Activation of Human Melanoma Reactive CD8+ T Cells by Vaccination with an Immunogenic Peptide Analog Derived from Melan-A/Melanoma Antigen Recognized by T Cells-1

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ABSTRACT

Purpose: As compared with natural tumor peptide sequences, carefully selected analog peptides may be more immunogenic and thus better suited for vaccination. However, T cells in vivo activated by such altered analog peptides may not necessarily be tumor specific because sequence and structure of peptide analogs differ from corresponding natural peptides.

Experimental Design: Three melanoma patients were immunized with a Melan-A peptide analog that binds more strongly to HLA-A*0201 and is more immunogenic than the natural sequence. This peptide was injected together with a saponin-based adjuvant, followed by surgical removal of lymph node(s) draining the site of vaccination.

Results: Ex vivo analysis of vaccine site draining lymph nodes revealed antigen-specific CD8+ T cells, which had differentiated to memory cells. In vitro, these cells showed accelerated proliferation upon peptide stimulation. Nearly all (16 of 17) of Melan-A-specific CD8+ T-cell clones generated from these lymph nodes efficiently killed melanoma cells.

Conclusions: Patient immunization with the analog peptide leads to in vivo activation of T cells that were specific for the natural tumor antigen, demonstrating the usefulness of the analog peptide for melanoma immunotherapy.

INTRODUCTION

The identification and molecular characterization of tumor antigens has opened the opportunity for specific immunotherapy in cancer patients (1–6), but it remains difficult to elicit protective immunity controlling tumor growth. It is important to identify the reasons for failure of immune protection from cancer. Tumor cells may have defects in expression or presentation of antigen (7–11). The weakness of tumor-specific immune activation must also be considered. Both spontaneous- (i.e., disease induced) and immunotherapy-induced activation of tumor antigen-specific T cells is only weak or even undetectable (12–16). This is in marked contrast to protective virus-specific T cells, which are often strongly activated and readily detected in vivo (17, 18). Thus, to obtain an efficient antitumor immune response, it is important to develop reagents activating the immune system more strongly and in a continuous manner (19). The missing piece of immunotherapy may be an early, strong, and continued activation of antitumor T cells (20, 21). How can this be achieved?

One can render tumor peptides more immunogenic by increasing the binding affinity to HLA through selective amino acid replacement (22). We have recently developed an analog to the melanoma differentiation antigen Melan-A/MART-123 (hereafter Melan-A) derived peptide Melan-A26–35, which has an Alanine-to-Leucine substitution at position two of the peptide. This analog peptide is strongly immunogenic in vivo (23) and in vivo in HLA-A*0201 transgenic mice (24). CD8+ T cells generated by stimulation of PBL in vitro with this Melan-A peptide analog recognize the natural peptide and Melan-A+/HLA-A2+ tumor cells. The question remained whether patient immunization with this analog peptide selectively activates T cells that are cross-specific for the natural self-peptide presented by tumor cells. It was necessary to address this question in vivo.

REFERENCES

1. M. A. was partially supported by a European Biomed Grant BMH4CT983589. M. J. P. was supported by the Swiss Cancer League Grant KFS 633-2-1998. A. Z. was supported by the Deutsche Forschungsgemeinschaft Grant Z688/1-1. This work was sponsored by the Ludwig Institute for Cancer Research and, in part, supported by a grant from the Leenaards Foundation.
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The abbreviations used are: MART-1, melanoma antigen recognized by T cells-1; IL, interleukin; LICR, Ludwig Institute for Cancer Research; LN, lymph node; mAb, monoclonal antibody; PBL, peripheral blood lymphocyte; rhIL, recombinant human interleukin; TILN, tumor-infiltrated lymph node; VSSN, vaccine site-sentinel lymph node.

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because T cells selected in vivo can reach higher specificity/avidity to antigen than those selected in vitro (25).

Untreated patients with Melan-A-positive melanomas frequently have activated Melan-A-specific T cells in tumor-infilt rated organs (26), and this activation is stronger in tissue with high tumor load (27). Although it is much more difficult to elic it a tumor antigen-specific immune response in absence of (detectable) tumor, vaccine efficiency should be tested in this setting to avoid uncontrolled immunogenicity by endogenous factors. Therefore, we selected melanoma patients with a high likelihood to be tumor free and immunized them with the Melan-A analog peptide in a healthy limb.

