Single cell analysis reveals similar functional competence of dominant and nondominant CD8 T-cell clonotypes

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Immune protection from infectious diseases and cancer is mediated by individual T cells of different clonal origin. Their functions are tightly regulated but not yet fully characterized. Understanding the contribution of each T cell will improve the prediction of immune protection based on laboratory assessment of T-cell responses. Here we developed techniques for simultaneous molecular and functional assessment of single CD8 T cells directly ex vivo. We studied two groups of patients with melanoma after vaccination with two closely related tumor antigenic peptides. Vaccination induced T cells with strong memory and effector functions, as found in virtually all T cells of the first patient group, and fractions of T cells in the second group. Interestingly, high functionality was not restricted to dominant clonotypes. Rather, dominant and nondominant clonotypes acquired equal functional competence. In parallel, this was also found for EBV- and CMV-specific T cells. Thus, the nondominant clonotypes may contribute similarly to immunity as their dominant counterparts.

immune dominance | T-cell repertoire | T-cell differentiation | T-cell function

Naturally acquired self-antigen (tumor)-specific T-cell responses can be detected in most patients with cancer who have advanced disease; however, they often fail to control or eliminate the disease, in contrast to many virus-specific CD8 T-cell responses (1, 2). This likely reflects the impact of central and peripheral tolerance in shaping self antigen-specific T-cell repertoires. Vaccination against cancer aims to generate and/or boost effective type 1 immune responses (Th1 and CD8 T-cell activation) to destroy tumor cells and prevent tumor progression. These goals are similar to those set for vaccines designed to treat chronic viral diseases. There is a great need to characterize and determine the biological similarities and differences between protective (e.g., EBV- and CMV-specific) and nonprotective (e.g., tumor-specific) T-cell responses.

Only limited data are available regarding T-cell clonotype dynamics in therapeutic vaccine settings. Clonotypic analyses provide great insight, mostly because T-cell clonotypes can be followed in a straightforward manner at any time and body location by using the T-cell receptor (TCR) as a clonotypic marker (3). For example, combined ex vivo analysis of T-cell differentiation and clonality allowed the identification of a naturally primed T-cell clone in a patient with melanoma (4). The progeny of this clone dominated the CD8 T-cell response to the tumor antigen Melan-A^{MART-1}, similarly to the clonal expansions observed in virus-specific T-cell responses (5–7). However, in several patients and healthy individuals, one can also find large numbers of low/nondominant T-cell clonotypes among tumor-specific (8) and virus-specific (6) CD8 T cells. Remarkably, not only the dominant, but also the subdominant, virus-specific clonotypes were maintained stably over several years, keeping the TCR repertoire composition constant (6). These observations raise the question whether dominant T-cell clonotypes alone are sufficient or if low/

nondominant clonotypes are also functionally competent and may contribute to immune protection.

Historically, research has mostly focused on dominant T-cell clonotypes, whereas the in vivo functions of nondominant clonotypes remain poorly characterized, likely because of technical limitations. Therefore, we developed methods suitable for the ex vivo functional assessment of individual T cells, combined with clonotypic characterization. With this strategy, we analyzed clonal responses of tumor antigen-specific T cells from patients with melanoma, in parallel to protective EBV- and CMV-specific T cells. The patients with melanoma had received potent low-dose synthetic vaccines composed of Melan-A_{26–35} peptide, CpG-ODN 7909, and incomplete Freund adjuvant (IFA). This vaccine consistently induced strong T-cell responses that were easily detectable by direct ex vivo analysis (9), not only when the Melan-A A27L analogue peptide (optimized for enhanced HLA-A*0201 binding) was used for vaccination, but also with the weakly antigenic native peptide (10). We have previously shown that the native peptide induced T-cell responses with increased capacity to recognize tumor cells and increased overall T-cell functionality (10). Here, we demonstrate that this superior tumor reactivity was associated with enhanced effector cell activation of nearly all individual T cells. Importantly, these properties were found in both dominant and nondominant tumor antigen-specific T-cell clonotypes. Our data reveal that T-cell functions are determined primarily by the antigen used for vaccination and the stage of T-cell differentiation, but are similar in dominant and nondominant clonotypes participating in a CD8 T-cell response.

