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Vaccination-induced functional competence of circulating human tumor-specific CD8 T-cells

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T-cells specific for foreign (*e.g.*, viral) antigens can give rise to strong protective immune responses, whereas self/tumor antigen-specific T-cells are thought to be less powerful. However, synthetic T-cell vaccines composed of Melan-A/MART-1 peptide, CpG and IFA can induce high frequencies of tumor-specific CD8 T-cells in PBMC of melanoma patients. Here we analyzed the functionality of these T-cells directly *ex vivo*, by multiparameter flow cytometry. The production of multiple cytokines (IFNγ, TNFα, IL-2) and upregulation of LAMP-1 (CD107a) by tumor (Melan-A/MART-1) specific T-cells was comparable to virus (EBV-BMLF1) specific CD8 T-cells. Furthermore, phosphorylation of STAT1, STAT5 and ERK1/2, and expression of CD3 zeta chain were similar in tumor- and virus-specific T-cells, demonstrating functional signaling pathways. Interestingly, high frequencies of functionally competent T-cells were induced irrespective of patient's age or gender. Finally, CD8 T-cell function correlated with disease-free survival. However, this result is preliminary since the study was a Phase I clinical trial. We conclude that human tumor-specific CD8 T-cells can reach functional competence *in vivo*, encouraging further development and Phase III trials assessing the clinical efficacy of robust vaccination strategies.

Key words: CD8 T-cell effector function, vaccination, CpG oligodeoxynucleotides, Melan-A/MART-1 peptide, melanoma

Abbreviations: CD8 T-cell: $CD8^{positive}$ T-lymphocyte; CpG: deoxycytidylate-phosphate-deoxyguanylate oligodeoxynucleotide; EBV: Epstein-Barr virus; GMFI: geometrical mean fluorescence intensity; HLA-A2: human leukocyte antigen A2 (an MHC class I molecule); IFA: incomplete Freund's adjuvant; IFNγ: interferon-γ; MART-1: melanoma antigen recognized by T-cells-1; Melan-A: melanoma antigen-A (identical to MART-1); PBMC: peripheral blood mononuclear cells; s.c.: subcutaneous; TNFα: tumor necrosis factor-α

Additional Supporting Information may be found in the online version of this article.

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Protection from disease by antigen-specific CD8 T-cells depends on their proper activation, with corresponding increase in T-cell frequency, effector and memory cell differentiation, and development of T-cell functions. Several properties of T-cells have been established as correlates of protection from infectious diseases. First, T-cells need to recognize cognate antigen efficiently[1–3] (*i.e.*, have good "functional avidity") which is controlled by T-cell receptors (TCRs) and coreceptors. Second, the precursor frequency of antigen-specific T-cells plays a critical role.[4,5] And third, protection requires functional competence of T-cells, which depends on intermediate-to-high functional avidity and additional parameters. Attempts for a more complete characterization of correlates of protection have focused on direct *ex vivo* functional profiling of T-cells.[6,7] These studies have indicated that strong cytotoxic activity and production of multiple cytokines correlates with improved disease outcome.[8,9]

Mouse models have suggested that tumor immunity may rely on similar principles as immunity to pathogens. Functional avidity appears to be essential, as well as the capacity of T-cells to recognize naturally processed and presented tumor antigens.[10] Moreover, the precursor frequency of naïve T-cells plays a key role.[11] Finally, it is important that T-cells are competent for proliferation, cell survival, homing, effector functions and generation of immunological memory.[8,12–14]

Activation of protective CD8 T-cells is best achieved by infection with natural pathogens. Among the commercially available vaccines, only live vaccines (*e.g.*, smallpox and yellow fever vaccines) are capable of inducing large numbers of pathogen-specific CD8 T-cells.[15] However, live vaccines are rarely available. As compared to natural infection, most

synthetic vaccines and cancer vaccines induce much weaker CD8 T-cell responses which are often unable to contain or eradicate tumors.[16] Among the available low-dose synthetic vaccines, formulations with antigenic peptides, CpG and IFA induce the strongest human CD8 T-cell responses.[17,18] CpG and IFA are also excellent adjuvants when using recombinant proteins instead of peptides, but CD8 T-cell responses are less potent.[19,20] In the present study, we analyzed CD8 T-cell responses of melanoma patients in unprecedented detail *ex vivo*, after vaccination with a low dose vaccine composed of Melan-A/MART-1 peptide and CpG 7909 (PF-3512676), emulsified in IFA. We have previously shown that this formulation induced high numbers of tumor antigen-specific T-cells in 8 of 8 HLA-A2^{pos} patients, and that the majority of the induced T-cells have high functional avidity and efficiently recognize tumor cells.[17] Here we confirm these findings in 24 patients and provide the first demonstration of tumor-specific CD8 T-cells that are multifunctional *in vivo*.

Methods

Patients, study protocol and eligibility criteria

This prospective Phase I clinical study (ClinicalTrials.gov Identifier NCT00112229) was performed by the Ludwig Institute for Cancer Research and the Multidisciplinary Oncology Center, upon approval by IRBs, Swissmedic and the LICR Protocol Review Committee. A total of 29 patients were included, and 24 patients were evaluable for this study, based on the availability of complete data sets and sufficient lymphocytes allowing direct *ex vivo* analysis of T-cell function. HLA-A2^{pos} patients with histologically proven metastatic (stage III/IV) melanoma of the skin expressing Melan-A/MART-1 (RT PCR or immuno-histochemistry) were included upon written informed consent. Inclusion criteria were: Karnofsky performance status of $\geq$70 %, normal CBC and kidney-liver function, no concomitant antitumor therapy or immunosuppressive drugs. Exclusion criteria were pregnancy, seropositivity for HIV-1 Ab or HBs Ag, uncontrolled bleeding, clinically significant autoimmune disease and symptomatic heart disease (NYHA III-IV). Study end points were toxicity and CD8 T-cell response.

Study treatment

Patients received monthly low dose vaccinations injected s.c., composed of CpG 7909 (PF-3512676) oligonucleotides and Melan-A/MART-1 peptide, emulsified in Montanide ISA-51, as stable emulsion prepared in a syringe. Vaccines for patients indicated as "without CpG" were prepared similarly but without CpG. Phosphorothioate backbone CpG 7909 (PF-3512676) oligonucleotides (TCGTCGTTTTGTCGTTTTGTCGTT) were provided by Pfizer and Coley Pharmaceutical Group, Wellesley MA. Clinically graded peptides were provided by the LICR, Melbourne (Australia) and NY (USA). Adjuvant IFA (Incomplete Freund's Adjuvant; Montanide ISA-51) consisted of mineral oil (Drakeol) and anhydro mannitol octadecanoate (Seppic, Paris, France). All components were prepared under GMP conditions, and had no detectable endotoxin by LAL assay.

