Unmodified self antigen triggers human CD8 T cells with stronger tumor reactivity than altered antigen

Daniel E. Speiser[†‡], Petra Baumgaertner[†], Verena Voelter[§], Estelle Devevre[†], Catherine Barbey[†], Nathalie Rufer[¶], and Pedro Romero[†]

[†]Division of Clinical Onco-Immunology, Ludwig Institute for Cancer Research and University of Lausanne, and [§]Multidisciplinary Oncology Center, Centre Hospitalier Universitaire Vaudois, 1001 Lausanne, Switzerland; and [¶]Swiss Institute for Experimental Cancer Research, 1066 Epalinges, Switzerland

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Human cancer vaccines are often prepared with altered ''analog'' or ''heteroclitic'' antigens that have been optimized for HLA class I binding, resulting in enhanced immunogenicity. Here, we take advantage of CpG oligodeoxynucleotides as powerful vaccine adjuvants and demonstrate the induction of high T cell frequencies in melanoma patients, despite the use of natural (unmodified) tumor antigenic peptide. Compared with vaccination with analog peptide, natural peptide induced T cell frequencies that were approximately twofold lower. However, T cells showed superior tumor reactivity because of (*i*) increased functional avidity for natural antigen and (*ii*) enhancement of T cell activation and effector function. Thus, novel vaccine formulations comprising potent immune stimulators may allow to circumvent the need for modified antigens and can induce highly functional T cells with precise antigen specificity.

analog peptides | CD8 T cell specificity | CpG oligodeoxynucleotides | immunotherapy | Toll-like receptor 9

Therapeutic vaccines against cancer and infectious disease aim to induce effective immune responses similar to protective anti-viral responses. The latter are characterized by profound activation of antigen specific T cells, resulting in numerous rounds of cell divisions and differentiation into memory and effector T cells, assuring long term persistence and strong effector function (1, 2). Unfortunately, T cell responses induced by current T cell vaccines are less efficient. Strategies for improvement focus on the three essential vaccine components, i.e., antigens, adjuvants, and delivery systems. Protective immune responses may be achieved when all vaccine components are carefully chosen and optimally composed.

Binding of natural cancer peptide antigens to HLA is usually unstable, which may result in low immunogenicity possibly hampering T cell priming and activation. Therefore, research has focused on the development of optimized "analog" or "heteroclitic" peptides with higher affinity binding to HLA. Obviously, structural modifications of peptides must be done without altering T cell receptor (TCR) binding moieties to ensure that vaccination-primed T cells are specific for natural antigen and efficiently recognize tumor cells. Development and application of altered (analog) peptides has been relatively successful. Analog peptides derived from various tumor antigens (e.g., gp100, Melan-A^{MART-1}, CEA, and NY-ESO-1) are now widely applied in clinical immunotherapy studies (3). Preclinical studies have confirmed that carefully designed analog peptides are indeed capable of inducing T cells with capacity of tumor cell recognition and killing (4–9). However, even when peptide modifications are selected very cautiously, by maximally avoiding changes in antigenic structure, peptide analogs may also trigger T cells bearing TCRs, which are unable to recognize tumor cells (10–14). Therefore, altered peptides continue to require careful reevaluation with regard to the risk of activating T cells with imprecise antigen specificity (15).

Analog peptides may induce human CD8 T cell responses more potently compared with unmodified tumor/self antigens. Indeed, vaccination of melanoma patients with Melan-A^{MART-1} analog peptide E*L*AGIGILTV more readily induces T cell responses than vaccination with natural (unmodified wild type) peptide EAAGIGILTV (16). When CPG oligodeoxynucleotide 7909 (PF-3512676; hereafter called CpG) is used as adjuvant, T cell frequencies reach even 10-fold higher levels, resulting in 10- to 1,000-fold higher T cell frequencies than with other clinically available low dose vaccines (9). Thus, the clinical introduction of CpGs as adjuvant, which trigger B cells, NK cells, and plasmacytoid dendritic cells through Toll-like receptor 9 (TLR-9), represents a milestone in the development of human T cell vaccination.