As a consequence of intense research in immunology and pharmaceutical production, the number of potential vaccine components (antigens, vectors, adjuvants, and additional immune stimulatory molecules) is steadily increasing. In addition, vaccines may be administered at different body sites, doses, and treatment schedules. A major challenge is to select optimal formulations and strategies for immunization. Therefore, we searched for a reliable method to evaluate the efficiency of experimental vaccines. Our aim was to investigate antigen specific CD8+ T cells at the site of primary activation, i.e., in local lymph nodes. For this, we took advantage of an existing technique allowing to identify and surgically remove the lymph node that drains a defined patient skin area. This so-called sentinel node technique is usually applied with the purpose of detecting metastatic disease (28). Tracing colloids (99technetium nanocolloids and patent blue) are injected in the skin in the primary tumor area. These markers subsequently accumulate in one or sometimes several draining lymph node(s), allowing to identify the so-called sentinel lymph node(s) known to bear the highest risk for micrometastases (29).

Patients with resected thick primary melanomas and with resected sentinel lymph node that is invaded by micrometastases have a high risk of progressive disease and must undergo lymph node dissection. Before this was done, the patients were included in the present study, applying the modified sentinel node technique to identify the vaccine site-sentinel node. The study procedure was performed in a lower limb not affected by the disease. HLA-A2-positive patients were injected with a mixture containing Melan-A analog peptide, Influenza peptide, and QS21-based immune adjuvant AS02B (Fig. 1). A booster injection was given 2 weeks later, and after another 2 weeks, the VSSN was surgically removed upon in vivo localization with tracing colloids. The study schedule was designed such that this VSSN resection could be performed during the surgery session for dissection of the tumor draining lymph nodes (which is part of standard patient care). After surgery, the patients received four additional biweekly vaccines with the aim to further enhance immunity.

Here, we present the detailed analysis of antigen-specific CD8+ T cells from the VSSN. The aim of the study was to investigate whether the applied vaccine was able to activate CD8+ T cells in tumor-free patients, to test T cell fine specificity, and to compare local with systemic immunity.

MATERIALS AND METHODS

Clinical Study Protocol. This Phase I study was performed at the Multidisciplinary Oncology Center, University Hospital of Lausanne and the Division of Clinical Onco-Immunology, LICR, Lausanne Branch, Switzerland, after approval by the LICR Protocol Review Committee (New York, NY) and the Lausanne University Hospital Ethics Committee. It was conducted as study LUD 98-009 under the supervision of the LICR Office of Clinical Trials Management. Endpoints were treatment toxicity and immune response. Eligibility criteria were: written informed consent; older than 16 years of age; HLA-A2 positive; Melan-A expressed by the melanoma; and histologically proven skin melanoma (initially staged as American Joint Committee on Cancer stage I T1N1M0, but upgraded to stage III T1N2M0, because of a previously identified positive, i.e., micrometastatic sentinel node).

Patients. Melanoma was diagnosed by histology upon excision of the primary tumor. Subsequent sentinel lymph node biopsy revealed presence of micrometastases in all three patients. After this, patients were included in the present study, and the vaccines were injected in a lower limb (Fig. 1). Three patients were studied: patient 1 (code LAU 359) had a primary skin melanoma at the left thigh. The contra-lateral LNs were resected (lymph node dissection) inguinal left and the VSSN inguinal right. Patient 2 (code LAU 371) had a primary melanoma in the left lumbar skin region. The contra-lateral LNs were resected inguinal left and the VSSN inguinal right. Finally, patient 3 (code LAU 445) had a primary melanoma at the right ear. The contra-lateral LNs were resected at the right axilla and the VSSN inguinal left. All resected contra-lateral LNs were found to be tumor negative by histology in all three patients.