Blood cells, HLA-A2/peptide tetramers and cytokine analyses

Ficoll-Paque centrifuged PBMC ($1–2 \times 10^7$) were cryopreserved in RPMI 1640, 40% FCS and 10% DMSO. Phycoerythrin-labeled HLA-A*0201/Melan-A/MART-1 A27L peptide$_{26-35}$ (ELAGIGILTV) and HLA-A*0201/EBV lytic protein BMLF1$_{280-288}$ (GLCTLVAML) tetramers were prepared as descried.[17] Anti-CD8, -CD28 and anti-CCR7 mAbs were purchased from BD PharMingen (San Diego, CA), anti-CD45RA from Beckman Coulter, goat anti-rat IgG Ab (for indirect staining of CCR7; Caltag Laboratories). All tetramer and antibody batches were titrated to determine optimal reagent concentrations. For all functional studies, cells were kept overnight in RPMI 10% FCS at 37°C and 5% CO_2. Before staining, CD8^{pos} T-cells were enriched using a MiniMACS device (Miltenyi Biotec, Bergisch Gladbach, Germany) resulting in >90% CD3^{pos} CD8^{pos} cells. Cells (10^6) were incubated with tetramers (1 μg ml^{-1}, 60 min, 4°C) and then with antibodies (30 min, 4°C). For intracellular assessment of cytokines, 10^6 MiniMACS purified CD8^{pos} T-cells were first prestained with PE-labeled tetramers for 30 min at 4°C. Without this prestaining, the visualization of antigen-specific T-cells would be inefficient due to TCR downregulation (Supporting Information Fig. 1), precluding comprehensive quantification of functional cells among antigen-specific T-cell populations. Then, they (4×10^5 T-cells) were incubated with 2×10^5 T2 cells at 37°C for 4 hr with or without 10 μM peptide (Melan-A/MART-1 ELAGIGILTV, or EBV GLCTLVAML) and anti-CD107a^{FITC} (BD PharMingen), whereby 10 μg ml^{-1} Brefeldin A (Sigma, St. Louis, MO) was added for the last 3 hr, in culture medium consisting of IMDM supplemented with 0.55 mM Arg, 0.24 mM Asn, 1.5 mM Gln, and 8% pooled human A^{pos} serum ("complete medium"). T2 cells were used because of their enhanced stimulatory capacity related to membrane expression of costimulatory molecules, and TAP deficiency resulting in high density of specific pMHC complexes after peptide loading. Subsequently, cells were stained at 4°C with PE-labeled tetramers, anti-CD8, anti-CD28 and anti-CD45RA antibodies. For dead cell exclusion, cells were stained with live/dead fixable dead cell violet stain (Molecular Probes/Invitrogen). Then, cells were permeabilized with 0.1% saponin at 4°C, washed, and stained for 40 min with anti-IFNγ$^{PE\text{-}Cy7}$, anti-TNFα^{Alexa700} and anti-IL-2^{allophycocyanin} (all BD PharMingen). CD8^{pos} T-cells/sample (5×10^5) was acquired with a flow cytometry LSR II^{TM} machine. The cytometer performance was checked daily using the CST (BD^{TM} Cytometer Setup and Tracking) quality control beads system according to the manufacturer's instructions. All flow cytometer data of this study were analyzed with FlowJo^{TM} software (TreeStar). Results of tetramer^{pos} T-cells were calculated and are indicated in percentages of