Results

Strong Expansion and Differentiation of Tumor Antigen-Specific CD8 T Cells Following Peptide Vaccination. Fifteen HLA-A2⁺ patients with advanced metastatic melanoma received monthly vaccinations consisting of the native Melan-A unmodified peptide (EAAGIGILTV, i.e., “EAA”) or the analogue Melan-A A27L modified heteroclitic peptide (ELAGIGILTV, i.e., “ELA”), combined with CpG-ODN 7909 and emulsified in IFA (Table S1). By using fluorescent HLA-A2/peptide multimers for ex vivo analysis, high frequencies of Melan-A-specific CD8 T lymphocytes were detected in all patients, and strongly increased compared with before vaccination (defined as time 0; Fig. 1A). We found progressive accu-

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Fig. 1. Analysis of TCR BV repertoire diversity and quantification of tumor-specific T-cell clonotypes. (A) Ex vivo analysis of circulating Melan-A₂₆₋₃₅-specific CD8⁺ T cells over time in 15 patients with melanoma vaccinated with the analogue A27L-modified ($n = 10$) or the unmodified native ($n = 5$) peptide, combined with CpG-ODN and IFA. Data are expressed as percentages of CD28^{neg} Melan-A-specific T cells. (B) cDNA pools (50–100 cells), ex vivo sorted from peripheral blood following vaccination with native or analogue peptide, were amplified by PCR by using 22 BV-specific primers, and subjected to electrophoresis. TCR BV repertoire diversity is expressed as the total number of amplified BV-CDR3-BC products of different lengths within each positive BV family. (C) Distribution of dominant and nondominant T-cell clonotypes within EM28⁺ and EM28⁻/EMRA T-cell subsets. Compiled data representing mean clonotypic frequencies of Melan-A-specific T cells after vaccination with the analogue ($n = 4$ patients) or native ($n = 4$) peptide and T cells specific for EBV ($n = 3$ healthy donors) and CMV ($n = 3$). Analyses were performed on large numbers of in vitro-generated T-cell clones ($n = 1,487$). Dominant clonotypes are defined as high (≥10%) or low (1–9%), whereas nondominant T-cell clonotypes are defined as less than 1% of epitope-specific T cells (* $0.01 < P < 0.05$, *** $P < 0.005$, two-tailed unpaired *t* test).

mulation of late-differentiated effector-memory EM28⁻ Melan-A-specific T cells (defined thereafter as “effector-like” cells), which appeared early in some patients and later in others, independent of whether patients received native or analogue peptide. The established T cells were maintained over extended periods of time while patients continuously received monthly booster vaccinations.

Vaccination-Induced T-Cell Responses Are Composed of Dominant and Nondominant Clonotypes. We characterized in depth the ex vivo TCR repertoire diversity resulting from clonal selection of tumor-

reactive T cells in patients vaccinated with EAA ($n = 5$) or ELA ($n = 10$; Fig. 1A), as described previously (4). Primed effector-memory EM28⁺ cells (also defined as early-differentiated cells) displayed large polyclonal TCR repertoires with a diverse use of the 22 different BV families studied, and high variability in CDR3 size. However, 12 of the 15 patients showed progressive restrictions in TCR BV/CDR3 diversity from EM28⁺ to EM28⁻ T-cell subsets (Fig. 1B), which was accompanied by preferential expansion of several tumor antigen-specific codominant clonotypes of intermediate (low) to high frequencies (Fig. 1C), irrespective of whether native or analogue peptide was used for vaccination. These clonotypes made up, on average, approximately 55% of the late-differentiated EM28⁻ effector-like subset, but only 25% of the early-differentiated EM28⁺ “effector-memory” T cells. Our data revealed the establishment of relatively few (between seven and 15) codominant tumor-specific T-cell clonotypes that were highly specific for each patient as defined by their private TCR BV CDR3 β signatures (Fig. S1A). Importantly, both subsets also contained nondominant T-cell clonotypes, but these were more frequent in EM28⁺ than in EM28⁻ T cells.

