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Decreased specific CD8$^+$ T cell cross-reactivity of antigen recognition following vaccination with Melan-A peptide

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The aim of T cell vaccines is the expansion of antigen-specific T cells able to confer immune protection against pathogens or tumors. Although increase in absolute cell numbers, effector functions and TCR repertoire of vaccine-induced T cells are often evaluated, their reactivity for the cognate antigen *versus* their cross-reactive potential is rarely considered. In fact, little information is available regarding the influence of vaccines on T cell fine specificity of antigen recognition despite the impact that this feature may have in protective immunity. To shed light on the cross-reactive potential of vaccine-induced cells, we analyzed the reactivity of CD8$^+$ T cells following vaccination of HLA-A2$^+$ melanoma patients with Melan-A peptide, incomplete Freund's adjuvant and CpG-oligodeoxynucleotide adjuvant, which was shown to induce strong expansion of Melan-A-reactive CD8$^+$ T cells *in vivo*. A collection of predicted Melan-A cross-reactive peptides, identified from a combinatorial peptide library, was used to probe functional antigen recognition of PBMC *ex vivo* and Melan-A-reactive CD8$^+$ T cell clones. While Melan-A-reactive CD8$^+$ T cells prior to vaccination are usually constituted of widely cross-reactive naive cells, we show that peptide vaccination resulted in expansion of memory T cells displaying a reactivity predominantly restricted to the antigen of interest. Importantly, these cells are tumor-reactive.

Key words: Antigens · Human · T cells · Vaccination

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Abbreviations: **DN:** CD45RA/CCR7-double-negative · **DP:** CD45RA/CCR7-double-positive · **IFA:** incomplete Freund's adjuvant · **NSFU:** normalized spot-forming unit · **ODN:** oligodeoxynucleotide · **PS-SCL:** positional scanning synthetic combinatorial peptide library · **SFU:** spot-forming unit

Introduction

Specific recognition of antigen-derived peptides is crucial in cellular immunity, as it dictates the activation and expansion of mature T cells and their role in mounting precise and adapted immune responses. This recognition occurs through a highly specific interaction between the TCR and self MHC molecules presenting the antigen, so that immunodominant CD8$^+$ T cell re-

sponses to natural viral antigens present usually a narrow epitope specificity [1–5]. Mature T cell recognition is so specific that minute structural changes of the epitope or the TCR can often profoundly affect it [6].

Nonetheless, some reports have shown that antigen-specific T cells can also be broadly cross-reactive [7–11] (*i.e.* have the capacity to recognize antigens that differ from their fine specificity or HLA restriction). Considering the relatively limited number of distinct specificities in the TCR repertoire (about 10^{12}), T cell cross-reactivity confers a selective advantage by virtue of further expanding the capacity to recognize the infinite variety of antigens [12]. In addition, cross-reactivity is central to the development of vaccines. For instance, in the case of HIV infection, a vaccine that would induce broadly cross-reactive T cells able to recognize multiple viral variants would certainly be advantageous [13]. However, on the other hand, vaccine-driven T cells with highly restricted specificity may be favored in order to avoid degeneracy of antigen recognition and undesirable hyper-responsiveness *(e.g.* auto- and/or alloreactivity) [14]. In any case, there is surprisingly little information available on this matter in humans. In particular, whether a T cell-based vaccine leads to the generation of highly specific or rather cross-reactive cells has remained barely studied.

To address this issue, we have used a selection of Melan-A-related peptides to probe the fine specificity repertoire of antigen recognition by $CD8^+$ T cells induced through vaccination with CpG/incomplete Freund's adjuvant (IFA) and optimized Melan-A peptide in humans. This constitutes a particularly informative system since a vaccine using Melan-A peptide in combination with CpG-oligodeoxynucleotide (CpG-ODN) 7909 and IFA as adjuvants induces strong *in vivo* expansions of Melan-A-specific $CD8^+$ T cells in melanoma patients [15]. This enables direct *ex vivo* studies of vaccine-boosted T cells in humans, including the assessment of their fine specificity repertoire. In addition, non-vaccinated melanoma patients are known to carry a heterogeneous population of circulating Melan-A-reactive $CD8^+$ T cell precursors that are largely cross-reactive (*i.e.* display multiple specificities) [16–18]. In this setting, vaccination acts directly on a generally cross-reactive population, which enhances the interest in studying the issuing T cell reactivity. Lastly, the study of cross-reactivity following vaccination with an optimized Melan-A peptide (*i.e.* ELAGIGILTV instead of native EAAGIGILTV), predicted to be more immunogenic [19], is of particular concern, since it has recently been hypothesized that such vaccine may induce cells that are not targeted to the peptide of interest, *i.e.* natural Melan-A, and therefore may display a limited tumor reactivity [20].

In order to perform such analysis, we have used a collection of 100 peptides retrieved from proteins of self or pathogen origin by means of biometric score matrix analysis of a positional scanning synthetic combinatorial peptide library (PS-SCL) screening [18, 21]. The PS-SCL approach has previously led to the successful study of T cell specificity with the identification of biologically active peptides [22–26]. The PS-SCL at the origin of these studies was composed of trillions of decapeptides systematically arranged in mixtures with defined natural amino acids at each position. A total of 180 mixtures were used to screen for their ability to stimulate a cytolytic response from a high-avidity Melan-A-specific CTL clone in a functional assay. Such analysis led to the prediction of peptide sequences from public protein databases, with ranked reactivity for Melan-A-specific TCR [27]. We have already provided evidence that some of the selected peptides were highly recognized by a series of Melan-A-reactive CTL clones and CTL lines [17, 18, 21].

To assess the *in vivo* impact of vaccination on T cell cross-reactivity in the Melan-A setting, we have performed *ex vivo* as well as *in vitro* analysis of the functional diversity of antigen recognition using this (PS-SCL-derived) Melan-A cross-reactive peptide collection on PBMC and on T cell clones from vaccinated donors. We report that from a heterogeneous and generally cross-reactive precursor population, Melan-A vaccination results mostly in $CD8^+$ T cells targeted to selective recognition of both optimized and natural Melan-A peptides.

Results

Ex vivo assessment of Melan-A T cell cross-reactivity in vaccinated patients

The 100-decapeptide sequences predicted as potential Melan-A-cross-reactive peptides included 22, 24 and 54 peptides from self, viral and bacterial proteins, respectively (Supplementary data Table 1). Due to the limited number of cells available (from vaccinated patients), this peptide collection was pooled in a matrix (of 20 pools of ten peptides; Supplementary data Table 2) in order to perform a screening directly on PBMC. For internal controls, the optimized peptide (ELA) was included in pools 5 and 13, and the native peptide (EAA) in pools 7 and 15, as well as in pools 2 and 13 (but at lower concentration; 100 times less concentrated). To assess the extent of T cell reactivity towards this peptide collection, the response to short-term exposure to them was measured by IFN-γ ELISPOT assays. As a relative measure of cross-reactivity, we counted the number of pools inducing a significant

IFN-γ response (*i.e.* at least 20% of the response obtained with ELA), but excluding those containing Melan-A peptides EAA or ELA (*i.e.* pools 2, 5, 7, 13 and 15).

