Induction and analysis of antigen-specific T cell responses in melanoma patients and animal model

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CHAPTER 2

Phase I clinical study with multiple peptide vaccines in combination with tetanus toxoid and GM-CSF in advanced-stage HLA-A*0201-positive melanoma patients.

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Abstract

Successful induction of functional tumor specific T cells by peptide vaccination in animal models has resulted in many clinical trials to test this approach in advanced-stage melanoma patients. In this phase I clinical trial, 11 end-stage melanoma patients were vaccinated intradermally with three peptides: MART-1 $_{(26-35)}$ E27L (ELAGIGILTV), tyrosinase $_{(368-376)}$ N375Q (YMDGTMSQV) and gp100$_{(209-217)}$ T210M (IMQVPFSV), admixed with Tetanus Toxoid and GM-CSF. The peptide vaccine was well tolerated at all tested doses, and led to grade 1-2 toxicity only. Although all patients did show a rise in anti-tetanus IgG titers, in only three patients peptide-specific CD8+ T cells were induced. In two cases the response was directed against MART-1(26-35) and consisted of 0.2% and 3.3% of the CD8+ population; however, in both instances these cells did not produce IFN-γ on stimulation with the unmodified peptide. The third patient mounted a small (0.1%) respons against GP100. In a fourth patient a non-functional tyrosinase specific response (0.6%) was found that was present prior to vaccination, but was not affected in size nor in function by the vaccine. None of the 11 patients responded clinically according to RECIST criteria. Although this study is a small scale phase I clinical trial, the efficacy that was observed was disappointingly low. In accordance with previously published peptide vaccination studies, these results add to the increasing evidence that peptide vaccination in itself is not potent enough as an effective melanoma immunotherapy in advanced-stage patients.

Introduction

Malignant melanomas express a range of tumor associated antigens (TAAs) [1] that can potentially serve as targets for T cell attack. Although tumor specific T cells can be detected in many end stage melanoma patients [2], the spontaneous occurrence of effective T cell responses, leading to tumor regression, is very rare. Therefore, the successful induction of functional tumor specific T cells in animal models using peptide vaccination [3;4], has resulted in various clinical trials to test this approach in melanoma patients.

In this phase I study, we tested a peptide vaccination strategy based on a novel combination of features to increase vaccine efficacy [5-10]. 1) The vaccine consisted of three separate melanocyte differentiation antigen derived peptide vaccines, in order to widen the tumor specific T cell repertoire stimulated by the vaccine [6;10] : MART -1 $_{(26-35)}$ E27L (ELAGIGILTV)[11], Tyrosinase $_{(368-376)}$ N375Q (YMDGTMSQV) [12] and GP100 $_{(209-217)}$ T210M (IMQVPFSV)[12]. The anchor residues in these peptide sequences are modified to improve HLA-A*0201 binding [7]. 2) As it has been shown to exert immunostimulatory functions in the treatment of end stage melanoma, granulocyte-monocyte colony stimulating factor (GM-CSF) was added to the vaccine [13]. 3) Additionally, in order to facilitate T cell help [14], Tetanus Toxoid was admixed as well. Tetanus Toxoid consists of formaldehyde treated, inactivated tetanus toxin and contains pan-DR epitopes (PADREs) [15] that can be presented in the context of multiple MHC class II molecules. 4) All injections were given subcutaneously [8] in adjuvant Montanide ISA-51 [16]. Furthermore, each of the peptide vaccines was injected on a separate location, to avoid competition for HLA-A*0201 binding during antigen presentation by antigen presenting cells (APCs) [5].

Considering the lack of correlation between disease prognosis and the presence of tumor specific cells after peptide vaccination [17;18], we assessed the effect of the vaccination by analyzing both the expansion and the functionality of the induced T cell populations. For this purpose, we performed tetramer and ELIspot analyses of blood samples collected before and after vaccination. In addition, we tested peptide specific delayed type hypersensivity (DTH) responses.

Material and Methods

Stage IV melanoma patients that were HLA-A*0201 positive, above 18 years of age, having progressive disease after chemotherapy with or without non-specific immunotherapy, were eligible for the study. Additional criteria were a WHO performance score of 0 or 1, adequate bone marrow, renal and liver function (WBC>3.0/nl, platelets>100/nl, creatinine clearance > 50 ml/min, bilirubin < 1.5xULN), a life expectancy of 3 months or more and signed informed consent.

