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Rapid and continued T cell differentiation into long-term effector and memory stem cells in vaccinated melanoma patients

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Abbreviations: IFA, Incomplete Freund's Adjuvant; TCR; T cell receptor; EM, effector-memory; EAA, Melan-A$^{MART\text{-}1}{}_{26\text{-}35}$ native peptide EAAGIGILTV; ELA, Melan-A$^{MART\text{-}1}{}_{26\text{-}35\ (A27L)}$ analog peptide ELAGIGILTV

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POG, PB, AH, EMI. Acquired and analyzed the data: POG, PB, DES and NR. Wrote the manuscript: POG, DES and NR.

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TRANSLATIONAL RELEVANCE

CD8 T lymphocytes bear high potential for destroying human cancers, as demonstrated by the recent great progress in immunotherapy. Robust T cell responses depend both on memory cells with long-term survival, self-renewal abilities and high proliferative potential, and on effector cells successfully migrating to disease sites and performing essential effector functions. We previously showed that melanoma patients treated with peptide/CpG-B/IFA vaccine mounted strong effector T cell responses. In the present study, we characterized the kinetics of these responses, and the underlying memory cells, with the aim to improve our understanding of anti-tumor T cell mediated immunity. We found that the vaccine-induced T cell responses occurred rapidly, leading to robust and long-lasting expansion of effector cells, displaying potent functions. In parallel, stem cell-like memory cells steadily expanded over time along vaccination, forming the basis for strong and long-term T cell responses and supporting the further development of CpG-B based cancer vaccines.

ABSTRACT

Purpose: Cancer patients benefit increasingly from T cell-based therapies, such as adoptive T cell transfer, checkpoint blockade or vaccination. We have previously shown that serial vaccinations with Melan-$A^{MART\text{-}1}_{26\text{-}35}$ peptide, CpG-B and IFA generated robust tumor-specific CD8 T cell responses in melanoma patients. Here, we describe the detailed kinetics of early- and long-term establishment of T cell frequency, differentiation (into memory and effector cells), poly-functionality and clonotype repertoire induced by vaccination.

Experimental design: Twenty-nine melanoma patients were treated with multiple monthly subcutaneous vaccinations consisting of CpG-B, and either the native/EAA (n = 13) or the analog/ELA (n = 16) Melan-$A^{MART\text{-}1}_{26\text{-}35}$ peptide emulsified in IFA. Phenotypes and functionality of circulating Melan-A-specific CD8 T cells were assessed directly *ex vivo* by multiparameter flow cytometry, and TCR clonotypes were determined *ex vivo* by mRNA transcript analyses of individually sorted cells.

Results: Our results highlight the determining impact of the initial vaccine injections on the rapid and strong induction of differentiated effector T cells in both patient cohorts. Moreover, long-term poly-functional effector T cell responses were associated with expansion of stem cell-like memory T cells over time along vaccination. Dominant TCR clonotypes emerged early and persisted throughout the entire period of observation. Interestingly, one highly dominant clonotype was found shared between memory and effector subsets.

Conclusions: Peptide/CpG-B/IFA vaccination induced powerful long-term T cell responses with robust effector cells and stem cell-like memory cells. These results support the further development of CpG-B based cancer vaccines, either alone or as specific component of combination therapies.

INTRODUCTION

The overarching goal of cancer immunotherapy is to generate a strong and persistent anti-tumor immune response leading to tumor growth control and elimination, while overcoming immune tolerance and suppression. Most immunotherapy-induced protective immune responses against cancer rely on CD8 T cells and their specific recognition, through the T cell receptor (TCR), of tumor-antigenic peptide presented on the cell surface by major histocompatibility complex I (MHC-I) molecules. Therapeutic peptide vaccines, in combinations with potent adjuvants, have been developed to induce CD8 T cell responses against the tumor by administering peptides mimicking the epitopes presented by MHC-I on cancer cells (1, 2). Over the last decade, a large number of clinical trials have demonstrated that this immunotherapeutic approach is feasible and safe, and may lead to increased frequencies, differentiation and survival of tumor-specific CD8 T cells (3-9) and eventually clinical efficacy (10). However, the majority of cancer vaccine trials failed (2), highlighting the strong need for more powerful vaccines and/or combinations with other effective agents such as immune checkpoint inhibitory antibodies (11, 12).

Antigenic peptides are poorly immunogenic by themselves. Therefore, vaccines containing synthetic peptides need to be administrated in conjunction to potent adjuvants. First, effective adjuvants should display a depot effect leading to prolonged antigen exposure allowing for efficient priming of the antigen-specific T cells (1, 2). Emulsifying agents such as mineral oils for emulsion formation, also defined as incomplete Freund's adjuvant (Montanide ISA-51; hereafter "IFA") are widely used for that purpose (13-16). Second, adjuvants should trigger the activation and maturation of dendritic cells (DCs) by innate immune stimulation e.g. via Toll-like receptors (TLRs) (1, 2). For instance, synthetic CpG oligodeoxynucleotides (ODNs) favor DCs activation and migration through TLR9 triggering, promoting the development of strong T helper cell responses, and supporting both antibody and CD8 T cell responses (17, 18). Previous clinical studies demonstrate that the CpG B-ODN 7909/PF-3512676 could elicit a strong adjuvant effect when combined with IFA and

HLA-A2 restricted Melan-$^{MART\text{-}1}_{26\text{-}35}$ (19) or NY-ESO-$1_{157\text{-}165}$ (20, 21) peptides, promoting the expansion of *ex vivo* detectable tumor-specific CD8 T cells in melanoma patients.

Analysis of the generation, function and long-term persistence of effector and memory CD8 T lymphocytes is of fundamental importance to our understanding of protective immunity and to improve T cell-based therapeutic strategies. While the properties of effector CD8 T cells are well characterized (22), the attributes constituting potent memory T cells remain less well understood. Recently, a rare subset of CD8 T stem cell-like memory (T_{SCM}) cells was characterized in pre-clinical models and in cancer patients (23, 24) and showed to possess enhanced potential of proliferation and persistence/survival as well as the capacity to produce large numbers of effector cell progeny of various differentiation states (23). Consequently, adoptive transfer of T_{SCM} cells shows promising results of tumor regression in mice (23, 25). The *in vivo* induction of T_{SCM} cells frequently occurs in natural infections such as Influenza, CMV, EBV, and HIV-1 (23, 26, 27). Furthermore, those vaccines capable of inducing strong CD8 T cell responses, namely the few existing live attenuated vaccines, may readily induce T_{SCM} cells, as demonstrated for the Yellow Fever vaccine 17D (26, 28). In contrast, subunit vaccines and cancer vaccines have not yet been shown to induce T_{SCM} cells.

Over the years, we studied thoroughly the impact of peptide-based therapeutic vaccination on the quality of the CD8 T cell responses in melanoma patients. We demonstrated that repeated vaccinations with peptides, CpG-B and IFA induced tumor-specific CD8 T cells with high functionality *in vivo* (29). Moreover, we showed that vaccination with a low dose of native Melan-A$^{MART\text{-}1}_{26\text{-}35}$ peptide (hereafter “native/EAA”) induced CD8 T cells with stronger tumor reactivity, increased polyfunctionality and higher TCR-pMHC structural avidity, compared to vaccination with the analog A27L peptide (hereafter “analog/ELA”) (30-33).

Here, we present a comprehensive and detailed analysis of the kinetics of anti-tumor-specific CD8 T cell responses from the previously published phase I study (29) with 29 melanoma patients vaccinated with low dose of either the native/EAA (n = 13) or the

analog/ELA (n = 16) Melan-$A^{MART\text{-}1}_{26\text{-}35}$ peptide mixed with CpG-B and IFA. Our data demonstrate an early and strong effect of this vaccine approach on tumor-specific CD8 T cells, with increased *in vivo* frequencies, differentiation to effector-memory cells, and acquisition of effector functions in nearly all patients of both cohorts, i.e. irrespectively of the peptide used for vaccination. However, native peptide vaccination predominantly promoted a stable and persisting dominant TCR clonotype repertoire. Interestingly, the robust and long-term effector T cell responses were associated with stem cell-like memory cells detectable at baseline and steadily expanding with vaccination. Collectively, our data support the further development of phase III trials for assessing the clinical efficacy of CpG-based cancer vaccines and understanding the interdependence of key differentiation subsets of responding CD8 T cells.

