

Priority Report

Ex vivo Detectable Human CD8 T-Cell Responses to Cancer-Testis Antigens

Petra Baumgaertner,¹ Nathalie Rufer,^{3,4} Estelle Devezve,¹ Laurent Derre,¹ Donata Rimoldi,¹ Christine Geldhof,¹ Verena Voelter,² Danielle Liénard,^{1,2} Pedro Romero,^{1,4} and Daniel E. Speiser^{1,4}

¹Division of Clinical Onco-Immunology, Ludwig Institute for Cancer Research; ²Multidisciplinary Oncology Center, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland; ³Swiss Institute for Experimental Cancer Research; and ⁴National Center for Competence in Research, Molecular Oncology, Epalinges, Switzerland

Abstract

Clinical trials have shown that strong tumor antigen-specific CD8 T-cell responses are difficult to induce but can be achieved for T-cells specific for melanoma differentiation antigens, upon repetitive vaccination with stable emulsions prepared with synthetic peptides and incomplete Freund's adjuvant. Here, we show in four melanoma patients that ex vivo detectable T-cells and thus strong T-cell responses can also be induced against the more universal cancer-testis antigens NY-ESO-1 and Mage-A10. Interestingly, all patients had ex vivo detectable T-cell responses against multiple antigens after serial vaccinations with three peptides emulsified in incomplete Freund's adjuvant. Antigen-specific T-cells displayed an activated phenotype and secreted IFN γ . The robust immune responses provide a solid basis for further development of human T-cell vaccination. (Cancer Res 2006; 66(4): 1912-6)

Introduction

A major goal in the development of active immunotherapy against cancer is the strong activation of self/tumor antigen-specific CD8 T-cells. For protection against disease, it is likely that frequencies of antigen-specific T-cells need to reach at least 0.01% to 1% of circulating CD8 T-cells and thus become detectable *ex vivo*, as is the case for antiviral CD8 T-cell responses (1-3). To protect from long-term disease, the (relatively) high levels of activated T-cells must be maintained and/or boosted over an extended period of time. Therefore, therapeutic vaccination should repetitively induce *ex vivo* detectable T-cell responses. It is not sufficient to show T-cell responses only after *in vitro* T-cell expansion through cultivation for several days to weeks because this only shows that T-cells can be (re-)activated *in vitro* upon stimulation, leaving the questions on eventual ongoing *in vivo* activity largely open.

For optimization of immunotherapy, it is necessary to compare the various candidate vaccines with respect to their capacity to activate CD8 T-cells. Unfortunately, methods and techniques applied to measure antigen-specific CD8 T-cells vary enormously, making it difficult to oversee the strengths and weaknesses of numerous novel vaccination approaches (4-10). For a more rational evaluation of vaccine efficacy, we apply a standardized and reproducible procedure measuring human antigen-specific CD8 T-cells *ex vivo* (11). Through comparison of different adjuvants

for peptide vaccination of melanoma patients, we found that stable emulsions with incomplete Freund's adjuvant are powerful to induce *ex vivo* detectable and thus strong activation of antigen-specific T-cells in ~50% of patients (12).

The primary goal of our present study was to achieve activation of T-cells specific for cancer-testis (CT) antigens, such as NY-ESO-1 and Mage-A10. In contrast to T-cells specific for melanocyte differentiation antigens (Melan-A/Mart-1, gp100, and tyrosinase), no clinical study with synthetic vaccines has yet reported *ex vivo* detectable CT antigen-specific T-cell responses despite the development of many new cancer vaccines (4-10). CT antigens are expressed in a variety of tumors, including melanoma, breast, ovarian, and lung cancers and sarcomas, with increasing expression in advanced-stage disease (13). Thus, CT-based immunotherapy is potentially applicable in large numbers of patients provided that it can be optimized to achieve clinical benefit.

