

Cancer Vaccine Enhanced, Non-Tumor-Reactive CD8⁺ T Cells Exhibit a Distinct Molecular Program Associated with “Division Arrest Energy”

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Abstract

Immune-mediated tumor rejection relies on fully functional T-cell responses and neutralization of an adverse tumor microenvironment. In clinical trials, we detected peptide-specific but non-tumor-reactive and therefore not fully functional CD8⁺ T cells post-vaccination against tumor antigens. Understanding the molecular mechanisms behind nontumor reactivity will be a prerequisite to overcome this CD8⁺ T-cell deviation. We report that these non-tumor-reactive CD8⁺ T cells are characterized by a molecular program associated with hallmarks of “division arrest energy.” Non-tumor-reactive CD8⁺ T cells are characterized by coexpression of CD7, CD25, and CD69 as well as elevated levels of Ick^{b505} and p27^{kip1}. *In vivo* quantification revealed high prevalence of non-tumor-reactive CD8⁺ T cells with increased levels during cancer vaccination. Furthermore, their presence was associated with a trend toward shorter survival. Dynamics and frequencies of non-target-reactive CD8⁺ T cells need to be further addressed in context of therapeutic vaccine development in cancer, chronic infections, and autoimmune diseases. [Cancer Res 2009;69(10):4346–54]

Introduction

Cancer vaccines are an attractive approach to treat malignant diseases, as the immune system is capable of recognizing tumor antigens and mounting productive antitumor immune responses leading to tumor regression (1). Yet, their clinical efficacy is under intense debate (2). In clinical trials, substantial tumor regression has been rarely induced, a fact that has been mainly attributed to inhibitory mechanisms within the tumor microenvironment (3). Notwithstanding, intrinsic defects of immune effector cells also need to be considered (4).

Recently, we have identified peptide-specific non-tumor-reactive CD8⁺ T cells after peptide vaccination against a well-characterized immunogenic peptide epitope of the NY-ESO-1 tumor antigen (5).

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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The lack of tumor reactivity of these antigen-specific CD8⁺ T cells might be due to differential antigen presentation, differences in functional avidity of T cells, or induction of tumor antigen-specific tolerance. Several mechanisms accounting for CD8⁺ T-cell unresponsiveness have been described: clonal deletion in the thymus, peripheral clonal anergy, activation-induced nonresponsiveness, loss of colocalization of the T-cell receptor (TCR) and CD8, as well as inhibition of CD8⁺ T cells through, for example, regulatory T cells (6–9). Persistence of antigen is another factor capable of generating peripheral tolerance during tumor development and most likely affecting the CD8⁺ T-cell repertoire shaped by peptide vaccination (10). More recently, it has been shown in a murine model that tumors might escape immune recognition and induce antigen-specific tolerance by inducing nitration of tyrosines in the TCR-CD8 complex (11).

Although numerous mechanisms of T-cell unresponsiveness have been identified in model systems, their role in the clinical setting is still unknown. For non-tumor-reactive CD8⁺ T cells, we establish a molecular program associated with hallmarks of “division arrest energy” by assessing CD8⁺ T-cell responses in context of NY-ESO-1 peptide vaccination in cancer patients. We provide evidence that such non-tumor-reactive CD8⁺ T cells exist *in vivo* before vaccination and that they can be increased in frequency following peptide vaccination. Presence of such cells was associated with a trend toward worse clinical outcome, which will need further investigations in the future.

Materials and Methods

Patient characteristics. Twenty NY-ESO-1⁺ HLA-A2⁺ cancer patients were enrolled in this study following institutional review board approval and informed written consent in one of the following clinical trials: LUD97-008 (12), LUD00-009 (13), LUD00-026 (NY-ESO-1-derived peptides alone or combined with polyarginine), and LUD02-007 (NY-ESO-1 p157-165 peptide combined with CpG7909 and Montanide ISA-51). From 17 patients, peripheral blood mononuclear cells were isolated before and after completion of the trial. CD8⁺ T cells were selected using CD8 MACS beads (Miltenyi Biotec). For flow cytometric assessment of prevalence of CD8⁺CD69⁺CD25⁺CD7⁺ T cells, sufficient material from 7 patients was available. Three patients with metastatic melanoma (NW1045, NW1789, and NW2608) could be assessed in further detail.

Presensitization of T cells by mixed lymphocyte peptide culture and mixed lymphocyte tumor cell culture. *In vitro* presensitization of CD8⁺ T cells with irradiated autologous T-cell-depleted peripheral blood mononuclear cells or tumor cells was done as described previously (14).

Generation of CD8⁺ T-cell clones. CD8⁺ T cells from *in vitro* prestimulated T cells were cloned as described previously (5). Proliferation of clones was lower after stimulation with tumor cells compared with

peptide-pulsed T2 cells. Tumor reactivity was considered positive if the percentage of specific lysis of tumor cells was >15% and the lysis of unpulsed T2 and K562 was <10%.

Purification of NY-ESO-1 peptide. NY-ESO-1 p157-165 peptide was further purified by reverse-phase high-performance liquid chromatography on a MultoHigh-Bio 300 RP4 5 μ m column (Chromatographie Service) using a 35 min linear gradient of acetonitrile in water (35-45%) containing 0.1% trifluoroacetic acid. Subsequent reanalysis of the purified SLLMWITQC monomer peak by reverse-phase high-performance liquid chromatography and mass spectrometry analysis routinely revealed purities >99%.