Altogether, these findings show that similarly to protective virus-specific responses (6), vaccination-induced tumor-specific T-cell responses are composed of dominant and nondominant clonotypes (Fig. 1C). Moreover, clonotype selection and expansion of tumor antigen-specific T-cells along cell differentiation resembled closely those observed in anti-EBV responses (6). These data were obtained following cell sorting with multimers bearing the analogue peptide. Similar results were obtained with multimers bearing the native peptide (Fig. S1B), thus indicating that the two multimers efficiently bound to all Melan-A-specific T cells with similar fine specificity to analogue or native peptide, in line with our previous report (10).

Enhanced Expression of Multiple Effector Mediators by Native Peptide Vaccination-Induced T Cells Closely Resembling Highly Differentiated CMV-Specific T Cells.

To qualitatively assess the effect of native versus analogue peptide vaccination, we developed techniques for combined ex vivo analysis of molecular and functional properties at the single-cell level, by using a modified RT-PCR protocol, enabling the detection of specific cDNAs after global amplification of expressed mRNAs from individual sorted T cells (Fig. 2 and Fig. S2A). We found that most early-differentiated EM28⁺ T cells issued from patients vaccinated with the analogue/ELA peptide contained low detectable levels of mRNA coding for effector mediators such as IFN- γ , perforin, granzyme B, and C-type killer cell lectin-like receptor CD94, while expressing measurable levels of mRNA coding for the costimulatory molecule CD27 and the cytokine receptor IL-7R α (CD127). Increased expression of effector mediators was observed in late-differentiated EM28⁻ T cells after analogue peptide vaccination, and was concomitant with down-regulation of costimulatory and cytokine receptors (Fig. 2A and B). EBV-specific T cells also presented distinct mRNA expression patterns along cellular differentiation, but globally these cells remained less differentiated than tumor- or CMV-specific T cells, in agreement with our recent report (6). A remarkable finding was that tumor antigen-specific T cells generated following native/EAA peptide vaccination exhibited similar detectable levels of effector mediators than those observed in CMV-specific T cells. Interestingly, such expression patterns were already found within the EM28⁺ compartment, despite the coexpression of early-differentiated CD27 and IL-7R α gene transcripts (Fig. 2A and B). These cells also showed more polyfunctional expression profiles compared with the corresponding EM28⁺ T cells specific for ELA and EBV epitopes (Fig. S2B).

A hierarchical clustering performed on a total of 529 single-cell samples identified five distinct clusters based on the differential coexpression patterns of the six studied genes (Fig. 2C). Heat-map analysis further allowed determination of the repartition of these clusters within the single-cell samples of each T-cell subset. Strikingly, it revealed that the early-differentiated EM28⁺ T cells induced by the native/EAA peptide were included within the

Fig. 2. Ex vivo gene expression profiling of individually sorted T cells. (A) Gene expression analysis was performed on cDNA obtained from individually sorted single-cell samples isolated from EM28⁺ and EM28⁻ T-cell subsets (patients vaccinated with analogue or native peptide), and from EM28⁺ and EMRA subsets (EBV- and CMV-specific T cells from healthy individual BCL8). Positive PCR products are depicted in red, negative ones in blue. Compiled data are shown per subset and per patient/healthy individual, with each line representing an individual T cell. (B) Complete sets of data of the proportion of each expressed gene in early- (EM28⁺) or late- (EM28⁻/EMRA) differentiated subsets from four patients vaccinated with analogue (LAU 618, ●; LAU 672, ■) and native peptide (LAU 1013, ○; LAU 972, □), and from two healthy donors BCL8 (EBV, ▲; CMV, ◆) and BCL6 (CMV, ▼). (C) A hierarchical clustering (Left; $n = 529$ single-cell samples) was performed based on Euclidean distance between samples with the Unweighted Pair Group Mean Arithmetic clustering method ([S1 Materials and Methods](#)) and allowed the identification of five distinct clusters. The repartition of these clusters within the single-cell samples of each EM28⁺ and EM28⁻ T-cell subset (specific for ELA, EAA, EBV, or CMV) was determined by heat-map analysis (Right).

second group formed by the late-differentiated EM28⁺/EMRA effector-like ELA-, EAA-, and CMV-specific T cells.