Excluded from the study were patients with brain metastases larger than 2 cm in diameter or a second malignant disease. Patients that were pregnant or lactating, taking systemic corticosteroids or requiring antibiotics for severe infections were also excluded, as were those with severe cardiac, respiratory or metabolic disease, or HIV seropositivity.

All patients received 4 vaccinations at 1 week intervals, each vaccination consisting of three separate subcutaneous injections of MART-$1_{(26-35)}$, $tyrosinase_{(368-376)}$ and $gp100_{(209-217)}$, mixed with 10 EI Tetanus Toxoid and 100 µg GM-CSF in Montanide ISA-51. To prepare the mixtures, the peptides were dissolved in a 0.9% NaCl water solution, and 100ug GM-CSF and 10IE Tetanus Toxoid were added, to a total of 1 ml. To this solution, 1.2 ml montanide ISA-51 was mixed by vigorous shaking, resulting in a homogeneous emulsion.

The injections were given subcutaneously, in the proximal part of a limb, in an area near draining lymph nodes (DLN). Patients that had undergone a lymph node dissection in the axilla region were vaccinated near the inguinal region, and vice versa. Two subsequent vaccinations were never placed in the same limb. The peptide dose was increased from 250µg in patients 1 to 4, to 500µg in patients 5 to 8, to a maximum dose of 1000µg in patients 9 to 11.

Before start of the vaccination (week 0) and 5 weeks after the start of the study, blood samples were collected for analysis. The samples were analyzed for the presence of tumor specific T cells by tetramer staining as described previously [(2)]. In short, thawed PBMC samples were incubated overnight at 37°C in Iscove' s medium supplemented with 10% FCS to recover PBMCs and to eliminate apoptotic cells. After washing, the cells were incubated for 5 min in cold PBS with 0.5% BSA and 1% normal mouse serum to block Fc receptors. Two million cells per sample were incubated for 10 min with 2 µg/ml phycoerythrin labeled MHC-tetramer at 37°C and $CD8^{pos}$ T cells were negatively selected by staining with a large set of FITC-labeled lineage marker antibodies (CD4, CD13, CD14, CD16, CD19; Becton-Dickinson). Cells were stained with propidium iodide to be able to gate out dead cells. Samples were analyzed by flow cytometry using a FACScalibur and Cell Quest software (Becton Dickinson).

Additionally, ELIspot assays were done, according to the instructions of the manufacturer (U-cytech biosciences, Utrecht, the Netherlands). Briefly, 96-well plates (Costar 3799) were incubated with coating antibody (U-cytech) overnight at 37^0 C., washed with phosphate buffered saline (PBS) and coated with coating buffer for 2 hours. Meanwhile, the patient PBL were thawed, washed and incubated 1 hour in Iscoves Modified Dulbecco's Medium (IMDM) supplemented with 5% Fetal Calf Serum (FCS) at $2x10^6$ cells/ml. Next, the coating buffer was removed from the plate, 100µl cell suspension was brought in each well, peptides were added at concentrations of 1, 0.5, 0.25 or 0.1 µg/ml and the plate was incubated overnight at 37^0C. After removal of the cell suspension the plate was washed with PBS and developed according to the instructions of the manufacturer. The plates were read using bioreader 4000 pro-X ELIspot reader (Bio-sys, Karben, Germany). Elispot and tetramer T cell counts higher than 3x the standard deviation of a panel of negative controls were considered positive.

Furthermore, also at week 0 and week 5, an ELISA was performed to determine the Tetanus Toxoid specific antibody titer, together with a DTH test for reactivity against any of the three peptides. The DTH tests were read 48 hours after

application, and an induration of more than 5 mm was scored as positive. In the first 2 of the 11 patients, TT was admixed to the peptides solutions used in the DTH tests. However, this caused considerable discomfort and was left out in the rest of the tests.

At 5 and 13 weeks after the vaccination, tumor progression was analyzed by Computer Assisted Tomography (CAT), Magnetic Resonance Imaging (MRI) or Positron Emission Tomography (PET) scans, and scored according to RECIST criteria.

The protocol was reviewed and approved by the Institutional Review Board of The Netherlands Cancer Institute / Antoni van Leeuwenhoek Ziekenhuis. All the patients signed for informed consent before participating in the clinical trial.

Results

11 patients were included in the study (table 1). All patients had unresectable stage IV disease, and had been treated with standard chemotherapy prior to enrollment. Four patients had received prior aspecific immunotherapy with GM-CSF, IFN-α and low dose IL-2 in combination with temozolomide.

