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Disease-driven T cell activation predicts immune responses to vaccination against melanoma

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Abstract

Tumor vaccines may induce activation and expansion of specific CD8 T cells which can subsequently destroy tumor cells in cancer patients. This phenomenon can be observed in approximately 5-20% of vaccinated melanoma patients. We searched for factors associated with T cell responsiveness to peptide vaccines. Peptide antigen-specific T cells were quantified and characterized *ex vivo* before and after vaccination. T cell responses occurred primarily in patients with T cells that were already pre-activated before vaccination. Thus, peptide vaccines can efficiently boost CD8 T cells that are pre-activated by endogenous tumor antigen. Our results identify a new state of T cell responsiveness and help to explain and predict tumor vaccine efficacy.

Introduction

Cytolytic CD8 T cells have a strong potential to eliminate tumor cells. Many efforts are being undertaken to understand and mobilize this potential, resulting in numerous approaches for immunotherapy (1, 2, 3, 4). Despite the large number of vaccinated melanoma patients, clinical efficacy and immune responses following treatment with synthetic vaccines remain unpredictable. Several reasons may account for this, for example tumor heterogeneity or variability in the immune responsiveness of patients. Naive T cells have the most stringent requirements for activation. While live vaccines (e.g. vaccinia virus) can activate naive (vaccinia) specific immunity, synthetic vaccines are usually unable to achieve this but are often capable of boosting previously activated lymphocytes. The type of immune status associated with synthetic vaccine responsiveness remains to be defined. In an attempt to approach this question, we determined whether pre-vaccine T cell activation differed between patients who responded versus those who did not respond to a peptide-based vaccine.

Results

Among 43 vaccinated patients, 12 responded to Melan-A peptide as revealed by increased frequencies of HLA-A2/Melan-A tetramer positive cells after vaccination. In responders, mean expansion of Melan-A-specific T cells (after vs. before vaccination) was 9.2-fold, as compared to 1.1-fold in non-responders (Table 1, Figure 1). Nine patients responded to influenza peptide vaccination with a mean increase of 6.7-fold, as compared to a 1.2-fold increase in non-responders. After vaccination, Melan-A- and influenza-specific T cells secreting interferon-gamma upon short term (16 h) *in vitro* peptide stimulation were detectable in the majority of responder patients but not in non-responder patients (data not shown), demonstrating that T cell effector function was confined to the former.

Table 1. Peptide-specific (tetramer positive) CD8 T cells from PBMCs analyzed *ex vivo*^a.

Patients	Antigen	^b Fold T cell expansion following vaccination (range)	^c Mean % of CD28 negative T cells before vaccination ± standard deviation
Immune responders	Melan-A	9.2 (2.3-24.0)	23 ± 25
	Influenza	6.7 (2.7-13.0)	23 ± 23
Non-responders	Melan-A	1.1 (0.0-2.0)	6 ± 5
	Influenza	1.2 (0.0-2.0)	8 ± 8

^aPBMCs were stained *ex vivo* with fluorescent HLA-A2/peptide tetramers, and CD8- and CD28-specific monoclonal antibodies (as for Figure 1). 12 patients responded to Melan-A and 9 patients to influenza peptides. 38 patients were evaluable for changes in the percentage of tetramer positive T cells before vs. after vaccination, and 23/31 patients (Melan-A/influenza) for CD28 negative T cells before vaccination.

^bThe percentage of Melan-A and influenza tetramer positive T cells was determined, and the value after vaccination divided by that prior to vaccination for each patient. Based on earlier results, patients were classified as immune responders if they had a >2-fold increase in the percentage of Melan-A- or influenza peptide-specific T cells following vaccination, a downregulation of CD45RA and/or CD28 by more than 20% of peptide-specific T cells, and/or a >2-fold increase in IFN-gamma Elispots.

^cShown are the percentages of CD28 negative cells among peptide-specific T cells before vaccination. The differences between immune responders and non-responders were statistically significant ($P < 0.01$ for Melan-A-specific T cells, $P < 0.005$ for influenza-specific T cells).

Correction: The sentence "The differences between immune responders and non-responders were statistically significant ($P < 0.01$ for Melan-A-specific T cells, $P < 0.005$ for influenza-specific T cells)" refers to the comparison of the mean % of CD28 negative T cells before vaccination among Melan-A- and influenza-specific cells, respectively. Originally this sentence was at the end of footnote a instead of footnote c.