The mRNA content correlated with protein expression of CD27, IL-7R α , IFN- γ , perforin, and granzyme B, as confirmed by extended ex vivo multiparameter flow cytometry analyses of T cells from 15 vaccinated patients with melanoma as well as seven healthy individuals with persistent herpes virus infections (Fig. 3 and Fig. S3). Of note, we observed differences in mRNA and protein expression by specific T cells from the same subset that may reflect biological variations among different individuals. The expression of CD57, a marker of cellular differentiation, perforin, and granzyme B, was found to be up-regulated even in the early-differentiated EM28⁺ T-cell subset in patients after native peptide vaccination (Fig. 3). Production of IFN- γ and up-regulation of LAMP-1 (CD107a) by tumor-specific T cells showed a tendency to increased effector properties after vaccination with native peptide (Fig. S3C). Altogether, our results indicate that vaccination with the native but not analogue tumor

antigen resulted in enhanced activation and polyfunctionality in vivo of virtually all specific CD8 T cells, independently of CD28, CD27, and IL-7R α coexpression, and resembling highly differentiated CMV-specific responses.

T-Cell Differentiation and Peptide Used for Vaccination Determine Functional Profile of Individual T-Cell Clonotypes. The powerful single-cell based approach allowed for analysis of in vivo gene expression of (multiple representatives of) individual T-cell clonotypes. By this strategy, we compared clonotype performance after vaccination with analogue versus native peptide based on their frequencies. The functional profiles of dominant T-cell clonotypes (Fig. 4A) largely overlapped with those of the corresponding early- or late-differentiated subset, respectively (Fig. S2B). For instance, the dominant EM28⁺ clonotypes from patient LAU 618/ELA were less polyfunctional and shared the gene expression profile found in early-differentiated EM28⁺ subsets from analogue peptide-vaccinated patients. Conversely, the profiles of

Fig. 3. Ex vivo expression of effector proteins within tumor- and virus-specific CD8 T-cell subsets by multiparameter flow cytometry. The proportions of CD57, perforin, and granzyme B protein expression was determined within antigen-specific EM28⁺ and EM28[−] T-cell subsets from 15 patients with melanoma following analogue (ELA, $n = 10$) or native (EAA, $n = 5$) peptide vaccination, and from seven healthy individuals with EBV ($n = 5$) and/or CMV ($n = 5$) specific T-cell responses (* $0.01 < P < 0.05$, ** $0.005 < P < 0.01$, *** $P < 0.005$, two-tailed unpaired t test). Tet+, multimer+ T cells.

the dominant native peptide vaccination-induced clonotypes from patient LAU 1013 were highly polyfunctional, and corresponded to the overall gene expression profiles found within respective EM28⁺ and EM28[−] subsets. Gene expression signatures of non-

dominant tumor-specific T cells (Fig. 4B) also correlated tightly with the subset of origin (e.g., EM28⁺ or EM28[−]) and the type of peptide used for vaccination (e.g., native or analogue peptide). Similar observations were made for the dominant and non-dominant clonotypes found in EBV- and CMV-specific immune responses. In summary, the functional profiles of dominant and nondominant T-cell clonotypes were primarily determined by antigen and differentiation stage, and not by their degree of prevalence.

Efficient Target Cell Killing by T Cells of Dominant and Nondominant Clonotypes from Patients Vaccinated with Native Peptide.

We assessed the ability of native and analogue peptide vaccination-induced tumor-specific T cells to recognize and kill tumor cells (Fig. 5). As expected, most T-cell clones derived from the late-differentiated effector-like EM28[−] subset were able to kill the Melan-A-expressing tumor cell line Me290, irrespective of the peptide used for vaccination. However, T-cell clones derived from the early-differentiated EM28⁺ subset following analogue peptide vaccination were often deficient in cytotoxic function. In contrast, a large majority of T-cell clones derived from the same EM28⁺ subset from patients vaccinated with the native peptide efficiently killed the Me290 cell line. Strikingly, this occurred regardless of their clonal prevalence (e.g., dominant or non-dominant frequencies; Fig. 5B). The results obtained by such analyses using in vitro generated clones are in full agreement with the ex vivo gene (Fig. 2) and protein (Fig. 3) expression patterns, thus allowing us to draw conclusions with respect to the in vivo status of T cells. Furthermore, we have previously demonstrated that these T-cell clones maintained their specific killing properties despite several weeks of in vitro culture (11). Indeed, high and low cytotoxic activity correlated well with high and low in vitro and in vivo expression of granzyme B and perforin, respectively (11).