At all tested doses the peptide vaccines were tolerated well, and led to grade 1-2 toxicity only. All patients developed a local redness at the site of injection, leading to some discomfort. Four patients experienced systemic side effects, in two cases leading to some difficulty in performing activities of daily living (ADL). (Table 2).

On vaccination, all patients except one showed a significant increase in tetanus toxoid specific antibody titers (Figure 1). On the contrary, peptide specific cellular T cell responses induced by the vaccines were much less consistent.

At 5 weeks after the start of the vaccination, 3 of the 11 patients (27%) showed a significant increase in T cells specific for one of the peptides in the vaccine. In 2 of these patients the response was present prior to vaccination and directed against MART-$1_{(26-35)}$ E27L (0.1% in patient I and 2.1% in patient II). In both patients, approximately a third of these cells were CD45RA negative, indicating recent antigen encounter. In patient I none of these pre-existing T cells could be detected in the ELIspot assay, but patient II showed some ELIspot reactivity prior to vaccination. The size of the tetramer positive populations in both patients increased upon vaccination with the MART-$1_{(26-35)}$ E27L peptide (0.2% and 3.3%), and in both patients the responses became clearly detectable in the ELIspot assay, indicating a change in the capacity to secrete IFN-γ (Fig. 2a/b).

The third patient that showed an increase in tumor specific T cells upon vaccination, did not have any circulating melanoma specific T cells prior to vaccination, but mounted a small gp$100_{(209-217)}$ specific response (0.1%, Fig. 2c) and a MART-$1_{(26-35)}$ E27L specific response on vaccination (0.2%) , detectable by tetramer stain and ELIspot assay.

Interestingly, in a fourth patient a sizable pre-existing tyrosinase$_{(368-376)}$ specific response was detected by tetramer staining (0.8%) that decreased on vaccination (0.7%) and could not be detected by ELIspot neither before nor after vaccination (Fig. 2d). This suggested that these cells resided in a non-functional state that could not be reversed by the vaccination, contrary to the MART-1$_{(26-35)}$ E27L specific T cells in patient I and II.

As an explanation for this difference we considered the fact that, in contrast to the modified MART-1$_{(26-35)}$ E27L epitope, the posttranslationally modified tyrosinase$_{(368-376)}$ N375Q epitope is naturally expressed by the tumor. Therefore, the tyrosinase$_{(368-376)}$ specific T cells could be more liable to tolerization by the tumor then the MART-1$_{(26-35)}$ E27L specific T cells. In order to assess the tumor cross-reactivity of the MART-1$_{(26-35)}$ E27L specific T cells, we repeated the MART-1$_{(26-35)}$ specific ELIspot and tetramer analyses, using the unmodified MART-1$_{(26-35)}$ peptide (EAAGIGILTV) and cognate tetramers, produced by "exchange" technology [19].

Similar pre-existing MART-1$_{(26-35)}$ specific T cell numbers were detected using the unmodified MART-1$_{(26-35)}$ tetramers (0.1% in patient I and 1.6% in patient II). The same applied for the MART-1$_{(26-35)}$ specific T cell counts after vaccination (0.3% in patient I and 2.1% in patient II). Hence, compared to the modified version the same pattern emerged using unmodified MART-1$_{(26-35)}$ tetramers. Strikingly however, using the unmodified peptide in the ELIspot assay no MART-1$_{(26-35)}$ specific T cell reactivity could be detected in both of the patients after vaccination (Fig. 3a/b), in analogy to the tyrosinase$_{(368-376)}$ response in patient IV.

Furthermore, none of the four patients scored positive in the DTH tests against any of the three peptides. Only the first two patients enrolled in the study, that received skin tests with Tetanus Toxoid admixed to the peptide, scored highly positive. As mentioned previously, this component was subsequently removed.

Finally, none of the detected immune responses coincided with any clinical response. Although in one patient of the lowest dosing group, the tumor evaluation at week 5 and week 13 showed stabilization of the disease, no tumor specific T cells could be detected in the blood of this patient. Though it may be that the disease stabilization was caused by the vaccination, it could well be part of the natural disease course in this patient.

The median survival of the patients after vaccination was 4 month (table 3), comparable to the survival in untreated stage IV melanoma cohorts [20].

Discussion

The results of the phase I peptide vaccination trial in end-stage melanoma patients described in this report are in accordance with previously published peptide vaccination studies. In short, the treatment may induce vaccine specific T cell responses in a minority of these patients (approx. 10-50%), but clinical responses are rare (approx. 2.5-5%)[21].