Here, we applied this vaccine formulation to directly compare vaccination with natural vs. analog peptide antigens. We show that vaccination with natural tumor/self antigen resulted in vigorous T cell responses easily detectable directly *ex vivo*, in peripheral blood of 6/6 melanoma patients. Detailed studies revealed that responding T cells expressed TCRs with high functional avidity for the natural antigen, conferring efficient tumor recognition. Tumor cell killing was further enhanced because of unexpected high levels of T cell activation, which was significantly superior compared with T cells from patients vaccinated with analog peptide. Thus, natural tumor antigens combined with strong adjuvants may elicit large numbers of T cells that are strongly activated and highly specific for tumor antigens.

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Results

Strong CD8 T Cell Responses to Vaccination with Natural Tumor/Self Antigen. Six HLA-A2^{pos} patients with advanced metastatic melanoma received four monthly s.c. vaccinations consisting of natural Melan-A peptide EAAGIGILTV and CPG 7909 (PF-3512676), emulsified in Montanide ISA-51 [incomplete Freund's adjuvant (IFA)]. Peripheral blood mononuclear cells (PBMCs) collected before and after vaccination were analyzed directly *ex vivo* by flow cytometry. After four vaccinations, all six patients exhibited frequencies of Melan-A specific CD8 T cells (0.11 to 1.47%) that were significantly ($P < 0.01$) higher than before vaccination (Fig. 1 *A* and *B*). Responses developed rapidly, because increased frequencies of Melan-A specific CD8 T cells were already observed after two vaccinations, which is exceptionally fast, because strong T cell responses to cancer vaccines most often take longer to develop, even when optimized analog peptides are used (16–19). These results demonstrate that vaccination with a natural (wild type) tumor/self peptide, when coadministered with CpG, can rapidly elicit high T cell frequencies in all patients of a small cohort. Earlier clinical studies with natural tumor peptides failed to trigger *ex vivo* detectable T cell

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[‡]To whom correspondence should be addressed. E-mail: daniel.speiser@hospvd.ch.

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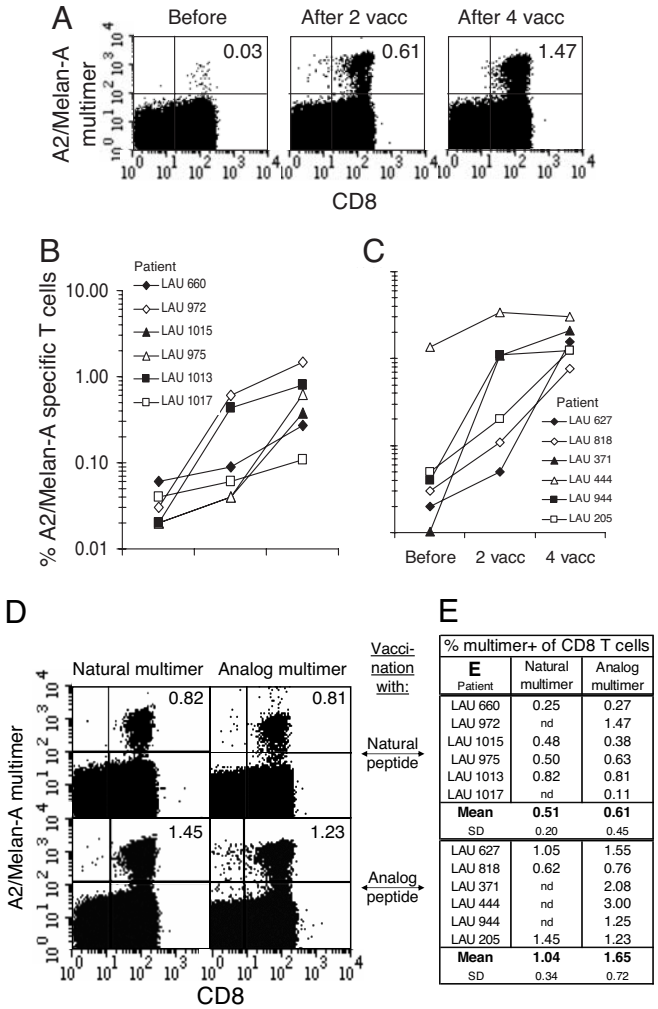
responses (20–23), which is the reason why until today, the majority of studies applied analog peptides, also allowing detailed *ex vivo* analysis of tumor antigen specific T cells. The strong responses in this study now opened the opportunity for detailed *ex vivo* analysis of T cells after vaccination with natural tumor antigen.

