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Early predictive value of multifunctional skin-infiltrating lymphocytes in anticancer immunotherapy

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Abbreviations: DC, dendritic cell; DTH, delayed-type hypersensitivity; KLH, keyhole limpet hemocyanin; SKIL, skin infiltrating lymphocyte, TAA, tumor-associated antigen.

Bioassays that predict clinical outcome are essential to optimize cellular anticancer immunotherapy. We have recently developed a robust and simple skin test to evaluate the capacity of tumor-specific T cells to migrate, recognize their targets and exert effector functions. This bioassay detects T cells with an elevated antineoplastic potential and hence rapidly identifies patients responding to immunotherapy.

Due to the strong immunostimulatory properties of dendritic cells (DCs) and their ability to elicit adaptive immune responses against malignant cells, novel anticancer therapies focus on the efficient generation and activation of this pivotal cell type. Our laboratory has conducted several clinical trials to test the therapeutic profile of DC-based vaccines over the past decade, mainly with autologous DCs generated and educated ex vivo to elicit efficient cellular immunity against neoplastic cells.[1-3] Robust T-cell responses against tumor-associated antigens (TAAs) were readily detected in a number of patients upon vaccination, providing a proof-of-principle in support of this immunotherapeutic approach. Despite enormous efforts in research and optimization, however, objective clinical responses could be detected in a minority of patients. Still, such responses were often long-lasting, indicating that enduring protection against neoplastic cells is achievable.[1] Interestingly, the fraction of patients who respond to different immunotherapeutic approaches is remarkably constant, pointing to the existence of an "immunologically reactive" subgroup of individuals.[4] The identification of such patients early in the course of treatment would greatly improve the clinical efficacy of these novel and costly therapeutic paradigms, but appropriate assays are lacking.

Anticancer immune responses are thought to be primarily mediated by $CD8^+$ T lymphocytes, which are able to trigger the apoptotic demise of neoplastic cells. Thus, current immunomonitoring approaches mainly focus on the assessment of cellular immunity using T cells isolated from the blood at various time points upon vaccination. The list of the biomarkers that are tested in this setting is long and includes the presence of TAA-specific $CD4^+$ and/or $CD8^+$ T cells, the fraction of T cells that secrete interferon γ (IFNγ) upon antigenic stimulation, and the presence of T cells or antibodies against exogenous antigens that are added as control antigens in a number of vaccination protocols, such as keyhole limpet hemocyanin (KLH).[5-7] So far, most attempts to predict objective clinical responses using these parameters failed, presumably because individual parameters were not combined in one assay. Moreover, the capacity of T cells to migrate into tissues, which is crucial for efficient anticancer immune responses, is usually not tested.

In order to address these issues, our lab conducted a pilot study in 2005 to investigate the potential value of skin-infiltrating lymphocytes (SKIL) obtained from delayed-type hypersensitivity reactions (DTHs) for predicting clinical responses in metastatic melanoma patients.[8] This approach was intended as a very comprehensive analysis of anticancer immunity, simultaneously assessing T-cell migration, effector functions and antigen recognition capability. After encouraging initial results, the SKIL test was included in subsequent vaccination protocols. Recently, we systematically analyzed the general

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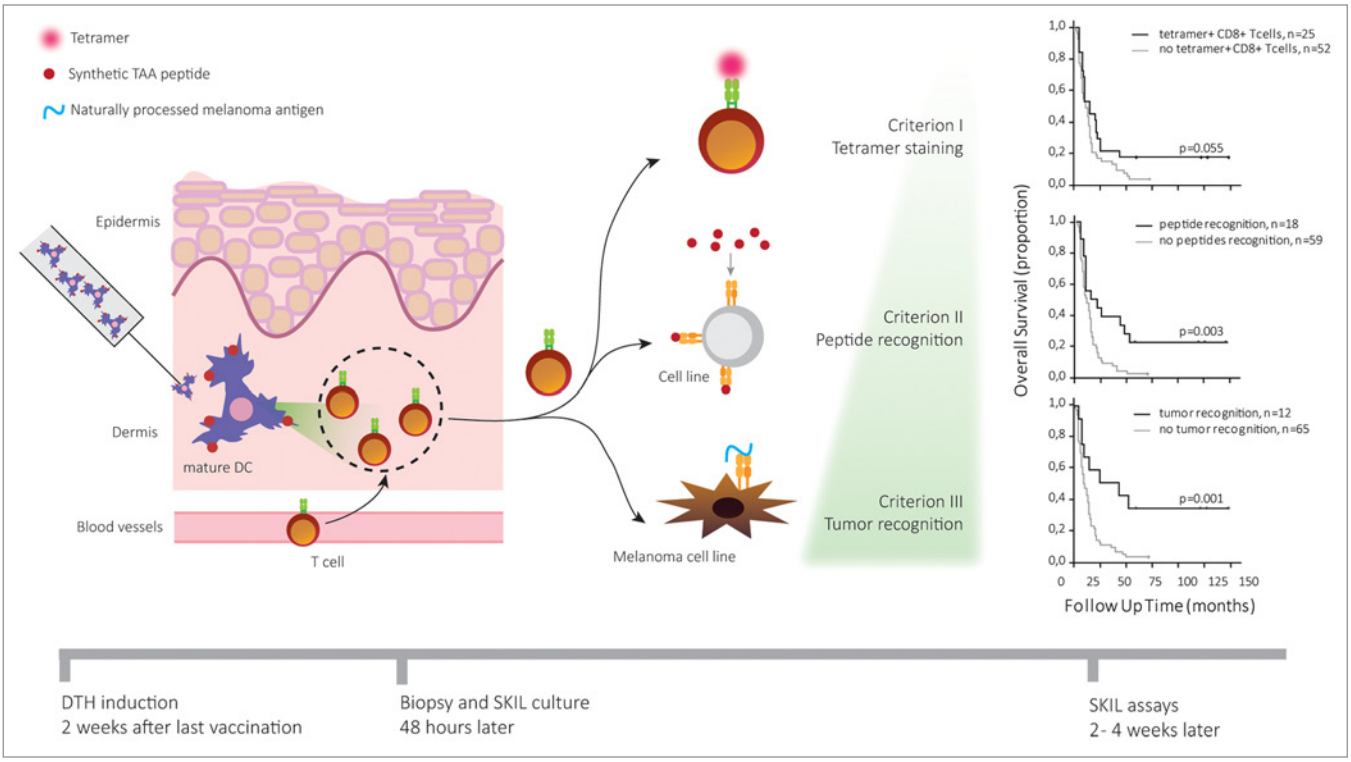