The meager clinical responses obtained in therapeutic tumor vaccination trials in general, and in this melanoma specific peptide vaccination trial in particular, are partially explained by the high tumor burden in the treated patients, which grows fast and may tolerize the tumor specific T cell repertoire [17]. This, in combination with the suboptimal affinity of these inherently autoreactive cells, makes active immunization in these patients a difficult feat.

The presence of vaccine induced T cells in the circulation and the occurrence of clinical responses hardly correlate [22]. Our data seem to suggest that non-functionality of tumor specific T cells may in part explain this paradox. In summary, after the vaccination three of eleven patients had a tumor specific response of a magnitude comparable to responses seen in viral infections. One directed against Tyr (0.7%) and the other two against (unmodified) MART-$1_{(26-35)}$ (0.3% and 2.1%). In all these cases however, the cognate ELIspot counts were negligible.

Interestingly, in the 2 patients with MART-$1_{(26-35)}$ specific T cells after vaccination, these cells did produce IFN-γ on stimulation with the altered MART-$1_{(26-35)}$ E27L peptide ligand. The high MART-$1_{(26-35)}$ specific precursor frequency [23] in humans may facilitate the emergence of these tumor non-cross reactive T cells upon vaccination with the modified peptide.

Although in this study the T cell responses after peptide vaccination neither induced DTH responses nor tumor regression, it is clearly established that T cells can induce tumor regression, when present in sufficiently high numbers and in the right activation status [24]. From this perspective, the induction of tumor specific T cells using current peptide vaccination strategies simply falls short.

Strategies to improve this success rate include co-injection of immunostimulatory adjuvants, such as TLR agonists and cytokines [25-27] or vaccination with peptide-loaded Dendritic Cells (DC) [28;29]. Another interesting approach is the use of peptides that are not constricted to a minimal epitope, but contain a larger part of the antigen sequence, spanning the entire protein as a pool [30]. In addition, recent developments in DNA vaccination hold a promise [31].

However, the gap between T cell numbers induced by any form of vaccination, and those necessary for tumor regression in end-stage melanoma patients [24] is large. Although abovementioned improvements could bridge this gap, it seems worthwhile to explore other means of cancer immunotherapy.

The transfer of autologous tumor infiltrating lymphocytes (TILs), propagated *ex vivo*, is the most effective current experimental immunotherapy in melanoma patients [23]. Moreover, the adoptive transfer of autologous PBL transduced with TCR genes, may lead to an equally potent, yet more versatile treatment. In view of the significantly greater success of adoptive therapies, it seems wise to focus on adoptive therapy for the treatment of melanoma. Possibly, the mechanisms revealed in such studies could lead to the design of more effective vaccination approaches in the distant future.

Table1, population characteristics

Demography			
Total number		11	
Caucasian		11	
Median age	(range)	45	(30-63)
Female / Male		3 / 8	
Prior treatment			
No prior treatment		0	
Prior radiation		3	
Prior chemotherapy		10	
Prior non-specific immunotherapy		4	
Site of primary tumor			
Skin		10	
Eye		1	
Site of metastasis			
Lung		4	
Skin		4	
Bone		2	
Liver		1	
Mediastinum		1	

Table 2, Toxicity according to CTC criteria

Local	
Grade 1: itching; erythema	6
Grade 2: Pain or swelling	11
Systemic	
Grade 1: Mild fatigue over baseline	5
Grade 1: Fever 38.0 - 39.0 degrees C	2
Grade 2: Moderate or causing difficulty performing ADL	2

Table 3, Median survival and blood levels of S100 and LDH before and 2 month after vaccination

	Median	***Range***
Survival	4 month	1 - >36+
S100 (µg/l)	before: 0.3 after 2.1	before: 0.1 - 0.7 after: 0.1 - 9.3
LDH (U/l)	before: 374 after 461	before:104 -1819 after: 100 - 2540

Survival, S100 and LDH levels in bloodsamples taken before and 5 weeks after the start of the vaccination.