PATIENTS AND METHODS

Patients, vaccination, and blood cell preparation

HLA-A*0201-positive patients with stage III/IV metastatic melanoma were included in a phase I prospective trial (ClinicalTrials.gov; Identifier: NCT00112229) (19, 30). Study protocols were designed, approved and conducted according to the relevant regulatory standards from (i) the ethical commission of the University of Lausanne (Lausanne, Switzerland), (ii) the Protocol Review Committee of the Ludwig Institute for Cancer Research (New-York) and (iii) Swissmedic (Bern, Switzerland). Patient recruitment, study procedures and blood withdrawal were carried out upon written informed consent prior to study inclusion. Primary endpoints were safety and tolerability, as well as detailed measurements of tumor-specific CD8 T cell responses over time.

Eligible patients received monthly low-dose vaccinations injected subcutaneously with 100 µg of either the unmodified native Melan-$A^{MART\text{-}1}{}_{26\text{-}35}$ peptide (EAAGIGILTV, native/EAA) or its heteroclitic analog Melan-$A^{MART\text{-}1}{}_{26\text{-}35\ (A27L)}$ peptide (ELAGIGILTV, analog/ELA), mixed with 0.5 mg CpG-B 7909 / PF-3512676 (Pfizer and Coley Pharmaceutical Group) and emulsified in Incomplete Freund's Adjuvant (IFA) (Montanide ISA-51; Seppic) (19). Half of the patients also received the $Tyrosinase_{368\text{-}376}$ (YMDGTMSQV) peptide (Supplementary Table S1). The responses of T cells specific for this peptide remained much lower than the responses to Melan-A peptide, precluding detailed phenotypic and functional analyses. Moreover, we did not observe any significant impact of vaccination with the tyrosinase peptide on the responses of Melan-A specific cells (data not shown). Lymphoprep (Axis-Shieldy) centrifuged peripheral blood mononuclear cells (PBMC) were cryopreserved in RPMI 1640, 40% fetal calf serum (FCS) and 10% DMSO before transfer into liquid nitrogen until further use.

Flow cytometry and direct ex vivo cell sorting

After thawing in a 37˚C water bath, PBMC were enriched immediately using anti-CD8-coated magnetic microbeads positive selection and a MiniMACS device (Miltenyi Biotech) resulting in > 90% CD3+/CD8+ cells. Purified CD8 T cells were stained with HLA-A*0201 analog/ELA Melan-$A^{MART\text{-}1}_{26\text{-}35\ (A27L)}$ multimers (TCMetrix Sàrl) in FACS sorting buffer composed of PBS, 0.2% BSA, 50 µM EDTA for 45 min at 4˚C and then with appropriate antibodies as described in the Supplementary Materials and Methods section. Tumor-specific CD8 T cells were analyzed on a LCRII cytometer (BD Biosciences) or a Gallios flow cytometer (Beckman Coulter).

Following cell surface marker and dead cell exclusion marker staining, individual or five-cell aliquots from multimer+ CD8+ T cells were directly sorted *ex vivo* into defined sub-populations of CD45RA+CCR7+, early-differentiated effector-memory CD28+ (EM28+) and late-differentiated CD28- (EM28-) using a BD FACSAria cytometer (BD Biosciences) or Astrios cytometer (Beckman Coulter).

IFNγ Elispot assay

Plates were coated overnight with human IFNγ-specific antibodies (Diaclone, Biotest) and 1.66 x 10^5 PBMC per well in 200 µl of complete medium and 10 µg/ml peptide were incubated for 16 h at 37°C (19). Assays were performed in six replicates, without peptide, or with native/EAA peptides. Cells were removed, and plates developed with a second (biotinylated) antibody to human IFNγ and streptavidin-alkaline phosphatase (Diaclone, Biotest, Switzerland). The spots were revealed with BCIP/NBT substrate (Sigma Tablets) and counted with an automatic reader (Bioreader 2000, BioSys GmbH). Percentage CD3+/CD8+ cells in PBMC was determined by flow cytometry on the same batch of cryopreserved cells. Results of both multimer+ T cells and Elispot forming T cells were calculated and are indicated in percentages of CD8+ T cells.

Stimulation for intracellular cytokine staining

Functional assays using peptide-pulsed T2 cells were performed as previously described (29). After thawing in a 37˚C water bath, PBMC were rested overnight in RPMI supplemented with 10% FCS, 10 mM Hepes, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% non-essential amino acids and 1% Na Pyruvate (Gibco Life Technologies) at a density of 1.5 $x10^6$ PBMC/ml. The next day, PBMC were positively enriched using anti-CD8-coated magnetic microbeads and a MiniMACS device (Miltenyi Biotech) resulting in > 90% CD3+/CD8+ cells. Cells were stained with PE-labeled analog/ELA multimers (TCMetrix Sàrl) in FACS sorting buffer composed of PBS, 0.2% BSA, 2 mM EDTA for 30 min at 4˚C. After washing, 100'000 CD8+ T cells were incubated in a 96-well V-bottom plate with FITC anti-CD107a, 10 µg/ml Brefeldin A (Sigma) and TAP-/- deficient T2 cells (HLA-A*0201+/Melan-A-) previously pulsed with 1 µg/ml of the native/EAA peptide with an effector-to-target ratio of 1:1. A "no peptide" control and stimulation with 1 µg/ml PMA (Sigma) and 0.25 µg/ml Ionomycin (Sigma) were used as negative and positive controls, respectively. After 4 hr, cells were harvested, stained with the analog/ELA multimers and subsequently with the aforementioned surface markers in 50 µl of FACS buffer (PBS, 5 mM EDTA (Gibco), 0.2% BSA (Merck) and 0.2% NaN_3 (Merck)) for 30 min at 4°C, followed by dead cell staining (Life Technologies) for 30 min. After this step, cells were fixed with 1% paraformaldehyde, 2% glucose and 5 mM NaN_3 in PBS at 4˚C overnight before intracellular staining using Alexa700 anti-IFNγ (Biolegends) at 4˚C during 30 min in FACS buffer and 0.1% saponin (Sigma).

Global cDNA preparation and amplification

Tumor-specific CD8 T cells of defined subsets were sorted directly *ex vivo* as single or five cell aliquots in 96-well V-bottom plates containing a "lysis/RT" mix and cDNA preparation and global cDNA amplification were performed as previously described (32). Briefly, following RT at 37˚C for 60 min, cDNA was transferred in 600 µl eppendorfs and precipitated overnight. The precipitated cDNA was resuspended in a homopolymeric 3'-oligo (dA) tailing reaction mix (Promega), which was followed by global cDNA amplification using oligo-(dT)

Iscove 61-mer primer. The resulting amplified cDNA was then analyzed by semi-quantitative PCR for TCR repertoire analyses.

TCR repertoire and clonotype analyses

CDR3 spectratyping, sequencing and clonotyping were performed as described (33, 34). Briefly, each cDNA sample was subjected to individual PCR using a set of previously validated fluorescent-labeled forward primers specific for the different known TRBV subfamilies and one unlabeled reverse primer specific for the corresponding C beta gene segment. Additionally, we characterized the alpha-chain repertoire by targeting the highly dominant TRAV12-1 sequence. PCR products visualized after electrophoresis on a 2.5% agarose gel and PCR products of interest were sequenced from the reverse primer (Fasteris SA). TRAV and TRBV segments were described according to the Lefranc nomenclature (35).

Statistical analyses

Data were analyzed using GraphPad Prism (v.6, GraphPad) by non-parametric Wilcoxon matched-pairs signed rank test, Mann-Whitney U-test and Spearman’s correlations as indicated throughout the manuscript.

Laboratory Environment

This work was conducted under GLP principles. The laboratory uses qualifies assays and participates in external Elispot and flow cytometry proficiency panels.