Direct Comparison to Vaccination with Analog Antigen. As reported in ref. 9, the first patient cohort in our clinical trial had been vaccinated with Melan-A analog peptide (E*L*AGIGILTV) instead of natural peptide (EAAGIGILTV). Otherwise, patients were selected and treated with identical procedures as patients receiving natural peptide, because the trial was designed for direct comparison. Similar to natural peptide, analog peptide vaccination induced rapid and strong T cell responses in all patients (Fig. 1*C*), whereby maximally reached T cell frequencies were approximately twice as high as after vaccination with natural peptide ($P < 0.05$). These data were obtained with multimers bearing the analog peptide. Subsequently, we directly compared results obtained with multimers constructed with natural vs. analog peptide (Fig. 1 *D* and *E*). Labeling with both multimers gave similar frequencies, showing high degree of cross-reactivity and confirming that the two multimers have comparable capacity to label Melan-A specific T cell clones, even when the clones express TCRs with preferential recognition for one or the other peptide (data not shown). Together, the data show that (*i*) vaccination with analog peptide induces T cell frequencies approximately twice as high as vaccination with natural peptide and (*ii*) T cell frequencies were comparable when analyzed with multimers constructed with natural and analog peptide.

Fine Antigen Specificity Differences. The precise characterization of TCR fine specificity and efficiency of antigen recognition cannot be determined directly *ex vivo*, because T cell populations are polyclonal and contain multiple fine specificities (24). Therefore, we generated T cell clones from three patients vaccinated with natural peptide and three patients vaccinated with analog peptide. The functional avidity of antigen recognition of these clones, as determined by cytotoxicity assays against HLA-A2^{pos} T2 cells in presence of decreasing peptide concentrations, showed that vaccination with natural peptide induced T cells recognizing natural peptide better or at least equally well as analog peptide (Fig. 2*A*). In contrast, the opposite was the case for clones after vaccination with analog peptide. It should be noted that the analog peptide binds ≈10 times more stably to HLA-A2 than the natural peptide (ref. 6 and unpublished data). This difference is important for T cell activation, but also for assessment of T cell function as shown here, because the stronger HLA binding of the analog peptide results in overestimation of TCR recognition efficacy. For example, clones with seemingly similar recognition efficiency of the two peptides, such as the clone shown for patient LAU 1013, bear TCRs with higher avidity to natural as opposed to analog peptide (25).

The summary of data from all cloned CTL (Fig. 2*B*) shows that the majority of clones obtained after vaccination with analog peptide displayed preferential reactivity to analog peptide, because it was recognized at lower concentrations compared with natural peptide. In sharp contrast, recognition of natural peptide was similar (patient LAU 1013) or superior (patients LAU 972 and LAU 975) after vaccination with natural peptide. These results correlated well with lysis of HLA-A2^{pos}/Melan-A^{pos} melanoma cells in absence of synthetic antigen: We found that such melanoma cells were strongly killed by 98% of clones from patients vaccinated with natural peptide, but only 62% of clones after analog peptide vaccination [supporting information (SI) Fig. 5]. Sequencing of TCRs expressed by the clones, and by T cells isolated directly *ex vivo*, indicate that the clones analyzed here are representative for *in vivo* expressed TCRs (ref. 25 and unpublished data). In summary, vaccination with the two studied Melan-A peptides resulted in significant fine specificity differences of *in vivo* circulating T cells.