We first analyzed the reactivity of precursor Melan-A-specific populations. Peripheral blood lymphocytes from non-vaccinated HLA-A2 donors, including a high proportion of melanoma patients, generally display a small but detectable population (about 0.08% of $CD8^+$ T cells) of A2/Melan-A tetramer$^+$ $CD8^+$ T cells. These cells are phenotypically and functionally naive, polyclonal and expected to be cross-reactive when probed with large collections of decapeptides from a combinatorial peptide library. However, it was not possible to assess their reactivity to Melan-A (or the peptide collection) in ELISPOT assays using PBMC from HLA-A2 healthy or non-vaccinated melanoma donors, since these donors' naive populations are small and do not produce IFN-γ in short-term assays (data not shown).

To overcome this limitation, we analyzed instead the reactivity of *in vitro* established T cell clones isolated from A2/Melan-A tetramer$^+$ $CD8^+$ T cells with the naive phenotype [CD45RA/CCR7-double-positive (DP)] obtained by flow cytometry-based cell sorting directly from blood lymphocytes of both healthy and melanoma donors (Fig. 1a). Our results confirmed the generally multi-reactive pattern and strong heterogeneity of these Melan-A-reactive naive populations [16–18]: on average, the clones had a relative cross-reactive score of 5 [*i.e.* responded to five cross-reactive peptide pools with a normalized spot-forming unit (NSFU) above 20% of the ELA value] (max: 13; min: 0). Of note, the reactivity of the clones with the native EAA peptide was relatively poor: half of these clones showed a reactivity to EAA below 20% of the ELA value.

We then analyzed the T cell reactivity of post-vaccination specific T cell populations directly *ex vivo* using PBMC from Melan-A-vaccinated patients. Seven out of nine individuals vaccinated with IFA + CpG + Melan-A were selected based on the availability of PBMC with both high percentages of A2/Melan-A tetramer$^+$ $CD8^+$ T cells and significant IFN-γ ELISPOT responses to ELA (sufficient to detect responses to potentially less reactive peptides) (Fig. 1b). The majority of vaccinated patients (six out of seven) showed a rather narrow specificity, responding mostly to pools 5, 7, 13 and 15, that is to say the pools containing the Melan-A peptides ELA or EAA (Fig. 1c). On average, these donors presented a cross-reactivity score of 1 (*i.e.* the response to only one pool was above 20% of the response to the ELA peptide). One exception was patient LAU 944, who showed a broader specificity (four cross-reactive pools) and responded less efficiently to the native EAA peptide (Fig. 1c).

To provide a direct *ex vivo* comparison of the degree of cross-reactivity between vaccinated donors' memory populations and a cross-reactive population, we analyzed the PBMC from one distinct healthy donor (BC26) who displayed an exceptional population of memory A2/Melan-A tetramer$^+$ $CD8^+$ T cells (Fig. 1b). These cells are considered to be Melan-A-reactive, but to exhibit a highly cross-reactive nature (the fine specificity of this population is currently being investigated). Indeed, BC26's PBMC displayed a broad reactivity in IFN-γ ELISPOTS (Fig. 1c), with a score of 9. Altogether, these data indicate that vaccination with Melan-A peptide resulted in $CD8^+$ T cell populations with reactivity targeted towards the EAA and ELA peptides, in contrast to the original naive precursor populations.

Effect of vaccination and *in vitro* assessment of cross-reactivity on Melan-A-reactive T cell clones

We next explored the potential relationship between vaccination and the evolution of cross-reactivity. As we have already reported [15], vaccination results in the *in vivo* expansion of Melan-A-reactive $CD8^+$ T cells, which is accompanied by a shift from a predominantly naive phenotype ($CD45RA^+/CCR7^+$) to a memory phenotype, as well as further differentiation (*e.g.* loss of CD28 expression) (Fig. 2a). Interestingly, vaccine-induced T cell expansion (or the size of tetramer$^+$ population) correlated with further differentiation of the cells (Fig. 2b), which is in keeping with observations done on natural $CD8^+$ T cell responses to viruses (HIV, CMV, EBV and Flu) [28, 29], and confirms the relationship between strong activation levels and further T cell differentiation.

To consider this relationship in the context of vaccine and cross-reactivity, we studied the reactivity of a series of T cell clones generated from *ex vivo* sorted A2/Melan-A tetramer$^+$ $CD8^+$ T cells at different stages of differentiation: naive-like (as defined with a DP phenotype), and antigen-experienced [selected on a CD45RA/CCR7-double-negative (DN) phenotype] either early differentiated $CD28^+$ ($DN28^+$) or more differentiated $CD28^-$ ($DN28^-$) (Fig. 2a). This analysis was performed on cells from one of the six patients who showed a restriction of its reactivity to the ELA and EAA peptides, from whom several stable CTL clones per subset were generated (Fig. 3a). As performed above, each clone was tested with the Melan-A cross-reactive peptide matrix in IFN-γ ELISPOTS (left-hand panel of the figure), in order to determine a relative score of cross-reactivity and assess reactivity to the native EAA peptide (right-hand panel of the figure). Interestingly, with increasing differentiation of derived clones, we found a decrease in cross-reactivity (mean cross-reactive scores of 5, 2 and 1 for DP (naive), $DN28^+$ and $DN28^-$