RESULTS

Patient characteristics and safety

In this study, 29 HLA-A2-positive patients with metastatic melanoma received serial monthly vaccines containing CpG-B, IFA and either the unmodified native Melan-A$^{MART\text{-}1}{}_{26\text{-}35}$ peptide (native/EAA) or its heteroclitic analog Melan-A$^{MART\text{-}1}{}_{26\text{-}35\ A27L}$ peptide (analog/ELA). Vaccination was done in cycles of 4 monthly injections, with 4-12 weeks intervals between cycles (Supplementary Fig. S1A). The patient's characteristics are shown in Supplementary Table S1 and S2. Vaccinations were well tolerated with side-effects severity comparable to previous reports on cancer vaccines (Supplementary Table S3). The main adverse effect being inflammatory granuloma at s.c. injection sites reported in 8 patients (27.6%), which was expected as IFA is documented to cause local inflammatory depots at the vaccination site allowing long-term local vaccine persistence and immune stimulation.

Rapid *in vivo* expansion of Melan-A-specific CD8 T cell responses following vaccination

The goal of this study was to characterize the detailed kinetics of the Melan-A$^{MART\text{-}1}$-specific CD8 T cell response following peptide vaccination with regards to T cell frequency, differentiation, functionality and composition of the TCR repertoire. From the *ex vivo* immuno-monitoring data with multimers, we could observe an important and significant increase in Melan-A-specific CD8 T cell frequencies following vaccination in all patients (Fig. 1A and B), as previously described on a smaller patient group (30). Prior to vaccination, tumor-specific CD8 T cells were present at low frequencies (<0.01% to 0.24% of total CD8 T cells), with the exception of patient LAU444 who had a frequency of 1.37% multimer+ CD8 T cells at the start of vaccination. The induction of Melan-A-specific CD8 T cells was readily significant in both cohorts of patients after only two vaccine injections (Supplementary Fig. S1B and C). There was also a trend for higher maximum multimer frequencies for patients

vaccinated with the analog/ELA (1.9% ± 2.1) than the native/EAA (1.1% ± 1.6) peptide (p = 0.055, Mann-Whitney U-test, Fig. 1B), which was already observed after two vaccines (p = 0.0176, Mann-Whitney U-test; Supplementary Fig. S1D).

Strong CD8 T cell differentiation occurs early after the start of vaccination

We characterized the differentiation of Melan-A-specific CD8 T cells based on the expression of CD45RA and CCR7, to discriminate between “naïve-like” (CD45RA+/CCR7+), central-memory (CM, CD45RA-/CCR7+), effector-memory (EM, CD45RA-/CCR7-) and effector-memory CD45RA+ (EMRA, CD45RA+/CCR7-) CD8 T cells (Fig. 1C). Prior to vaccination, considerable proportions of Melan-A-specific CD8 T cells displayed a naïve-like phenotype. Following two vaccine injections, the relative percentages of these T cells rapidly decreased due to the frequency increase of differentiated Melan-A-specific CD8 T cells, dominated by EM cells (Fig. 1D). We could also observe a continuous increase in differentiated multimer+/CD28- CD8 T cells. On average, the frequencies of CM and EMRA cells remained relatively constant throughout the monitoring period, even though some patients showed considerable changes. Together, these data demonstrate a strong effect of peptide/CpG/IFA vaccination with increased *in vivo* frequencies and phenotypic shift to differentiated EM cells, rapidly following the initiation of treatment, leading to their persistence for many months. Importantly, this vaccine-induced effect occurred in all patients, regardless of the peptide used for vaccination.

Enrichment of Melan-A-specific stem cell-like memory CD8 T cells along vaccination

Stem cell-like memory T cells (T_{SCM}) have been described as T cells possessing enhanced persistence and renewal capacities, which would endow them with very long-term therapeutic potential (23, 24, 36). Such cells have been described in infectious disease and after vaccination with live attenuated replication competent viruses. In contrast, they have

never been shown to be induced in patients by a cancer vaccine. Consequently, we analyzed T_{SCM} in subsets of our patients vaccinated with the native/EAA (n = 4) or the analog/ELA (n = 4) peptide (Fig. 2). Melan-A-specific T_{SCM} were defined as CD45RA+/CCR7+/CD95+, as characterized previously (36) (Fig. 2A). Prior to vaccination, the frequencies (Fig. 2B) and total numbers (Fig. 2C) of T_{SCM} cells varied from patient to patient, but no statistical differences were found between patients vaccinated with native/EAA and analog/ELA peptides (data not shown). Interestingly, vaccination led to significant expansion over time of T_{SCM} cells, associated to the enhanced frequencies or maintenance of Melan-A-specific T cells observed at late time-points (Supplementary Fig. S2A and S2B). The total numbers of T_{SCM} cells were also significantly enhanced late (> 4 vaccines and > 6 months) but not early (2-4 vaccines and ≤ 3 months) after the start of vaccination (Fig. 2C). We further stained for CD11a, also shown to be expressed by T_{SCM} (23), and found a similar increase in T_{SCM} cells upon vaccination (Fig. 2D; Supplementary Fig. S2C). Collectively, our data show for the first time that a cancer vaccine could expand tumor-specific CD8 T_{SCM} cells, supporting long-lasting memory T cell responses.

Early induction of effector function following vaccination

We monitored IFNγ spot forming Melan-A specific CD8 T cells triggered with the native/EAA peptide in a 16h *ex vivo* assay, in all 29 patients throughout the vaccination period. Again, we observed an increase early after the start of vaccination in both native/EAA and analog/ELA vaccinated patients (Fig. 3A and B), which was only significant for the latter patients (Fig. 3C). There was no significant differences in the maximum fold induction between the native/EAA and analog/ELA vaccinated cohorts, nor in the kinetics of induction. We did observe a strong correlation between IFNγ producing and multimer+ Melan-A-specific CD8 T cells frequencies (Fig. 3D), confirming that higher frequencies of antigen-specific CD8 T cells generate more spots.

Vaccination rapidly promotes functionally matured T cell subpopulations

Next, we analyzed the *ex vivo* functionality of circulating tumor-specific CD8 T cells by flow cytometry and intracellular cytokine staining (ICS) after short-term stimulation with T2 cells pulsed with the native/EAA peptide. We characterized CD107a and IFNγ expression, within three subsets of effector-memory CD8 T cell differentiation: EM28+, EM28- and $EMRA^{INT}$ (Fig. 4 with the gating strategy in Supplementary Fig. S3). For all patients analyzed (EAA; n = 4 and ELA; n = 5), we selected samples from pre-vaccine, early and late time-points. Strikingly, we found rapid and strong acquisition of effector functions following vaccination (pre-vacc vs early time-points) in the three characterized CD8 T cell subpopulations of almost all patients (Fig. 4A). Both native/EAA and analog/ELA peptide vaccination promoted the differentiation of effector cells expressing CD107a and/or IFNγ early after treatment. At late time-points, the frequencies of CD107a+, IFNγ+, and dual CD107a+/IFNγ+ Melan-A-specific CD8 T cells either plateaued or decreased. Increasing functional differences towards the native/EAA vaccinated patients were observed over time when comparing the Melan-A-specific $EMRA^{INT}$ CD8 T subsets from both cohorts of patients (Fig. 4B). These results support our previously published work demonstrating that the native/EAA peptide vaccine favors the differentiation of Melan-A-specific CD8 T cells with increased effector functions compared to the analog/ELA peptide vaccine (30-33). Importantly, our data indicate that the quality of effector functions (i.e. polyfunctionality) is rapidly determined after the initiation of vaccination.

Furthermore, we found that the early-differentiated EM28+ T cells rapidly acquired effector competence in line to the kinetics observed for the late-differentiated EM28- and $EMRA^{INT}$ T cells (Fig. 4A). Taking into consideration the expression of CD127 (the cytokine receptor IL-7Rα) in longitudinal analyses, we further observed that vaccination induced the fast differentiation of EM28+/CD127+ to EM28+/CD127- CD8 T cells (Supplementary Fig. S4A and B). This was more prominent for the native/EAA peptide vaccinated patients. When functional differences were evaluated along T cell differentiation, we found that Melan-A-

specific CD8 T cells were progressively capable to express CD107a and IFNγ from EM28+/CD127+ to EM28+/CD127- to EM28-/CD127- and to $EMRA^{INT}$ (Supplementary Fig. S4C and D). This was true for both early and late time-points and for both the native/EAA and analog/ELA peptide vaccinated cohorts, albeit the acquisition of effector functions was always more pronounced for the native/EAA patients.