Figure 1. Analysis of skin-infiltrating lymphocytes allows for early prediction of clinical responses in cancer patients treated with immunotherapy. Dendritic cells (DCs) generated, activated, and loaded with tumor-associated antigens (TAAs) ex vivo were injected in the dermis of melanoma patients 1 to 2 wk after vaccination. The resulting delayed type hypersensitivity (DTH) reaction promotes the migration of TAA-specific T cells into the skin, which were isolated via punch biopsy 48 h after injection. Isolated skin-infiltrating lymphocytes (SKILs) were expanded and tested for cancer-specificity using tetramer staining (criterion 1). Patients whose SKIL cultures contained antigen-specific $CD8^+$ T cells showed a survival benefit over patients not fulfilling this criterion. Additionally, the specific recognition of TAA-derived peptides by SKILs (criterion II) was measured in terms of cytotoxic activity or secretion of T_H1 cytokines, such as interleukin (IL)-2, tumor necrosis factor α (TNFα) and interferon γ (IFNγ), coupled to the release of no T_H2 cytokines (i.e., IL-4, IL-5, IL-13). Finally, the ability of SKILs to specifically recognize TAAs naturally processed and presented by a melanoma cell line (criterion III) was assessed. Combining criterion 2 and 3 to criterion 1 further increased the predictive value of our assay.

feasibility of the SKIL analysis for the routine immunomonitoring of patients treated with DC-based vaccination in the context of a clinical trial.[9]

In this study, patients with metastatic melanoma were allocated to receive DCs that have been generated, activated, and pulsed with TAAs plus KLH ex vivo. Patients received 3 intradermal, intravenous or intranodal injections of the vaccine in a biweekly cycle. One to 2 wk after the last injection, mature, autologous DCs pulsed with TAAs were injected intradermally in the back of vaccinated patients to induce DTH reactions. After 48 h, punch biopsies were taken and SKILs emigrating from these tissues were cultured and analyzed for specificity, antigen recognition capability and functionality. In addition, peripheral blood mononuclear cells (PBMCs) collected on the same day than biopsies were analyzed for their ability to proliferate and secrete IFNγ in response to KLH.

We found that neither the KLH-induced proliferation of PBMC-derived $CD4^+$ T cells nor their ability to release IFNγ correlated with the overall survival of patients. This demonstrates that monitoring KLH-elicited responses can indicate the immunological competence of individual patients but does not provide an adequate means to assess antitumor immune responses.

To analyze the potential value of SKILs for predicting clinical response, lymphocytes within skin biopsies were expanded. After 2–4 wk, 80% of SKIL cultures yielded sufficient cell numbers for an extensive assessment of T-cell function and specificity. Thus, the SKIL test appears to be feasible in a large fraction of patients. We developed 3 increasingly stringent criteria to predict clinical responses: (1) the presence of TAA-specific $CD8^+$ T cells in SKIL cultures, (2) the ability of SKIL cultures to specifically recognize cells pulsed with TAA-derived peptides, and (3) the ability of SKIL cultures to specifically recognize cell lines that naturally process and present TAAs. Strikingly, we found that patients whose SKIL cultures contained TAA-specific $CD8^+$ T cells (criterion 1) displayed a survival benefit over patients whose SKIL cultures failed to do so. By adding functional properties to the assessment (criteria 2 and 3), the accuracy of prediction could be further improved (**Fig. 1**).

Remarkably, within SKIL tests we were able to detect distinct T-cell cytokine profiles upon antigenic stimulation. Whereas most of the cultures secreted T_H1 cytokines such as IFNγ, tumor necrosis factor α (TNFα) and interleukin (IL)-2, tumor-specific SKILs from two patients were found to secrete IL-5, coinciding

with rapid disease progression. This finding highlights the importance of assessing T-cell functionality for the prediction of disease outcome. Interestingly, studies on intracellular pathogens revealed the importance of multifunctional T cells, i.e., individual T cells that are able to exert cytotoxic, pro-inflammatory, and proliferative functions, for disease control.[10] Naturally, these T cells would be highly desirable for anticancer therapy as well. Future studies need to clarify which impact multifunctional T cells have on anticancer immunity and what their added value as biomarker for clinical responses is.

In conclusion, we developed a robust and simple assay for the evaluation of the migratory behavior, antigen recognition capability, and effector function of tumor-specific T cells. By identifying highly functional T cells with elevated anticancer potential, this assay allows for the early and reliable prediction of clinical responses in large-scale trials testing the clinical profile of multiple immunotherapeutic interventions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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