Vaccine Preparation and Treatment Schedule. Patients received Melan-A analog and Influenza peptides. The Melan-A analog peptide26-35 A27L ELAGIGILTV (with Alanine-to-Leucine substitution at position two; Ref. 23) and the Influenza matrix peptideE5A-E6A GILGFVFITL were synthesized by Multiple Peptide Systems (San Diego, CA). The lyophilized peptides were diluted in sterile PBS at a concentration of 330 μg/ml and distributed in 0.5-ml vials according to Good Manufacturing Practice quality criteria by the LICR Biological Pro-
duction Facility (Melbourne, Australia). Vials were stored at −80°C and thawed just before vaccine preparation and injection. As immune adjuvant, AS02B provided by GlaxoSmithKline Biologicals (Rixensart, Belgium) was used. AS02B includes the three components QS21 (a natural saponin), monophosphoryl lipid A (MPL), and an oil-in-water emulsion composed of an organic phase made of two metabolizable oils (a-tocopherol and squalene), an aqueous phase (PBS), and an emulsifier (TWEEN 80). Patient immunization was done as follows: 100 μg of each peptide were mixed with 600 μl adjuvant AS02B and injected i.m. at a limb not affected by the disease. Each immunization was followed by a booster injection consisting of intradermal peptide injection without adjuvant 2 weeks later. The first immunization was performed on day 0, followed by a booster injection on day 14 (Fig. 1). Lymph node dissection and removal of the VSSN were done on day 28: the skin site of vaccine injection had been marked with a permanent pen, where tracing colloids (°technetium nanocolloids and patent blue) were injected, and the VSSN was resected. Patients received four additional injections on study days 42, 56, 70 and 84. Treatment toxicity was evaluated according to the National Cancer Institute Common Toxicity Criteria scale. Tumor staging was carried out by physical examination and computed tomography scans.

Lymph Node and Blood Cells. Lymph nodes were dissociated to obtain sterile single cell suspensions in RPMI 1640 supplemented with 10% FCS, washed, and cryopreserved in RPMI 1640, 40% FCS, and 10% DMSO. Vials containing 5–10 × 10⁶ cells were stored in liquid nitrogen. TILs were prepared similarly. They were obtained from patient 1 (after disease progression) and six other HLA-A2-positive melanoma patients who had no irradiation, chemotherapy, or immunotherapy for a minimum of 12 weeks before surgical dissection. PBLs were separated from heparinized blood by centrifugation over Ficoll-Paque (Pharmacia), washed three times, and cryopreserved similarly as the lymph node cells. All experiments were done with previously frozen cells. This allowed standardization and repetition of testing, as well as proper comparison between samples obtained at different dates.

mAbs and Tetramers. The mAbs were obtained from Becton Dickinson (Mountain View, CA), except anti-CD28°RFP (Immunotech, Marseille, France) and goat-antirat APC (Caltag, Burlingame, CA). The rat IgG2a mAb 3D12 (anti-CCR7) was kindly provided by Reinhold Förster and Martin Lipp, Max Delbrueck-Center for Molecular Medicine (Berlin, Germany; Ref. 30). Tetraper complexes were synthesized as described previously (26, 31). The peptides used to construct tetramers were GILGFVFTL (Influenza matrix protein peptide58–66), ELAGIGILTV (Melan-A analog peptide36–35 A27L), and ILKEPVHG (HIV-1 polymerase (pol) peptide476–484), the latter were validated in HIV-1-positive patients.

Flow Cytometry. For ex vivo analysis, frozen cells were thawed and kept in culture medium overnight and processed after washing. Cells (5–10 × 10⁶) were stained by first incubating them with tetramers (50 µg/ml) for 60 min at room temperature and then adding the fluorescent mAbs for 30 min at 4°C, all in 50 µl of PBS containing 2% BSA and 0.2% azide. Cells were washed once in the same buffer and analyzed immediately by flow cytometry (FACSCalibur; Becton Dickinson). Data acquisition and analysis was performed using Cell Quest software. Only cells falling in the lymphocyte gate were analyzed; this gate was defined by forward/side scatter settings corresponding to a cell population expressing >98% CD45 and <1% CD14 (as determined by control CD45/CD14 stainings). The stainings of PBLs were done with previously purified CD8+ T cells prepared by two rounds of positive cell sorting using a magnetic Minimacs device (Millenyi Biotec GmbH, Bergisch Gladbach, Germany), resulting in >98% CD3+ CD8+ cells. Statistical analyses were done using the t test for two samples with equal variance.