Antibodies and flow cytometry. The phenotype of CD8⁺ T-cell clones was defined by flow cytometry using specific antibodies (Supplementary Table S1). Tetramer staining with phycoerythrin-labeled HLA-A2 multimers was done as described previously (5).

CDR3 sequencing. TCR sequences were amplified by PCR using specific primers for C β and V β region (Supplementary Table S2), sequenced, and compared with TCR sequences in the IMGT database.

Assessment of TCR downstream signaling. Seven days post-antigen stimulation, phosphorylation of TCR downstream targets was measured using antibodies against CD3 ζ , ZAP70, Ick^{p505}, extracellular signal-regulated kinase (ERK), p38, and nuclear factor- κ B (BD Transduction Laboratories) after stimulation with either CD3 (OKT3) and CD28 antibodies (9.3) cross-linked by an anti-IgG antibody or phorbol myristate acetate (Sigma-Aldrich) at optimal time points established for each protein in CD8⁺ T cells (data not shown).

Western blot analysis for p27^{kip1} expression. To analyze differences in p27^{kip1} protein expression, we assessed CD8⁺ T-cell clones after either polyclonal or antigen-specific TCR stimulation with magnetic beads coated

with CD3, CD28, and anti-MHC class I (W6/32) antibodies or CFSE-stained NY-ESO-1 p157-165 pulsed T2 cells or autologous tumor cell lines. CD8⁺ T cells were purified using a FACS Vantage (BD Biosciences) with purities >95%.

Post-stimulation, cell lysates were assessed with the following antibodies: anti-p27^{kip1} (Santa Cruz Biotechnology), anti- β -actin (Chemicon), and anti-mouse IgG horseradish peroxidase (DAKO Cytomation).

Cytometric bead arrays. For assessment of cytokine release, concentrations of IFN- γ , tumor necrosis factor- α , and interleukin-4 were analyzed using the human Th1/Th2 Cytokine kit II (BD Pharmingen).

Gene expression analysis by microarray. Comparative microarray analysis was used to confirm differences in gene expression patterns. Therefore, total RNA was analyzed using high-density oligonucleotide microarrays (HG-U133A arrays; Affymetrix) after cRNA generation using the Two-Cycle cDNA Synthesis kit. For data assessment and normalization, dCHIP 1.3 was used. Selection of differentially expressed genes was done using the following filter criteria: fold change \geq 1.5, absolute difference in signal intensity between group means \geq 50, and $P \leq$ 0.05. For visualization and gene ontology assessment, we used GenMAPP and MAPPfinder. All heat maps were visualized using MAYDAY. All microarray data can be accessed under GSE11188.

Real-time reverse transcription-PCR for differentially expressed genes. Quantitative real-time PCR with LightCycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics) was used to validate microarray data (primers listed in Supplementary Table S3). Five replicate reactions were done; all data are normalized to GAPDH.

ELISA for XCL1. XCL1 in cell supernatants from CD8⁺ T-cell clones was measured by XCL-1 ELISA kit (Antigenix America). All samples were analyzed at least in triplicates.