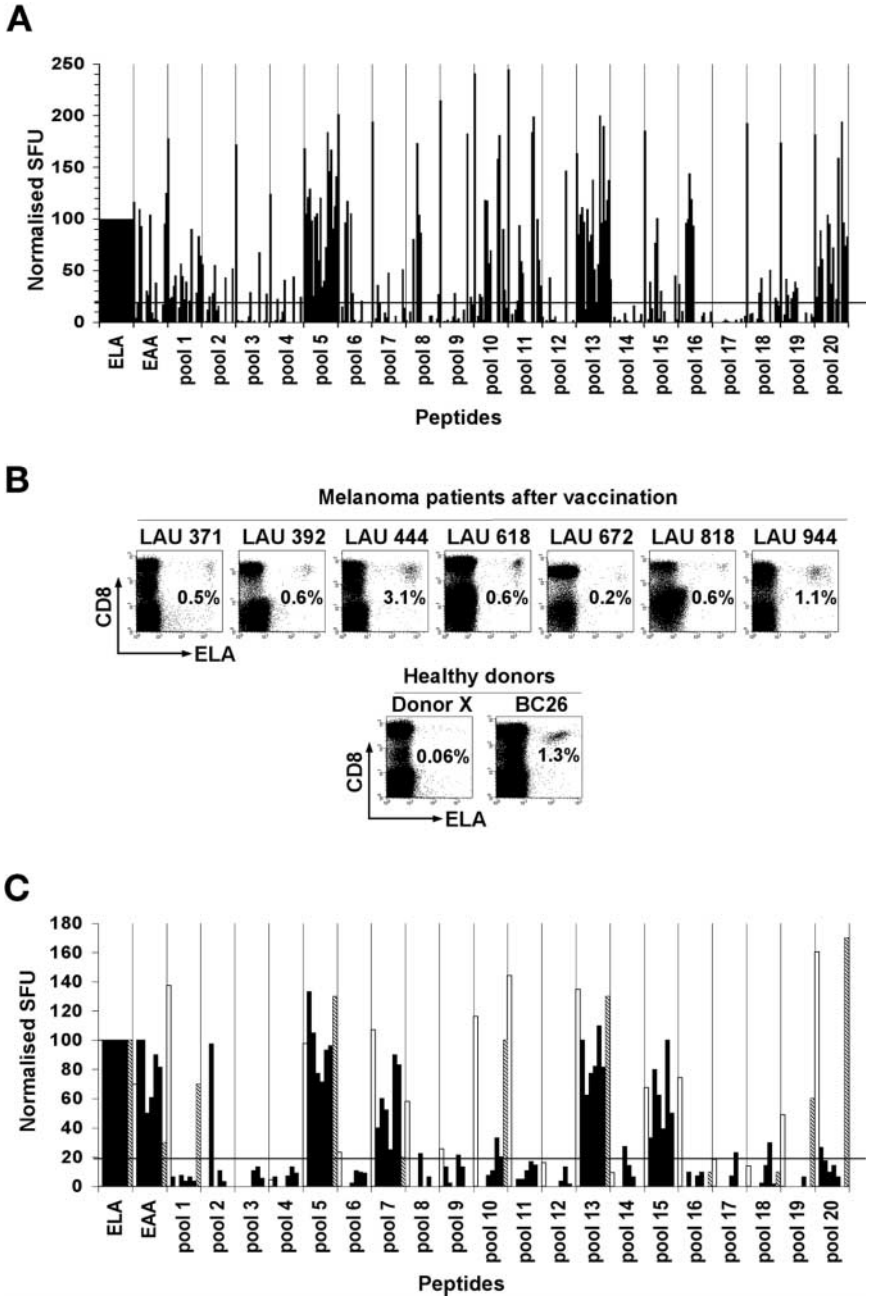

Figure 1. Analysis of T cell reactivity in Melan-A-vaccinated melanoma patients in comparison to healthy or non-vaccinated melanoma donors. (A) Reactivity of T cell clones generated from both healthy donor (eight clones) or melanoma patient (12 clones) A2/Melan-A tetramer$^+$ CD8$^+$ T cells with a naive phenotype. IFN-γ ELISPOT assays were performed to examine the reactivity of each clone to the ELA and EAA peptides as well as to the pools of Melan-A cross-reactive peptides obtained from a combinatorial peptide library analysis. Results are expressed in SFU normalized to ELA values (background subtracted) for each clone. Each bar represents one clone (mean of duplicates). Absolute SFU numbers ranged from 34 to 215 (mean at 118) for ELA-specific responses, and from 5 to 330 (mean at 81) for the peptide pools; no peptide background values ranged from 0 to 30 (mean at 10). (B) *Ex vivo* identification of A2/Melan-A tetramer$^+$ CD8$^+$ T cells in Melan-A peptide-vaccinated melanoma patients and healthy donors. Tetramer and CD8 stainings on PBMC are shown for the seven selected Melan-A-vaccinated patients as well as one representative healthy donor and one particular healthy donor (BC26) with a known A2/Melan-A tetramer$^+$ CD8$^+$ T cell cross-reactive population. Percentages of CD8$^+$ tetramer$^+$ T cells are shown. (C) *Ex vivo* analysis of T cell reactivity in Melan-A-vaccinated melanoma patients and BC26. PBMC were tested in IFN-γ ELISPOT assays with ELA and EAA peptides as well as pools of Melan-A-cross-reactive peptides obtained from a combinatorial peptide library analysis. Results are expressed in SFU normalized to ELA peptide values (background subtracted) for each donor. Each bar represents one donor (mean of duplicates); healthy donor BC26: white bars; vaccinated patient LAU 944: crossed bars; other vaccinated patients (n=6): black bars. Absolute SFU numbers ranged from 20 to 67 (mean at 35) for ELA responses, and from 5 to 82 (mean at 20) for the peptide pools; no peptide background ranged from 0 to 13 (mean at 5).

(effector-memory) derived clones, respectively), associated with an increased reactivity to the native EAA peptide (Fig. 3a). This is in line with the observations made *ex vivo*, indicating that vaccination resulted in the activation and expansion of cells with targeted Melan-A reactivity.

To provide a comparative analysis, we also generated clones from LAU 944, the patient who displayed broader post-vaccine reactivity, and performed a similar analysis. In this case, we found no relationship between differentiation and targeted cross-reactivity (Fig. 3b), also in line with the *ex vivo* observations of cross-reactivity and lesser native EAA peptide reactivity for LAU 944. Of note, the four cross-reactive pools that came forward with LAU 944 PBMC, were also strongly recognized by the DN28$^-$ derived T cell clones, illustrating the weight of the CD28$^-$ Melan-A-reactive T cells in the *ex vivo* assay.

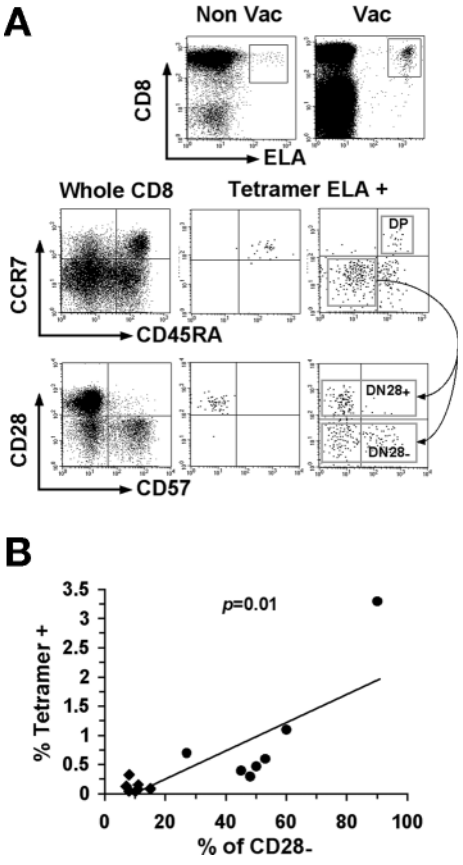

Figure 2. Relationship between vaccination-driven expansion and differentiation of Melan-A-reactive CD8$^+$ T cells. (A) Representative staining for the expression of CD45RA/CCR7 and CD28/CD57 on A2/Melan-A tetramer$^+$ CD8$^+$ T cells in one non-vaccinated and one vaccinated melanoma patient. The rectangles DP, DN28$^+$ and DN28$^-$ are fictive gates to illustrate the populations that were sorted on a flow cytometer for the generation of CD8$^+$ T cell clones from distinct differentiation subsets. (B) Positive correlation between size (percentage of tetramer$^+$ CD8$^+$ T cells) and differentiation (percentages of CD28$^-$ tetramer$^+$ cells) of Melan-A-reactive CD8$^+$ T cells from non-vaccinated (diamonds) and vaccinated (circles) melanoma patients. The *p* value was obtained using the non-parametric Spearman rank correlation test.