Lymphocyte Stimulation Assay. LN cells were cultured in CTL medium (Iscove Dulbecco’s medium with 0.55 mm Arg, 0.24 mm Asn, 1.5 mm Glu, and 8% human serum) supplemented with rhIL-2 (50 units/ml) and rhIL-7 (10 ng/ml). Melan-A analog or Influenza peptide was added at a concentration of 1 μg/ml. After 9–12 days, cultures were stained with tetraper and anti-CD8 mAb and analyzed with a FACSCalibur machine.

Fluorescence-activated Cell Sorting and T-Cell Cloning. LN cells derived from VSSN were thawed and kept in CTL medium overnight. Cells were stained with tetraper and anti-CD8 mAb, and CD8+ tetramer+ T cells were isolated by fluorescence activated cell sorting using a FACSVantage machine (Becton Dickinson). Subsequently, the sorted cells were expanded as polyclonal T-cell lines and under limiting dilution conditions in the presence of irradiated allogeneic feeder cells, phytohemagglutinin (1 μg/ml) and rhIL-2 (150 units/ml). No peptides nor tumor cells were used to stimulate the cultures before or during the generation of T-cell lines and clones to avoid antigen-driven selection of particular clonotypes in vitro.

Chromium Release Assay. The cytolytic activity was tested in °Cr release assays against HLA-A2-expressing T2 cells, and the melanoma cell lines Me 290 (established in our laboratory from a surgically excised melanoma metastasis from patient LAU 203 as described previously (23)), and NAB-MEL (a gift from Dr. Francine Jotereau, U211 Inserm, Nantes, France). Target cells were radiolabeled with Na°CrO₄ for 1 h at 37°C, washed, and coincubated in V-bottomed microwells at the indicated effector to target ratio (10³ target cells/well). After 4 h at 37°C/5% CO₂, supernatants were collected and counted in a Top count (Canberra Packard) gamma counter. The percentage of specific lysis was calculated as [experimental release – spontaneous release] × 100/(total release – spontaneous release).

RESULTS

In Vivo Activation and Expansion of Peptide-specific T Cells in VSSNs. We prepared single cell suspensions from the excised lymph nodes and performed flow cytometry ex vivo with fluorescent HLA-A°*0201 tetramers. To establish background values, we applied a tetramer detecting HIV-1 polymerase-specific cells and found <0.01% CD8+ tetraper+ cells as shown in patient 2 (Fig. 2). In the VSSN of this patient, we found 0.08% Melan-A-specific and 0.96% Influenza-specific cells, demonstrating that the frequency of Melan-A-specific cells was at least ×8 and Influenza specific cells at least ×96 above background (Fig. 2). In a contra-lateral lymph node from the same patient, 0.05% of CD8+ T cells were Melan-A spe-
Tumor Reactive T Cells Activated by Analog Peptide

To test specificity and function of the induced cells in local draining lymph nodes. Flow cytometry with fluorescent tetramers and CD8 specific mAbs revealed increased percentages of specific CD8+ T cells in VSSN as compared with contralateral LNs. The numbers indicate the percentages of cells among total CD8+ cells. Table 1 shows the results for all patients.

Specific Expansion of VSSN T Cells upon in Vitro Peptide Stimulation. To investigate T-cell responsiveness, the lymph node cells were in vitro stimulated with Influenza or Melan-A peptides in presence of IL-2 and IL-7. After 9–12 days, tetramer staining and flow cytometry analysis revealed a strong expansion of Influenza- and Melan-A-specific T cells derived from the VSSNs, which was significantly stronger as compared with contralateral LN cultures (Table 2). Thus, the data show accelerated responses of VSSN-derived lymphocytes to Influenza and Melan-A peptides, indicating that the Melan-A + Influenza peptide vaccine effectively activated specific T cells in local draining lymph nodes.