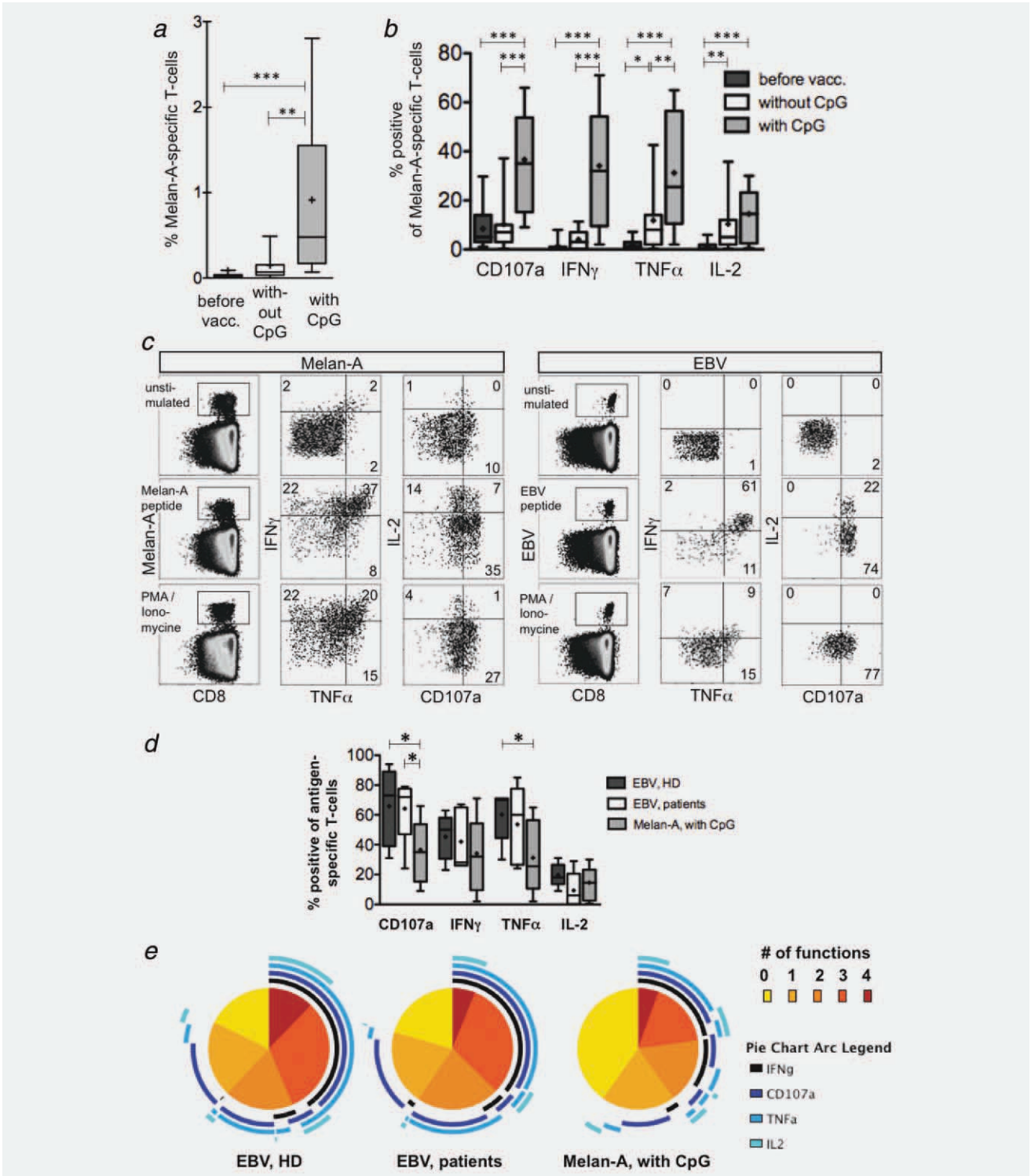

Figure 1. T-cell frequency, cytokine production and degranulation. Melanoma patients received monthly peptide vaccinations. PBMCs were prepared from blood withdrawn 1 week after booster vaccination, and analyzed by flow cytometry *ex vivo*. (*a*) Frequencies of tumor-specific T-cells before vaccination, after vaccination without CpG (11 patients), and with CpG (24 patients). (*b*) Direct *ex vivo* analysis of tetramer gated T-cells for cytokine production (IFNγ, TNFα, IL-2) and degranulation (CD107a/LAMP-1), after 4 hr incubation with peptide pulsed T2 cells. (*c*) Representative example dot plots. Left side: Melan-A-specific T-cells from patient LAU 1264 (2.88% A2/Melan-A tetramer^{pos} cells, withdrawn 1 week after four vaccinations); right side: EBV-specific T-cells from healthy donor (HD) BCL3 (0.23% A2/EBV tetramer^{pos} cells). (*d*) Comparison of tumor-specific T-cells (after vaccination with CpG) with EBV-specific T-cells from untreated HDs and patients. EBV (BMLF1) specific T-cells were analyzed from healthy donors (HD) and from the 24 patients after vaccination "with CpG" [the latter results are identical to (*b*)]. (*e*) Cytokine coexpression and degranulation was analyzed with SPICE software.[21] Data are from CD8^{pos} T-cells withdrawn after a mean of 6 ± 4 monthly vaccinations, at the time of (nearly) maximal frequency of A2/Melan-A tetramer^{pos} cells reached per patient (Supporting Information Table 1A). *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$; no symbol, not significant.

circulating $CD8^{pos}$ T-cells. Geometrical mean fluorescence intensity (GMFI) was used to quantify TCR and CD8 expression levels. To compensate for day-to-day variations, the values were normalized based on the GMFI of tetramer-negative CD8 T-cells.

Detection of pSTAT1 and pSTAT5

PBMCs were stained first with tetramers for 30 min, washed and stained with anti-CD4 and anti-CD8 mAbs for 20 min. Cells were resuspended in RPMI 10% FCS and left for 1 hr at 37°C and 5% CO_2. Then cells were stimulated for 15 min with either IL-2 (100 ng ml^{-1}) and IL-15 (10 ng ml^{-1}) or IFNα (1,000 U ml^{-1}) and IFNγ (1,000 U ml^{-1}). Samples were fixed by addition of equal volumes of fixing solution (1% formaldehyde, 2% glucose and 5 mM NaN_3) for 15 min at 37°C and 5% CO_2, washed and permeabilized with Perm Buffer III (BD Biosciences) for 30 min at 4°C. The samples were washed two times, resuspended in 15 μl staining buffer and 5 μl of each BD Phosflow anti-STAT1(pY701)-Alexa 647 and anti-STAT5(pY694)-A488 (BD Biosciences) were added for 30 min at 4°C. Finally, cells were washed and analyzed on a Gallios Flow cytometer (Beckman Coulter).

CD3 zeta chain analysis

CD8 T-cells were enriched using the CD8 MiniMACS system from Miltenyi. Isolated CD8 T-cells were stained first with tetramers for 30 min, washed and stained with anti-CD8 antibody for 20 min. To exclude dead cells, samples were incubated with live/dead fixable aqua stain (Invitrogen), followed by intracellular staining of CD3 zeta chain antibody/CD247 (AbD serotech) in presence of 0.1% saponin. As control we used the corresponding IgG2a isotype antibody. Samples were washed and analyzed on the Gallios Flow cytometer.

Tetraphosphoflow assay

We developed a novel technique for *ex vivo* detection of pERK1/2 in antigen-specific T-cells. CD8 T-cells were enriched using the CD8 MiniMACS system from Miltenyi, followed by staining first with tetramers for 30 min, washing and staining with anti-CD8 antibody for 20 min. Cells were cultured in RPMI 10% FCS for 1 h at 37°C and 5% CO_2. In the meantime, T2 stimulator cells were pulsed with 10 μM peptides for 1 hr, washed and resuspended in RPMI 10% FCS. CD8 T-cells were coincubated with T2 stimulator cells (after 1 min centrifugation) for 10 min, in absence or presence of IL-2 (100 ng ml^{-1}). As controls, CD8 T-cells were incubated with unpulsed T2 cells, or with 80 nM PMA. Samples were fixed immediately with BD Cytofix fixation buffer for 15 min at 37°C and 5% CO_2, washed and permeabilized with Perm Buffer III (BD Biosciences) for 30 min at 4°C. Samples were washed two times, resuspended in 20 μl staining buffer and 5 μl of BD Phosflow anti-ERK1/2 (pY202/pY204) antibody were added for 30 min at room temperature. Samples were washed and analyzed on a Gallios Flow cytometer.

Data analysis and statistics

Analysis of coproduction of cytokines and degranulation in Figure 1*e* was performed with SPICE software version 5.1.[21] With the aim of ranking patients with respect the multifunctional T-cells, we calculated a "functional T-cell score" based on the four functions analyzed (IFNγ, TNFα, IL-2 and CD107a). The formula was: score = 1×% cells with one function + 2×% cells with two functions + 4×% cells with three functions + 8×% cells with four functions. Statistical significance was assessed by the nonparametric Mann–Whitney test or the parametric student *t* test, where appropriate. *p* values less than 0.05 were considered significant. Box-and-whisker plot representations were designed with boxes indicating 25–75 percentiles, whiskers 10–90 percentiles, and horizontal bars and "+" within the box median and mean values, respectively.