In this study, we aimed to elicit T-cell responses against multiple antigens by vaccination with a formulation containing three different peptides (two CT antigens + one differentiation antigen) mixed together in one single emulsion. Here, we show that this approach induced *ex vivo* detectable CT antigen-specific T-cell responses against multiple epitopes.

Patients and Methods

Patients and study protocol. HLA-A2+ patients with metastatic skin melanoma were included after written informed consent in this phase I prospective trial, approved by the local institutional review boards and the Ludwig Institute for Cancer Research Protocol Review Committee. Patients had a Karnofsky performance status of $\geq 70\%$, normal blood cell counts and kidney-liver function, and no concomitant antitumor therapy or immunosuppressive drugs. Tumor antigen expression before study inclusion was assessed on resected metastases. Expression of NY-ESO-1 and Melan-A was detected in all patients. Expression of Mage-A10 was detected in two patients (LAU 50 and 701). Vaccinations were given s.c. in monthly intervals. Vaccines were composed of $3 \times 500 \mu\text{g}$ peptide [NY-ESO-1₁₅₇₋₁₆₅ SLLMWITQA analogue (14), Mage-A10₂₅₄₋₂₆₂ GLYDGM EHL (15), and Melan-A₂₆₋₃₅ ELAGI-GILTV analogue (16)] and 1 mL Montanide ISA-51, prepared altogether in one syringe as a stable emulsion (2 mL injection volume). Peptides were provided by ClinAlfa, MerckBiosciences (Läufelfingen, Switzerland), and adjuvant (incomplete Freund's adjuvant; Montanide ISA-51) was provided by Seppic (Paris, France). Patient LAU 940 received four vaccinations; the other three patients received 12 vaccinations.

Flow cytometry. Ficoll-Paque centrifuged peripheral blood mononuclear cells (PBMC; 10^7) were cryopreserved in RPMI 1640, 40% FCS, and 10% DMSO. Phycoerythrin-labeled HLA-A*0201/peptide multimers (originally called tetramers) were prepared as described (12). Five color stains were done with HLA-A2/peptide multimers, anti-CD28^{FITC}, anti-CD45RA^{PCD}, anti-CD8^{APC-Cy7} reagents, and anti-CCR7 monoclonal antibody (mAb) followed by goat anti-rat IgG^{APC} mAb. Cells (10^6) were incubated with multimers (1 $\mu\text{g}/\text{mL}$, 60 minutes, room temperature) and then with mAbs (30 minutes, 4°C). CD8⁺ T-cells (5×10^5) per sample were acquired with a

Requests for reprints: Daniel Speiser, Division of Clinical Onco-Immunology, Ludwig Institute for Cancer Research, Hôpital Orthopédique, Niveau 5 Est, Av. Pierre-Decker 4, CH-1005 Lausanne, Switzerland. Phone: 41-21-314-01-82; Fax: 41-21-314-74-77; E-mail: daniel.speiser@hospd.ch.

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FACSVantage SE machine and data were analyzed with CellQuest software (BD Biosciences, San Jose, CA).

Functional assays. Multimer+ CD8⁺ T-cells were *ex vivo* sorted by flow cytometry and cloned as described (12). Antigen-specific lysis was assessed in 4-hour ⁵¹Cr release assays (12). IFN γ Elispot assay plates were done by incubating untreated PBMCs for 18 hours at 37°C with and without peptides, including HIV-1 polymerase peptide ILKEPVHGV as negative control. Results of both multimer+ T-cells and Elispot-forming T-cells were calculated and are indicated in percentage of CD8⁺ T-cells.

