challenges were performed with mature DC. In most patients (35 of 38 patients tested), positive DTH reactions with indurations up to 33 mm were observed. However, both unloaded DC and DC pulsed with KLH and/or peptides mounted a positive DTH (Fig 2A). No correlation was observed between the induration size and the type of injected antigen (data not shown). Therefore, in our setting, the induration at the DTH site was not predictive for vaccine-induced immune responses.

DTH: Immunohistochemistry, T-Cell Outgrowth, and Detection of KLH-Specific T Cells

Infiltrating cell clusters were observed in biopsies taken from DTH sites of peptide- and/or KLH-loaded and -unloaded DC but not in control skin biopsies (Fig 2). The majority of cells (90%) in these clusters were CD2 and CD3 T lymphocytes (15 biopsies of five patients). Approximately 50% to 70% of the cells were CD4, and 30% to 50% were CD8 T cells (Fig 2). Only a few CD16 cells were found scattered between the T-cell clusters, and no CD20, CD23 (B lymphocytes), or CD56 (natural killer) cells were discerned (data not shown).

Fig 2. Analyses of delayed-type hypersensitivity (DTH) biopsies in situ. Staining of the DTH sections of a vaccinated patient (group 4; Table 1) revealed infiltrating clusters of (A) CD8 and (B) CD4 T cells. In situ tetramer staining on cryosections of biopsies: sections were stained with CD8 in green (C and E) in combination with tetramer tyrosinase (D) or gp100:280 (F) in red. All sections (A through F) were counterstained with 4',6-diamidino-2-phenylindole, resulting in blue fluorescent nuclei.
Leukocytes from DTH biopsies were further characterized after culture with low amounts of IL-2 without the addition of antigen. Outgrowth of DIL occurred in 70% of the biopsies induced by antigen-loaded DC (32 patients) and in only 55% of the biopsies induced by unloaded DC. DIL were mainly T cells, and their amount varied between biopsies (Table 1). The rates of CD4⁺ and CD8⁺ T cells derived from DIL cultures varied among DIL cultures and between patients (65% ± 21% CD4⁺ and 27% ± 20% CD8⁺). No correlation was observed between the amount of T-cell outgrowth, the induration, and the presence of KLH. Interestingly, functional analysis of DIL from KLH-pulsed DC demonstrated a KLH-specific response (Fig 1). No anti-KLH response was detected in DIL from a DTH site injected with unpulsed DC (data not shown). Thus, KLH-reactive T cells were specifically detected in DTH sites injected with KLH-loaded DC, and positive induration was not predictive for T-cell reactivity.

**DTH: Detection of gp100- and Tyrosinase-Specific T Cells**

To determine the potential of DIL as a source for immunomonitoring of gp100- and tyrosinase-specific CD8⁺ T cells, we performed tetramer stainings, the cytokine bead array, or cytotoxicity assays. In 11 (six stage III patients and five stage IV patients) of 22 patients tested, one or more tetramer-positive T-cell population was readily detected after one DC vaccination cycle in biopsies from DTH performed with peptide-loaded DC (Fig 3, Table 2). In five additional patients, antigen-specific CTL in DIL cultures were detected after additional vaccination cycles (Table 2). No tetramer-positive T cells were detected in DTH biopsies injected with unloaded DC (n = 4) or KLH-loaded DC (n = 3; Fig 3). Because significant numbers (up to 45% of total T cells) of tetramer-positive T cells were present in the cultured DTH biopsies (Table 2), we also analyzed the presence of tetramer-positive T cells directly in blood at the same time point. Strikingly, in six of seven patients, no tetramer-positive cells were found in their PBMC, whereas in five of these six patients, T-cell staining with two different tetramers was readily detected in their DIL cultures. In only one of seven patients, a minor amount of gp100:154 tetramer-positive (0.1%) and tyrosinase:369 tetramer-positive (0.03%) T cells was detected in peripheral blood.

In 15 patients, sufficient DIL were available to determine their cytokine production on coculture with peptide-loaded target cells as well. In nine of 15 patients (27 biopsies), specific cytokine release was detected (Fig 4, Table 2). Moreover, DIL from five patients produced cytokines on coculture with target cells endogenously expressing the tyrosinase or gp100 protein (Fig 4). Especially large amounts of IFN-γ and IL-2 were produced, and in some cultures, IL-5, but not IL-4 or IL-10, was detected. Importantly, the observed cytokine production by DIL correlated with the presence and specificity of the tetramer reactivity of the DIL (Fig 3).

Finally, DIL of 23 biopsies from 10 patients were available to determine their cytotoxicity. Six DIL cultures (four patients) efficiently lysed peptide-loaded HLA-A2.1-positive target cells. Again, the observed cytotoxicity corresponded with the presence and specificity of the tetramer reactivity of the DIL (Figs 5A to 5C). Collectively, these data demonstrate that significant numbers of tetramer-positive T cells accumulated at the DTH site and that these T cells specifically produced cytokines and were cytotoxic for tumor antigen–expressing target cells.