Collectively, these data show differential cytotoxicity of analogue peptide-generated T-cell clones depending on the in vivo differentiation stage. In contrast to analogue peptide vaccination, strong cytolytic activity was observed after vaccination with the native peptide, regardless of the type of subset (i.e., clones isolated from EM28⁺ or EM28[−]). Similar efficient target cell killing was observed between dominant and nondominant T-cell clonotypes in cytotoxic assays against HLA-A2^{pos} T2 cells pulsed with graded peptide concentration (Fig. S4). Our findings reveal a surprising functional competence not only of the frequent clonotypes, but also of the rare clonotypes among epitope-specific T-cell populations.

Discussion

T cells from peptide-vaccinated patients with melanoma provide an ideal opportunity to assess possible effects of small antigenic differences in defined therapeutic vaccines on memory and effector cell functions, stage of differentiation, and clonotype

Fig. 4. Coexpression of effector molecules by T-cell clonotypes. Polyfunctional gene expression profile was determined for dominant (A) and nondominant (B) tumor- and virus-specific T-cell clonotypes upon ex vivo single-cell sorting of EM28⁺ and EM28[−]/EMRA T cells. Dominant clonotypes (A) are defined according to their relative frequencies in epitope specific T-cell subsets as high (>10%) or intermediate (1–9%), and non-dominant TCRs (B) as frequencies lower than 1%. The pie charts illustrate coexpression of CD94, IFN- γ , perforin, and granzyme B as detected in single-cell samples from four patients vaccinated with the analogue ($n = 2$) and native ($n = 2$) peptide and two healthy individuals.

Fig. 5. Tumor cell killing and efficiency of antigen recognition. Tumor-specific T-cell clones were in vitro generated from four patients following analogue ($n = 2$) or native ($n = 2$) peptide vaccination. (A) Tumor reactivity for melanoma cell lines Me290 (A2*/Melan-A*) and NA8 (A2*/Melan-A*) in the absence or presence of synthetic Melan-A analogue peptide (1 μ M), at an effector-to-target ratio of 30:1. Each line represents an individual clone derived from EM28⁺ and EM28⁻ subsets after analogue ($n = 153$) or native ($n = 80$) peptide vaccination. (B) Complete set of data representing specific lysis of the Melan-A* Me290 tumor cell line without exogenous peptide (effector-to-target ratio, 10:1) by T-cell clones derived from EM28⁺ and EM28⁻ subsets following analogue ($n = 2$ patients; gray whiskers) or native ($n = 2$; empty whiskers) peptide vaccination. To allow direct comparison, clones were divided into dominant (frequencies >1%) and nondominant (frequencies <1%) subgroups (**0.0001 < P < 0.001, *** P < 0.0001, two-tailed unpaired t test). Tet+, multimer+ T cells.

selection. Here, we demonstrate that vaccination with native peptide induced functional T cells in nearly all dominant and low/nondominant clonotypes. In contrast, many T cells remained poorly functional after vaccination with analogue peptide, despite similar clonotype-dependent differentiation. The present study shows that single-cell analysis provides reliable quantification of individual cells expressing particular genes, as confirmed by protein expression analyses. Moreover, our strategy was highly sensitive to identify tumor-reactive T-cell clonotypes and to assess their frequencies. Finally, our results from these direct ex vivo analyses were in excellent agreement with the data obtained with large numbers of T-cell clones generated in vitro (6). An analogous approach has been previously and successfully applied to study cell heterogeneity during in vivo CD8 T-cell differentiation (12, 13). Recently, single-cell gene expression profiling was shown to allow identification of qualitative differences in CD8 T-cell responses elicited by different gene-based vaccines (14).