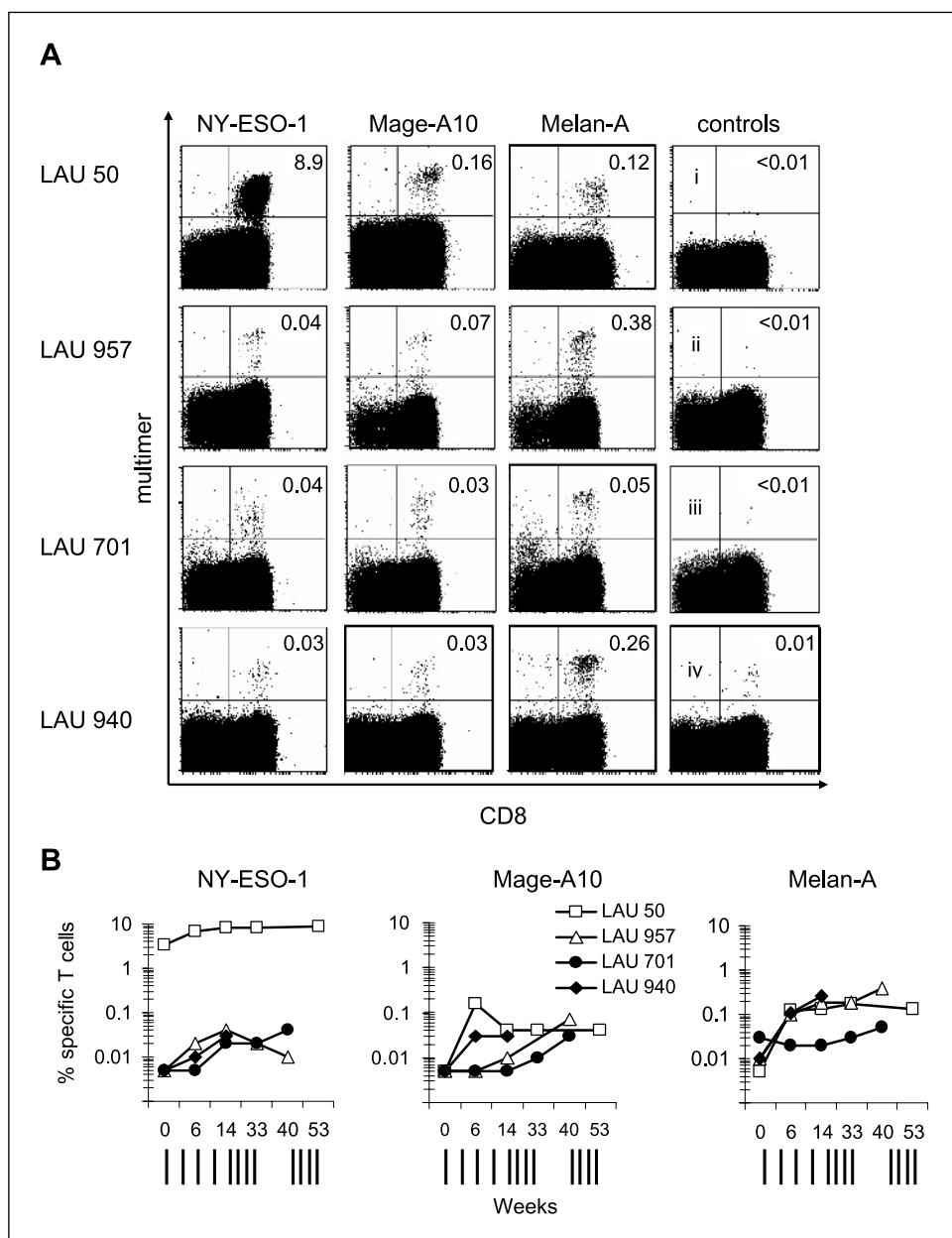
Results and Discussion

Frequent and strong *ex vivo* detectable responses of CD8 T-cells specific for NY-ESO-1, Mage-A10, and Melan-A/Mart-1.