% multimer+ of CD8 T cells		
E Patient	Natural multimer	Analog multimer
LAU 660	0.25	0.27
LAU 972	nd	1.47
LAU 1015	0.48	0.38
LAU 975	0.50	0.63
LAU 1013	0.82	0.81
LAU 1017	nd	0.11
Mean	**0.51**	**0.61**
SD	0.20	0.45
LAU 627	1.05	1.55
LAU 818	0.62	0.76
LAU 371	nd	2.08
LAU 444	nd	3.00
LAU 944	nd	1.25
LAU 205	1.45	1.23
Mean	**1.04**	**1.65**
SD	0.34	0.72

Fig. 1. Melan-A specific T cell responses detected *ex vivo* after vaccination with peptide, CpG, and IFA. PBMCs were analyzed *ex vivo* before vaccination and after two and four vaccinations (vacc) by flow cytometry, using CD8 specific antibodies and HLA-A2/Melan-A^{MART-1} multimers (formerly called tetramers). (*A*) Representative data from patient LAU 972 showing low percentages of multimer^{pos} CD8 T cells before vaccination (*Left*) followed by strongly increased T cell frequencies after two (*Center*) and four (*Right*) vaccinations. (*B*) Corresponding data from six patients vaccinated with Melan-A natural peptide EAAGIGILTV. As an exception, patient LAU 660 received only three instead of four vaccinations. (*C*) Comparable data from six patients vaccinated with Melan-A analog peptide E*L*AGIGILTV. (*D* and *E*) Analysis with multimers constructed with natural vs. analog peptide for representative patients vaccinated with natural peptide (LAU 1013) (*D Top*) and analog peptide (LAU 205) (*D Lower*) and for all 12 patients (*E*). Data from *A*–*C* were generated with analog peptide multimers. All values are percentages of multimer^{pos} cells of CD8^{pos} T cells (equal to 100%). nd; not done.

***In Vivo* Expression of Perforin and Granzyme B.** Effector T cells were analyzed *ex vivo*. Multiparameter flow cytometry allowed to gate for CD8^{pos} and A2/Melan-A multimer^{pos} cells, combined with exclusion of naïve phenotype cells. Because the vast majority of T cells responding to vaccination were CD45RA^{neg}/CCR7^{neg} [so-called effector memory (EM) cells (9)], we gated on this population and found that perforin and granzyme B were highly expressed in Melan-A specific cells responding to natural peptide vaccination