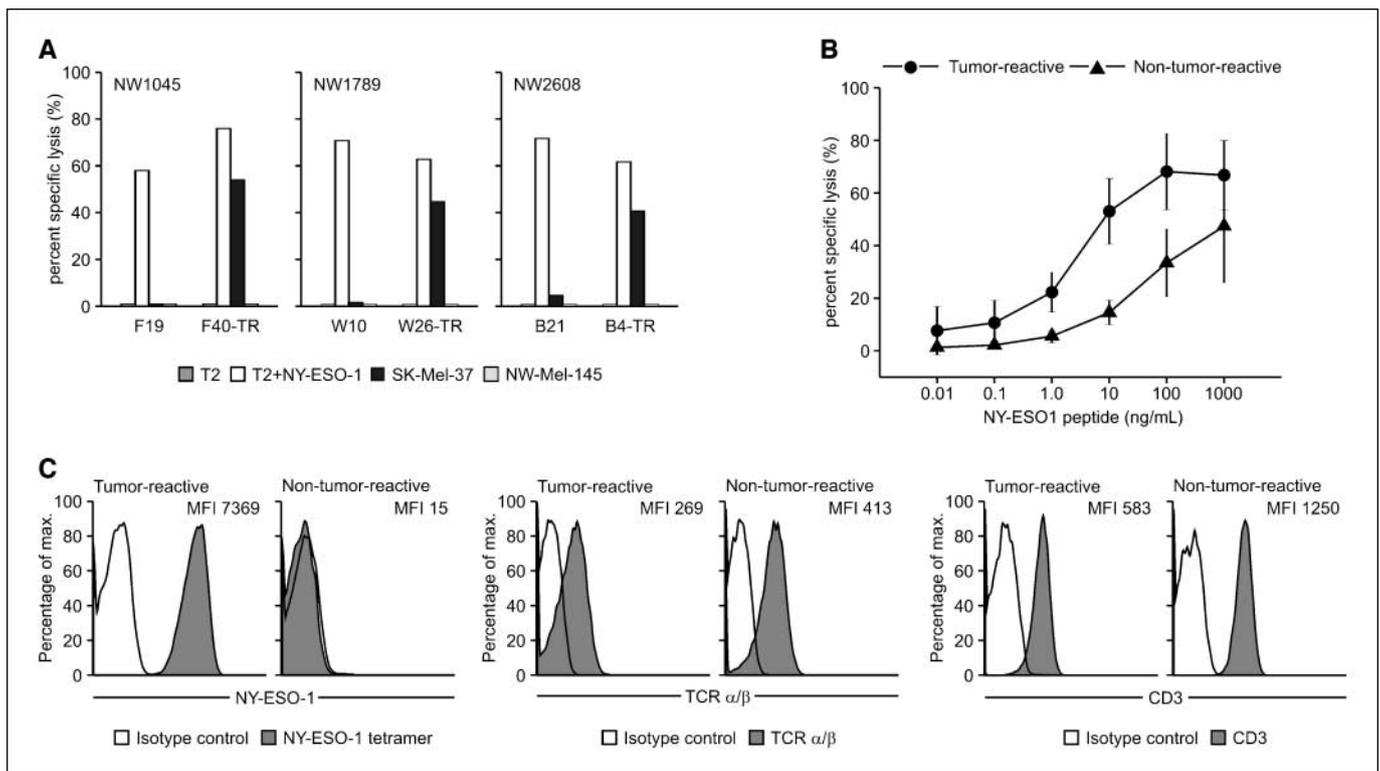


Figure 1. Tumor-reactive and non-tumor-reactive CD8⁺ T-cell clones differ in TCR-CD3 complex expression and affinity. **A**, lytic activity of CD8⁺ T-cell clones from patient NW1045 (left, F19 and F40-TR), NW1789 (middle, W10 and W26-TR), and NW2608 (right, B21 and B4-TR) against NY-ESO-1 p157-165 peptide-pulsed T2 cells (white columns), NY-ESO-1⁺ SK-Mel-37 (black columns), unpulsed T2 cells (dark gray columns), and NY-ESO-1⁻ NW-Mel-145 (light gray columns) was assessed by ⁵¹Cr-release assay. Mean of at least two independent experiments with at least two replicates for each condition. TR, tumor-reactive. **B**, to assess functional avidity of tumor-reactive (●) and non-tumor-reactive (▲) CD8⁺ T-cell clones, their ability to lyse T2 cells pulsed with NY-ESO-1 p157-165 peptide at different concentrations was tested for >8 individual clones each. Values represent percent of specific lysis and correspond to a 10:1 effector-to-target ratio. **C**, flow cytometric analysis of tumor-reactive (left) and non-tumor-reactive CD8⁺ T-cell clones (right) with HLA-A2 NY-ESO-1 p157-165 tetramer, TCR α/β , and CD3 (gray fill) and isotype control (black line). Top right, mean fluorescence intensity values. All experiments were done at least four times.

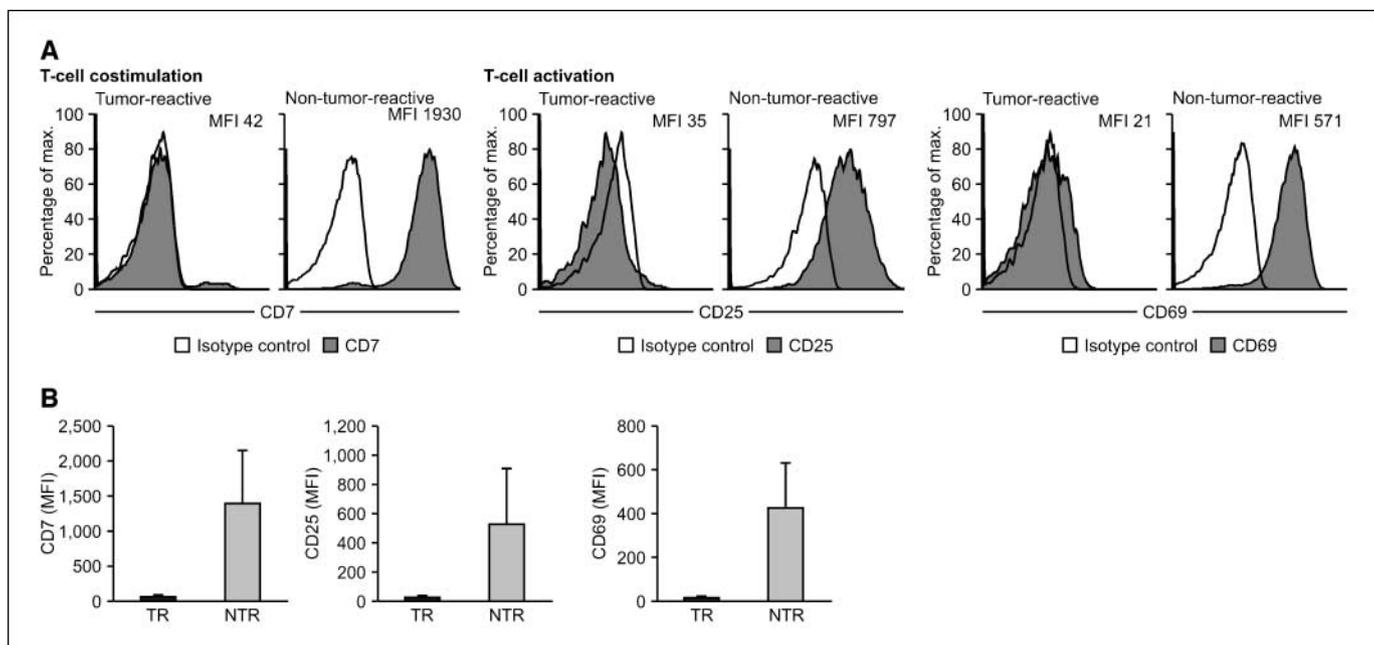


Figure 2. Phenotypic analysis of non-tumor-reactive CD8⁺ T-cell clones. *A*, flow cytometric analysis of CD7 (left), CD25 (middle), and CD69 (right). Top right, mean fluorescence intensity (MFI) values. One of four representative experiments. *B*, mean \pm SD expression of CD7 (left), CD25 (middle), and CD69 (right) on tumor-reactive and non-tumor-reactive CD8⁺ T-cell clones.