Results

Functionally competent tumor-specific T-cells induced by vaccination with peptide and CpG

Melanoma patients received monthly s.c. injections of Melan-A/MART-1 $peptide_{26-35}$ and CpG, emulsified in IFA. This vaccine induced high frequencies of circulating Melan-A/MART-1 specific T-cells[17] (hereafter called "tumor-specific T-cells"). T-cell frequencies were very much higher than before vaccination, and also much higher (about 10×) than in patients vaccinated with the same peptide without CpG (Fig. 1*a* and Ref. [17]). To investigate the functional competence of T-cells, we performed multiparameter flow cytometry assays after 4 hr triggering with peptide, allowing simultaneous assessment of cytokine production and degranulation. Many tumor-specific T-cells were functionally competent, as they produced IFNγ, TNFα and IL-2, and upregulated CD107a (LAMP-1), a marker for degranulation and cytotoxicity[8,9] (Figs. 1*b* and 1*c* and Supporting Information Table 1A). T-cell function was highly significantly increased as compared to T-cells from before vaccination, and after vaccination without CpG (Fig. 1*b*). As a reference, we analyzed T-cells specific for the HLA-A2-restricted epitope GLCTLVAML derived from the EBV lytic protein BMLF1, and found relatively similar functional competence (Figs. 1*c* and 1*d* and Supporting Information Table 1B). IFNγ and IL-2 productions were similar, but degranulation and TNFα production were less frequent in tumor- than in virus-specific T-cells (Fig. 1*d*). Interestingly, the function of EBV-specific T-cells was similar in patients and untreated healthy donors (Fig. 1*d*), which was also revealed by the evaluation of cells with 0, 1, 2, 3 or all of the 4 functions analyzed (Fig. 1*e*). Typical for CD8 T-cells, IL-2 was less frequently produced, which was the case in both tumor- and virus-specific T-cells.

With the aim to obtain a single numeric value characterizing the functional competence of each T-cell population, we

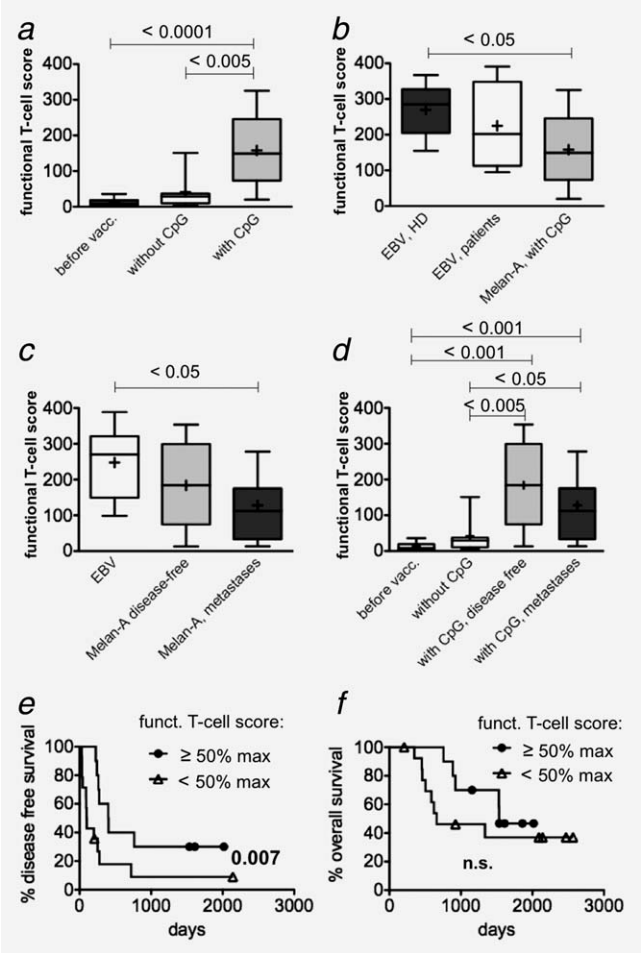

Figure 2. Comparisons based on a functional T-cell score, and correlation with patient survival. For each T-cell population, we determined a score, *i.e.*, a quantitative value for the combined IFNγ, TNFα, IL-2 and CD107a expression (described in Methods) (*a*,*b*) T-cell scores from patients and healthy donors, generated from the same data as shown in Figs. 1*b* and 1*d*, respectively, confirming the identified differences between cohorts. (*c*) Comparison of T-cells specific for EBV *vs.* Melan-A from disease-free patients ($n = 13$) and patients with metastases ($n = 11$). (*d*) Comparison of scores from Melan-A specific T-cells from patients "before vaccination," after vaccination "without CpG," and after vaccination "with CpG," depending on absence or presence of metastases at the time of T-cell analysis (from the same data as shown in Figs. 1*b* and 2*c*). (*e*,*f*) Kaplan–Meier curves for disease-free survival (*e*) and overall survival (*f*) from patients with T-cells scoring equal or above *vs.* below 50% of the highest score reached (10 *vs.* 14 patients, respectively). The 50% max score was 181 (Supporting Information Table 1). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; no symbol, not significant.

calculated a "functional T-cell score" (described in Methods). Using this score, the data again showed enhanced T-cell function when CpG was added to the vaccine formulation (Fig. 2*a*), confirming the results obtained for the four individual T-cell functions (Fig. 1*b*). The scores also confirmed that EBV-specific T-cells were of similar functional competence in healthy controls and patients, whereas the tumor-specific T-cells were slightly inferior (Fig. 2*b*). The individual patient data showed large interindividual differences, from strong to modest, and in one patient (LAU 936) even "nonfunctional" tumor-specific T-cells (Supporting Information Table 1). We analyzed patients without *vs.* with metastases at the time of T-cell analysis, and found that only the latter had lower scores of tumor-specific T-cells when compared to virus-specific T-cells (Fig. 2*c*). Therefore, the overall slightly inferior functionality of tumor- as compared to EBV-specific T-cells (Fig. 1*d*) was likely due to the reduced function of tumor-specific T-cells from tumor bearing patients (Fig. 2*c*). However, after CpG-based vaccination even these patients generated T-cells with highly significantly enhanced function as compared to before vaccination, and to patients after vaccination without CpG (Fig. 2*d*). Finally, we analyzed the patient's clinical outcome, and found that disease-free survival was significantly increased in patients who had T-cells above the half maximal score (Fig. 2*e*). The results for overall survival of patients pointed in the same direction, but the difference to patients with T-cells below the half maximal score did not reach statistical significance (Fig. 2*f*).