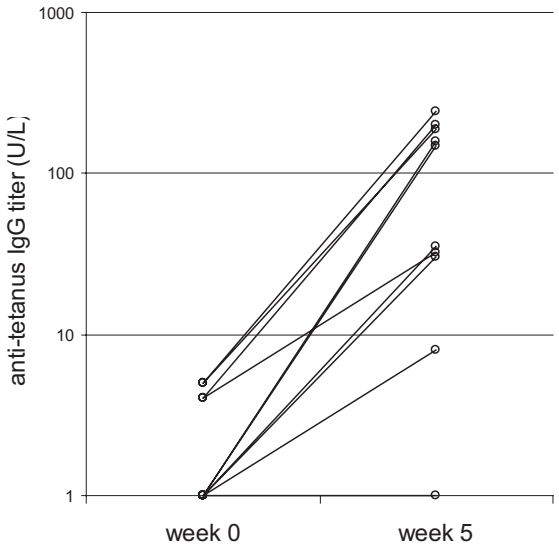


Figure 1

Tetanus toxin specific IgG titers of the enrolled patients before and after vaccination. One of the eleven patients could not be tested at week 5.

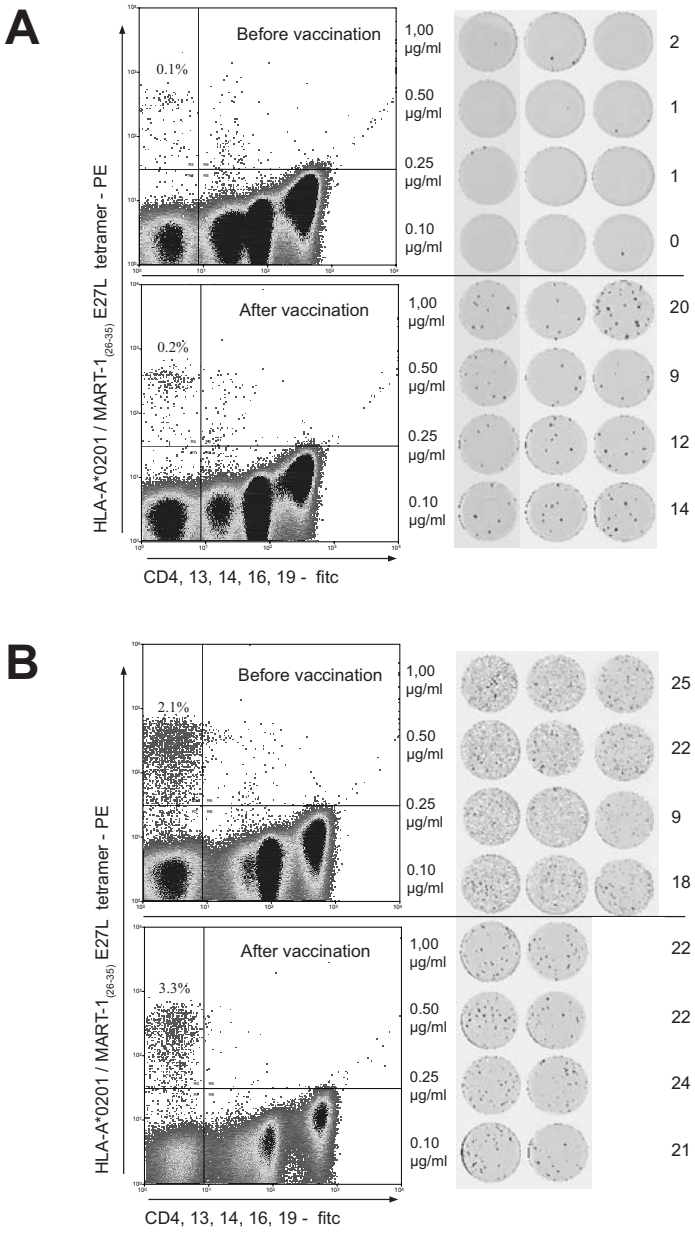
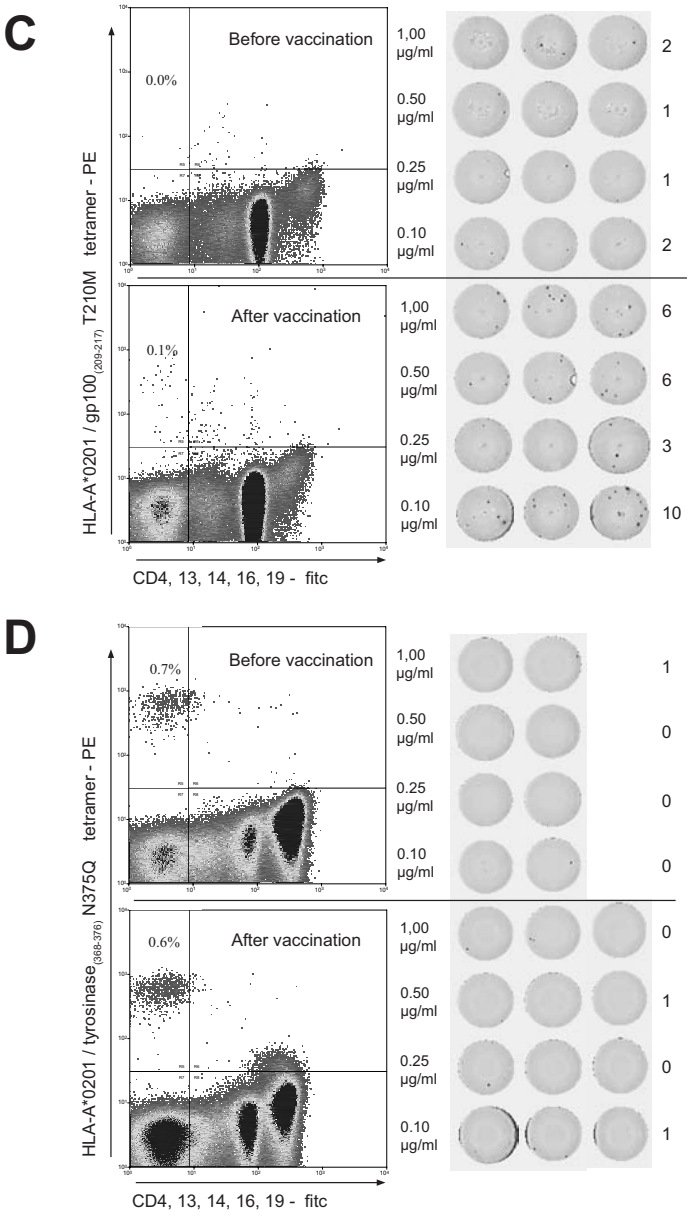