In addition, we tested the capacity of these clones to recognize the tumor. DP, DN28$^+$ and DN28$^-$ derived clones that exhibited cytolytic capacities (*i.e.* were able to kill Melan-A peptide-pulsed cell lines) were selected and assessed for their ability to lyse HLA-A2$^+$ melanoma cell lines that express naturally Melan-A (Fig. 4). Similar to the observations on cross-reactivity, the naive DP derived clones exhibited a heterogeneous pattern, with at least half of the clones failing to recognize the Melan-A-expressing tumor cell line. In contrast, the majority of antigen-experienced (DN28$^+$ and DN28$^-$) derived clones could recognize this cell line. No major difference was observed between the clones generated from the two patients, which suggests that breadth of reactivity and tumor recognition are independent. Nonetheless, these data confirmed that vaccination could lead to the expansion of cells with Melan-A reactivity and tumor recognition capacities.

Identification of commonly recognized peptides

The results obtained with the different peptide pools were used to identify individual peptides commonly recognized by Melan-A-cross-reactive CD8$^+$ T cell clones. The different peptide pools were ranked according to their mean NSFU responses and numbers of responding clones (out of 40 clones tested); pools 1, 2, 5, 7, 9, 10, 11, 13, 15, 18, 19, 20 were selected for having the highest values (*i.e.* recognized by at least 25% of the clones and with an NSFU >50%) (Supplementary data Table 3).

Based on the assessment of these pools and corresponding values positioned in the matrix (Supplementary data Table 2), as well as matching reactivity for individual clones, five peptides emerged as commonly recognized peptides (Fig. 5a). It is important to appreciate that this is not an exhaustive list, as other peptides may also be recognized by certain clones, although less frequently. Responses to the single peptides were then tested in IFN-γ ELISPOT assays on some cross-reactive clones to check that they were indeed recognized, as exemplified in Fig. 4b. In addition, peptide titrations were performed in chromium-release assays with the same clones to determine the relative recognition efficiency (Fig. 5b). The recognition efficiency for some of these peptides (in particular 10, 90 and 91) could match that of the Melan-A peptide, showing that cross-reactive recognition obtained with these peptides was significant.

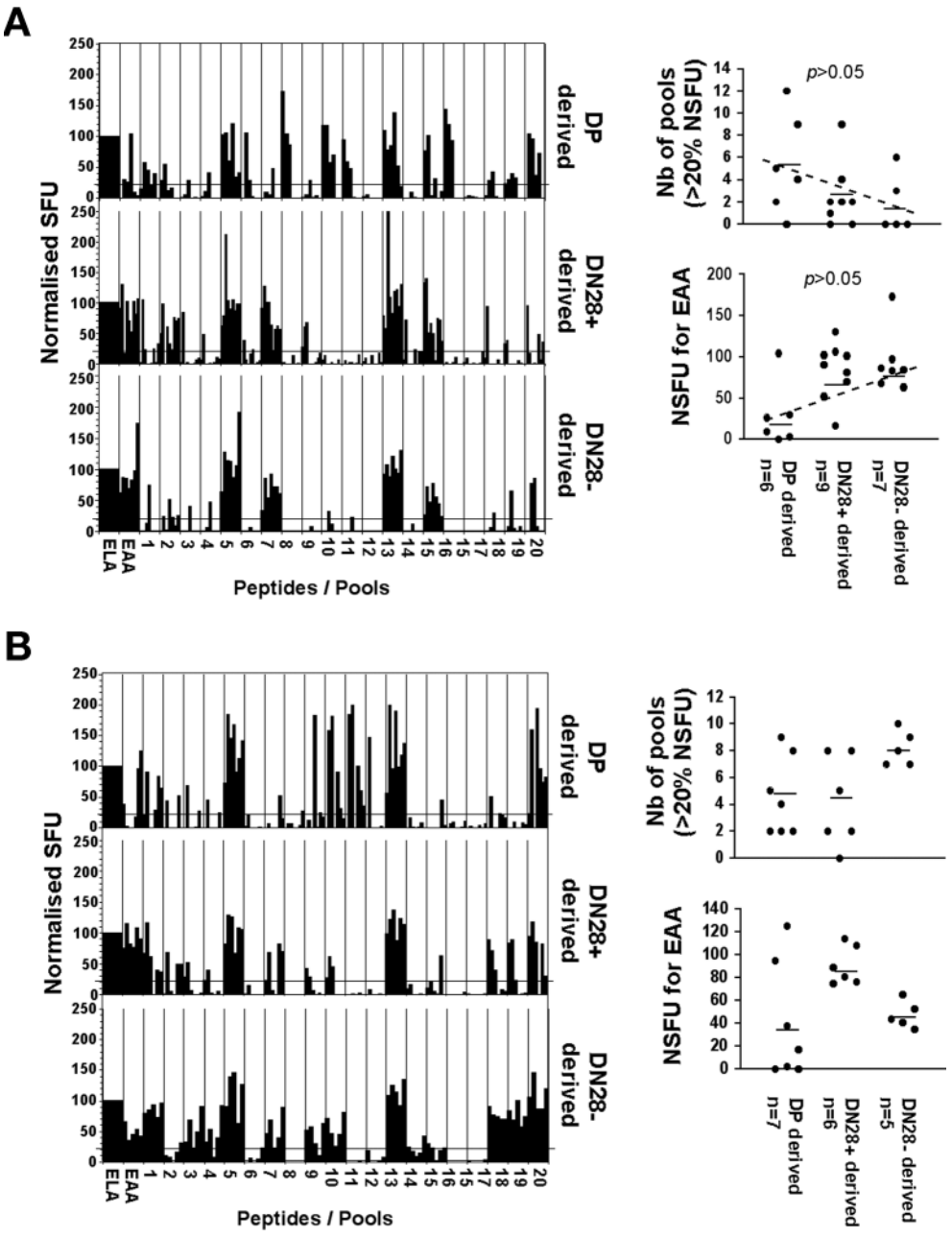

Figure 3. Reactivity analysis of CD8$^+$ T cell clones generated from two Melan-A-vaccinated melanoma patients. Melan-A-reactive CD8$^+$ T cell clones were derived from distinct differentiation subsets [naive-like DP cells, antigen-experienced DN CD28$^+$ (DN28$^+$) or CD28$^-$ (DN28$^-$) cells] and tested in IFN-γ ELISPOT assays using the pools of Melan-A-cross-reactive peptides (left-hand panels). Clones from one vaccinated patient (LAU 371) who showed *ex vivo* Melan-A-targeted reactivity (A) and from the vaccinated patient (LAU 944) who showed *ex vivo* Melan-A broad cross-reactivity (B) were analyzed; each bar represents one clone (mean of duplicates). On the right-hand panels, cross-reactive scores as well as responsiveness to the natural Melan-A peptide EAA (NSFU) are plotted for each clone (black circles), grouped in differentiated subset category (hyphens show means). *p* values were obtained using a simple regression. The number of clones analyzed is indicated (*n*).