Frequencies and percentages of $CD28^{neg}$ Melan-A/MART-1 specific T-cells correlate with function

Strong CD8 T-cell responses are known to be associated with enhanced effector cell differentiation and function.[15] Not surprisingly, we found that patients exhibiting high percentages (>0.5%) of tumor-specific T-cells had significantly enhanced functions on a per T-cell basis, for CD107a, IFNγ and TNFα. By contrast, IL-2 production was similar to patients reaching only lower (<0.5%) percentages of tumor-specific T-cells (Fig. 3*a*). Effector cell differentiation can be assessed by changes of cell surface receptors. We have previously shown that most antigen-specific T-cells became $CCR7^{neg}$ $CD45RA^{neg}$ effector memory cells after vaccination with peptide and CpG.[17] More extended phenotyping revealed that variable proportions of vaccine responding T-cells were $CD28^{neg}$ (Fig. 3*b*) which is characteristic for advanced differentiation of virus-specific CD8 T-cells.[22] This is not usually observed after T-cell vaccination, except after vaccination with peptide and CpG. When comparing patients with strong T-cell responses (>0.5% of tumor-specific cells) with those with weaker T-cell responses, we found that the former contained significantly higher proportions of $CD28^{neg}$ cells (Fig. 3*b*), which were more often positive for CD107a and IFNγ, and a similar trend for TNFα (Fig. 3*c*), confirming strong effector function for $CD28^{neg}$ cells. Finally, we analyzed TCR and CD8 expression levels. As compared to $CD28^{pos}$ cells, $CD28^{neg}$ cells showed similar TCR but lower CD8 expression, possibly related to their enhanced function associated with more pronounced "tuning" *via* CD8 downregulation *in vivo*.[23]

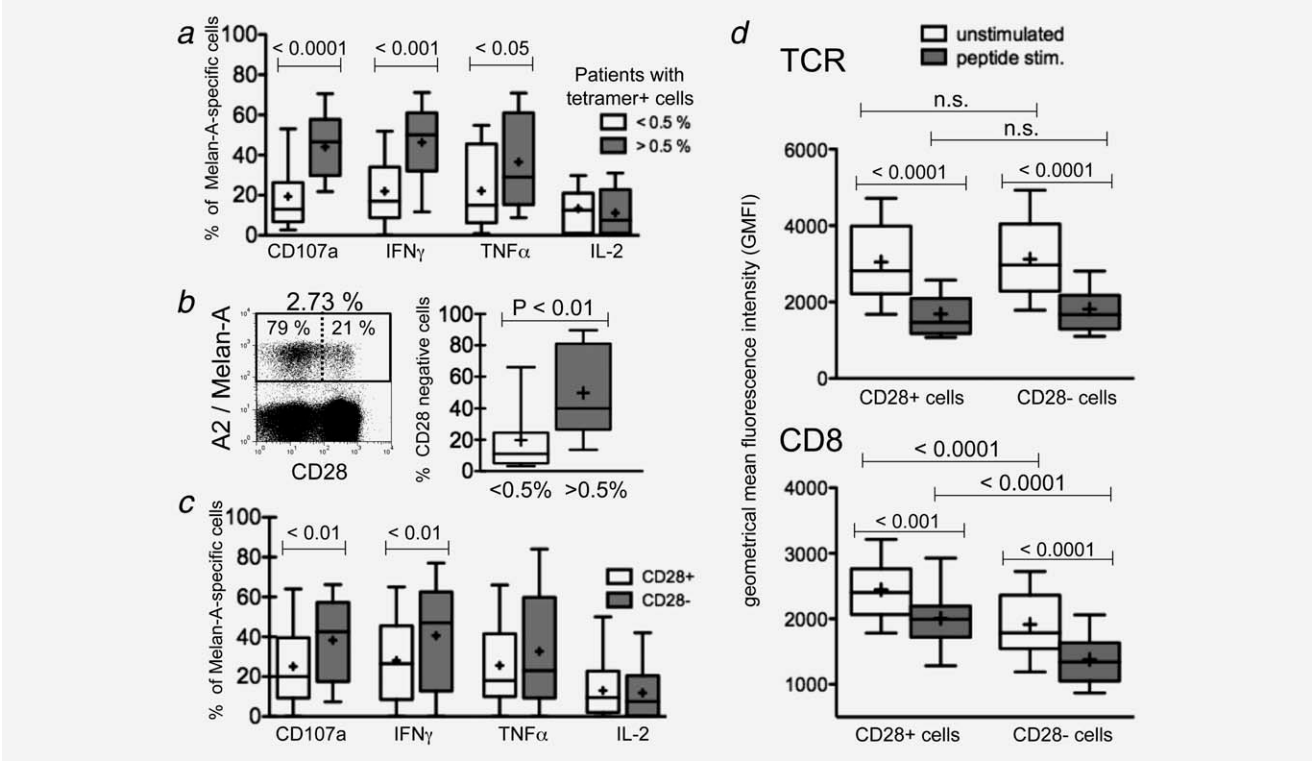

Figure 3. Enhanced function of strongly expanded T-cells, concomitant with increased percentages of $CD28^{neg}$ T-cells. (*a*) Comparison of function [degranulation (CD107a) and production of cytokines] by T-cells from patients with low (<0.5%) *vs.* high (>0.5%) percentages of A2/Melan-A $tetramer^{pos}$ T-cells in PBMC. (*b*) Example dot plot from $CD8^{pos}$ gated T-cells from patient LAU 1106, with 2.73% A2/Melan-A $tetramer^{pos}$ T-cells, including the two subpopulations of $CD28^{neg}$ (79%) and $CD28^{pos}$ (21%) cells. Significantly increased ($p < 0.01$) frequency of $CD28^{neg}$ cells among A2/Melan-A $tetramer^{pos}$ T-cells in patients with high frequencies (>0.5%) of A2/Melan-A $tetramer^{pos}$ T-cells. (*c*) Comparison of function by $CD28^{pos}$ *vs.* $CD28^{neg}$ T-cells. (*d*) Mean fluorescence intensity of TCR (tetramer) and CD8, expressed by $CD28^{pos}$ and $CD28^{neg}$ Melan-A/MART-1 specific T-cells. Although desirable, such analysis (*e.g.*, of subpopulations of antigen-specific T-cells) from before immunotherapy and after vaccination without CpG was not possible because of low T-cell frequencies.

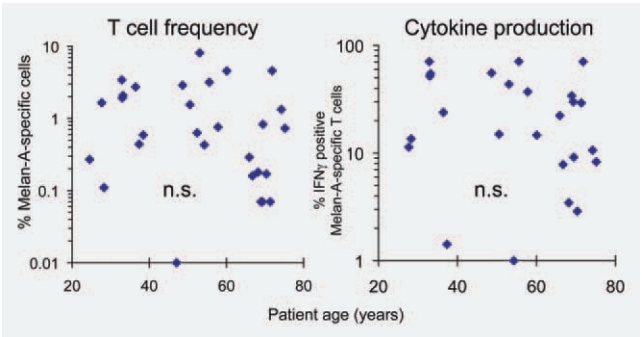

Figure 4. T-cell frequency and function in relation to patient age. T-cell frequency and function are shown at the time point of maximally reached % A2/Melan-A $tetramer^{pos}$ T-cells per patient, *i.e.*, after a mean of 8 (±5) monthly vaccinations. Patient age did also not correlate with the other three functional parameters, *i.e.*, CD107a, TNFα and IL-2 of Melan-A specific T-cells (not shown). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

After 4-hr triggering with peptide, we found significant downregulation, which was evident for both TCR and CD8, and similar for $CD28^{pos}$ and $CD28^{neg}$ T-cells (Fig. 3*d*).