In vivo detection of non-tumor-reactive CD8⁺ T cells. To assess the frequency of CD8⁺ T-cell clones *in vivo*, we designed primers specific for the corresponding genomic CDR3 region of the *in vitro* analyzed CD8⁺ T-cell clones. We performed quantitative PCR with the Universal ProbeLibrary Assay (Roche Diagnostics) on DNA isolated from purified total CD8⁺ T cells from patient NW1789 with normalization to CD8 DNA for absolute quantification. A known CDR3 sequence of a CD8⁺ T-cell clone from a HLA-A2⁺ patient recognizing a mycobacterial heat shock protein 60-derived peptide was used as negative control (primers listed in Supplementary Table S4; ref. 15). Assessment of patients NW1045 and NW2608 was technically unfeasible, as no specific CDR3 primers could be designed.

Assessment of CD8⁺ T-cell reactivity after mixed lymphocyte peptide culture. CD8⁺ T-cell reactivity post-peptide vaccination was tested in ⁵¹Cr-release assays against a NY-ESO-1⁺ tumor cell line and peptide-pulsed T2 cells (5). At least 24 individual CD8⁺ T-cell cultures were done for each patient. Overall reactivity is calculated as the percentage of reactive cultures per patient. No reactivity was defined as $\geq 20\%$ lysis of T2 or tumor cells in $\leq 12.5\%$ of wells analyzed, nontumor reactivity as $\geq 75\%$ of reactive wells with $\geq 20\%$ lysis of only peptide-pulsed T2 cells, and tumor reactivity as $\geq 20\%$ tumor cell lysis in $\geq 25\%$ of reactive wells. Individuals were classified according to these criteria.

Statistical analysis. Statistical analyses were done using Student's *t* test with *P* < 0.05 considered statistically significant. Overall patient survival was the interval between diagnosis and death. Data were censored at the last follow-up for patients who were alive at the time of analysis. To visualize median survival times, we used Kaplan-Meier methods. Differences in survival functions were assessed using the log-rank test.

Results

Identification of non-tumor-reactive but peptide-reactive T cells post-vaccination. Two distinct types of NY-ESO-1 p157-165-specific CD8⁺ T-cell clones were generated from three HLA-A*0201⁺ melanoma patients after peptide vaccination: tumor-reactive T-cell clones lysed NY-ESO-1 peptide-pulsed T2 cells and the NY-ESO-1⁺ tumor cell line SK-Mel-37 (Fig. 1A; Supplementary Fig. S1), whereas non-tumor-reactive T-cell clones were not able to

kill SK-Mel-37 or NY-ESO-1-expressing autologous melanoma cell lines (data not shown) despite their reactivity against peptide-pulsed T2 cells. To confirm that non-tumor-reactive CD8⁺ T-cell clones indeed recognize the SLLMWITQC NY-ESO-1 peptide and not a potential contaminant, the NY-ESO-1 p157-165 peptide was further subfractionated by reverse-phase high-performance liquid chromatography. Analysis of CD8⁺ T-cell clones with these subfractions showed reactivity only against the main peak representing the now ultrapure SLLMWITQC peptide (Supplementary Fig. S2). To further confirm specificity, we performed additional experiments with irrelevant NY-ESO-1 p159-167 peptide-pulsed T2 cells (Supplementary Fig. S3) as well as IFN- γ -pretreated as well as NY-ESO-1 p157-165 peptide-pulsed tumor cells (Supplementary Fig. S4). Non-tumor-reactive CD8⁺ T-cell clones were only able to detect either T2 or tumor cells pulsed with the NY-ESO-1 p157-165 peptide, whereas tumor-reactive CD8⁺ T-cell clones efficiently lysed tumor cells independently of IFN- γ pretreatment or peptide loading. To exclude a preferential induction of non-tumor-reactive CD8⁺ T-cell clones by polyclonal stimulation of CD8⁺ T cells with peptide-pulsed antigen-presenting cells, we generated CD8⁺ T-cell clones from non-tumor-reactive T-cell lines stimulated with antigen-presenting cells pulsed with different doses of NY-ESO-1 peptide (Supplementary Fig. S5) and could generate tumor-reactive as well as non-tumor-reactive CD8⁺ T-cell clones.

To determine the relationship of non-tumor-reactive and tumor-reactive CD8⁺ T-cell clones, sequence comparison of the specific TCR- β chains (TCR β) was done. Interestingly, for two patients (NW1045 and NW1789), we observed identical V β joining, and constant regions (Supplementary Table S5). Sequencing of the CDR3 regions, however, revealed distinct sequences for non-tumor and tumor-reactive CD8⁺ T-cell clones, clearly indicating that these T cells are of different origin. We observed identical CDR3 sequences in independently generated tumor-reactive clones from patient NW1045. Similarly, we were able to detect