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### Table 2. Summary of Immunomonitoring Data of Positive DIL Cultures

<table>
<thead>
<tr>
<th>Measure</th>
<th>gp100:154</th>
<th>gp100:280</th>
<th>tyrosinase:369</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetramer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean, %</td>
<td>2.6</td>
<td>7.3</td>
<td>3.3</td>
</tr>
<tr>
<td>Range, %</td>
<td>0.1-20.0</td>
<td>0.1-45.0</td>
<td>0.6-10.9</td>
</tr>
<tr>
<td>No. positive</td>
<td>28</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>IFN-γ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean, pg/mL</td>
<td>3,776</td>
<td>1,707</td>
<td>3,375</td>
</tr>
<tr>
<td>Range, pg/mL</td>
<td>480-10,000</td>
<td>427-5,000</td>
<td>433-10,000</td>
</tr>
<tr>
<td>No. positive</td>
<td>16</td>
<td>16</td>
<td>15</td>
</tr>
</tbody>
</table>

**Abbreviations:** DIL, delayed-type hypersensitivity–infiltrated lymphocytes; IFN-γ, interferon gamma.  
*Mean of the positive tetramer DIL.  
†Not all assays performed on every DIL culture  
‡Mean of IFN-γ production of the positive DIL.
Detection of gp100- and Tyrosinase-Specific T Cells in Single-Antigen DTH Reactions

To further discriminate the specific T-cell reactivity in the DTH sites, DTH reactions were induced with mature DC pulsed either with the gp100 peptides or the tyrosinase peptide with or without KLH. Both tetramer and functional analysis demonstrated that the specificity of the accumulated T cells corresponded with the peptide loaded on the injected DC (Fig 5). DIL derived from gp100 peptide–induced DTH sites (47 tested, 20 positive) were specific for gp100 and did not recognize tyrosinase and vice versa (47 tested, 10 positive). These data provide further support for the hypothesis that CD8+ T cells accumulate at DTH sites in accordance with the specificity of the DTH challenge.

In Situ Tetramer Analysis of DTH Biopsies

We performed in situ tetramer analysis on cryosections of the biopsies to exclude the possibility that the observed T-cell reactivity was induced in vitro. Tetramer-positive cells were clearly detected with the gp100 or tyrosinase tetrarmers but not with control tetrarmers (21 biopsies of nine patients). Interestingly, the tetramer-positive cells were specifically present in T-cell clusters (Figs 2C to 2F), and their specificity fully correlated with the specificity of the peptide loaded on the injected DC. No gp100 or tyrosinase tetramer–positive cells were detected in unloaded (n = 3) and KLH-loaded (n = 3) DTH biopsies. CD8 double staining further demonstrated that most of the tetramer-positive T cells expressed CD8.

Fig 4. The delayed-type hypersensitivity (DTH)–infiltrated lymphocytes (DIL) of which the tetramer staining is depicted in Figure 3 was also tested for cytokine production. Dendritic cells (DC) + three peptides (A to C and G to I); DTH: DC loaded + keyhole limpet hemocyanin (KLH) + three peptides (D to F and J to L); DIL cultures did not recognize control BLM-G250 (G, J) whereas BLM-gp100 was recognized (H, K). Both DIL cultures recognized MEL624, a melanoma cell line expressing gp100 and tyrosinase (I, L). IL, IL, interleukin; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma.
In 71% of the patients exhibiting a positive staining in situ (one half of the biopsy), the specificity correlated with the specificity in the DIL cultures derived from the other half of the biopsy. These findings demonstrate that the antigen-specific T-cell reactivity reflects the in vivo situation and is not artificially induced by in vitro cultures.

**Correlation With Clinical Outcome**

To correlate the presence or absence of vaccine-related T-cell responses and clinical outcome, patients were evaluated for clinical response according to Response Evaluation Criteria in Solid Tumors. Of 26 stage IV melanoma patients, 15 had progressive disease, 10 showed stable disease of at least 4 months in duration, and one patient with multiple liver metastases showed a complete remission. One of the patients with stable disease had salvage surgery and is now disease free at 52+ months.

No tumor-reactive DIL were observed in 13 of 15 patients with progressive disease (Fig 6). In six of 10 patients with stable disease or better, vaccine-related T-cell responses were observed in their DIL, including one patient with a complete response (Fig 6). The median progression-free survival time of these six patients was 30 months (range, 4 to 52+ months). For comparison, in the six patients with stable disease without tumor-reactive DIL, the median progression-free survival time was 6 months (range, 4 to 11 months). The median progression-free survival times of stage IV patients with and without tumor-reactive DIL were 17 and 2 months, respectively. Although the number of patients was limited, this difference was highly statistically significant ($P = .0012$; Fig 6).