T-cell responses independent of patient age and gender

Previous studies have suggested that T-cell responses decline with age.[24] Because our study population varied largely with respect to patient age, it was possible to analyze its impact. However, we found no significant correlations with frequency of tumor-specific T-cells after vaccination with CpG, IFA and peptide. Similarly, we found no significant differences of T-cell function between younger and older individuals (Fig. 4). Finally, patient gender did not correlate with frequency and function of tumor-specific T-cells (not shown).

Efficient phosphorylation of STAT1 and STAT5

Previous studies have shown that T-cells of cancer patients may have functional defects in cytokine signaling.[25,26] Because STATs play a central role,[27] we analyzed STAT phosphorylation after 15-min stimulation with the common-γ chain cytokines IL-2 and IL-15. Interestingly, tumor-specific T-cells after vaccination with CpG showed similar frequencies of STAT5(pY694) positive cells as EBV-specific cells (Figs. 5*a* and 5*c*). Total CD8 T-cells from patients were slightly lower, and CD4 T-cells higher. As expected, this

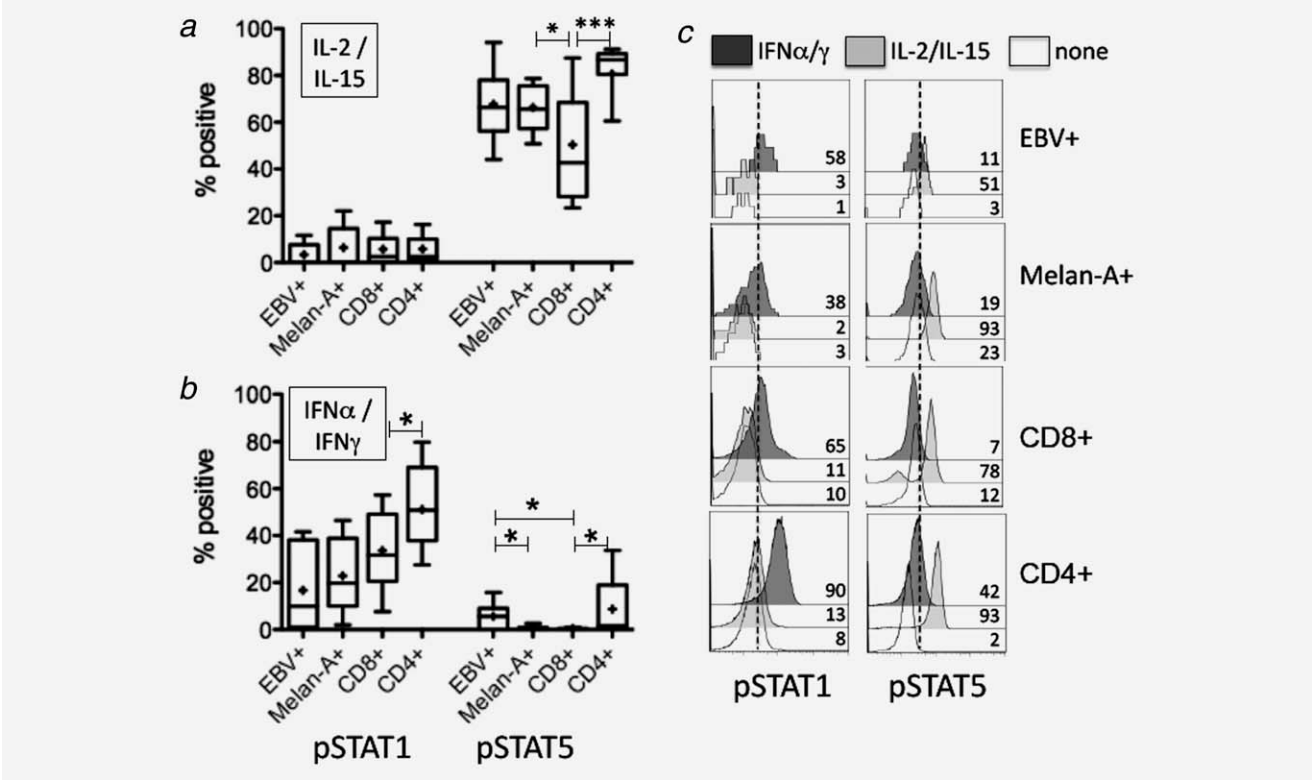

Figure 5. STAT phosphorylation. Percent positive cells after 15 min stimulation with (*a*,*c*) IL-2 plus IL-15, or (*b*,*c*) IFNα plus IFNγ, as described in Methods. Data on tumor-specific, and total CD8+ and CD4+ T-cells are from 19 patients, and on EBV-specific CD8+ T-cells from four healthy donors and three patients, *i.e.*, whenever enough cells were available for analysis. (*c*) Representative histograms from patient LAU 972 and HD BCL3 (for EBV), with indication of percent positive cells. No significant differences were found in EBV-specific T-cells from untreated HD *vs.* patients (not shown).

stimulation with IL-2/IL-15 induced stronger phosphorylation of STAT5 (pY694) than STAT1 (pY701). In contrast, stimulation with IFNα and IFNγ triggered stronger phosphorylation of STAT1 than STAT5 (Figs. 5*b* and *c*). There was no difference between EBV- and tumor-specific T-cells. Together, these data demonstrate intact STAT1/5 signaling by tumor antigen-specific T-cells after vaccination with peptide and CpG. Note that with the exception of data labeled with "before vaccination" or with "without CpG" (Figs. 1*a* and 1*b*, 2*a* and 2*d*), all data for tumor-specific T-cells in this article are from the 24 evaluable patients vaccinated with CpG. Although we attempted to also characterize T-cells from patients before vaccination or after vaccination without CpG, there were not enough tumor-specific T-cells available for performing all the functional assays used in this study. These restrictions apply also to most other studies of T-cell based therapy in humans.[28]

Expression of CD3 zeta chain and phosphorylation of ERK1/2

T-cells from cancer patients have been suggested to express abnormally low levels of the CD3 zeta chain,[29] which was however not confirmed in another study.[30] We found similar intracellular CD3 zeta chain expression by total CD8 T-cells and tumor- and virus-specific T-cells (Figs. 6*a* and 6*b*). In turn, the importance of the MAP kinase for T-cell responses is well known.[31] Therefore, we developed a novel "tetraphosphoflow assay" combining the known phosphoflow technology[32] with tetramers, allowing the identification of ERK1/2 (pY202/pY204) positive cells. Ten minutes after stimulation with peptide, distinct populations of pERK1/2^{pos} T-cells were detected with remarkably bright staining (Figs. 6*c* and 6*d*). Earlier and later time points showed lower percentages and intensities for pERK1/2 staining (data not shown). Both tumor-specific and EBV-specific T-cells were significantly more frequently pERK1/2^{pos} after stimulation with cognate peptide antigen as compared to unstimulated CD8 T-cells. Comparable results were obtained upon addition of IL-2; there was a trend for increased phosphorylation in EBV-specific T-cells which was however not significant. Control stimulation with PMA showed significantly higher percentages of pERK1/2^{pos} cells, likely due to its powerful triggering of multiple signaling pathways. Together, our data suggest efficient TCR signaling, consistent with the overall data showing functional competence of tumor-specific T-cells after peptide/CpG vaccination.