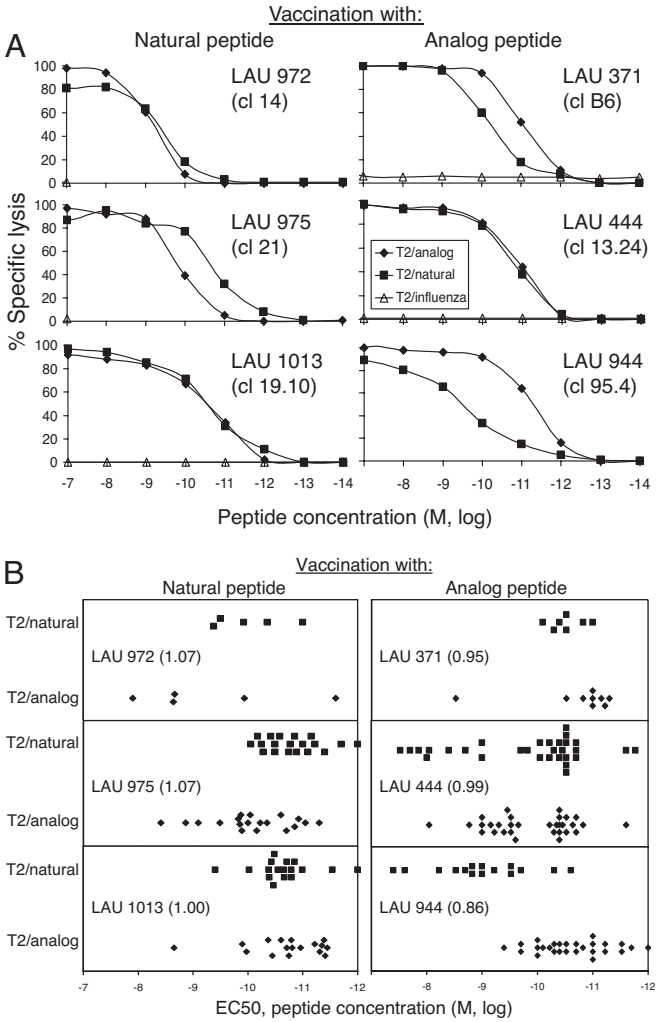


Fig. 2. Fine antigen specificity analysis of T cell clones. Clones were generated from three patients vaccinated with natural peptide (*Left*) and three patients vaccinated with analog peptide (*Right*). Cytotoxicity assays with T2 target cells were performed with decreasing peptide concentrations, as shown for representative clones (*A*) and all cytotoxic clones (*B*). Each data point represents an individual clone and its peptide concentration required for half maximal lysis (log EC_{50}). Squares, natural peptide; diamonds, analog peptide. The ratio of log EC_{50} for natural/analog peptide recognition was calculated for each clone (data not shown); the mean of these values for each patient are indicated in parenthesis. Mean ratios for all cytotoxic clones after vaccination with natural peptide was 1.04; with analog peptide, it was 0.95, indicating that the mean difference was approximately 10-fold. Indeed, the comparison of these ratios from all clones from natural vs. analog vaccinated patients revealed a statistically significant difference ($P < 0.001$).

(Fig. 3). In comparison, expression of these proteins was lower in T cells from patients after vaccination with analog peptide. Intriguingly, these data reveal that natural peptide vaccination, in conjunction with CpG, was more potent for *in vivo* induction of cytotoxicity compared with vaccination with analog peptide. Interestingly, these results are in accordance with our observation that Melan-A specific T cell clones from patients vaccinated with analog but not natural peptide were often deficient of cytotoxic function (ref. 26 and unpublished observation).

IFNγ Production. To further characterize T cell function, we measured IFNγ production by Melan-A specific T cells after 4 h triggering of PBMCs with peptide. Among $multimer^{pos}$ gated cells, the percentages of $IFN\gamma^{pos}$ cells were higher when triggered with natural than analog peptides after natural peptide vaccination, and the opposite was observed after analog peptide vaccination (Fig. 4*A*). Interestingly, the highest percentages were found when both triggering and vaccination were done with natural peptide (Fig. 4 *A* and *B*). Similar results were obtained with IFNγ Elispot assays (SI Fig. 6). These results confirm that vaccination with natural peptide leads to more robust T cell activation with enhanced expression of lytic proteins. This conclusion is further supported by the observed peptide stimulation-induced TCR down-regulation (reduced multimer fluorescence), which was stronger after natural than after analog peptide vaccination (Fig. 4*A*). Unfortunately, however, TCR down-regulation cannot be precisely quantified *ex vivo*, because T cells with low level TCR expression are no longer detectable in PBMCs by fluorescent multimers or by any other available technique.