Discussion

The primary aim of T cell vaccines is to trigger the expansion of antigen-specific memory T cells in order to confer protection against pathogens or tumors. In this context, it is important to consider the specificity of antigen reactivity of these cells, as for instance, a broad cross-reactivity of vaccine-boosted T cells may be regarded as being either advantageous or disadvantageous according to the situation. Whether vaccination modulates such reactivity and how it may affect it remain open questions.

This issue is particularly relevant when the immunogen incorporates amino acid substitutions: while these

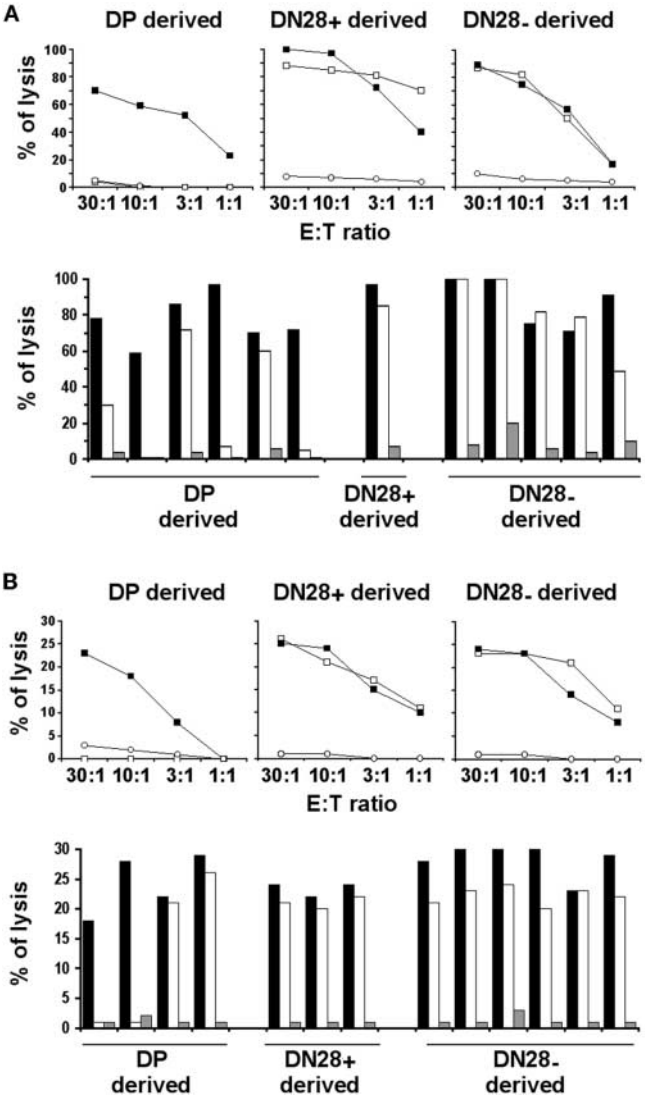

Figure 4. Tumor recognition by Melan-A-reactive $CD8^+$ T cell clones generated from two Melan-A-vaccinated melanoma patients. Tumor reactivity of Melan-A-specific $CD8^+$ T cell clones generated from vaccinated patients LAU 371 (A) and LAU 944 (B) was tested in chromium-release assays on HLA-A2 melanoma cell lines. Top panels show representative examples of tumor recognition by one clone derived from the distinct differentiation subsets [naive-like DP cells, antigen--experienced DN $CD28^+$ ($DN28^+$) or $CD28^-$ ($DN28^-$) cells] at different effector-to-target cell ratios. In the lower panels, results are shown for several clones at 10:1 effector-to-target cell ratio. The different melanoma cell lines tested are: non-pulsed $A2^+$/Melan-A^+ (white squares/white bars), Melan-A peptide (ELA)-pulsed $A2^+$/Melan-A^+ for positive control (black squares/black bars), and non-pulsed $A2^+$/Melan-A^- for negative control (white circles/gray bars).

changes may help overcome tolerance, they may also induce partially cross-reactive T cells. To shed light on such issue, we have investigated for the first time the effect of vaccination on T cell cross-reactivity in humans. The strong *in vivo* T cell expansion induced by a Melan-A peptide analog + IFA + CpG-ODN vaccine in melanoma patients has enabled us to perform direct *ex vivo* analysis. This represents an advantage over the sole analysis of *in vitro* stimulated T cell lines, which can introduce bias difficult to control. In addition, the specificity of antigen recognition of vaccine-induced cells was probed with a battery of highly selected peptides, predicted to cross-react with Melan-A-specific T cells.

Using this peptide collection in IFN-γ ELISPOT assays, we were able to confirm the Melan-A cross-reactive nature of one healthy donor $CD8^+$ T cell memory population, as well as the heterogeneous reactivity of Melan-A-reactive naive cells (CTL clones) in healthy donors and melanoma patients. In contrast, we observed that *in vivo* stimulation of this heterogeneous and broadly cross-reactive precursor $CD8^+$ T cell population with a peptide-based vaccine resulted in T cells with a reactivity predominantly restricted to the antigen of interest. This seems to be due to the selective activation, from the pool of precursors, of T cells with restricted reactivity that expand and further differentiate, as indicated by the loss of CD28 expression.

These results were supported by the analysis of Melan-A-reactive $CD8^+$ T cell clones derived from one patient. Patients vaccinated with the optimized Melan-A peptide generally displayed a stronger reactivity for ELA peptide analog than for native EAA peptide. This is in keeping with a superior recognition efficiency for ELA compared to EAA (1 log difference on average), as usually observed with Melan-A-reactive T cell clones [19]. Nonetheless, the reactivity of the vaccine-induced $CD8^+$ T cell populations was mostly focused on the Melan-A antigen (both ELA and EAA peptides), and these cells were able to recognize melanoma cell lines (as assessed using antigen-experienced cell-derived clones). This is in contrast with the hypothesis proposed by Lee and colleagues [20] that vaccination with an optimized Melan-A peptide would result in the induction of T cells with a non-focused reactivity associated with a weak tumor activity. Here, our data suggest that an optimized peptide-based vaccine can result in the expansion of T cells that are not only Melan-A antigenic peptide-targeted, but also tumor-reactive.

One vaccinated patient (LAU 944) though showed a contrasting profile from the *ex vivo* and *in vitro* results obtained with the other donors. Vaccine-induced cells from this donor displayed a broad reactivity, including to peptides with a recognition efficacy equivalent to those obtained with Melan-A antigens. Interestingly, all the $DN28^-$ derived T cell clones analyzed from this patient exhibited the same TCR, reflecting therefore the expansion of a single clonotype (N.R., manuscript in preparation). This clonotype was also dominant *ex vivo*, *i.e.* within the circulating $CD28^-$ Melan-A-reactive $CD8^+$

T cell population of the patient. This indicates that the cross-reactive profile observed *ex vivo* in this patient (as well as with the DN28$^-$ derived clones) is due to the preferential expansion of a single, highly cross-reactive T cell clone *in vivo*, that is particularly influential on the whole response.