Lysis of Melanoma Cells by Cytotoxic T Cells Derived from VSSN. To test specificity and function of the induced response, specific T cells from patients 2 and 3 were isolated by tetramer-guided cell sorting ex vivo from VSSN and polyclonal monospecific T-cell lines as well as T cell clones were generated as described in “Materials and Methods.” T-cell lines and 16 of 17 Melan-A specific clones lyed the A2+ Melan-A+ Me 290 melanoma cell line in the presence and absence of synthetic Melan-A peptide (Fig. 3a). The A2+ Melan-A-negative NAI-

MEL melanoma cell line was only lysed in presence of synthetic peptide. Both targets were lysed by 15 of 15 generated Influenza matrix-specific T-cell clones and lines upon addition of Influenza peptide. In general, the T-cell clones showed similar specificity as the representative lines, with the exception of clone 1E6 that failed to kill melanoma cells. Peptide titration experiments (Fig. 3b) revealed half maximal lysis (EC50) at low peptide concentrations between 10^-10 and 10^-13 molar, demonstrating that the tumor reactive T-cell receptors were of high avidity for the melan-A analog peptide (ELAGIGILTV). The natural Melan-A peptides EAAAGIGILTV (decamer) and AA-

GIGILTV (nonamer) were less efficiently recognized, similarly to what had been observed in T-cell clones generated from TILNs or PBLs from untreated melanoma patients (23). Among the total of 17 Melan-A-specific clones tested, 14 were of high avidity similarly as e.g., clone 1B7 or 1E2. The clones 1E6, 1C8, and a third clone (data not shown) were of low avidity. Thus, 3 of 17 clones were of low avidity and 1 of 17 clones (clone 1E6) did not kill melanoma cells expressing the tumor antigen Melan-A. As expected, the Influenza-specific clones had a high avidity of peptide recognition (EC50 = 10^-13 molar), confirming that virus-specific T cells usually reach higher avidity than tumor (self) antigen-specific T cells. Representative clones of both specificities also produced large amounts of IFN-γ upon antigen stimulation (data not shown). In summary, the CD8+ T cells induced in vivo by peptide vaccination and derived from VSSN recognized and killed target cells in an antigen-specific manner, and the Melan-A-specific cells showed remarkably efficient killing of tumor cells naturally processing and presenting endogenous Melan-A antigen. Importantly, the T cells efficiently recognized tumor cells, although they had been primed in vivo with the Melan-A analog peptide.

Phenotypic Characterization of VSSN T Cells ex Vivo. Influenza-specific and Melan-A-specific T cells from all lymph nodes were analyzed for expression of three different surface molecules (Fig. 4) characteristic for various T-cell differentiation stages (30–37). Antigen experienced T cells may down-regulate CD45RA (32) and undergo additional phenotypic changes in vivo (30–37). We found that Influenza-specific cells were CD45RA negative in contralateral LNs and VSSNs (Fig. 4), which is in agreement with the notion that these cells are antigen experienced in the majority of adult individuals. About half of the Melan-A-specific T cells were CD45RA positive both in contralateral LNs and VSSNs. For comparison, TILNs from seven melanoma patients with more advanced disease were used for similar characterization of Melan-A-specific T cells. Interestingly, many Melan-A-specific cells in TILNs showed down-regulation of the chemokine receptor CCR7 and some had also reduced expression of the costimulatory molecule CD28 known to be associated with differentiation toward effector cells (30, 33). No such down-regulation was observed in contralateral LNs and VSSNs. We conclude that contralateral LNs and VSSNs contained significant percentages of antigen-experienced T cells, but they remained CCR7+ and CD28+ in contrast to TILN where the tumor antigen-specific T cells showed down-regulation of CCR7 and in some TILN also of CD28.
The treatment was well tolerated with no or minor removal. The treatment was well tolerated with no or minor before and four vaccines after lymph node dissection and VSSN vaccine injections according to the study protocol (two vaccines compared with before vaccination. We also determined IFN-γ Elispot forming cells after short-term (18 h) stimulation of PBLs with peptides. The results were compatible with tetramer data because no Melan-A specific spots were obtained and only minor changes between pre- and postvaccination were found for the percentages of Influenza-specific spots (data not shown). Thus, activation of circulating T cells was borderline detectable in two patients and only for Influenza but not Melan-A-specific cells, indicating that systemic immune activation was weak to undetectable.