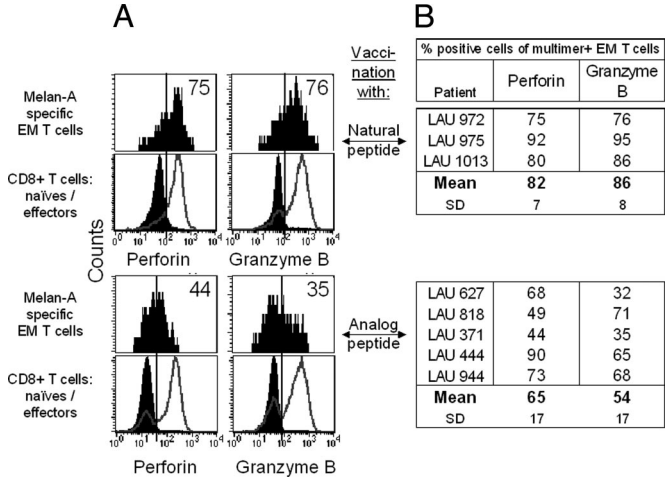


Fig. 3. Enhanced expression of lytic proteins after vaccination with natural peptide. (*A*) Expression of perforin and granzyme B in Melan-A-specific and CD8+ T cells, respectively, from representative patients, after natural peptide vaccination (patient LAU 972) (*Upper*), and analog peptide vaccination (patient LAU 371) (*Lower*). Numbers indicate percentages of positive cells. To exclude naïve T cells, a gate was set (data not shown) on effector memory cells positive for CD8 and A2/Melan-A multimers and negative for CD45RA and CCR7, representing the vast majority of T cells responding to vaccination (9). For calibration, histograms below show results from $multimer^{neg}$ $CD8^{pos}$ T cells, gated on naïve phenotype cells ($CD45RA^{pos}/CCR7^{pos}$; filled histograms) and on effector phenotype cells ($CD45RA^{pos}/CCR7^{neg}$; open histograms), allowing to precisely position the threshold between cells negative vs. positive for perforin and granzyme B for each patient. (*B*) Percentages of perforin and granzyme B expression by Melan-A specific EM T cells of patients after natural (*Upper*) and analog (*Lower*) peptide vaccination, respectively, with a statistically significant difference for granzyme B ($P = 0.02$), and a trend for perforin expression.

Discussion

Our data show that vaccination with natural tumor antigen can induce strong CD8 T cell responses, thanks to the use of CpG and IFA as adjuvants. Direct comparison of vaccination with natural vs. analog peptide revealed that the natural peptide induced lower T cell frequencies. However, T cell responses were of better quality, with superior tumor recognition and a surprising enhancement of T cell activation *in vivo*, resulting in stronger cytotoxicity and cytokine production.

Besides the Melan-A analog peptide studied here, several other modified peptides are increasingly used for clinical immunotherapy. This strategy has been applied for the tumor antigens gp100 and NY-ESO-1 (27, 28). Studies in HLA-A2 transgenic mice showed that gp100 analog peptides were much more immunogenic (4, 5). Yet, T cells obtained after patient vaccination with gp100 analog peptide were not always capable to

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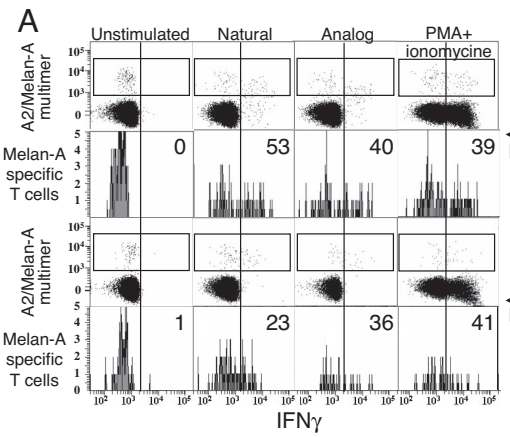

B

Vaccination with:	% IFNγ+ cells of multimer+ T cells		
	Patient	triggered with natural peptide	triggered with analog peptide
Natural peptide	LAU 972	53	40
	LAU 1015	29	23
	LAU 975	74	27
	LAU 1013	76	61
	Mean	**58**	**38**
	SD	19	15
Analog peptide	LAU 818	23	36
	LAU 444	31	40
	LAU 944	9	19
	LAU 205	23	18
	Mean	**22**	**28**
	SD	8	10

Fig. 4. IFNγ production of Melan-A specific T cells. PBMCs from natural and analog peptide vaccinated patients were left unstimulated or triggered for 4 h with natural or analog peptides or with PMA + ionomycine and analyzed with A2/Melan-A multimers and IFNγ- and CD8-specific antibodies. (*A*) Representative results after natural peptide vaccination (patient LAU 972) (*Upper*) and analog peptide vaccination (patient LAU 818) (*Lower*), respectively. (*B*) Percentages of IFNγ^{pos} cells after vaccination with natural (*Upper*) or analog (*Lower*) peptide and triggering with natural vs. analog peptide. The numbers are percentages of IFNγ^{pos} cells of CD8^{pos}/multimer^{pos} cells. Note TCR down-regulation, as revealed by reduced multimer fluorescence, particularly among IFNγ^{pos} cells after vaccination with natural peptide.

recognize tumor cells (10, 11, 13). However, these and further analog peptides have not yet been tested in a stringent and comparative manner, i.e., with vaccine formulations that induce strong T cell responses. Importantly, such studies are necessary to determine whether vaccination with carefully designed analog peptides may frequently induce T cells with imprecise fine specificity and reduced functionality. Even small changes in epitopes may have complex and unpredictable functional effects.