We found that antitumor T-cell function was primarily determined by the type of peptide used for vaccination (native versus analogue) and was not inherent to the degree of prevalence of a given T-cell clonotype (dominant vs. nondominant). Moreover, similar distributions in memory/effector gene expression signatures were found between T-cell clonotypes issued from the same subset of differentiation. Comparable observations were made for EBV- and CMV-specific T cells, indicating that the functional profile of individual T cells is also influenced by the differentiation status. As a consequence, most if not all clonotypes from a given subset of differentiation and participating in a tumor (i.e., self) or viral (i.e., nonself) T-cell response may be equally competent. Selection of a T-cell repertoire composed of low/nondominant and dominant clonotypes could serve to promote clonotypic diversity, while maintaining functional competence. These results are consistent with the report by Messaoudi and colleagues (15) that a diverse TCR repertoire but not a restricted one can mobilize protective antigen-specific T cells of high avidity and efficient target cell killing.

Our data also show that a synthetic vaccine [i.e., decapeptide, CpG-ODN, and IFA (9)] was able to trigger a similar composition of T-cell clonotypes as in viral infections (5, 7), and particularly in EBV-specific T-cell responses (6). Responding T cells were composed of clonotypes of varying frequencies (dominant, subdominant, and nondominant), yet their clonal prevalence was dependent on the differentiation stage. Late-differentiated EM28⁻ (i.e., effector-like) cells consisted primarily of patient-specific dominant (i.e., high-frequency) and subdominant (i.e., low-frequency) clonotypes with long-term persistence. In contrast, the early-differentiated EM28⁺ subset (that includes effector-memory cells) contained mostly nondominant clonotypes with only a small fraction of codominant T-cell clonotypes. Thus, clonotypic diversity was mostly found within the effector-memory EM28⁺ subset, whereas a selection was observed with advanced differentiation as certain clonotypes were enriched whereas others were not. This process is likely clonotype-dependent, consistent with studies on T cells specific for persistent viruses (5, 7), and highlighting the importance of the structural composition of the TCR repertoire for T-cell differentiation. Moreover, many tumor-specific clonotypes identified in the late-differentiated effector-like subset were also found within the pool of less-differentiated effector-memory cells, albeit at much lower frequencies. Coexistence of identical clonotypes as both effector-memory and effector-like T cells has also been described for human T cells specific for influenza matrix protein peptide (16) and HIV epitopes (7). Together, these observations are in line with recent findings (17), showing that individual naive T cells have multiple fates and can differentiate into memory and effector T-cell subsets.

Our single-cell analysis confirmed that vaccination with native peptide was more efficient at inducing the expression of multiple effector molecules, and shows that this occurred in nearly all Melan-A-specific T-cell clonotypes. This correlated with efficient target cell killing. Consequently, both T-cell subsets (EM28⁺ and EM28⁻) induced by native-peptide vaccination closely resembled each other, and the highly differentiated CMV-specific T cells. These data refine our current view of the process of T-cell differentiation, as they indicate that progressive up-regulation of cytolytic activity does not necessarily require the stepwise loss of costimulatory (CD27, CD28) and cytokine receptor (IL-7R α) expression. Previous reports have described a hierarchical order of T-cell differentiation stages, from naive to CM, EM28⁺, EM28⁻, and EMRA cells (12, 18–20). Specifically, it was shown that cellular differentiation (loss of CCR7/CD27/CD28 expression) was associated with progressive up-regulation of multiple “killer” mediators by the same cell (12). This report, together with our own observations (20), demonstrated tight correlations between particular cell surface markers (e.g., CCR7^{+/+}, CD28^{+/+}, and CD27^{+/+}) and T-cell functional properties. Although these studies were performed on circulating “bulk” CD8 T cells irrespective of antigen specificity, an alternative picture became apparent when assessing virus antigen-specific T cells. Indeed, despite

showing distinct expression patterns along with cell differentiation, CMV-specific cells also expressed globally higher levels of CD94, CD57, IFN- γ , perforin, and granzyme B than EBV-specific cells (6). The reasons behind this higher degree of differentiation and TCR clonotypic repertoire restriction are not fully understood. For example, it could be influenced by the differences in the biology of these viruses (21). Nevertheless, the fact that enhanced effector functions can be acquired without losing expression of CD27, CD28, and IL-7R α is likely important, as these receptors contribute significantly to memory cell function, essential for protection from disease.