non-tumor-reactive CD8⁺ T-cell clones from patient NW1789 with identical CDR3 sequences, suggesting that the same CDR3 region will be found either in the tumor-reactive or non-tumor-reactive group but not in both (data not shown). Because CDR3 regions determine the binding avidity of the TCR/MHC-peptide complex, the observed sequence differences most likely lead to functional differences (16–18). Using peptide titration assays, we show that tumor-reactive CD8⁺ T-cell clones exhibit a higher avidity for NY-ESO-1 p157-165 peptide than non-tumor-reactive CD8⁺ T-cell clones (Fig. 1B). To corroborate these findings, we performed tetramer staining (Fig. 1C; Supplementary Figs. S1 and S6). There was no binding of the specific NY-ESO-1 tetramer on non-tumor-reactive T-cell clones even when assessed at later time points post-antigen restimulation (data not shown). Lack of tetramer binding

as a function of low-affinity TCR/MHC-peptide interaction has been recently observed in murine models (19). To exclude that this effect was due to internalization of the TCR/CD3 complex after repeated antigen stimulation, we assessed extracellular TCR α / β chain (Fig. 1C) and CD3 protein expression (Fig. 1C). Interestingly, non-tumor-reactive CD8⁺ T-cell clones exhibited an even higher surface expression of the TCR/CD3 complex than tumor-reactive CD8⁺ T-cell clones.

Non-tumor-reactive CD8⁺ T-cell clones exhibit a partially activated phenotype. T cells stimulated with peptides inducing suboptimal TCR stimulation are characterized by a phenotype associated with partial activation (6). To test this hypothesis, we assessed well-known T-cell activation and differentiation markers. As expected, both CD8⁺ T-cell clones had an effector-memory