We had also the opportunity to analyze the TCR repertoire of Melan-A-reactive CD8$^+$ response from five other vaccinated patients, and observed similar expansions of dominant clones in four of them (N.R. and V.A., manuscript in preparation). However, the difference between LAU 944 and the others seems to reside in the highly cross-reactive character of LAU 944's expanded clone. Overall, this shows that while there may be selection and expansion of CD8$^+$ T cells with a reactivity restricted to the vaccine antigen, with respect to Melan-A peptide vaccination, this is not a general rule, and single clones with broad reactivity can eventually become dominant following vaccination. Further work is needed to understand the mechanisms involved in the selection of particular clones following vaccination and the shaping of vaccine-induced T cell antigen repertoire. In our studies, we did not find any correlation between the recognition efficacy of the different clones to ELA or EAA peptides (assessed through peptide titration experiments using chromium release as readout) and their degree of cross-reactivity (*i.e.* cross-reactive score) (data not shown).

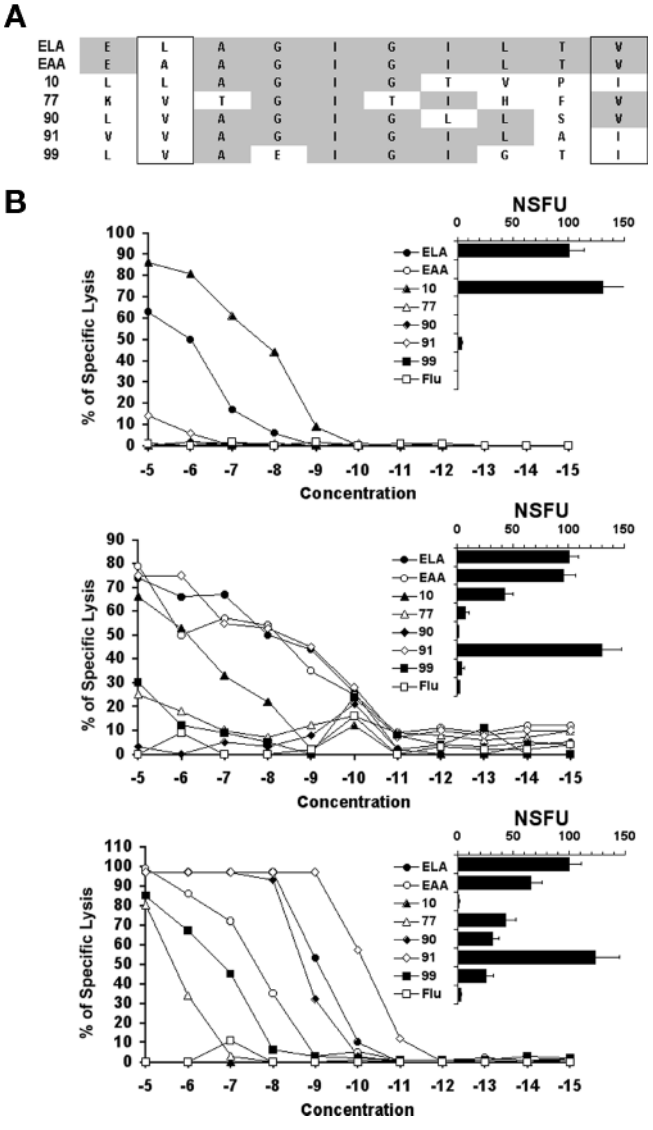

Figure 5. Melan-A-homologous peptides frequently recognized by Melan-A-reactive CD8$^+$ T cells. (A) Sequence alignment of Melan-A peptides and five frequently recognized homologues. Amino acids common to the natural Melan-A peptide are in gray. Amino acids of reported or conservative anchor residues are highlighted/framed. (B) Examples of peptide titration chromium-release assays on three cross-reactive clones (two DP and one DN28$^-$ from top to bottom) from LAU 944 and corresponding NSFU results from IFN-γ ELISPOT assays (with 1 μM peptide).

To conclude, it is interesting to observe that, in the case of our Melan-A peptide vaccine, the stimulation of broadly cross-reactive T cells can result in vaccine-induced cell populations with a rather targeted reactivity towards the antigen of interest and the tumor. It is tempting to speculate that a similar outcome could arise with other vaccines, for instance HIV vaccines. However, no conclusions can be reached on this point since the Melan-A epitope may be atypical among many antigens, and different vaccines (*i.e.* formulations, antigens, but also precursors) may lead to different outcomes in terms of T cell reactivity. Our work represents only a first step in the study of post-

We identified peptides that were commonly recognized by Melan-A-reactive clones: one peptide from a human protein (peptide 10 – prostaglandin receptor) and four peptides from bacterial proteins (peptides 77, 90, 91 and 99 – *Thermotoga maritima*, *Shewanella violacea*, *Mycobacterium tuberculosis* and *Cyanobacterium synechocystis,* respectively). There was a strong sequence homology between Melan-A and these peptides, in particular among residues that are localized in the central part of the peptides and likely to interact with the TCR. Of note, these peptides were not those with the highest score of predicted reactivity from the PS-SCL analysis. This is particularly obvious in the case of peptide 91, which was highly recognized by Melan-A-reactive clones (about 60%) but stands in 32nd position in the ranking of predicted peptides of bacterial origin. This may simply be due to the statistical nature of such analysis, but also to the fact that this analysis was originally based on the functional assessment of a single Melan-A-reactive CTL clone (with good recognition efficiency and tumor cell reactivity). Performing PS-SCL analysis using a large numbers of clones for comparison may provide more accurate predictive ranking and values.

vaccination cross-reactivity, which requires strong attention if one aims at developing effective and safe vaccines.

Materials and methods

Samples and T cell clones

PBMC samples were separated from patients' heparinized blood and cryopreserved for subsequent studies. The patients were HLA-A2$^+$ individuals with histologically proven metastatic melanoma of the skin expressing Melan-A/MART-1 (RT-PCR or immuno-;histochemistry) enrolled in a phase I trial of the Ludwig Institute for Cancer Research [15] to receive monthly vaccinations s.c. composed of 500 μg of CpG--ODN 7909, 100 μg of Melan-A-optimized peptide ELAGIGILTV ("ELA") and 300 μL Montanide ISA-51. VaxImmune CpG--ODN 7909 (TCGTCGTTTTGTC-GTTTTGTCGTT) was produced by Coley Pharmaceutical Group (Wellesley, MA). Patients presenting a strong Melan-A-reactive CD8$^+$ T cell population post-vaccination were selected for this study. Samples from HLA-A2$^+$ healthy donors and non-vaccinated melanoma patients were obtained from volunteers attending the clinic. The relevant local Institutional Review Boards and Ethics Committees approved this study LUD 00-018 sponsored by the LICR.