Clinical Evolution. The three patients received all six vaccine injections according to the study protocol (two vaccines before and four vaccines after lymph node dissection and VSSN removal). The treatment was well tolerated with no or minor local pain after injection, and no systemic toxicities were observed. Patients 2 and 3 remained tumor free (current status 24 and 15 months after study termination). Patient 1 had progressive disease after study termination, for which he underwent additional left iliac lymph node dissection, whereby 9 of 10 lymph nodes were found tumor positive (and analyzed as TILNs in this study, Fig. 4). This patient underwent surgery and isolated limb perfusion with Dacarbazine and Fotemustine for numerous in transit metastases. Overall, the clinical results of the patients in this study were comparable with untreated historical controls. However, evaluation of clinical vaccine efficacy was not an aim of this study.

DISCUSSION
To characterize the biological effects induced by our vaccine formulation, we measured lymphocyte activation both locally and systemically. We focused on Melan-A- and Influenza-specific T cells in part because they can readily be detected in the majority of healthy HLA-A2-positive humans despite that these cells are naive and have not undergone antigen driven clonal expansion (38, 39). Influenza-specific T cells are present at

### Table 1 Antigen-specific CD8+ T cells detected ex vivo

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<th>A2/Influenza+</th>
<th>A2/Melan-A+</th>
<th>A2/HIV+</th>
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<tr>
<td></td>
<td>Contralateral LN</td>
<td>VSSN</td>
<td>Contralateral LN</td>
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<tr>
<td>Patient 1</td>
<td>0.14</td>
<td>3.10</td>
<td>0.05</td>
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<tr>
<td>Patient 2</td>
<td>0.17</td>
<td>0.96</td>
<td>0.05</td>
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<tr>
<td>Patient 3</td>
<td>0.47</td>
<td>1.40</td>
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<tr>
<td>Mean</td>
<td>0.26</td>
<td>1.82</td>
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<tr>
<td>SD</td>
<td>0.18</td>
<td>1.13</td>
<td>0.02</td>
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P < 0.05 n.s.

a Flow cytometry results obtained with fluorescent tetramers and CD8 specific mAbs (similarly as in Fig. 2) from all lymph node samples from the three patients. The data are individual experimental results or mean values when multiple contralateral LNs/VSSNs per patient were recovered and tested (i.e., 3 contralateral LNs in patient 1, 2 contralateral LNs in patient 2, and 2 VSSNs in patient 3). The results are representative for two repeated experiments. The difference between contralateral LNs and VSSNs was significant for Influenza-specific cells but not for Melan-A- and HIV-1 polymerase-specific cells (the latter allowing to estimate background levels).

### Table 2 In vitro expansion of antigen-specific CD8+ T cells

<table>
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<th>A2/Melan-A+</th>
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<td>81.6</td>
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<tr>
<td>Patient 2</td>
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<td>Mean</td>
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<tr>
<td>SD</td>
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P < 0.01

a Lymph node cells were peptide stimulated in vitro and cultured for 9–12 days in presence of IL-2 and IL-7 and analyzed with tetramers and CD8-specific antibodies. Statistically significant differences between contralateral LNs and VSSNs were found for Influenza- and Melan-A-specific cells. One hundred percent is the total CD8+ lymphocytes. All cultures had <0.01% HIV-1 polymerase-specific cells as assessed by tetramers (data not shown). Additional cultures stimulated with the HIV-1 polymerase peptide also had <0.01% HIV-1 polymerase-specific cells (data not shown).
elevated levels because of prior expansion and persistence as so-called memory cells in most adults (31, 40).

One explanation why tumor-specific T-cell activation is absent or only weak may be that most tumor antigen-specific T-cell receptors are of low avidity. However, at least some T cells efficiently recognize tumor antigens such as Melan-A (22). Once activated, T cells may exert their effector functions even against target cells that bear antigens to which they are of relatively low avidity. Analogue peptides such as the Melan-A analog studied here have a stronger potential to activate specific T cells (23). However, when analog peptides are generated, great care must be taken to maintain the original specificity such that the activated T cells are clearly tumor specific. Because T-cell fine specificity is dominantly determined during in vivo priming (25), this issue needs to be tested in vivo.