T cells elicited with both types of Melan-A peptides are highly cross-reactive, as demonstrated by their shared capacity to recognize target cells loaded with high to intermediate peptide concentrations. It was not surprising that cross-reactivity was most evident with multimers constructed with natural and analog peptide, because multimers bind with comparable efficiency to TCRs with different fine specificity and avidity (data not shown). However, extensive clonal analysis of multimer+ CD8 T cells in cytotoxicity assays at low peptide concentrations revealed distinct fine specificity differences. Indeed, clones from the three patients vaccinated with natural peptide revealed excellent tumor cell recognition and preference for natural as opposed to analog peptide. Clones from the three patients vaccinated with the analog peptide were in part less potent: Although the majority of clones from patients LAU 444 and LAU 371 recognized tumor cells, patient LAU 944 quite dramatically illustrates a response where the majority of clones were unable to recognize tumor cells, associated with strong preference for analog but not natural peptide. Studies showed successful tumor cell recognition after immunization with the Melan-A analog peptide used here (6–9). Our present findings do not contradict these studies but rather refine previous statements as follows: Melan-A analog peptide indeed triggers tumor reactive T cells, and, in the majority of patients, these cells are more frequent than those unable to recognize tumor cells. By contrast, vaccination with natural peptide induces close to 100% tumor reactive T cells.

It will be important to determine whether the increased proportion of tumor recognizing cells after vaccination with natural peptide is due to optimal priming through vaccination or preferential boosting of spontaneously primed T cells. It was recently reported that spontaneously arising T cells are more likely to recognize tumor cells as opposed to peptide vaccine induced T cells (14, 29), confirming that current vaccines require improvement to recruit T cells with better TCRs. This can best be done by direct comparison of immune responses induced with different vaccine formulations, as performed in this study. To reveal the impact of vaccination on TCR selection, T cells must obviously be triggered by the vaccine, rather than endogenously by tumor derived antigen. The consistent high proportion of T cell clonotypes with fine specificity corresponding to the antigen used for vaccination strongly indicates that vaccination was indeed the dominant driving force. In the majority of cancer vaccine studies, however, the situation is different, because vaccine driven T cell responses were much weaker (22), and such responses were primarily composed of T cells primed by tumor derived antigen (30).

At first, it seems paradoxical that the "less immunogenic" natural peptide induced more strongly functional T cells. However, previous animal and clinical studies were not suitable to challenge the assumption that stronger antigenicity (of analog peptides) correlates with enhanced T cell function. Using large amounts of peptide antigens with strong HLA binding likely results in high antigen density, and a central question is whether this approach is appropriate for the generation of efficient T cell responses. Clearly, this strategy allows to activate large numbers of T cells, which may show appropriate function in various *in vitro* assays. However, this mechanism is inefficient for immune defense against viral diseases, because CD8 T cells must be able to recognize low amounts of viral peptide antigen for protection (31). *In vitro*, high peptide concentrations and peptides with stable MHC binding recruit T cells with reduced TCR avidity and reduced protective capacity (32–34). More recently, *in vivo* experiments in mice showed that the peptide concentration used for DC labeling and priming inversely correlated with the avidity of TCRs of memory cells (35). Thus, one may conclude that vaccination should be done with low peptide doses and/or peptides with low HLA binding stability (provided that one can still elicit a reasonably strong T cell response).