Melan-A-reactive CD8$^+$ T cell clones were derived from PBMC of patients LAU 371 and LAU 944 as well as unvaccinated healthy HLA-A2 donors. Single A2/Melan-A (ELA) tetramer$^+$ CD8$^+$ T cells were sorted using a FACSVantage according to their phenotype (*i.e.* expression of CD45RA, CCR7, CD28) and expanded in the presence of irradiated allogeneic PBMC, 1 μg/mL PHA and human rIL-2 (150 IU/mL). Cells were subsequently expanded by periodic (3- to 4-wk) restimulation in microtiter plates together with irradiated feeder cells in the presence of PHA and human rIL-2 (no peptide was added to the culture). The HLA-A2$^+$ human mutant cell line T2, used as APC in cytolytic and IFN-γ ELISPOT assays, was cultured in RPMI 1640 medium containing 10% FCS.

Peptide collection

Decapeptides predicted to be stimulatory to Melan-A-specific CD8$^+$ T cells were ranked using a scoring matrix generated from data previously obtained by screening an amidated C terminus decapeptide PS-SCL with one high-avidity Melan-A-specific CTL clone (expanded *in vitro* from melanoma patient PBMC) in a functional chromium-release assay [21]. Briefly, a Z-scoring matrix was generated using the average and SD of the percentage of specific-lysis values of multiple experimental data obtained for each mixture defined with one of 18 natural L-amino acids in each of the ten positions of the decamer library [27]. Based on the assumption of independent and additive contribution of the individual amino acids at each position of a peptide to the peptide's activity, the score of each individual peptide was calculated by adding individual stimulatory values of the composing amino acids. A program was designed to use the matrix to score all overlapping decapeptides contained in the GenPept protein database (ftp://ftp.ncicfr.gov/pub/genpept) and thus identify sequences with the highest predicted stimulatory scores [27]. Individual peptides were synthesized at Mixture Science Inc. (San Diego, CA) by the simultaneous multiple peptide synthesis method. Purity and identity of each peptide were characterized using an electrospray mass spectrometer interfaced with a liquid chromatography system.

ELISPOT assays

Single or pooled synthetic peptides were used at a concentration of 10 μM in IFN-γ ELISPOT assays on PBMC (1.5×10^5 cells per well) or CD8$^+$-enriched T cells (5×10^4 cells per well) to define CD8$^+$ T cell responses directly *ex vivo*, as previously described [30]. IFN-γ ELISPOT kits were purchased from Diaclone Biotest. PHA was always included as a positive control. Spots were counted with an automatic reader (Bioreader 2000; BioSys GmbH). Assays were performed in duplicates that showed good similarity. Spot-forming unit (SFU) values were normalized to the SFU values obtained with the optimized ELA peptide analog (with background subtracted). Responses were considered as positive when we counted five clean spots (and three times above background), which corresponded to normalized values beyond 20% of ELA SFU response values. For assessing CD8$^+$ T cell clone reactivity, 500 cells were stimulated with 1 μM peptide and in the presence of 3×10^4 T2 cells (tests on several clones showed that 1 μM yielded to equivalent results as 10 μM).

Chromium-release assays

Chromium-release experiments were performed as follows: target cells were labeled with ^{51}Cr for 1 h at 37°C and washed three times. Target cells (1000 cells/well) were T2 cells (A2$^+$/Melan-A$^-$) for peptide titration assays, and the melanoma cell lines Me 275 (A2$^+$/Melan-A$^+$) and NA8-MEL (A2$^+$/Melan-A$^-$) for tumor cell recognition assays [19]. Labeled T2 cells were incubated in the presence of titrated amounts of peptides for 15 min at room temperature before the addition of effector cells at a lymphocyte-to-target cell ratio of 10:1. Peptide-pulsed (1 μM) melanoma Me 275 cells were used as positive control. Chromium release was measured in supernatants harvested after 4-h incubation at 37°C. Data are expressed as percentage of lysis [100 × (experimental – spontaneous release)/(total – spontaneous release)] and represent the mean value from three technical repeats (with occasional outlier single values being discarded) in single experiments.

Flow cytometry – cell sorting

HLA-A2/Melan-A-ELAGIGILTV tetramers were synthesized as described previously [31]. Anti-CD8-peridinin chlorophyll protein, anti-CD28-allophycocyanin, anti-CD45RA-fluorescein isothiocyanate (FITC), anti-CCR7-Alexa and anti-CD57-FITC antibodies were purchased from Becton Dickinson Pharmingen (San Diego, CA). Briefly, titrated tetramers (PE-conjugated) were added to PBMC, followed by addition of a panel of titrated antibodies. Cells were then washed before single cell

sorting on a Becton Dickinson FACSVantage or stored in Cell Fix™ buffer (Becton Dickinson) at 4°C until analysis on a Becton Dickinson FACSCANTO, after compensation was checked using freshly stained PBMC.

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References

1 **Hedrick, S. M., Matis, L. A., Hecht, T. T., Samelson, L. E., Longo, D. L., Heber-Katz, E. and Schwartz, R. H.,** The fine specificity of antigen and Ia determinant recognition by T cell hybridoma clones specific for pigeon cytochrome c. *Cell* 1982. **30:** 141–152.

2 **Carbone, F. R., Fox, B. S., Schwartz, R. H. and Paterson, Y.,** The use of hydrophobic, alpha-helix-defined peptides in delineating the T cell determinant for pigeon cytochrome c. *J. Immunol.* 1987. **138:** 1838–1844.

3 **Gotch, F., McMichael, A. and Rothbard, J.,** Recognition of influenza A matrix protein by HLA-A2-restricted cytotoxic T lymphocytes. Use of analogues to orientate the matrix peptide in the HLA-A2 binding site. *J. Exp. Med.* 1988. **168:** 2045–2057.

4 **Burrows, S. R., Silins, S. L., Moss, D. J., Khanna, R., Misko, I. S. and Argaet, V. P.,** T cell receptor repertoire for a viral epitope in humans is diversified by tolerance to a background major histocompatibility complex antigen. *J. Exp. Med.* 1995. **182:** 1703–1715.

5 **Hausmann, S., Biddison, W. E., Smith, K. J., Ding, Y. H., Garboczi, D. N., Utz, U., Wiley, D. C. and Wucherpfennig, K. W.,** Peptide recognition by two HLA-A2/Tax11-19-specific T cell clones in relationship to their MHC/peptide/TCR crystal structures. *J. Immunol.* 1999. **162:** 5389–5397.

6 **Baker, B. M., Gagnon, S. J., Biddison, W. E. and Wiley, D. C.,** Conversion of a T cell antagonist into an agonist by repairing a defect in the TCR/peptide/MHC interface: Implications for TCR signaling. *Immunity* 2000. **13:** 475–484.

7 **Huesmann, M., Scott, B., Kisielow, P. and von Boehmer, H.,** Kinetics and efficacy of positive selection in the thymus of normal and T cell receptor transgenic mice. *Cell* 1991. **66:** 533–540.

8 **Ignatowicz, L., Kappler, J. and Marrack, P.,** The repertoire of T cells shaped by a single MHC/peptide ligand. *Cell* 1996. **84:** 521–529.