Discussion

Multifunctional cancer-specific human T-cells have been described previously, but after ten or more days of *in vitro*

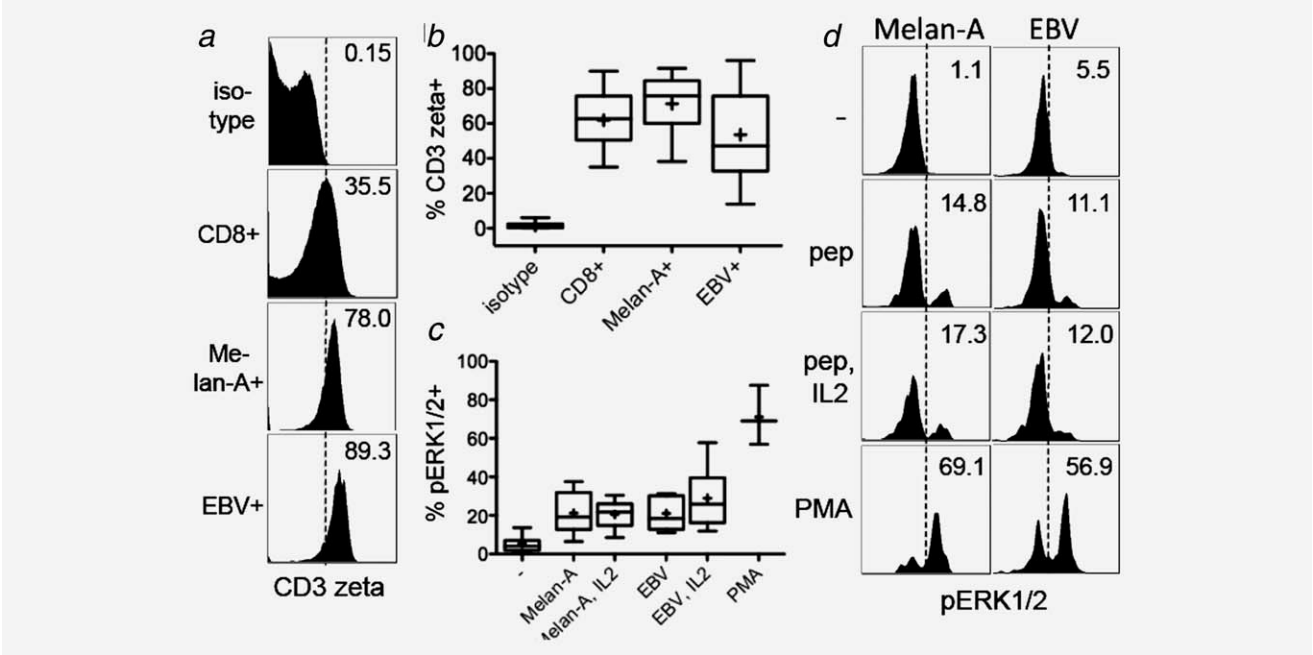

Figure 6. Expression of CD3 zeta and phosphorylation of ERK1/2. (*a*,*b*) Staining for CD3 zeta chain was done *ex vivo*, after cell membrane permeabilization, and analyzed by gating on total CD8+ T-cells and T-cells positive for A2/Melan-A and A2/EBV tetramers. (*a*) Representative histograms from patient LAU 1106 and HD BCL3 (for EBV). (*b*) Percent CD3 zeta positive cells from 16 patients and 5 healthy donors. Isotype control values were statistically significantly lower ($p < 0.001$) than CD8+, Melan-A and EBV-specific T-cells; no significant differences between the latter three. (*c*,*d*) To determine ERK phosphorylation, T-cells were analyzed *ex vivo*, by a novel "tetraphosphoflow assay." (*c*) Percentages of phosphoERK1/2+ T-cells after 10-min incubation with unlabelled T2 cells ("-"), or T2 cells pulsed with cognate peptide, in absence or presence of IL-2 (100 ng ml^{-1}). Data are from 14 patients and 11 healthy donors. The values from Columns 2, 3, 4, and 5 are statistically significantly higher than Column 1 ($p < 0.001$), and lower than Column 6 ($p < 0.05$). (*d*) Representative histograms from patient LAU 1106 for Melan-A (left) and HD BCL7 for EBV-specific T-cells (right), with indication of percentages of pERK1/2 positive cells. No significant differences were found in EBV-specific T-cells from HD *vs.* patients (not shown). All the data in this article are from experiments performed directly *ex vivo*, *i.e.*, with T-cells without keeping them in culture for (many) days.

stimulation.[33] In contrast, multifunctional virus-specific T-cells have been shown by direct *ex vivo* analyses.[34] With the latter approach, we show here multiple functions of human tumor-specific CD8 T-cells in most patients of our study. In disease-free patients, T-cell functions were comparable to virus-specific CD8 T-cells, suggesting protective potential. The present data are in agreement with our previous report[17] that vaccination with peptide, CpG and IFA induced higher CD8 T-cell numbers and more pronounced effector differentiation than vaccination with DNA, recombinant viral vectors, or peptides and proteins in vaccine formulations without CpG.[22] Following multiple booster vaccinations, T-cell responses were maintained at high levels over extended periods of time, confirming previous data on cancer-specific human CD8 T-cells.[35,36]

The functionality on a per T-cell basis was increased in patients with high T-cell frequencies, as observed previously in mice and humans.[35,37] Moreover, high frequency T-cell populations exhibited high proportions of $CD28^{neg}$ cells, which showed enhanced effector function as compared to $CD28^{pos}$ cells (the latter including memory cells), indicating that $CD28^{neg}$ populations are enriched for effector cells. Strong and continued T-cell activity was associated with persistence of codominant clonotypes, which were not limited by replicative senescence or functional alterations ([36] and manuscript in preparation). Thus, strong T-cell responses dominated by advanced differentiated $CD28^{neg}$ T-cells may be highly functional and can persist over many years.[35,36]

It has been suggested that increased incidence of infection and cancer of elderly individuals may be related to functional T-cell deficiencies.[24] A recent mouse study indicated that the appropriate choice of state-of-the-art vaccine adjuvants may overcome such deficits.[38] Indeed, our findings of vigorous self/tumor-specific T-cell responses even in elderly patients confirm that adjuvants such as CpG successfully promote strong T-cell responses independent of age.