Native peptide vaccination predominantly promotes a stable and persisting dominant TCR clonotype repertoire

We previously demonstrated minor differences between the clonotype repertoires of native/EAA and analog/ELA vaccinated patients (34) and similar effector profiles of the non-dominant and dominant clonotypes (31). In this study, our goal was to further understand whether the choice of peptide used during vaccination impacted the kinetics of the establishment of a dominant TRBV clonotype repertoire. We performed TRBV spectratyping at the single cell level on tumor-specific CD8 T cells sorted at early versus late time-points from four native/EAA- and three analog/ELA-vaccinated patients (Supplementary Fig. S5). Our single cell data highlighted a relatively high level of plasticity and diversity, which was patient specific (Fig. 5A and B). Comparable TRBV efficiencies were obtained between native/EAA and analog/ELA vaccinated cohorts (Fig. 5C). We could detect dominant clonotypes in all patients and time-points (Fig. 5D). However, these T cells emerged earlier and were more frequently identified in the native peptide-induced vaccination when compared to the analog peptide (Fig. 5D, Supplementary Fig. S6). Interestingly, some dominant clonotypes persisted throughout the vaccination period while others appeared or disappeared over time. Extended clonotypic studies performed on a large library of *in vitro* generated T cell clones (n = 150; (33)) combined to the current single cell identified clonotype database (n = 479) revealed the predominance of a persisting dominant TCR clonotype repertoire after vaccination with the native/EAA peptide (Fig. 5E). Finally, one melanoma patient presented a uniquely strong frequency of one TRAV/TRBV clonotype,

365 which was highly dominant in the differentiated EM28- subset and persisted over time during
366 vaccination. Interestingly, this clonotype could also be detected in the pool of less-
367 differentiated EM28+ cells as well in the CD45RA+CCR7+ subset containing the T_{SCM} cells,
368 albeit at much lower frequencies (Fig. 5F). In conclusion, while dominant clonotypes were
369 present early and persisted throughout the entire monitored period, this was favored by the
370 native peptide vaccination.

371

372 **Clinical outcome**

373 We plotted survival curves, even though patient survival was not an endpoint of this phase I
374 trial. We did not observe statistically significant differences in progression-free survival (Fig
375 6A) nor overall survival (Fig. 6B) between the patients vaccinated with the native/EAA and
376 the analog/ELA peptides. In both groups, some patients survived for many years. Overall,
377 the clinical outcome was favorable when compared to other trials in similar patients (10), but
378 it remains important to note that comparisons with other trials does not allow to draw firm
379 conclusions, because of patient selection effects and other potentially confounding factors.

DISCUSSION

To date, there is good evidence that peptide-based vaccines can break immune tolerance and successfully induce tumor-reactive CD8 T cell responses in cancer patients (1, 2). However, a major challenge is to generate a robust and targeted immune response. In that regard, the formulation of CpG B-ODN 7909 with short peptides and IFA represents the currently most potent synthetic vaccine for the generation of high frequencies of tumor-specific CD8 T cells (19, 20, 30). Importantly, such vaccines can induce *in vivo* functionally competent T cells (29), correlating with a favorable clinical outcome (21). However, prospective phase III trials are necessary to demonstrate clinical benefit. Patients immunized with this vaccine also provide the unique opportunity to study the effects of early/initial versus late/sustained rounds of vaccination on (i) the T cell expansion and differentiation into effector and memory subsets and (ii) the TCR clonotype selection and maintenance over time.

Extending on previous observations (7, 8, 19, 20, 30), we first demonstrated the fast (after only 2 vaccine injections) and high magnitude of tumor-specific T cell responses in the large majority of the twenty-nine patients of the trial. This was associated with robust acquisition of effector functions detectable directly *ex vivo* (IFNγ production and CD107a upregulation), which also occurred rapidly during the first cycle of vaccination (between 2 to 4 vaccines and ≤3 months after the start of immunization). The addition of CpG-B to Montanide/IFA is likely essential, since CD8 T cell responses from patients vaccinated with Melan-A/peptide and IFA without CpG-B show much lower frequencies and slower kinetics of T cell responses (16, 19). In a recent preclinical mouse study, Perret and colleagues showed that CpGs preferentially amplify effector T cells over regulatory T cells (37). However, more studies are needed to further understand the precise role of CpGs as powerful adjuvants on the generation and maintenance of antigen-specific T cell responses.

We next assessed the impact of vaccination with peptide/CpG-B/IFA on the T cell priming and repertoire selection early after the start of vaccination and its evolution during repeated

vaccination cycles. We previously found that, at late time-points (i.e. >8 months after the start of vaccination), Melan-A-specific CD8 T cell responses were generally composed of highly (i.e. dominant) as well as of less frequent (i.e. non-dominant) T cell clonotypes (31). Herein, we show that rapid vaccine-induced T cell responses were likewise associated with the generation of patient-specific co-dominant TCR clonotypes. Strikingly, and despite some level of plasticity in the repertoire selection, many of the most prevalent TCR clonotypes appeared early (≤3 months after the start of vaccination) and persisted throughout the entire monitored period.

In line with these observations, individual T cell clonotypes with high avidity to cognate tumor antigens could be detected over extended periods of time in melanoma patients with favorable disease outcome (38, 39) as well as in a patient with a known pre-existing dominant clonotype, efficiently boosted by Melan-A peptide/CpG/IFA vaccination (40). Altogether, these data indicate that once established, the clonal composition of tumor-specific T cells can be kept stable along vaccination or in naturally occurring anti-cancer immune responses. Such long-lasting tumor-specific T cell clonotypes may play an important role in mediating tumor control and/or regression as exemplified in several adoptive T cell transfer trials (41-43) or following allogeneic hematopoietic stem cell transplantation (44, 45). In most studies, objective clinical responses positively correlated with the degree of persistence of transferred T cell clonotypes (41-43, 45).

A better understanding of the parameters influencing the *in vivo* selection and persistence of those dominant tumor-specific CD8 T cell clonotypes remains of the upmost importance. While this was not directly addressed in this report, preliminary data suggest that the relative avidity of the TCR-pMHC binding interactions could be involved. Using the new NTAmer-based technology, which quantifies the TCR-pMHC dissociation rates (46), we found that two of the three analyzed and long-term persisting clonotypes shared the highest binding avidity (33). Furthermore, the type of peptide (i.e. native/EAA versus analog/ELA) can induce different T cell responses with enhanced functional competence (31) and stronger

TCR repertoire avidity (33) at late time-points after vaccination with the native/EAA as opposed to the analog/ELA peptide. Here, we further report on the differential impact observed between both peptides with the predominance of a persisting dominant clonotype repertoire within native/EAA peptide vaccinated patients. Nevertheless, it still remains to be determined whether vaccination with the native/EAA peptide is also a contributing factor for the early selection of a superior TCR avidity repertoire.

It will also be important to validate the possibilities of priming with the native/EAA peptide to fix the CD8 T cell repertoire onto the highest avidity and boosting with the analog/ELA peptide to efficiently drive the expansion and differentiation of the primed clones (1). Alternatively, it is possible that priming with the analog/ELA peptide followed by boosting with the native/EAA peptide may be preferable, as the former is more likely to recruit large numbers of naïve precursor cells. However, the success of this approach may depend on subsequent avidity maturation, i.e. that the native/EAA peptide can selectively boost the high avidity clonotypes despite that the priming with the analog/ELA peptide had previously recruited and activated also many lower avidity tumor-specific T cells.

Finally, our results show for the first time that a subunit vaccine can lead to increased frequencies of stem cell-like memory T cells. As mentioned above, the vaccine formulation used in our study also induced strong and long lasting effector T cell responses, unlike most other cancer vaccines. Using the TCR as a clonotypic marker, we were further able to follow, along T cell differentiation, one individual tumor-specific CD8 T cell clonotype from melanoma patient LAU944. Remarkably, this particular clonotype dominated the differentiated effector-memory (EM28-) subset, and could readily be detected, though at much lower frequencies, in the CD45RA+CCR7+ compartment, known to include the T_{SCM} cells. These results are in line with our previous observations showing the co-existence of identical Melan-$^{MART\text{-}1}{}_{26\text{-}35}$ (31, 33) and NY-ESO-$1_{157\text{-}165}$ (39) -specific CD8 T cell clonotypes in early-differentiated EM28+ and late-differentiated EM28- subsets.