After vaccination, the VSSN contained activated specific T cells that showed enhanced proliferative responses upon Melan-A and Influenza peptide stimulation in vitro. Melanoma cell recognition was of high specificity, as demonstrated by the fact that 16 of 17 Melan-A-specific clones were able to kill melanoma cells. This is remarkable because Melan-A-specific clones generated from normal individuals frequently fail to kill melanoma cells because of low efficiency in antigen recognition, as revealed by half-maximal lysis requiring natural decamer peptide concentrations of >10⁻⁸ molar (41). Thus, we provide direct evidence that the T-cell receptor repertoire recruited by the analog peptide is highly specific for the naturally processed antigen leading to efficient melanoma cell killing. This supports the notion that the Alanine-to-Leucine substitution at position two of the Melan-A analog peptide does not significantly alter the T-cell receptor binding sites (42). Rather, the mechanism of enhanced immunogenicity of this analog peptide is the enhanced binding to HLA-A*0201 (23).

Several studies in tumor patients suggest that tumor antigen-specific T-cell responses are usually only detectable in PBLs from patients with multiple and/or large metastases, even after specific vaccination. Thus, tumor tissue and synthetic antigen appear to have a synergistic effect in the activation of specific T cells. In this study, we took the challenge to immunize tumor-free patients because it was important to test vaccine efficacy in a situation with low likelihood of T cells being activated by tumor tissue. Disappointingly, tetramers and IFN-γ Elispot assays did not reveal T-cell activation in PBLs after vaccination. This is similar to our experience in 25 other melanoma patients treated with the same vaccine as used in this study.5 It is important to note that, in contrast to our ex vivo

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In this vaccination study, statistically significant differences \((P < 0.001)\) after study termination and from six melanoma patients not included in TILNs of patient 1 (resected upon disease progression that occurred after study termination) and from six melanoma patients not included in this vaccination study. Statistically significant differences \((P < 0.001)\) are marked with an asterisk. n.s., not significant.

**Fig. 4** Phenotype of antigen-specific T cells from contralateral LN, VSSN, and TILN \(ex\ viva\). Expression of CD45RA, CCR7, and CD28 by antigen-specific T cells. The histograms represent mean percentages (+ SD) in tetramer+ lymphocytes expressing the indicated cell surface molecules, whereby 100% are the total CD8+ tetramer+ lymphocytes. The data are from all VSSNs and LNs from patients 1–3 and from TILNs of patient 1 (resected upon disease progression that occurred after study termination) and from six melanoma patients not included in this vaccination study. Statistically significant differences \((P < 0.001)\) are marked with an asterisk. n.s., not significant.

Analysis strategy, most other groups are analyzing specific T cells after \(in\ vito\) stimulation for one or more weeks. In fact, none of the published studies with synthetic vaccines (13, 15, 43, 44) showed \(ex\ viva\) detectable activation of Melan-A-specific T cells in PBLs.

We have withdrawn and analyzed the lymph nodes after only two vaccinations, although additional vaccinations may have increased the likelihood of lymphocyte activation. For medical reasons, lymph node dissection needs to be performed shortly after diagnosis of lymph node metastases, leaving only a short time window for vaccination. Ethical reasons also excluded the possibility to excise the VSSN at a later time point because this would have required an extra surgery session.

Our data confirm that for the assessment of CD8+ T-cell responses to the vaccine, PBLs were less sensitive than vaccine site-draining lymph node cells (45). Thus, immune activation is more readily detectable locally than systemically. This may possibly be explained by dilution of specific T cells taking place when they enter circulation, which significantly reduces the likelihood that they can be revealed by tetramers (detection limit of \(\sim 1\) in 10,000 CD8+ lymphocytes).