Patients received repetitive monthly vaccinations with the three synthetic peptides emulsified in incomplete Freund's adjuvant. PBMCs collected before and after vaccination were analyzed

ex vivo by flow cytometry using CD8-specific mAb and HLA-A2/peptide multimers (Fig. 1A) in a validated immune-monitoring approach identifying real frequency changes of circulating T-cells (11). All four patients of this study exhibited increased percentages of antigen-specific CD8⁺ T-cells reaching *ex vivo* detectable levels (0.03-8.9%), and thus had much higher frequencies of CT antigen-specific T-cells compared with previous reports (4-6). Analysis over time revealed that, already after 4 monthly vaccinations, increased frequencies were found in four of four patients for NY-ESO-1-specific T-cells, in two of four patients for Mage-A10, and in three of four patients for Melan-A-specific T-cells (Fig. 1B). Therefore, T-cell responses developed more rapidly than in the majority of published cancer vaccine studies (7, 8). For Mage-A10, two of four patients remained initially negative, but nevertheless became strong responders after eight vaccinations. The present results show for the first time in humans that vaccination with peptide in

Figure 1. *Ex vivo* detectable T-cell responses specific for NY-ESO-1, Mage-A10, and Melan-A/Mart-1. After vaccination, PBMCs were collected and analyzed *ex vivo* by flow cytometry with fluorescent peptide/HLA-A*0201 multimers and CD8-specific mAb. A, numbers indicate percentages of NY-ESO-1, Mage-A10, and Melan-A-specific cells of total CD8⁺ T-cells. Data are the highest reached percentages after vaccination for each patient and antigen. Dot plots in the right column, representative examples of controls: Before vaccination, NY-ESO-1-specific (ii, patient LAU 957) and Mage-A10-specific (iii, patient LAU 701) T-cells were undetectable, similar to HIV polymerase-specific T-cells after vaccination (i, patient LAU 50). In contrast, and as frequently observed (12), Melan-A-specific T-cells can be detected *ex vivo* even before vaccination (iv, patient LAU 940). B, response kinetics of T-cells specific for NY-ESO-1, Mage-A10, and Melan-A. Multimer+ T-cells in percent of circulating CD8⁺ T-cells (similarly as in A), demonstrating increased values (11) after vaccination compared with before, for all three antigens in all four patients, with the exception of the nonresponding Melan-A-specific T-cells of patient LAU 701. Vertical lines, time points of monthly vaccinations.



incomplete Freund's adjuvant, when coadministered in a large volume of stable emulsion, can frequently elicit *ex vivo* detectable CT antigen-specific CD8⁺ T-cell responses in melanoma patients. The high values of NY-ESO-1-specific T-cells in patient LAU 50 represent an astonishing rare exception (17). This patient had strongly increased percentages already before immunotherapy, with 3.4% of NY-ESO-1-specific T-cells shortly before study inclusion (Fig. 1B). In >99% of HLA-A2+ individuals, CT antigen-specific T-cells are not detectable *ex vivo* (4, 5, 17), as also confirmed before vaccination for all other patients and for all Mage-A10-specific T-cells (Fig. 1A and B).

Differentiation to effector memory T-cells. For characterization of T-cell differentiation, we assessed the expression of CD45RA, CCR7, and CD28. These receptors are well expressed on the cell surface of naïve T-cells and are progressively down-regulated upon T-cell activation and differentiation to effector and/or memory cells (18, 19). *Ex vivo* analysis by multicolor flow cytometry and gating on multimer+ T-cells revealed that after vaccination, the majority of antigen-specific T-cells expressed only low levels of CD45RA and CCR7 (Fig. 2). Thus, the vaccine-induced T-cells displayed an effector memory phenotype (18). This result is similar to our previous observation in Melan-A-specific T-cells that were strongly activated by vaccination of melanoma patients with peptide, incomplete Freund's adjuvant, and CpG oligonucleotides (12). Interestingly, the vast majority of antigen-specific T-cells remained CD28 positive, which was the case in all patients and for all T-cell specificities, except for the very strongly activated NY-ESO-1-specific T-cells of patient LAU 50. To assess T-cell function, PBMC were analyzed with validated (11) IFN γ Elispot assays by overnight incubation with antigenic peptides. Antigen-specific IFN γ responses were found against NY-ESO-1, Mage-A10, and Melan-A (Fig. 3A), confirming differentiation to effector cytokine-secreting cells. Unfortunately, it was not possible to determine whether T-cells had been CD45RA+ and CCR7+ and thus presumably naïve before vaccination, because CT antigen-specific T-cells are rarely detectable *ex vivo* even in patients with advanced cancers. Thus, the question remains open whether T-cells had been primed by vaccination. Alternatively, T-cells may have been primed