We further noted that, in all stage III melanoma patients tested (10 of 13 patients), a specific T-cell response was readily detected in their DIL. However, because long-term follow-up is required for these patients, no correlation between clinical outcome and the presence or absence of tumor-reactive DIL can be made for this patient group at this time.

**DISCUSSION**

Immunomonitoring is an essential step in the development of evidence-based immunotherapy. In this study, we report that sampling of DTH sites is an effective novel approach to detect vaccine-related T cells predictive for clinical outcome. We demonstrated the following: antigen-specific T cells specifically accumulated in the DTH and their specificity corresponded with the specificity of the DTH challenge; after a brief culture step, up to 45% of the CD8+ cells were antigen specific, produced cytokines, and demonstrated cytotoxic activity towards antigen-positive target cells; and the presence of antigen-specific T cells is predictive for the clinical outcome ($P = .0012$).
Prediction of clinical efficacy based on immunologic monitoring is crucial for the rational design of cancer vaccination studies as well as for defining the correlates of protection. Several vaccination studies in cancer patients have reported T-cell responses in the peripheral blood but usually in a minority of patients or only after prolonged re-stimulation with antigen in culture. One approach to accumulate T cells in vivo is to provide a local antigenic challenge by means of a DTH reaction. Measuring the degree of induration on DTH has frequently been used to assess vaccine-related immune responses. In some studies, a correlation with clinical outcome was reported, whereas in other studies, a positive DTH reaction was not predictive for a successful response to vaccination. In our vaccination settings, the application of a DTH consisting of mature DC with or without antigen induced local indurations and erythema and was not predictive for the presence of vaccine-related T cells. The reason for the DTH response to unloaded DC is not clear but could be explained by the vast amount of chemokines produced by mature DC. However, this cannot be the sole explanation because patients with a positive DTH reaction after DC vaccination did not develop induration at the site of the first ID injection of up to 25-fold higher numbers of DC.

Antigen-specific T cells were readily detected in the DTH biopsies, whereas at the same time, these T cells were largely undetectable in blood. Antigenic stimulation of blood lymphocytes at limiting dilution conditions, as described by Coulié et al., demonstrated that antigen-specific T cells were detected in blood at low frequencies in our responding patients (range, 1.2 to 7.7 \times 10^{-5} of the CD8+ cells; three patients). Coulié et al. also reported low T-cell frequencies in blood and implied that even these low numbers of CTL in blood (5 \times 10^{-3} of the total CD8+ T cells) correlated with the rejection of a large volume tumor. In our study, the presence or absence of these T cells in the DTH biopsies nicely correlated (P = .0012) with the progression-free survival of the stage IV melanoma patients. Part of the explanation for this highly significant correlation might be that the conditions to obtain vaccine-specific DIL are more stringent. These specific cells have to migrate and proliferate in vivo, and this might select for high-quality CTL capable of eradicating tumor in vivo. The explanation of why induration at DTH sites per se is not a specific indicator for the induction of vaccine-related responses in many studies, including ours, remains unclear. We note that, after vaccination, but not before vaccination, simply culturing PBMC in the absence of antigen is sufficient to induce a low level of proliferation in most patients. This finding might indicate that vaccines with potent immune-activating properties, like DC, also induce a certain level of general immune activation sufficient for nonspecific T-cell accumulation, resulting in a positive DTH reaction. Detailed analysis of short-term cultures of DIL generated from biopsies of vaccinated patients revealed that a KLH response was only detected when KLH was used as a challenge, indicating specific accumulation of KLH-reactive T cells. Moreover, gp100/tyrosinase-specific CD8+ T cells in the DIL cultures corresponded with the DTH challenge (ie, gp100-specific T cells were only found in biopsies of DTH sites induced by gp100 peptide–loaded DC). No tetramer-positive cells were detected in T-cell cultures derived from DTH sites with unloaded DC or DC loaded with KLH without the peptides. In situ tetramer staining further demonstrated that specific T cells, corresponding with the DTH challenges, accumulated in vivo and, thus, were not induced in vitro. The DIL cultures containing tetramer-positive CTL were also functionally active because they produced cytokines (predominantly IFN-γ) and induced lysis of cells expressing the appropriate target antigen.

Specific accumulation of antigen-specific T cells requires the presence of antigen, resulting in specific retention of these T cells. Culturing biopsies from DTH sites unmasks the presence of vaccine-related T cells. The authors indicated no potential conflicts of interest.

**Authors’ Disclosures of Potential Conflicts of Interest**

The authors indicated no potential conflicts of interest.

**REFERENCES**


5786
DTH T Cells Correlate With Clinical Outcome