The contralateral LNs were taken from the diseased limb and were tumor free according to histological analysis. We have no definitive proof that they were indeed completely tumor free because small numbers of tumor cells cannot be entirely excluded. However, it is unlikely that small numbers of tumor cells can significantly activate tumor antigen-specific T cells because this can only be detected in presence of substantial tumor infiltration (26, 27). Although one should investigate disease free LNs from multiple body sites and from patients without melanoma, ethical considerations limit the amount of healthy tissue that can be withdrawn from patients.

Besides measuring numbers of antigen-specific T cells, we determined their \(ex\ viva\) surface phenotype known to be associated with functional stages (30–37). Our data suggest that the T cells from VSSNs were less activated and/or less differentiated as compared with T cells from TILNs because only the latter showed down-regulation of CCR7 (and occasionally of CD28) by Melan-A-specific T cells. Because effector functions (cytokine production and cytotoxicity) are associated with CCR7 down-regulation (30), our results suggest that the vaccines did not induce effector cells in the VSSN. That nearly all of the cells were CCR7+ was, however, not surprising because CCR7 expression is characteristic for T cells residing in lymph nodes (46). One possibility is that vaccine-activated T cells have left the VSSNs and differentiated to effector cells elsewhere.

Immune adjuvants are usually necessary to immunize T cells \(in\ viva\), but molecular pathways need to be elucidated in more detail. An important element is activation of dendritic cells, which occurs through various Toll-like receptors triggered by microbial products such as lipoproteins, peptidoglycans, lipopolysaccharides, and bacterial DNA (47, 48). Another way to activate dendritic cells is via CD40, which is triggered by CD40L expressed on activated T cells (49, 50). The consequences on antigen-specific immune responses are intensively studied (51, 52) and will need to be tested in humans. Hopefully, the corresponding immune stimulatory substances become available for clinical use within the near future and will allow to activate human T cells more efficiently.

Taken together, our findings show that T-cell priming \(in\ viva\) with the analog peptide resulted in highly tumor-specific T cells. However, although the analog peptide is more immunogenic than the natural one, it was not sufficiently strong to induce systemic T-cell activation detectable \(ex\ viva\) after administration with immune adjuvant. Thus, this Melan-A analog peptide needs to be combined with new generation immune stimulatory molecules that promote immune activation more

**Table 3** Antigen-specific CD8+ T cells detected in peripheral blood \(ex\ viva\)

<table>
<thead>
<tr>
<th>Day</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A2/Influenza+</td>
<td>A2/Melan-A+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A2/Influenza+</td>
<td>A2/Melan-A+</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.46</td>
<td>0.04</td>
<td>0.19</td>
</tr>
<tr>
<td>27</td>
<td>0.42</td>
<td>0.05</td>
<td>0.31</td>
</tr>
<tr>
<td>70</td>
<td>0.33</td>
<td>0.03</td>
<td>0.12</td>
</tr>
<tr>
<td>98</td>
<td>0.26</td>
<td>0.04</td>
<td>0.11</td>
</tr>
<tr>
<td>0</td>
<td>0.08</td>
<td>0.06</td>
<td>0.19</td>
</tr>
<tr>
<td>27</td>
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<td>0.06</td>
<td>0.31</td>
</tr>
<tr>
<td>70</td>
<td>0.12</td>
<td>0.06</td>
<td>0.13</td>
</tr>
<tr>
<td>98</td>
<td>0.11</td>
<td>0.06</td>
<td>0.16</td>
</tr>
</tbody>
</table>

\(a\) PBLs were obtained before (day 0) and after (days 27, 70, and 98) vaccination and analyzed \(ex\ viva\) for positive staining with Influenza- and Melan-A-specific tetramers. One hundred percent is the total CD8+ T lymphocytes.
strongly, resulting in strong systemic immunity and possibly in protection from tumor progression.

In conclusion, the VSSN approach provided detailed information on local and systemic immunity. The technique will be applied to evaluate and compare new vaccine formulations. In addition, it can be used to test immune responses against multiple cancer peptide antigens in individual patients and to adapt the subsequent immunotherapy for each patient by selecting those peptides to which the patient is responsive.

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REFERENCES


Activation of Human Melanoma Reactive CD8+ T Cells by Vaccination with an Immunogenic Peptide Analog Derived from Melan-A/Melanoma Antigen Recognized by T Cells-1