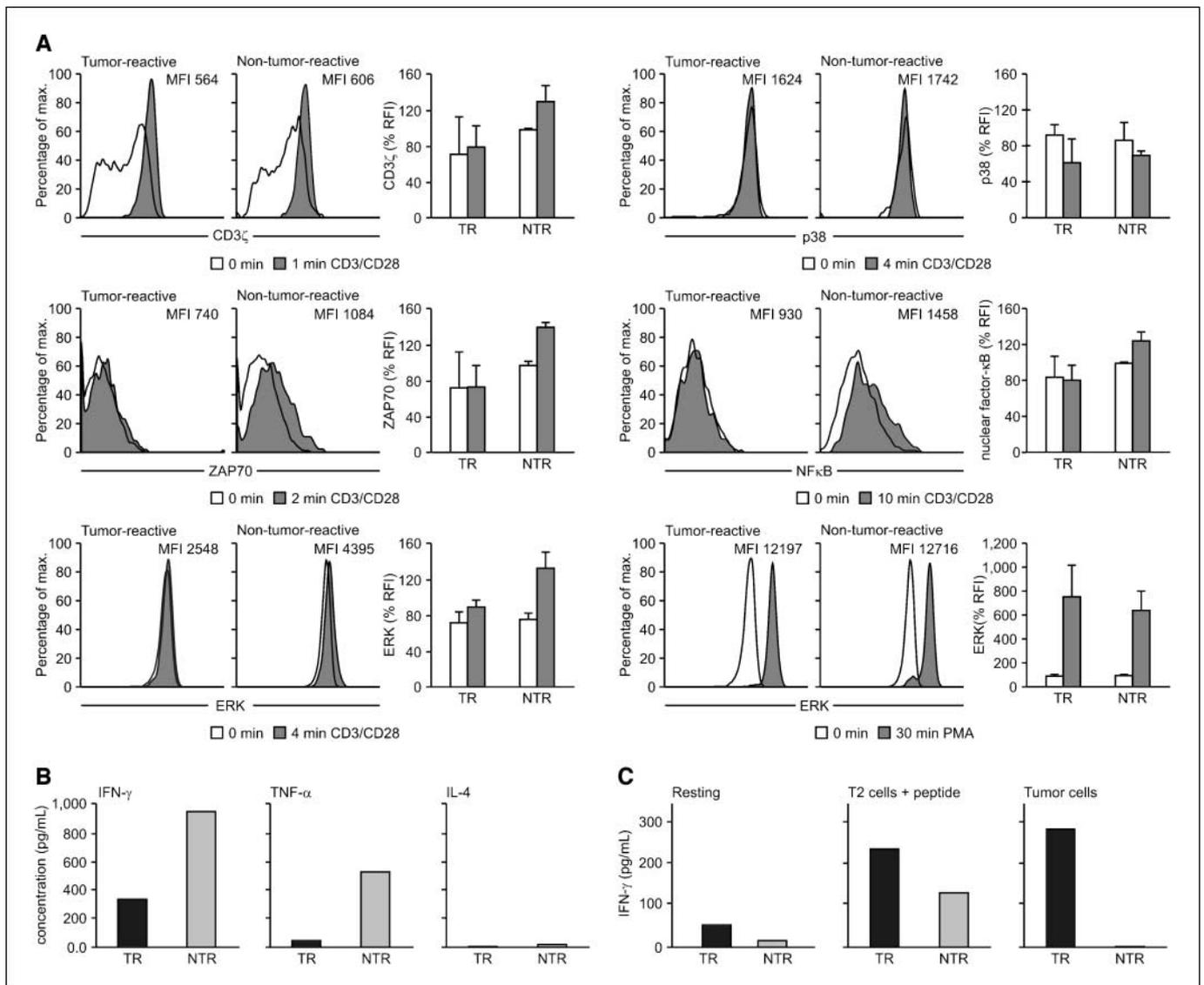


Figure 3. TCR-mediated signaling and function after polyclonal or antigen-specific stimulation. **A**, tumor-reactive and non-tumor-reactive CD8⁺ T-cell clones were stimulated by CD3 and CD28 and phosphorylation of CD3 ζ , ZAP70, ERK, p38, and nuclear factor- κ B was assessed at optimal time points established for each protein as well as phosphorylation of ERK after phorbol myristate acetate (PMA) stimulation, and nuclear factor- κ B was assessed at optimal time points established for each protein as well as phosphorylation of ERK after phorbol myristate acetate (PMA) stimulation. *Gray fill*, phosphorylation; *black line*, baseline steady-state phosphorylation. For each protein, one representative experiment of at least two. *Right*, mean \pm SD of at least two clones. Phosphorylation after stimulation was normalized to that before stimulation (set as 100%) and is presented as relative fluorescence intensity (RFI). **B**, cytokine secretion by CD8⁺ T-cell clones in response to CD3 and CD28 cross-linking. **C**, IFN- γ production by CD8⁺ T-cell clones in response to stimulation with peptide-pulsed T2 cells or HLA-A*0201⁺ NY-ESO-1⁺ tumor cells. IFN- γ was measured by cytometric bead array.

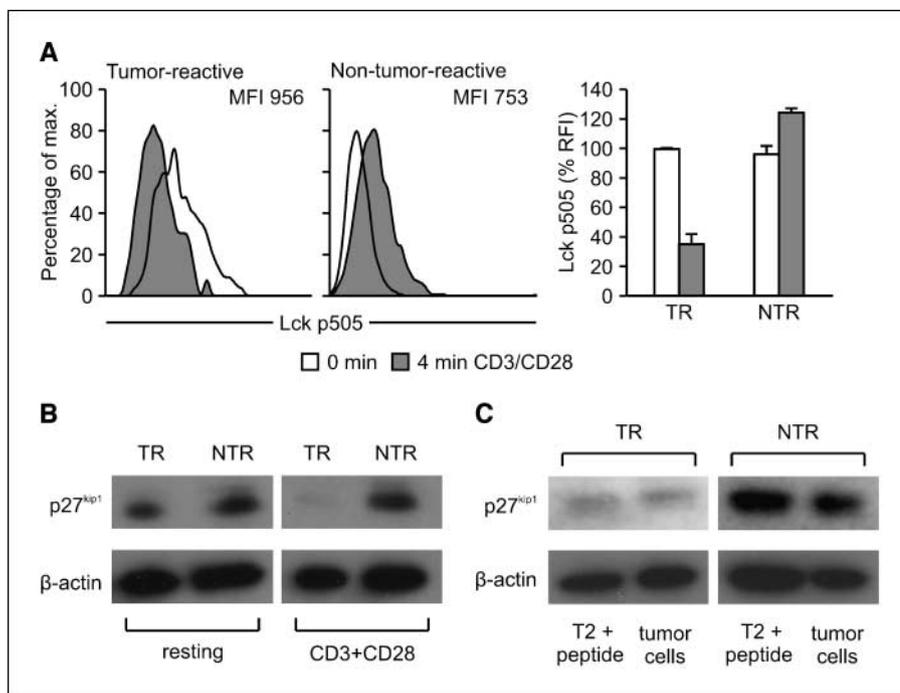


Figure 4. Differential regulation of Ick^{p505} and p27^{kip1} in tumor-reactive and non-tumor-reactive CD8⁺ T-cell clones. **A**, phosphorylation of Ick^{p505} in tumor-reactive (*left*) and non-tumor-reactive (*middle*) CD8⁺ T-cell clones was assessed 4 min after CD3 and CD28 cross-linking. *Gray fill*, phosphorylation; *black line*, baseline steady-state phosphorylation. One representative experiment of at least two. *Right*, mean \pm SD of at least two clones. Phosphorylation after stimulation was normalized to that before stimulation (set as 100%) and is presented as relative fluorescence intensity. **B** and **C**, Western blot analysis of p27^{kip1} in tumor-reactive (*TR*) and non-tumor-reactive (*NTR*) CD8⁺ T-cell clones after stimulation for 24 h at 37°C with either (**B**) CD3 and CD28 monoclonal antibody-coated magnetic beads or (**C**) peptide-pulsed T2 cells or autologous tumor cell lines.

T-cell phenotype (Supplementary Fig. S7), whereas CD8 and CD5 expression were inversely correlated (Supplementary Fig. S8).

Non-tumor-reactive CD8⁺ T cells showed high expression of CD7, which has been recently associated with lack of full effector function (ref. 20; Fig. 2*A* and *B*). In contrast, all tumor-reactive CD8⁺ T-cell clones were negative for CD7, suggesting that these cells are fully differentiated.

In addition, we observed significant differences in the expression of early activation markers between both CD8⁺ T-cell clones. Whereas tumor-reactive CD8⁺ T-cell clones were negative for CD25 and CD69, all non-tumor-reactive T-cell clones expressed high levels of CD25 and CD69 (Fig. 2*A* and *B*). This phenotype was stable throughout the culture and independent of restimulation, which is in line with a partial activation phenotype of non-tumor-reactive CD8⁺ T-cell clones (21, 22).

Increased phosphorylation and cytokine production in non-tumor-reactive CD8⁺ T-cell clones on polyclonal stimulation. Next, we assessed whether the partial activation status of non-tumor-reactive CD8⁺ T-cell clones is directly linked to reduced TCR signaling and can be reversed by polyclonal stimulation. Surprisingly, non-tumor-reactive CD8⁺ T-cell clones showed no defect in phosphorylation of CD3 ζ chain and ZAP70 on stimulation with CD3 and CD28 (Fig. 3*A*). These findings argue against a state of anergy in non-tumor-reactive CD8⁺ T-cell clones associated with reduced phosphorylation of early TCR signaling molecules as has been described previously for CD4⁺ T cells (6).