In addition, we determined CD45RA and CD28 expression by the antigen-specific (tetramer positive) T cells. CD8 T cells that have never encountered antigen ("naive" T cells) express CD45RA (5) and CD28 on the cell surface (Figure 1, healthy donor and patient LAU 392). Upon activation, T cells downregulate CD45RA (5). Further activation/differentiation is necessary for the development of cytolytic effector cells. During this process, T cells downregulate CD28 and undergo additional molecular events (6, 7, 8, 9).

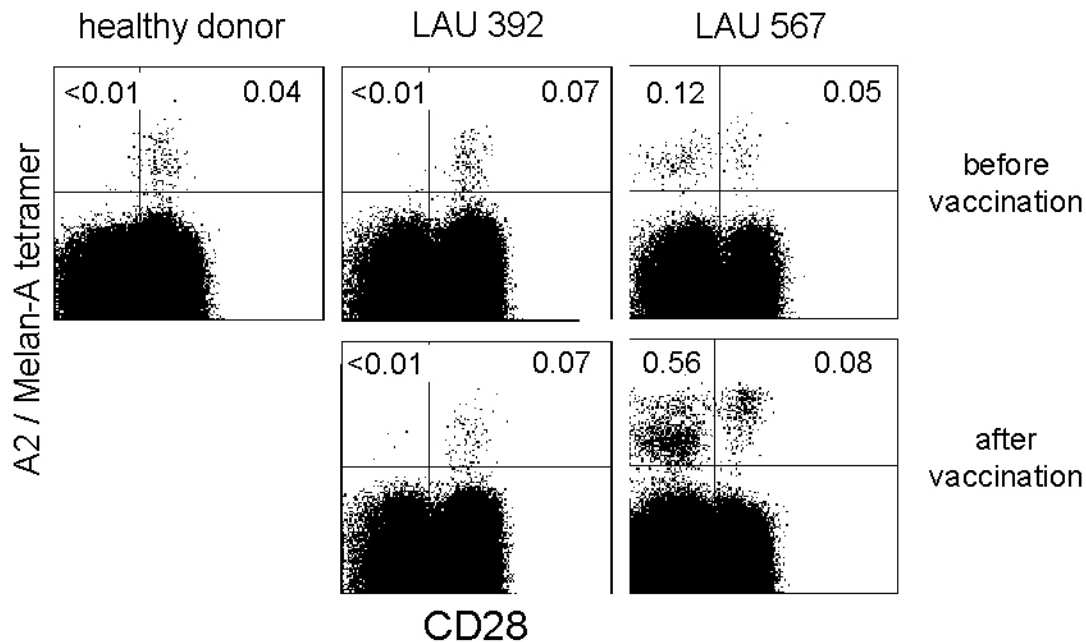


Figure 1. Ex vivo analysis of peptide-specific CD8 T cells before and after vaccination.

Peripheral blood mononuclear cells were stained *ex vivo* with fluorescent HLA-A2/Melan-A tetramers and monoclonal antibodies, and analyzed by flow cytometry. The dot plots show CD8 gated T cells (100% corresponds to the total number of CD8+ lymphocytes). The percentages of tetramer positive cells that are CD28 negative (upper left quadrants) or CD28 positive (upper right quadrants) are indicated. In healthy donors, all tetramer positive (Melan-A-specific) cells are CD28 positive. This was also the case for patient LAU 392 who did not respond to vaccination since Melan-A-specific cells were not more frequent following vaccination. In contrast, patient LAU 567 responded by activation of Melan-A-specific T cells, which were expanded 3.8-fold after vaccination ($0.12 \pm 0.05\%$ before vaccination, $0.56 \pm 0.08\%$ after vaccination).

Several patients had a relatively high percentage of pre-activated (CD28 negative) Melan-A-specific T cells already before vaccination, for example patient LAU 567 (Figure 1). We analyzed whether immune responder patients differed from non-responder patients with respect to this natural (tumor-driven) pre-activation that is never seen in healthy individuals (10). Indeed, responder patients had a significantly higher percentage of CD28 negative cells ($23 \pm 25\%$) than non-responder patients ($6 \pm 5\%$) before vaccination ($P < 0.01$; Table 1). A statistically significant correlation was also found for CD28 negative, influenza-specific T cells and responsiveness to influenza peptide vaccine ($P < 0.005$). Interestingly, there was no significant correlation between the pre-vaccination status of CD45RA negative T cells and vaccine responsiveness (data not shown).