The natural Melan-A peptide binds less stably to HLA-A2. Otherwise, its capacity to trigger TCRs is not compromised, i.e., the binding strength of the peptide/HLA/TCR complex is not affected (but depends, of course, on the TCR). The increased peptide/HLA stability of the analog peptide is likely to increase the number of cells presenting the peptide, at higher peptide/

MHC concentrations and for a longer period. Bevan *et al.* (36) recently described that prolonged duration of TCR stimulation leads to higher magnitude but not increased functionality of murine T cell responses. Indeed, the induction of strong effector function can be achieved after very short TCR interactions. By contrast, longer TCR triggering is required to induce efficient proliferation (37). Our results fit well with these observations, because the magnitude but not functionality was increased with analog peptide. *In vitro*, the analog peptide provides longer T cell stimulation than the natural peptide (data not shown), but we have no proof that the same is true *in vivo*. Further studies, designed for controlled and individual assessments of peptide/MHC stability, concentration, and duration of antigen presentation, are necessary.

Together, the most likely explanation for our finding of increased T cell functionality after vaccination with natural peptide is that the latter recruited T cells with superior TCR affinity. Unfortunately, this point remains unproven, because the precise measurement of TCR affinity is still impossible for large series of TCRs such as those elicited after vaccination.

In summary, our results were obtained due to (*i*) strong *in vivo* T cell activation and (*ii*) extended and detailed T cell analysis. CpGs allow to achieve much higher T cell frequencies than previously possible, which is the case even with natural tumor antigens that are only weakly immunogenic when used with conventional adjuvants or when expressed by recombinant viruses. To enhance vaccine efficacy, antigens (peptides, proteins, DNA, and recombinant vectors) have been modified, but this strategy bears the risk to alter T cell recognition and function unfavorably. The present findings support the use of natural antigen, in conjunction with potent new generation adjuvants, holding promise for future immunotherapy (38, 39).

Patients and Methods

Patients, Blood Cells, HLA-A2/Peptide Multimers, and Flow Cytometry. Patients were recruited and vaccinated as described in ref. 9 and *SI Text*. Ficoll-Paque centrifuged PBMCs ($1–2 \times 10^7$) were cryopreserved in RPMI medium 1640, 40% FCS, and 10% DMSO. Phycoerythrin-labeled HLA-A*0201/peptide multimers (originally called tetramers) were prepared as described in refs. 40 and 41. Before staining, $CD8^{pos}$ T cells were enriched by using a MiniMACS device (Miltenyi Biotec) resulting in >90% $CD3^{pos}$ $CD8^{pos}$ cells. Cells (10^6) were incubated with multimers (1 μg/ml, 60 min, 4°C) and then with antibodies (30 min, 4°C). For intracellular assessment of IFNγ, $\approx 10^6$ $CD8^{pos}$ cells purified by MiniMACS were resuspended in culture medium and incubated for 4 h with 1 μM peptide. After 1 h, 10 μg/ml Brefeldin A (Sigma) was added. Intracellular staining was done as follows: Cells were permeabilized with saponin at 4°C, washed, and stained for 40 min at 4°C with PE-labeled multimers along with $IFN\gamma^{FITC}$ (PharMingen) and CD8 specific antibodies. Cells to be activated were stained with PE-labeled multimers for 30 min at 37°C before activation. $CD8^{pos}$ T cells (5×10^5 per sample) were acquired with a FACSVantage SE™ machine, and data were analyzed with CellQuest software (BD Biosciences). All samples were analyzed by applying a lymphocyte forward/side scatter gate.

T Cell Cloning and Cytotoxicity Assay. T cells stained with fluorescent multimers and antibodies were sorted by flow cytometry, cloned by limiting dilution, and expanded with PHA and allogeneic feeder cells in culture medium supplemented with 150 units/ml hrIL-2. They were periodically (3–4 weeks) restimulated with PHA, irradiated feeder cells, and hrIL-2. Lytic activity and antigen recognition were assessed functionally with T2 target cells ($A2^{pos}$/Melan-A^{neg}) in 4-h ^{51}Cr release assays (41). Percentage specific lysis was calculated as follows: 100 × (experimental − spontaneous release)/(total − spontaneous release).

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