by tumor-derived antigen and subsequently boosted by vaccination, which was the case for NY-ESO-1-specific T-cells from patient LAU 50 (17).

Cross-reactivity to natural NY-ESO-1 and Melan-A antigens.

For vaccination, we had decided to use NY-ESO-1 and Melan-A analogue peptides, because these carefully selected analogue peptides bind more stably to HLA-A*0201 and are more immunogenic compared with the wild-type peptides (14, 16). It was important to assess whether the vaccination induced T-cells were capable to recognize natural antigen. This was indeed the case because IFN γ Elispot assays showed comparable activity against analogue and natural peptides (Fig. 3B). The data for Melan-A confirm previous *in vitro* and *in vivo* studies showing that the majority of Melan-A-specific T-cells primed by the analogue peptide were capable to recognize natural peptides and melanoma cells (16). To assess killing of tumor cells, we generated 18 NY-ESO-1-specific clones from postvaccination PBMC of patient LAU 50. The clones successfully killed autologous Me 275 and allogenic Me 290 melanoma cells (A*0201+/NY-ESO-1+). As expected, NA8-MEL cells (A*0201+/NY-ESO-1-) were not recognized unless exogenous peptide was added to the cytotoxicity assay (Fig. 3C). Together, the data show that after vaccination with these analogue peptides, T-cells are often capable to recognize natural antigens and tumor cells based on physiologic antigen presentation.

Favorable clinical evolution in three of four patients.

Vaccination caused no major side effects. Minor systemic side effects were transient and included mild myalgia and fatigue (two patients). As observed earlier (12), patients developed local inflammatory signs (induration, erythema, and mild to moderate pain) at s.c. injection sites (three patients). Interestingly, one patient (LAU 957) showed local skin depigmentation and regenerated inflammatory signs at several previous injection sites after booster injections at distant sites. Three patients had progressive disease at study entry. Patient LAU 940 developed further metastases during treatment and died 6 months after study inclusion. Patient LAU 50 experienced a complete remission with regression of s.c. lesions as detected by positron emission tomography scans, lasting 12+ months. Multiple skin metastases