460 At present, it would be interesting to determine whether other identified clonotypes were
461 present within the T_{SCM} cells. However, such studies are likely only possible in those rare
462 patients with strong and almost monoclonal T cell responses. Moreover it would require
463 improving the efficiency of our single cell approach as well as much larger blood volumes
464 due to the very low frequencies of T_{SCM} cells. This last limitation also forced us to reduce our
465 T_{SCM} cell analysis to only a subset of the 29 patients of this trial, unfortunately precluding any
466 conclusions on the potential clinical benefits of T_{SCM} cells. Nevertheless, it seems not
467 surprising that strong effector cell responses were associated with relatively high (i.e.
468 detectable) T_{SCM} cell frequencies, likely because the latter may contribute to continued
469 effector cell production. Altogether, our current data support the notion that only a fraction of
470 the diverse pool of less differentiated EM28+ memory cells are selected to populate the
471 often-larger pool of differentiated EM28- T cell clonotypes. Whether those are the ones that
472 preferentially depend on T_{SCM} cells remains to be determined. In any case, our data
473 demonstrate that T_{SCM} cells are amplified by vaccination with CpG-B/peptide/IFA. Monitoring
474 T_{SCM} cells becomes increasingly important in the assessment of human immune responses
475 and evaluation of novel immunotherapy approaches (47).

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FIGURE LEGENDS

Figure 1. Frequencies and cell differentiation of circulating Melan-A-specific CD8 T cells following peptide vaccination detected directly *ex vivo.* (A, B) Blood samples of vaccinated melanoma patients were harvested before (0) and at regular time-points following vaccination. Melan-A-specific CD8 T cell frequencies were quantified *ex vivo* by multimer staining following CD8 enrichment. **(A)** Tumor-specific T cells for native/EAA (red, n = 13) and analog/ELA (blue, n = 16) vaccinated patients according to the vaccination cycle. Lines link samples from the same patient. **(B)** Maximum Melan-A-specific CD8 T cell frequencies reached during the study compared with pre-vaccination levels (native/EAA patient with red squares and analog/ELA patients with blue circles). *p*-values by Wilcoxon matched-pairs signed rank test. **(C, D)** Characterization of CD8 T cell differentiation by CCR7, CD45RA and CD28 expression: Naïve (CD45RA+/CCR7+), Central Memory (CM, CD45RA-/CCR7+), Effector Memory (EM, CD45RA-/CCR7-), Effector Memory CD45RA+ (EMRA, CD45RA+/CCR7-) and CD28- (Melan-A+/CD8+/CD28-). **(C)** Lines link samples from the same patient according to vaccination cycles. **(D)** Comparison of the Melan-A-specific CD8 T cell frequencies highlighting differences during the initial vaccine injections between native/EAA- and analog/ELA-vaccinated patients (red and blue bars, respectively). *p*-values by Mann-Whitney U-test.

Figure 2. Peptide vaccination promotes the *in vivo* differentiation and expansion of CD8 stem cell-like memory T cells over time. (A) Gating strategy for one patient at three time-points (pre-vaccination, early and late) for the characterization of tumor-specific CD8 T_{SCM} cells within enriched CD8 T cells (live cells, multimer+/CD3+, CD45RA+/CCR7+, and CD95+). **(B)** Frequencies according to the gated CD45RA+/CCR7+ subpopulation. **(C)** Counts according to 10E6 CD3+ T cells of CD8+multimer+ T_{SCM} cells. Comparisons between pre-vaccination and early (2-4 vaccines and ≤ 3 months; left panel) or late time-points (> 4 vaccines and > 6 months after the start of vaccination; right panel) are shown. **(B,**

C) *p*-values by Wilcoxon matched-pairs signed rank test. Native/EAA-vaccinated patients (n = 4; open squares) and analog/ELA-vaccinated patients (n = 4; black circles). **(D)** Characterization of tumor-specific CD8 T_{SCM} cells for one patient at three time-points (pre-vaccination, early and late) according to a second gating strategy (live cells, multimer+/CD8+, CD45RA+/CCR7+, and CD95+/CD11a+).

Figure 3. Quantification of IFNγ production by circulating Melan-A-specific CD8 T cell following peptide vaccination detected directly *ex vivo*. Mean IFNγ production quantified by Elispot following 16h incubation with the native/EAA peptide as described in Materials and Methods. Frequencies of IFNγ+ within Melan-A-specific CD8 T cells for native/EAA (red, left panel) and analog/ELA (blue, right panels) vaccinated patients according to **(A)** the vaccination cycle or **(B)** the number of vaccine injections. **(A)** Lines link samples from the same patient. **(B)** Mean (black line) frequencies of IFNγ+ Melan-A-specific CD8 T cells with each dot representing an individual patient. **(C)** Maximum IFNγ production reached during the study compared with pre-vaccination levels. Left panel; EAA patients, right panel; ELA patients. *p* values by Wilcoxon matched-pairs signed rank test. **(D)** Correlation between maximum IFNγ production (*x*-axis) and maximum Melan-A-specific CD8 T cell frequencies (*y*-axis) quantified during the study period. *r and p*-value by Spearman correlation. Native/EAA (red squares) and analog/ELA (blue circles) vaccinated patients.

Figure 4. Acquisition of effector functions occurs early following the start of peptide vaccination. (A) Kinetics of the acquisition of effector functions by flow cytometry data from *ex vivo* enriched CD8 T cells stimulated by T2 cells pulsed with the native/EAA peptide for 4 hr at 37˚C. The analysis is depicted for multimer+/CD8+ T cells in the EM28+, EM28- and $EMRA^{INT}$ subsets with regards to the expression of CD107a at the surface (left panels), intracellular IFNγ expression (middle panels) and dual CD107a+/IFNγ+ (right panels).

Native/EAA patients (n = 4, red lines) and analog/ELA patients (n = 5, blue lines). **(B)** Quantitative comparison of T cells from patients vaccinated with native/EAA peptide (n = 4, red bars) and analog/ELA (n = 5, blue bars) peptide, showing CD107a, IFNγ and dual CD107a/IFNγ expression found within the $EMRA^{INT}$ T cell subset at different time-points. Pre-vaccine; prior to vaccination, early; between 2 to 4 vaccines after the start of vaccination, and late time-points; >4 vaccines and >6 months after the start of vaccination. *p*-values by Mann-Whitney U-test. Of note, a significant increase in IFNγ and dual CD107a/IFNγ was observed at pre-vaccine time-points in the patients who received the analog/ELA peptide vaccine, which could eventually be explained by the fact that those patients did have more treatments prior to the start of vaccination than patients vaccinated with the native/EAA peptide (see Supplementary Table 1).

Figure 5. Early establishment and long-term persistence of dominant TRBV clonotypes following peptide vaccination. (A, B) Pie charts for three native/EAA **(A)** and three analog/ELA **(B)** vaccinated patients illustrating the specific PCR reactions performed against defined TRBV families and sequencing data obtained from *ex vivo* individual tumor-specific CD8 T cell samples at early (between 2 to 4 vaccines; n = 753) versus late (>8 months after the start of vaccination; n= 512) time-points. Dominant TRBV clonotypes are defined by identical BV-CDR3-BC and TRAV12-1 sequences and those found at >1 time-point are expanded from the main pie chart and described by a distinct color code. Single non-dominant clonotypes are depicted as dark gray. Un-identified TRBV clonotypes (depicted as light gray) represent single cell samples for which no positive TRBV sequence was found based on the selection of performed TRBV family PCRs. **(C)** Ratio of identified TRBV clonotypes versus total single cell samples analyzed and compared between tumor-specific CD8 T cells from native/EAA (red bar) and analog/ELA (blue bar) vaccinated patients. **(D)** Ex vivo quantification of the dominant TRBV clonotypes found within single cell samples of tumor-specific CD8 T cells in total (pooled early and late time-points), at early

and at late time-points. Native/EAA patients (n = 4, red bars) and analog/ELA patients (n = 3, blue bars). **(E)** Quantification of the persistence of dominant TRBV clonotypes found at early (between 2 to 4 vaccines) and late (>6 months after the start of vaccination) time-points and compared between native/EAA (n = 5) and analog/ELA (n = 6) vaccinated patients. *p* values by Mann-Whitney U-test. **(F)** Quantification of a dominant TRAV/BV clonotype from patient LAU944 among memory (i.e. CD45RA+CCR7+ or EM28+) and effector (EM28-) Melan-A-specific CD8 T cell subsets and over time following peptide/CpG/IFA vaccination, based on calculations from five-cell pools analyzed directly *ex vivo*.