As exemplified for ERK, p38, and nuclear factor- κ B (Fig. 3*A*), we also assessed whether downstream targets show an increased activation on optimal stimulation. For nuclear factor- κ B and ERK, an increase in phosphorylation in non-tumor-reactive CD8⁺ T-cell clones was observed on polyclonal stimulation, whereas phosphorylation of p38 was comparable between non-tumor-reactive and tumor-reactive T cells. When using the mitogen phorbol myristate acetate, similar results were obtained (Fig. 3*A*).

To assess whether increased TCR signaling following polyclonal stimulation would also be associated with increased function, we

analyzed cytokine production of the T-cell clones 18 h post-stimulation. Secretion of IFN- γ was significantly higher in non-tumor-reactive CD8⁺ T-cell clones after stimulation with CD3 and CD28 monoclonal antibodies (Fig. 3*B*). Similar results were obtained for tumor necrosis factor- α , whereas the levels of interleukin-4 were very low in both types of CD8⁺ T-cell clones (Fig. 3*B*).

We obtained opposite results when stimulating with NY-ESO-1⁺ tumor cell lines (Fig. 3*C*). Only tumor-reactive CD8⁺ T-cell clones produced IFN- γ under these conditions. Stimulation with NY-ESO-1 peptide-pulsed T2 cells induced IFN- γ in both types of CD8⁺ T-cell clones albeit somewhat less in the non-tumor-reactive CD8⁺ T-cell clones. However, this might be due to the lower background expression of IFN- γ by non-tumor-reactive CD8⁺ T cells, as the fold increase of IFN- γ secretion was comparable between both types of clones. Overall, these data show that non-tumor-reactive CD8⁺ T-cell clones are fully functional when providing strong polyclonal TCR signals; however, NY-ESO-1 peptide presented by tumor cells induces only suboptimal TCR signaling.

Increased phosphorylation of Ick^{p505} and up-regulation of p27^{kip1} in non-tumor-reactive CD8⁺ T-cell clones. We postulated that additional mechanisms are operative preventing non-tumor-reactive T cells from becoming fully functional in response to NY-ESO-1⁺ tumor cells. The lack of dephosphorylation of Ick at the inhibitory tyrosine residue (Ick^{p505}) has been associated with induction of anergy in CD4⁺ T cells (23). Resting T cells contain high levels of phosphorylated Ick^{p505}. On dephosphorylation of Ick^{p505}, Ick is activated and subsequently activates downstream targets (24). When assessing phosphorylation of Ick^{p505} on stimulation with CD3 and CD28, Ick^{p505} was clearly dephosphorylated in tumor-reactive clones, whereas, in non-tumor-reactive T-cell clones, there was even a trend toward increased phosphorylation post-stimulation (Fig. 4*A*).

Previous data suggested a role of Ick^{p505} in the induction of p27^{kip1} expression in CD4⁺ T cells, thereby resulting in inhibition of T-cell function (25). We therefore assessed p27^{kip1} expression

(Fig. 4B). Before stimulation, both types of clones expressed p27^{kip1}, although the level of expression was always lower in tumor-reactive clones. However, after stimulation, tumor-reactive clones almost completely down-regulated p27^{kip1}. In contrast, in non-tumor-reactive CD8⁺ T-cell clones, the expression of p27^{kip1} remained at high levels similar to recent observations in anergic CD4⁺ T cells (26–28). Because non-tumor-reactive CD8⁺ T-cell clones showed differential regulation of p27^{kip1} after polyclonal stimulation, we postulated that antigen-specific stimulation would also lead to differential regulation of these proteins. To address this question, CD8⁺ T-cell clones were stimulated with either NY-ESO-1 peptide-pulsed T2 cells or autologous melanoma tumor cell lines. Indeed, expression of p27^{kip1} was significantly higher in non-tumor-reactive CD8⁺ T-cell clones independent of the cell type used for stimulation (Fig. 4C).

RNA fingerprint of non-tumor-reactive CD8⁺ T-cell clones.

Because tumor-reactive and non-tumor-reactive CD8⁺ T-cell clones showed distinct phenotypic and functional properties, we postulated that both types of clones also induce different molecular programs. Genome-wide transcriptional profiles were established 7 days post-antigen stimulation. We first addressed cell cycle regulation and visualized differentially expressed genes by using an adapted cell cycle map from GenMAPP (Supplementary Fig. S9). Non-tumor-reactive CD8⁺ T-cell clones showed profoundly reduced cyclin D2 expression, one of the most important inducer of G₁-S-phase transition. Up-regulation of SMAD3 gene expression (Supplementary Fig. S9) in combination with high levels of p27^{kip1} and reduced cyclin D2 expression points toward mechanisms previously observed for tolerance induction in murine CD4⁺ T cells (29). Interestingly, many regulatory proteins of later cell cycle checkpoints were expressed at elevated levels in non-tumor-reactive CD8⁺ T-cell clones. It will be interesting to further study whether these are compensatory mechanisms of cell cycle regulation because it was previously reported that p27^{kip1} is a universal cyclin-dependent kinase inhibitor (30).