Unfortunately, it still remains unknown why the native peptide induced qualitatively better T-cell responses than the analogue peptide. We still favor the hypothesis that the native peptide recruited T cells with superior TCR affinity/avidity, selected to overcome the lower peptide binding to MHC [the analogue peptide binds approximately 10 times more stably to HLA-A2 than the native peptide (22)]. Alternatively, vaccination with weak antigens may induce more profound T-cell activation through non-TCR-mediated signals, e.g., enhanced costimulatory and/or reduced inhibitory pathways that are maintained in T-cell progeny. Therefore, we are currently analyzing additional T-cell properties. Our preliminary data revealed relatively similar expression of the inhibitory receptors TIM-3, PD-1, CD160, CTLA-4, and BTLA among tumor-specific T cells induced by native and analogue peptide vaccination (Figs. S3 and S5).

In the future, it will be important to clarify which of the observed properties are already present before vaccination. T cells from healthy individuals should be analyzed in parallel before and after vaccination. However, these are major challenges, as clinical studies in healthy individuals are difficult to conduct. Furthermore, the frequencies of tumor-specific T cells are very low in nonvaccinated individuals, or after vaccination without CpG-ODN, even in patients with melanoma, thus severely limiting direct ex vivo analysis.

We conclude that nondominant clonotypes may display similar functions as their dominant counterparts, both for tumor- and virus-specific T cells. High-resolution characterization of individual T cells at the clonotype level provides the basis to identify the biological benchmarks associated with protective T-cell immunity, contributing to the rational development of vaccines.

Materials and Methods

Patients and Healthy Blood Donors. Fifteen HLA-A*0201-positive patients with stage III/IV metastatic melanoma were included in a phase I prospective trial at the Ludwig Institute for Cancer Research and the Multidisciplinary Oncology Center, which was approved by institutional review boards and regulatory agencies (9, 10). Patients received monthly low-dose vaccinations whereby they were injected s.c. with 100 μ g of the Melan-A^{MART-1}_{26–35} unmodified native peptide (EAAGIGILTV) or the Melan-A^{MART-1}_{26–35} analogue A27L peptide (ELAGIGILTV), mixed with 0.5 mg CpG-ODN 7909/PF-3512676 (Pfizer/Coley Pharmaceutical Group) and emulsified in IFA (Montanide ISA-51; Seppic) (9). Leukapheresis from two healthy donors (BCL6 and BCL8) with positive EBV- and CMV-specific CD8 T-cell responses were collected upon informed consent.

Cell Preparation and Flow Cytometry. Samples were collected and processed as described in *SI Materials and Methods*. Purified CD8 T cells were stained with HLA-A2/peptide multimers and then with appropriate mAbs, and sorted into defined subpopulations on a FACSVantage SE or FACSAria device (BD Biosciences) or immediately analyzed on a LSR II flow cytometer (BD Biosciences) by using CellQuest (BD Biosciences) or FlowJo (TreeStar) software.

Generation of T-Cell Clones and Functional Cytolytic Assays. HLA-A2/multimer⁺ CD8⁺ T-cell subsets (EM28⁺, EM28[−], and EMRA) were sorted by flow cytometry, cloned by limiting dilution, and expanded as described in *SI Materials and Methods*. Tumor cell killing and efficiency of antigen recognition by in vitro generated T-cell clones was analyzed as detailed in *SI Materials and Methods*.

cDNA Preparation, Gene Expression, and TCR BV Analysis. cDNA preparation, cDNA amplification and PCR were performed on individually sorted single-cell samples as described in *SI Materials and Methods*. Cells (10⁵) from T-cell clones were processed through direct lysis and cDNA synthesis without undergoing the global cDNA amplification procedure. Specific PCR reactions and TCR BV CDR3 spectratyping, sequencing, and clonotyping were performed as described in ref. 23 and in *SI Materials and Methods*.

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