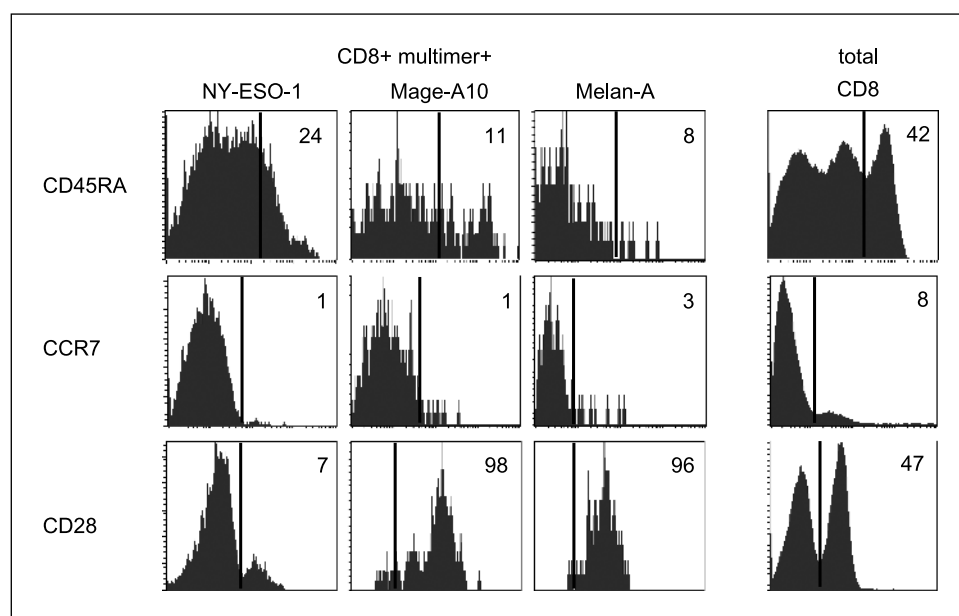
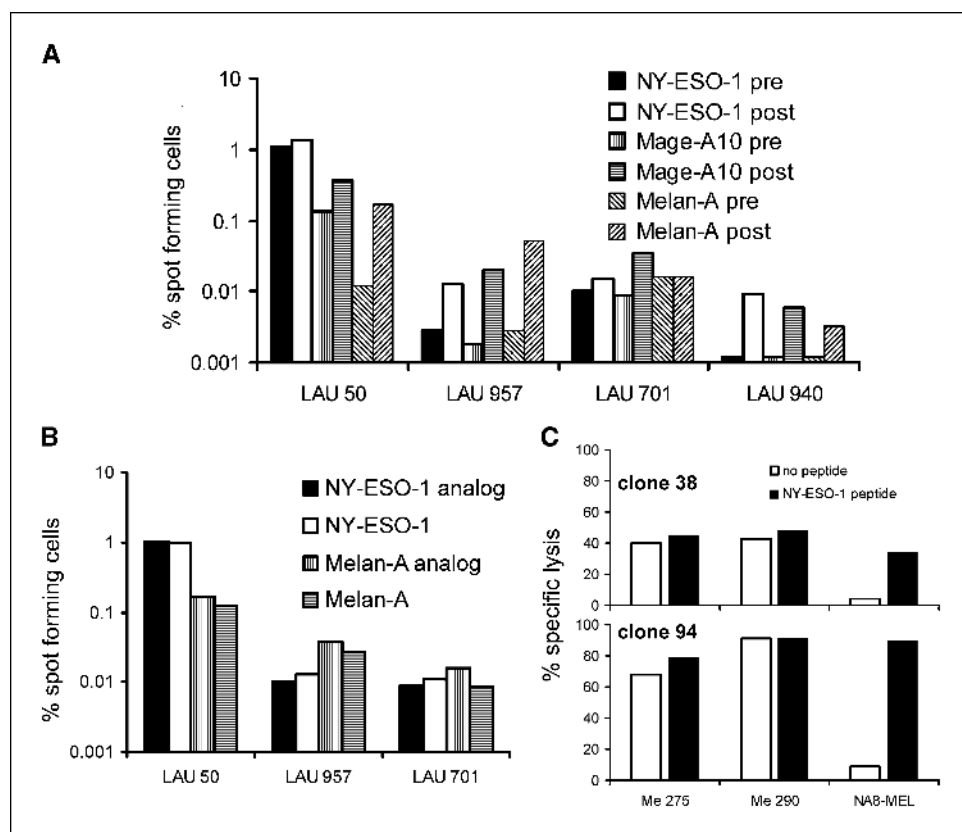


Figure 2. Dominant effector memory phenotype of tumor antigen-specific T-cells. Expression of CD45RA, CCR7, and CD28 is shown for antigen-specific (multimer+ gated) CD8⁺ T-cells and for comparison of total CD8⁺ T-cells (histograms to the right). Results from PBMC analyzed *ex vivo* from patient LAU 50 after vaccination. NY-ESO-1-, Mage-A10-, and Melan-A-specific T-cells from the three other patients were predominantly CD45RA⁻, CCR7⁻, and CD28⁺ (data not shown). Numbers, percentages of cells positive for CD45RA, CCR7, or CD28.