No definitive conclusions can be drawn about a possible effect of vaccine-induced immune responses on the clinical course of the melanoma (data not shown). While patients who did not exhibit an immune response had no tumor regression, two of the Melan-A-specific immune responder patients experienced regression of metastases in skin, lymph nodes and lungs. Interestingly, these two patients belonged to a small group of five immune responder patients who had $>75\%$ CD28 negative cells among the Melan-A-specific T cells after vaccination. However, the numbers of patients and clinical responses were small, and the study was not designed for statistical analysis of clinical outcome.

Discussion

Immune responses to tumor vaccines are often delayed, i.e. they become detectable only many months after starting immunotherapy (11). This fact, and the unpredictability of immune responses, suggested that vaccine responder patients might primarily be those with spontaneous (i.e. tumor-driven) immune pre-activation. Our results are the first to confirm this hypothesis and to identify a feature of such immune activation, i.e. the presence of CD28 negative, antigen-specific T cells in PBMCs before vaccination.

Previous studies of tumor infiltrating lymphocytes and tumor infiltrated lymph nodes revealed that melanoma cell - lymphocyte vicinity is associated with a marked natural activation of tumor antigen-specific T cells, many of which are CD45RA and CD28 negative (10, 12, 13). Furthermore, we have recently found that local tumor-driven T cell activation is frequently associated with the presence of activated T cells in the peripheral blood, i.e. with systemic immune activation.

Our data indicate that significant activation and expansion of antigen-specific T cells may be the result of combined or sequential stimulation by antigen provided by the tumor and the vaccine. Presumably these two "steps" comprise different, and in part complementary, events required for a T cell response. Indeed, T cell activation occurs through a cascade of cellular and molecular interactions involving antigen, antigen-presenting cells (dendritic cells, DCs) and lymphocytes. An efficient vaccine should contain at least two components: antigens and molecule(s) activating DCs. The latter are critical, since only activated DCs (14) can stimulate differentiation to protective effector CD8 T cells (15) (i.e. those that proliferate efficiently, secrete cytokines and kill infected or malignant cells). CD4 helper T cells may also contribute to a continued CD8 T cell activation (16, 17, 18) by activating DCs, producing cytokines, and possibly also by direct triggering of CD8 T cells (19). We are currently investigating lymph node biopsies in an attempt to determine whether immune responder patients had tumor-driven activation of DCs and CD4 T cells.

An increase in antigen-specific, CD28 negative T cells after immunization, as observed in patient LAU 567, was seen in 6 patients. Overall, there was a tendency towards an increase in the percentage of CD28 negative T cells among Melan-A-specific cells after vaccination as compared to before, but the difference was not statistically significant (data not shown). This suggests that peptide vaccination only partially induced differentiation to CD8 effector T cells, despite the fact that this treatment led to considerable proliferation of antigen-specific T cells. Further studies are in progress to obtain more data on post-vaccine T cell phenotype and function, which are issues critical to the development of vaccines.

No correlation was found between the presence of CD45RA negative T cells and vaccine responsiveness. The majority of patients had CD45RA negative, peptide-specific T cells before treatment, yet only a minority responded to the vaccine. This is surprising, since CD45RA negative cells are so-called memory cells known to require less stimulation for activation than naive cells. Apparently, responsiveness to peptide vaccines requires a more advanced immune activation state, e.g. the one associated with the presence of CD28 negative T cells. However, it remains to be elucidated whether vaccine responses involved the identified CD28 negative T cells directly, or whether the latter were simply reflecting an immune status associated with vaccine responsiveness involving T cells with distinct properties. Indeed, it may well be that T cells responded and expanded in lymph nodes, a process that is believed to involve CD28 positive T cells. These questions can be addressed through T cell receptor sequence determination, followed by the design of clonotypic primers, enabling individual T cell populations to be monitored both before and after vaccination, as well as at different body sites. Such studies are ongoing in our laboratory with selected patients (20).