Figure 6. Clinical outcome of the twenty-nine melanoma patients vaccinated with CpG-B and the native/EAA or the analog/ELA peptide, emulsified in IFA. (A, B) Kaplan-Meier analyses of **(A)** progression-free and **(B)** overall patient survival. Native/EAA patients (n = 13, red line) and analog/ELA patients (n = 16, blue line).

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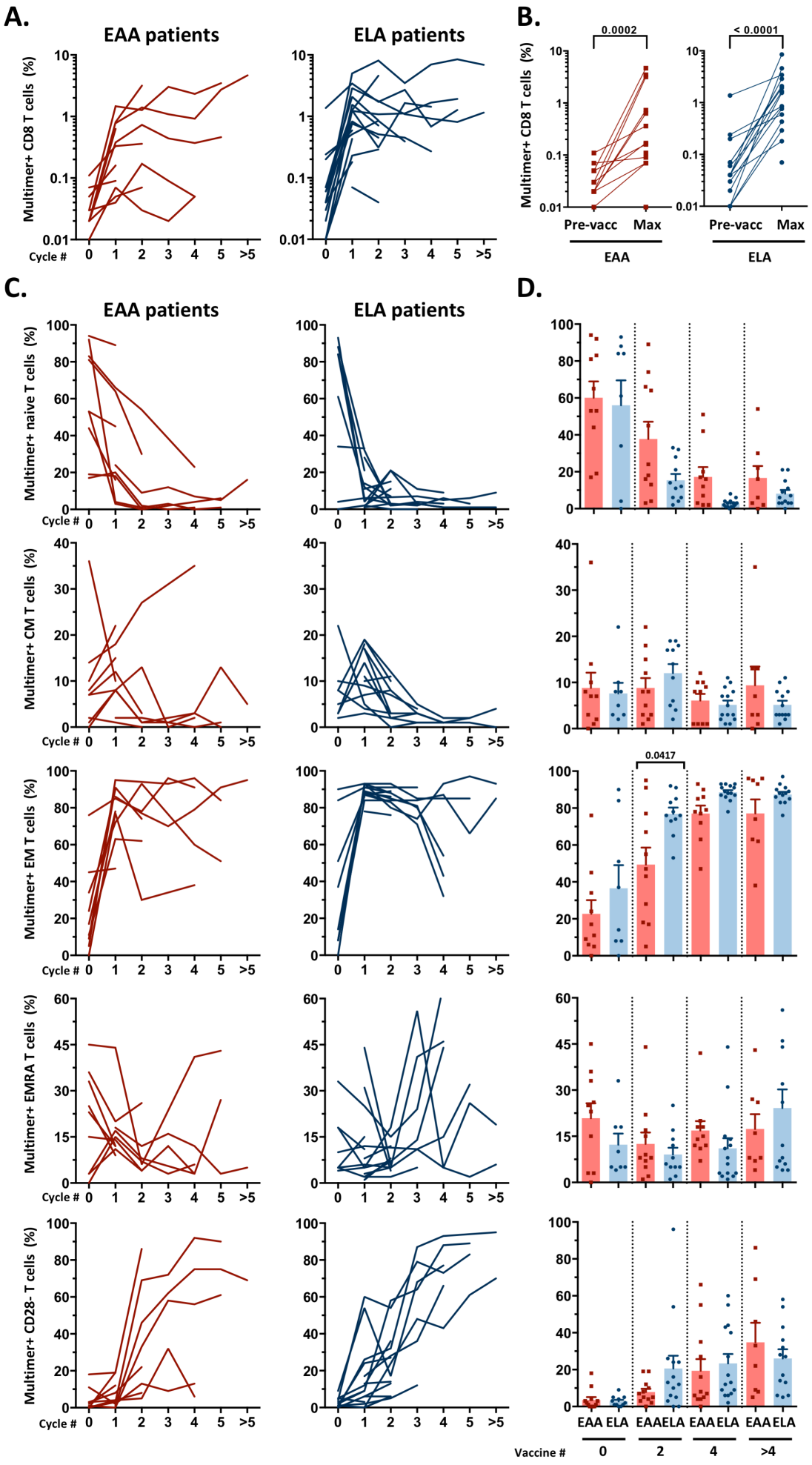
A.
EAA patients
ELA patients
Multimer+ CD8 T cells (%)
Cycle #
B.
0.0002
< 0.0001
Multimer+ CD8 T cells (%)
Pre-vacc
Max
EAA
ELA
C.
EAA patients
ELA patients
Multimer+ naive T cells (%)
Multimer+ CM T cells (%)
Multimer+ EM T cells (%)
Multimer+ EMRA T cells (%)
Multimer+ CD28- T cells (%)
Cycle #
D.
0.0417
EAA ELA EAA ELA EAA ELA EAA ELA
Vaccine #
0
2
4
>4

Figure 1.

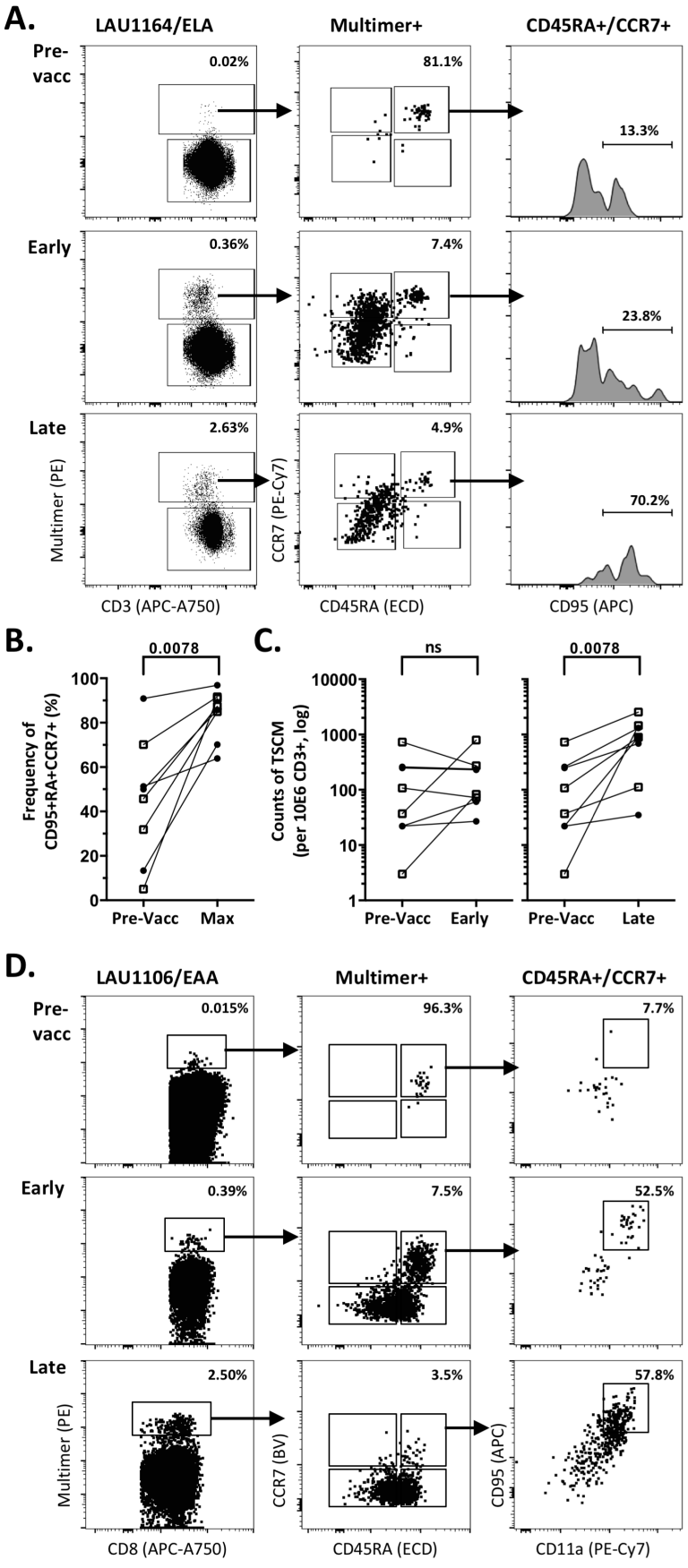
A.
LAU1164/ELA
Multimer+
CD45RA+/CCR7+
Pre-vacc
Early
Late
0.02%
81.1%
13.3%
0.36%
7.4%
23.8%
2.63%
4.9%
70.2%
Multimer (PE)
CCR7 (PE-Cy7)
CD3 (APC-A750)
CD45RA (ECD)
CD95 (APC)
B.
0.0078
Frequency of CD95+RA+CCR7+ (%)
Pre-Vacc
Max
C.
ns
0.0078
Counts of TSCM (per 10E6 CD3+, log)
Pre-Vacc
Early
Pre-Vacc
Late
D.
LAU1106/EAA
Multimer+
CD45RA+/CCR7+
Pre-vacc
Early
Late
0.015%
96.3%
7.7%
0.39%
7.5%
52.5%
2.50%
3.5%
57.8%
Multimer (PE)
CCR7 (BV)
CD95 (APC)
CD8 (APC-A750)
CD45RA (ECD)
CD11a (PE-Cy7)