We analyzed STAT phosphorylation, since recent studies had demonstrated defective signaling upon stimulation with interferon[25] and with IL-2[26] in circulating total CD8 and CD4 T-cells from melanoma patients. In contrast, our data revealed similar STAT phosphorylation in tumor- and virus-specific T-cells. Furthermore, functional T-cell competence depends on intact TCR signaling, whereby the MAP kinase pathway plays a major role.[31] Our novel method allowing

identification of pERK1/2 positive cells among antigen-specific T-cells revealed similar results in tumor- and virus-specific T-cells. Thus, after CpG based vaccination, these signaling events appear intact. However, direct comparison with antigen-specific T-cells from nonvaccinated cancer patients is still lacking, due to the limited availability of un-manipulated cells, *i.e.*, cells that have not been cultured for days or weeks. Further technical improvements are necessary for comprehensive characterization of signaling pathways in small numbers of tumor-specific T-cells. In general, many more studies are required for understanding T-cell signaling, including its differences between humans and mice.[31]

With regard to clinical results, we found that T-cell function correlated significantly with disease-free survival of patients. A trend was also found for overall survival, but without reaching statistical significance. However, our study was a Phase I clinical trial and not randomized, indicating that these findings represent preliminary evidence. Nevertheless, our data are compatible with the notion that functionally competent tumor-specific CD8 T-cells may be clinical meaningful.

Patients with massive tumor burden are less likely to respond to immunotherapy,[39] a notion that becomes increasingly acknowledged together with identification of various mechanisms used by tumors to suppress anti-tumor immune responses.[40–42] Therefore, immunotherapy may preferentially be successful in patients with minimal residual disease, after early diagnosis and/or after complete surgical tumor resection. Although the majority of our patients had functionally competent T-cells, CD8 T-cells showed tendencies of lower cytokine production and degranulation in patients bearing metastases as compared to disease-free patients. Future studies will focus on patient heterogeneity and identify mechanisms responsible for the impaired T-cell responses.[40–42] In this regard, more detailed characterization of immune cell function is necessary not only for circulating cells, but also for cells directly isolated from tumor tissue. These aims were beyond the scope of the present manuscript and require collection of large amounts of tumor tissue.

We have previously reported significantly different T-cell responses in patients after vaccination with the analog E*L*A-GIGILTV decapeptide as opposed to the natural EAAGI-GILTV decapeptide. The analog peptide induced frequencies of Melan-A/MART-1 specific T-cells that were about twice as high than vaccination with the natural peptide. In contrast, the natural peptide triggered T-cells with higher functional avidity TCRs and enhanced IFNγ production.[43] However, a minority of patients responded only very weakly to the natural peptide. In this study, 10/24 patients were vaccinated with the natural, and 14/24 patients with the analog peptide (Supporting Information Table 1A). It was important to determine whether T-cell function depended on vaccination with natural *vs.* analog peptide. However, this was not the case, since degranulation and cytokine production were similar in the two patient groups (Supporting Information Table 1A). This was not surprising, since functional testing by triggering the T-cells with the analog Melan-A peptide does not reveal significant differences, whereas testing with natural peptide demonstrates superior functionality after vaccination with natural as opposed to analog vaccination.[43] In this study we used the analog peptide for functional testing because of the increased peptide/MHC stability important for assay reproducibility. Unfortunately, analyzing all patients and samples in all functional assays with both analog and natural peptide was not possible due to the limited availability of cells. Therefore, we currently develop novel molecular and functional assays allowing the reliable assessment of T-cell function with small cell numbers. These assays will be applied for all patients with available cells. Thus, it remains to be determined whether vaccination with the natural peptide results in superior T-cell function beyond the known enhanced IFNγ production.[43]

The natural and analog peptides represent very highly cross-reactive epitopes compatible with the similar configuration of the two peptide/MHC complexes.[44] We generated >1,000 T-cell clones, and did not find a single clone binding tetramers constructed with one but not the other peptide/MHC complex.[43,45] Similarly, cross-reactivity was always observed in functional assays, despite the quantitative differences found in peptide titration studies, with enhanced recognition of target cells labeled with natural peptide by T-cells from patients after vaccination with natural peptide.[43] Nevertheless, this system reveals a very high degree of cross-reactivity to self/tumor antigen. The characterization of fine differences of T-cell receptors and T-cell function after vaccination with natural *vs.* analog peptide remains challenging,[45] but is important in order to determine whether analog peptide vaccination remains an option in patients not responding to the natural peptide, or for enhanced boosting after priming with natural peptide.

Our findings of functionally competent self/tumor-specific T-cells are in disagreement with results showing that mechanisms of self-tolerance may impair T-cell function.[46,47] T-cells with high affinity TCRs to self are eliminated in the thymus, which is likely also happening to some Melan-A/MART-1 specific T-cells,[35] resulting in deficiency of high affinity TCRs. We continue to study the TCR repertoires of human cancer-specific T-cells; including the concern that immunotherapy may activate low affinity T-cells that are unable to recognize naturally presented antigen. However, the majority of T-cells analyzed in this study have competent TCRs capable to recognize tumor cells.[43] Furthermore, recent studies suggest that low/intermediate avidity T-cells may have protective potential.[48] Many studies have shown that T-cells may be tolerized in the periphery,[49] *e.g.*, by mechanisms of anergy and/or deletion of self-specific T-cells. Very recently, we demonstrated that circulating Melan-A/MART-1 specific T-cells had a gene expression profile of functional effector cells after vaccination with CpG, which was largely different in melanoma metastases where they showed significant

molecular hallmarks of exhausted cells.[50] In contrast, after vaccination without CpG or in absence of immunotherapy, T-cells were poorly functional. Thus, self/tumor-specific T-cells may be functionally deficient due to insufficient T-cell stimulation, which is unfortunately the case for the majority of human T-cell vaccines, hence the need for improvement to achieve therapeutic efficacy.[15] Our data provide the rational basis for Phase III clinical trials with efficient T-cell vaccines, with the aim to determine whether this type of immunotherapy is indeed capable of improving clinical outcomes.

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