Figure 3. IFN γ secretion, recognition of natural and analogue peptides, and tumor cell lysis. **A**, Elispot assays were done with PBMC withdrawn before (*pre*) and after (*post*) vaccination to determine the percentage of spot-forming cells of CD8 $^{+}$ circulating T-cells specific for NY-ESO-1 analogue, Mage-A10, and Melan-A analogue peptide. **B**, IFN γ Elispot assays comparing reactivity to analogue versus natural peptides (NY-ESO-1 analogue SLLMWITQA, natural SLLMWITQC; Melan-A analogue ELAGIGILTV, natural EAAGIGILTV). **C**, NY-ESO-1-specific T-cells from patient LAU 50 were sorted by flow cytometry and cloned, and cytotoxicity was tested against melanoma cell lines Me 275 (NY-ESO-1+), Me 290 (NY-ESO-1+), and NAB-MEL (NY-ESO-1-) in the absence (*white columns*) or presence (*black columns*) of synthetic NY-ESO-1 analogue peptide (1 μ mol/L). Clone 38 was generated from blood obtained 9 days after the 12th vaccination, and clone 94 from blood was obtained 10 days after the 4th vaccination. Further 11 clones were capable to lyse tumor cells in the absence of synthetic peptides, whereas five other clones killed peptide-loaded but not unloaded tumor cells (not shown).



of patient LAU 701 partially reduced in size between the 2nd and 7th study month, with consecutive surgical resection. Finally, patient LAU 957 had no evidence of disease at study entry and remained so (12+ months). However, as phase I studies are not designed to assess clinical efficacy, it is necessary to perform trials with larger patient numbers and longer follow-up to obtain representative data on clinical responses.

In previous studies, T-cell responses to NY-ESO-1 in cancer patients did not reach frequencies of $\geq 0.01\%$ circulating CD8 T-cells (4–6, 10), despite considerable efforts using diverse vaccine formulations (recombinant viruses, DNA, peptides, proteins, adjuvants, etc). Several reasons may account for the improved vaccine efficacy found in the present study: We administered the two CT peptides together with a differentiation antigen (the Melan-A peptide), leaving the possibility that responses to one (differentiation) antigen may have helped T-cells with specificity to further antigens. Second, we have used doses of peptides (0.5 mg) and incomplete Freund's adjuvant (1 mL), which were slightly increased compared with previous studies. Third, we have optimized the vaccine formulation with the goal to maximize emulsion stability. The relative volumes of the components (incomplete Freund's adjuvant and PBS/peptides) were precisely adapted, and the amount of DMSO (0.3 mL) used to solubilize the NY-ESO-1 peptide was kept as low as possible. The emulsion was prepared with a syringe, resulting in superior stability as opposed to the frequently applied vortex method. Consequently, our emulsions remained stable for >1 month of storage at room temperature. Finally, the large volume of the vaccine (2 mL) may have contributed to the long persistence in the s.c. tissue, and the strong immunogenicity of this vaccine formulation.

It is important to elucidate the mechanisms underlying strong T-cell responses in humans, by evaluating hypotheses of immune activation implicating natural killer and dendritic cells, CD4 T-cell help, cytokines, and more. On this road, *ex vivo* detectable T-cells are a central benchmark, not only because they represent reasonably high T-cell frequencies, but also because they allow direct (i.e., *ex vivo*) molecular and functional investigation of antigen-specific T-cells. This is the strategy of choice to avoid the need for *in vitro* T-cell expansion, which leads to nonphysiologic alterations in T-cell repertoire, activation, and differentiation stage. Currently, we are characterizing large numbers of individual T-cells and dominant clonal bursts *ex vivo*, because we believe that this approach represents an important technical step toward a better understanding of protective immunity against cancer and infection (20).

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