Influenza peptide was included in this study based on the hypothesis that influenza-specific T cells may respond more readily than Melan-A-specific T cells. Surprisingly, this was not the case. It became clear that patient

responsiveness varied depending on the pre-vaccine influenza immune status. One may speculate that those patients with a higher percentage of CD28 negative, influenza-specific T cells before vaccination had encountered influenza virus relatively recently or in a more pronounced fashion. In any case, similar to Melan-A, the influenza-specific immune status varied from patient to patient, correlating with responsiveness to peptide vaccination.

Six patients responded only to Melan-A, 3 patients only to influenza, and 6 patients to both antigens. Among the latter, there was an interval of more than 1 month between the responses to Melan-A and to influenza in 4 patients, and only 2 patients exhibited responses to both antigens within the same month. Also, the percentage of pre-activated (CD28 negative) cells in Melan-A-specific T cells differed largely from that in influenza-specific T cells per patient (data not shown). Immune responses were therefore antigen-specific, and not patient-specific. Thus the patients did not suffer from general immunosuppression.

To be eligible for vaccination with the HLA-A2 binding peptide Melan-A₂₆₋₃₅, patients must express HLA-A2 and their tumor must be Melan-A positive. Based on our findings, one may consider adding the existence of activated antigen-specific CD8 T cells as a further criterion for the selection of patients for vaccination. This may be envisaged with the current types of synthetic peptide vaccines, which apparently have mainly the potential to induce expansion of previously activated, antigen-specific T cells, and may also be used as recall vaccines in prime - boost strategies. It is a great challenge to develop new generations of synthetic vaccines that activate (naive) CD8 T cells independently from tumor-driven stimulation, and that induce effector T cells that can efficiently destroy tumor tissue in cancer patients. In this respect, it is evident that precise *ex vivo* monitoring of antigen-specific T cell responses contributes to the rational development of vaccines by providing key information on the quantity and quality of the induced T cells.

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Materials and methods

Patients

Forty-three HLA-A2 positive stage III/IV melanoma patients with Melan-A/MART-1 positive tumors were treated at least four times with vaccines containing 100 µg Melan-A/MART-1₂₆₋₃₅ peptide and 100 µg influenza matrix protein₅₈₋₆₆ peptide. Peptides were given at monthly intervals either in saline, in 600 µl adjuvant AS02 (GlaxoSmithKline, Belgium) or in 600 µl adjuvant Montanide ISA-51 (Seppic, France) as described (12). The immunization protocols were approved by medical and ethical committees, and the patients were treated upon written informed consent. A detailed report on immune responses and clinical outcome will be published in a separate paper (21).

Immune monitoring

To monitor antigen-specific T cells, we developed and applied a standardized flow cytometry technique which combined the use of fluorescent HLA-A2/peptide tetramers (22) and antibodies specific for CD8, CD28 and CD45RA. Briefly, heparinized blood was collected before and after vaccination, and peripheral blood mononuclear cells (PBMCs) were isolated and frozen. Staining with anti-CD28^{FITC}, tetramers, anti-CD45RA^{ECD}, anti-CD8^{APC} and 4',6-diamidino-2-phenylindole (DAPI) was performed as follows. PBMCs were thawed and CD8+ T cells were purified by positive sorting using a MiniMACS device (Miltenyi Biotech Inc). Cells (10⁶) were incubated with tetramers (50 µg/ml) for 60 min at room temperature (in 50 µl PBS, 2% bovine serum albumin and 0.2% azide) and subsequently with fluorescent antibodies for 30 min at 4°C. Cells were washed once in the same buffer. DAPI (Molecular Probes, Eugene, OR) was then added at 1 µg/ml for 30 min. Cells were analyzed in a FACSVantage™ (five color) machine (Becton Dickinson, Mountain View, CA). Data analysis was performed using CellQuest software. Electronic gates were set for lymphocytes according to forward and side scatter characteristics, and for viable (DAPI negative) cells. In this way we exploited at least two major advantages of this technique: First, Melan-A- and influenza-specific T cells were identified and characterized not only after vaccination but also before, and second, T cells were investigated *ex vivo* (10) which is crucial to determine ongoing changes in immunity *in vivo*.

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