Figure 2

Analysis of vaccine specific T cell responses before and after vaccination. (a) MART-1 $_{(26-35)}$ E27L specific Tetramer staining (right) and Elispot assay (left), before (above the black line) and 5 weeks after start of vaccination (below the black line) in patient I. (b) The same display for patient II.



(c) $Gp100_{(209-217)}$ specific T cell response in patient III and (d) $tyrosinase_{(368-376)}$ specific responses in patient IV. The percentage in the upper left corner of the scatterplot represents the fraction of tetramer positive cells in the CD8 positive lymphocyte population. In the ELIspot assay 4 peptide concentrations were tested in triplicate or duplicate. The average number of spots per well for each tested pepide concentration is depicted at the right side of the figure.

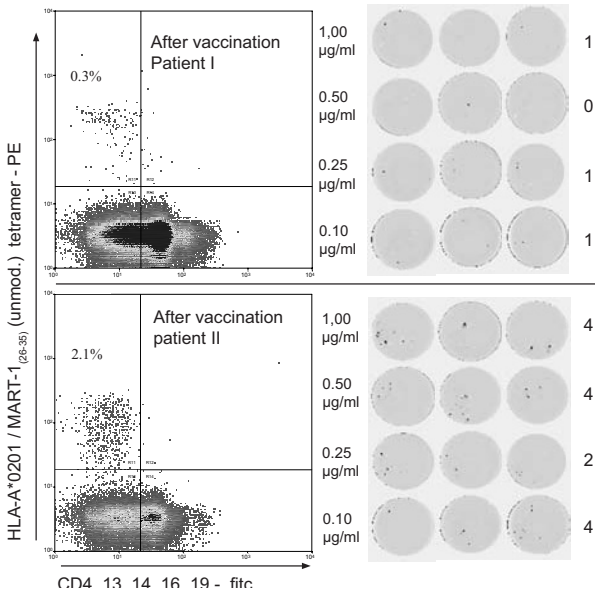


Figure 3

MART-1 $_{(26-35)}$ specific responses after vaccination in patient I and II, measured using the unmodified MART-1$_{(26-35)}$ (EAAGIGILTV) peptide for ELIspot and tetramer analysis. The percentage in the upper left corner of the scatterplot represents the fraction of tetramer positive cells in the CD8 positive lymphocyte population. In the ELIspot assay 4 peptide concentrations were tested in triplicate. The average number of spots per well for each tested pepide concentration is depicted at the right side of the figure.

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