Figure 2.

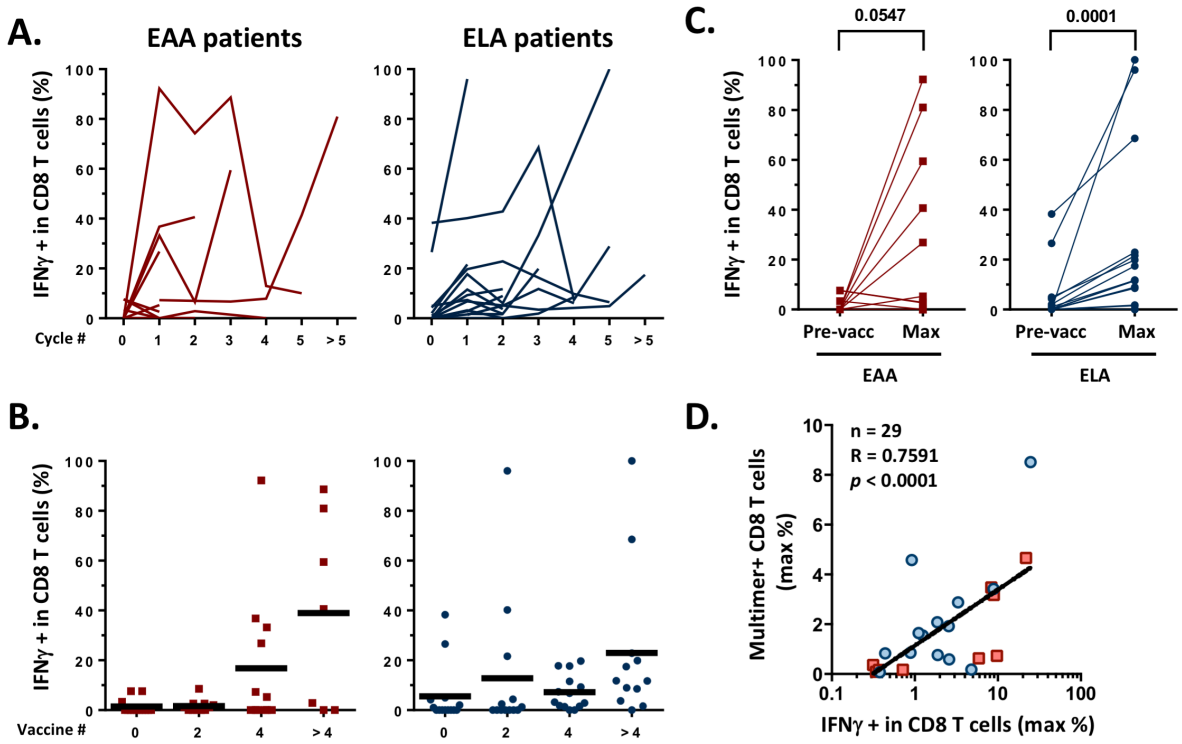
A.
EAA patients
ELA patients
IFNγ + in CD8 T cells (%)
Cycle #
0 1 2 3 4 5 > 5
B.
Vaccine #
0 2 4 > 4
C.
0.0547
0.0001
Pre-vacc
Max
EAA
ELA
D.
n = 29
R = 0.7591
p < 0.0001
Multimer+ CD8 T cells (max %)
IFNγ + in CD8 T cells (max %)

Figure 3.

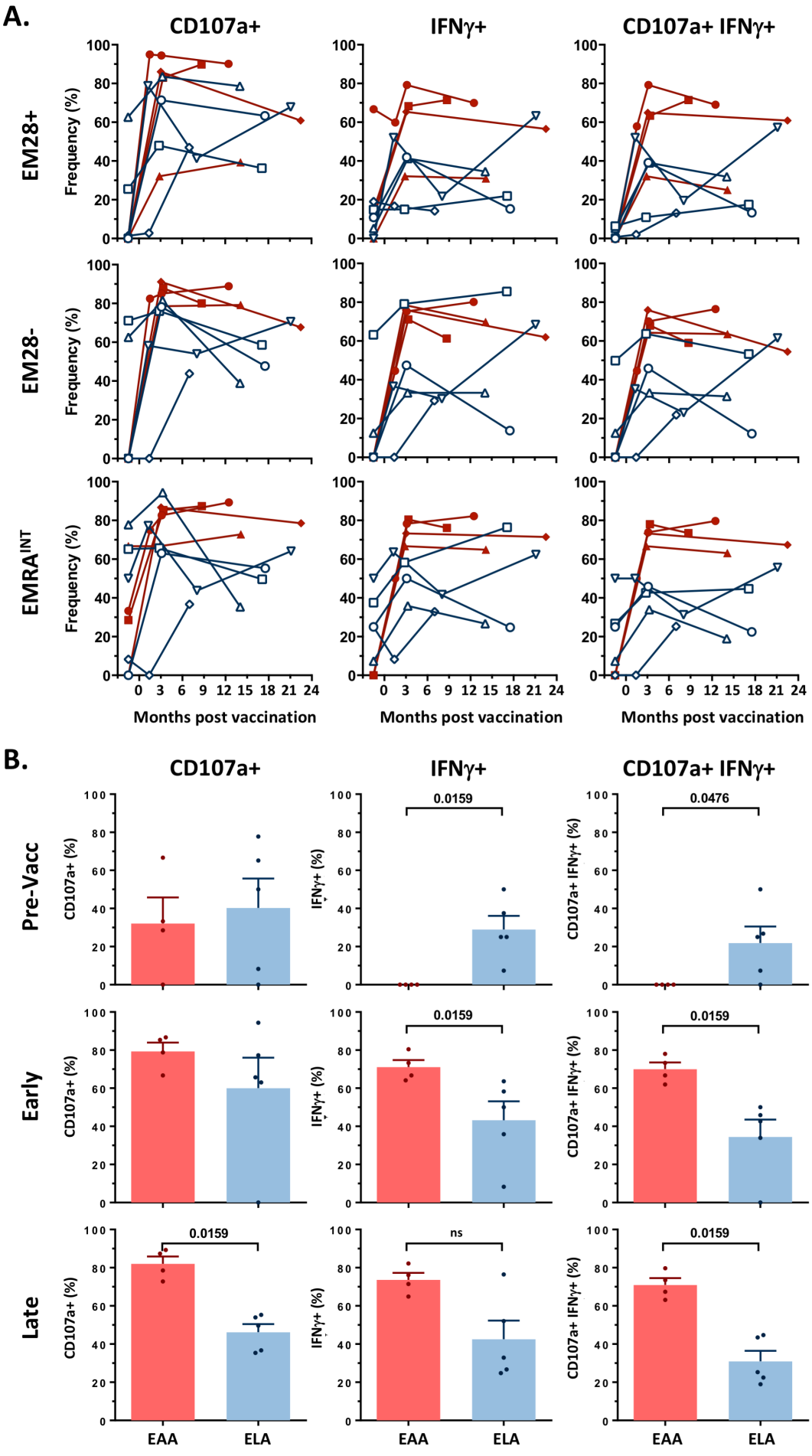
A.
CD107a+
IFNγ+
CD107a+ IFNγ+
EM28+
EM28-
EMRA INT
Frequency (%)
Months post vaccination
B.
CD107a+
IFNγ+
CD107a+ IFNγ+
Pre-Vacc
Early
Late
CD107a+ (%)
IFNγ+ (%)
CD107a+ IFNγ+ (%)
0.0159
0.0476
ns
EAA
ELA

Figure 4.