9 **Burrows, S. R., Silins, S. L., Khanna, R., Burrows, J. M., Rischmueller, M., McCluskey, J. and Moss, D. J.,** Cross-reactive memory T cells for Epstein-Barr virus augment the alloresponse to common human leukocyte antigens: Degenerate recognition of major histocompatibility complex--bound peptide by T cells and its role in alloreactivity. *Eur. J. Immunol.* 1997. **27:** 1726–1736.

10 **Lang, H. L., Jacobsen, H., Ikemizu, S., Andersson, C., Harlos, K., Madsen, L., Hjorth, P. *et al.*,** A functional and structural basis for TCR cross-reactivity in multiple sclerosis. *Nat. Immunol.* 2002. **3:** 940–943.

11 **Lee, J. K., Stewart-Jones, G., Dong, T., Harlos, K., Di Gleria, K., Dorrell, L., Douek, D. C. *et al.*,** T cell cross-reactivity and conformational changes during TCR engagement. *J. Exp. Med.* 2004. **200:** 1455–1466.

12 **Mason, D.,** A very high level of crossreactivity is an essential feature of the T-cell receptor. *Immunol. Today* 1998. **19:** 395–404.

13 **McMichael, A. and Hanke, T.,** The quest for an AIDS vaccine: Is the $CD8^+$ T-cell approach feasible? *Nat. Rev. Immunol.* 2002. **2:** 283–291.

14 **Selin, L. K. and Welsh, R. M.,** Plasticity of T cell memory responses to viruses. *Immunity* 2004. **20:** 5–16.

15 **Speiser, D. E., Lienard, D., Rufer, N., Rubio-Godoy, V., Rimoldi, D., Lejeune, F., Krieg, A. M. *et al.*,** Rapid and strong human $CD8^+$ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909. *J. Clin. Invest.* 2005. **115:** 739–746.

16 **Zippelius, A., Pittet, M. J., Batard, P., Rufer, N., de Smedt, M., Guillaume, P., Ellefsen, K. *et al.*,** Thymic selection generates a large T cell pool recognizing a self-peptide in humans. *J. Exp. Med.* 2002. **195:** 485–494.

17 **Dutoit, V., Rubio-Godoy, V., Pittet, M. J., Zippelius, A., Dietrich, P. Y., Legal, F. A., Guillaume, P. *et al.*,** Degeneracy of antigen recognition as the molecular basis for the high frequency of naive A2/Melan-A peptide multimer(+) CD8(+) T cells in humans. *J. Exp. Med.* 2002. **196:** 207–216.

18 **Rubio-Godoy, V., Dutoit, V., Zhao, Y., Simon, R., Guillaume, P., Houghten, R., Romero, P. *et al.*,** Positional scanning-synthetic peptide library-based analysis of self- and pathogen-derived peptide cross-reactivity with tumor-reactive Melan-A-specific CTL. *J. Immunol.* 2002. **169:** 5696–5707.

19 **Romero, P., Valmori, D., Pittet, M. J., Zippelius, A., Rimoldi, D., Levy, F., Dutoit, V. *et al.*,** Antigenicity and immunogenicity of Melan-A/MART-1 derived peptides as targets for tumor reactive CTL in human melanoma. *Immunol. Rev.* 2002. **188:** 81–96.

20 **Stuge, T. B., Holmes, S. P., Saharan, S., Tuettenberg, A., Roederer, M., Weber, J. S. and Lee, P. P.,** Diversity and recognition efficiency of T cell responses to cancer. *PLoS Med.* 2004. **1:** e28.

21 **Pinilla, C., Rubio-Godoy, V., Dutoit, V., Guillaume, P., Simon, R., Zhao, Y., Houghten, R. A. *et al.*,** Combinatorial peptide libraries as an alternative approach to the identification of ligands for tumor-reactive cytolytic T lymphocytes. *Cancer Res.* 2001. **61:** 5153–5160.

22 **Dooley, C. T. and Houghten, R. A.,** The use of positional scanning synthetic peptide combinatorial libraries for the rapid determination of opioid receptor ligands. *Life Sci.* 1993. **52:** 1509–1517.

23 **Eichler, J., Lucka, A. W. and Houghten, R. A.,** Cyclic peptide template combinatorial libraries: Synthesis and identification of chymotrypsin inhibitors. *Pept. Res.* 1994. **7:** 300–307.

24 **Pinilla, C., Martin, R., Gran, B., Appel, J. R., Boggiano, C., Wilson, D. B. and Houghten, R. A.,** Exploring immunological specificity using synthetic peptide combinatorial libraries. *Curr. Opin. Immunol.* 1999. **11:** 193–202.

25 **Rubio-Godoy, V., Ayyoub, M., Dutoit, V., Servis, C., Schink, A., Rimoldi, D., Romero, P. *et al.*,** Combinatorial peptide library-based identification of peptide ligands for tumor-reactive cytolytic T lymphocytes of unknown specificity. *Eur. J. Immunol.* 2002. **32:** 2292–2299.

26 **Tumenjargal, S., Gellrich, S., Linnemann, T., Muche, J. M., Lukowsky, A., Audring, H., Wiesmuller, K. H. *et al.*,** Anti-tumor immune responses and tumor regression induced with mimotopes of a tumor-associated T cell epitope. *Eur. J. Immunol.* 2003. **33:** 3175–3185.

27 **Zhao, Y., Gran, B., Pinilla, C., Markovic-Plese, S., Hemmer, B., Tzou, A., Whitney, L. W. *et al.*,** Combinatorial peptide libraries and biometric score matrices permit the quantitative analysis of specific and degenerate interactions between clonotypic TCR and MHC peptide ligands. *J. Immunol.* 2001. **167:** 2130–2141.

28 **Papagno, L., Spina, C. A., Marchant, A., Salio, M., Rufer, N., Little, S., Dong, T. *et al.*,** Immune activation and CD8(+) T-cell differentiation towards senescence in HIV-1 infection. *PLoS Biol.* 2004. **2:** E20.

29 **Gamadia, L. E., van Leeuwen, E. M., Remmerswaal, E. B., Yong, S. L., Surachno, S., Wertheim-van Dillen, P. M., Ten Berge, I. J. and Van Lier, R. A.,** The size and phenotype of virus-specific T cell populations is determined by repetitive antigenic stimulation and environmental cytokines. *J. Immunol.* 2004. **172:** 6107–6114.

30 **Lalvani, A., Brookes, R., Hambleton, S., Britton, W. J., Hill, A. V. and McMichael, A. J.,** Rapid effector function in $CD8^+$ memory T cells. *J. Exp. Med.* 1997. **186:** 859–865.

31 **Romero, P., Dunbar, P. R., Valmori, D., Pittet, M., Ogg, G. S., Rimoldi, D., Chen, J. L. *et al.*,** *Ex vivo* staining of metastatic lymph nodes by class I major histocompatibility complex tetramers reveals high numbers of antigen--experienced tumor-specific cytolytic T lymphocytes. *J. Exp. Med.* 1998. **188:** 1641–1650.