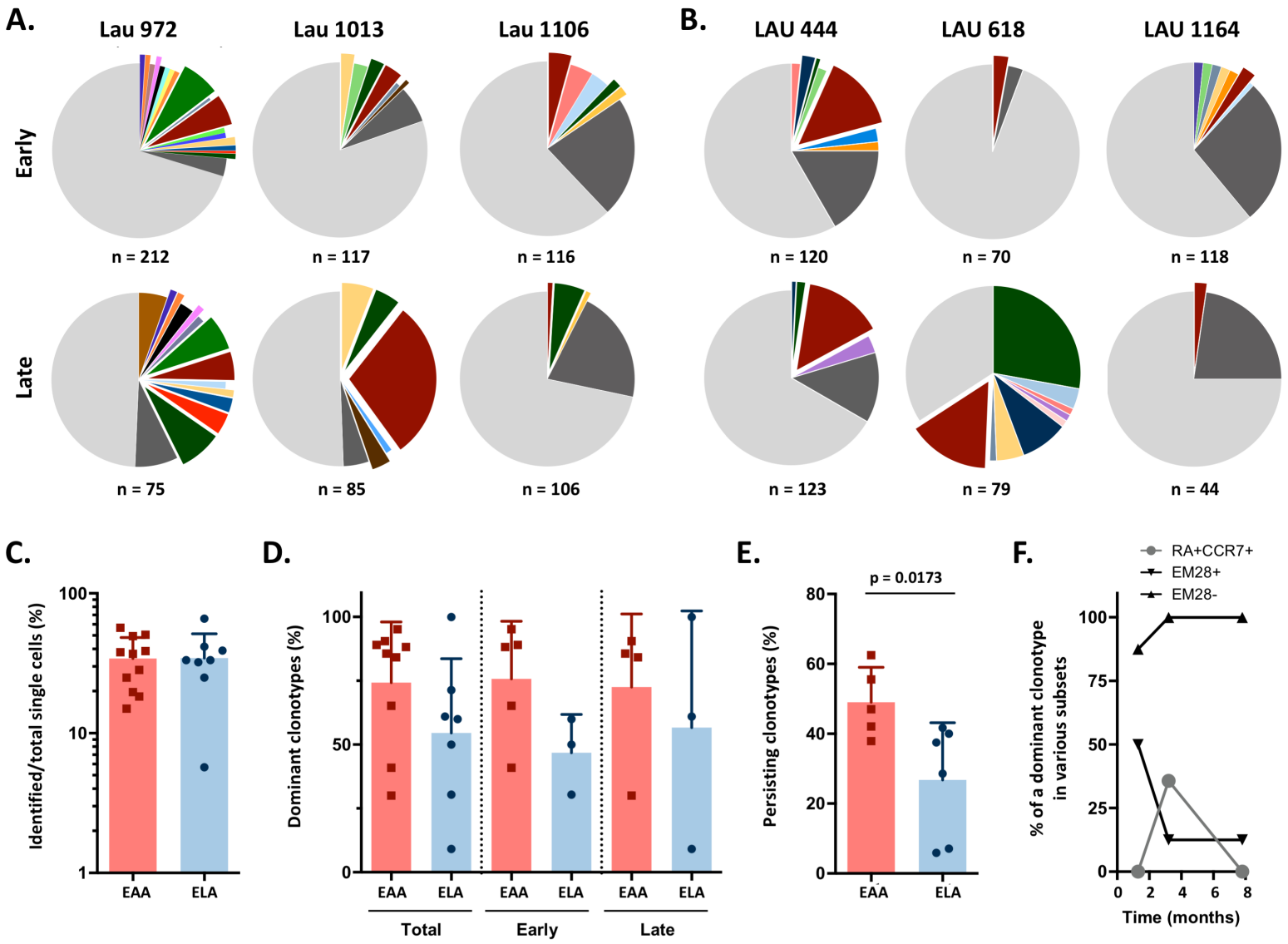
A.
Lau 972
Lau 1013
Lau 1106
Early
Late
n = 212
n = 117
n = 116
n = 75
n = 85
n = 106
B.
LAU 444
LAU 618
LAU 1164
n = 120
n = 70
n = 118
n = 123
n = 79
n = 44
C.
Identified/total single cells (%)
EAA
ELA
D.
Dominant clonotypes (%)
Total
Early
Late
E.
p = 0.0173
Persisting clonotypes (%)
F.
RA+CCR7+
EM28+
EM28-
% of a dominant clonotype in various subsets
Time (months)

Figure 5.

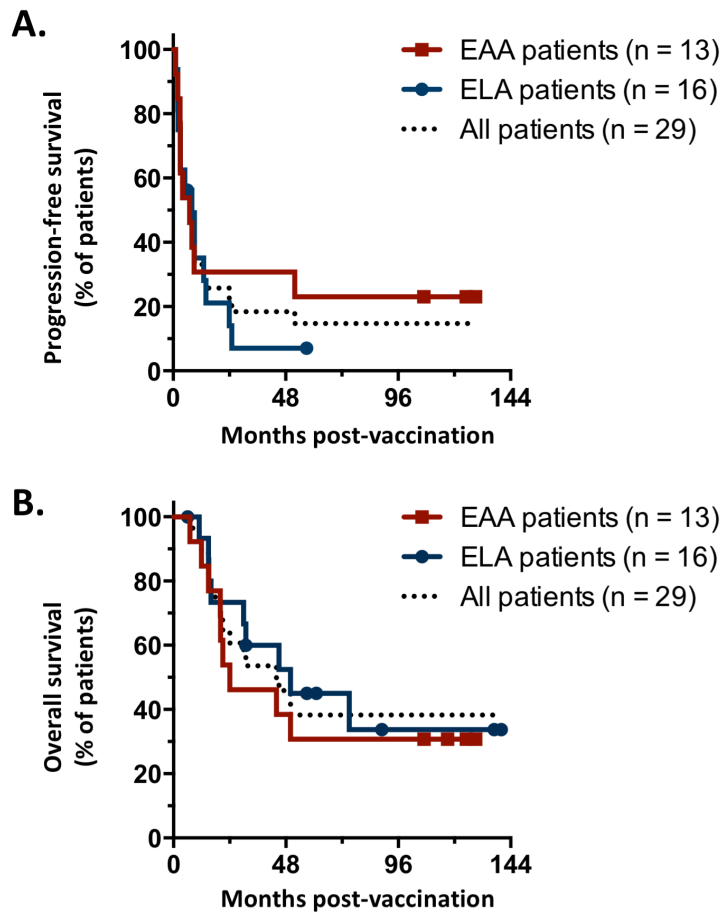
A.
Progression-free survival (% of patients)
100
80
60
40
20
0
0
48
96
144
Months post-vaccination
EAA patients (n = 13)
ELA patients (n = 16)
All patients (n = 29)
B.
Overall survival (% of patients)
100
80
60
40
20
0
0
48
96
144
Months post-vaccination
EAA patients (n = 13)
ELA patients (n = 16)
All patients (n = 29)

Figure 6.