To further dissect the molecular program of tumor-reactive and non-tumor-reactive CD8⁺ T-cell clones, we defined their specific RNA fingerprints as described previously for other T-cell subsets (25, 31, 32). Interestingly, the number of genes differentially expressed between the two types of CD8⁺ T-cell clones was rather small; 13 genes were expressed at lower levels in non-tumor-reactive CD8⁺ T-cell clones, whereas 12 genes were expressed at higher levels (Fig. 5A). To validate the microarray results, we performed quantitative reverse transcription-PCR for 14 of the differentially expressed genes and confirmed the differential expression for all 14 genes (Fig. 5B). Similarly, genes known to be changed on protein level (e.g., CD69, CD25, or CD7) were higher expressed in non-tumor-reactive CD8⁺ T-cell clones; however, this did not reach statistical significance (Supplementary Fig. S10). For XCL1 (lymphotactin), both significant differences of mRNA expression and protein expression were shown (Fig. 5C). XCL1 expression has been described in CD8⁺ T-cell clones stimulated with agonist peptide, whereas no significant expression was observed in unstimulated CD8⁺ T cells or T cells stimulated with irrelevant ligand (33).

It was a rather unexpected finding that several genes with differential expression in non-tumor-reactive CD8⁺ T-cell clones have already been associated with decreased T-cell function, T-cell inhibition, or even tolerance (Supplementary Results). Interestingly, the genes differentially expressed were not part of the profiles established for conventional CD8⁺ T-cell anergy (34) or CD8⁺ T-cell

exhaustion during chronic viral infection (35), further highlighting that we identified a unique molecular profile for non-tumor-reactive CD8⁺ T cells (data not shown).

Increase of non-tumor-reactive CD8⁺ T-cell clones in cancer patients. Next, we were interested whether non-tumor-reactive

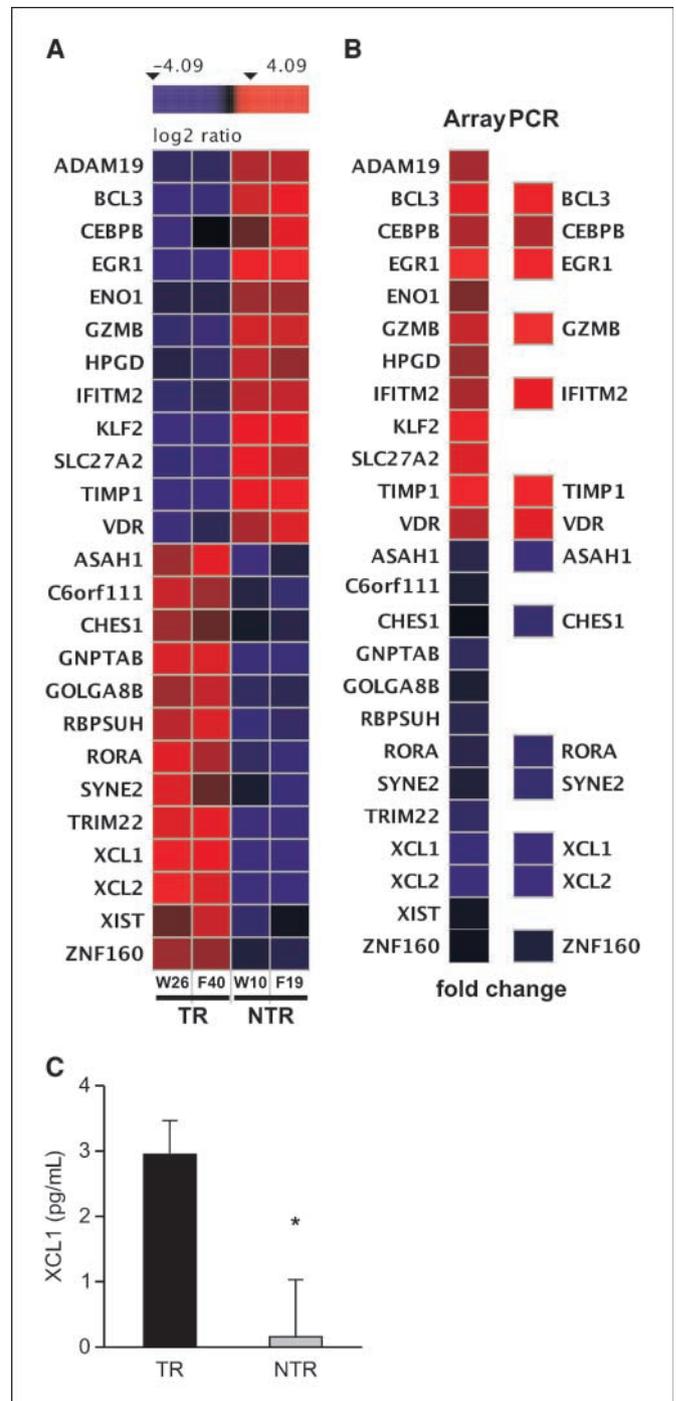


Figure 5. Gene expression differences in non-tumor-reactive CD8⁺ T-cell clones. *A*, visualization of differentially expressed genes as assessed by microarray analysis. *B*, comparative visualization of array-based results and quantitative reverse transcription-PCR-based results for 14 selected genes also assessed by quantitative reverse transcription-PCR. *Red*, genes with increased expression in non-tumor-reactive CD8⁺ T-cell clones; *blue*, those with decreased expression. *C*, ELISA to assess XCL1 release in tumor-reactive and non-tumor-reactive CD8⁺ T-cell clones. *, *P* < 0.05.

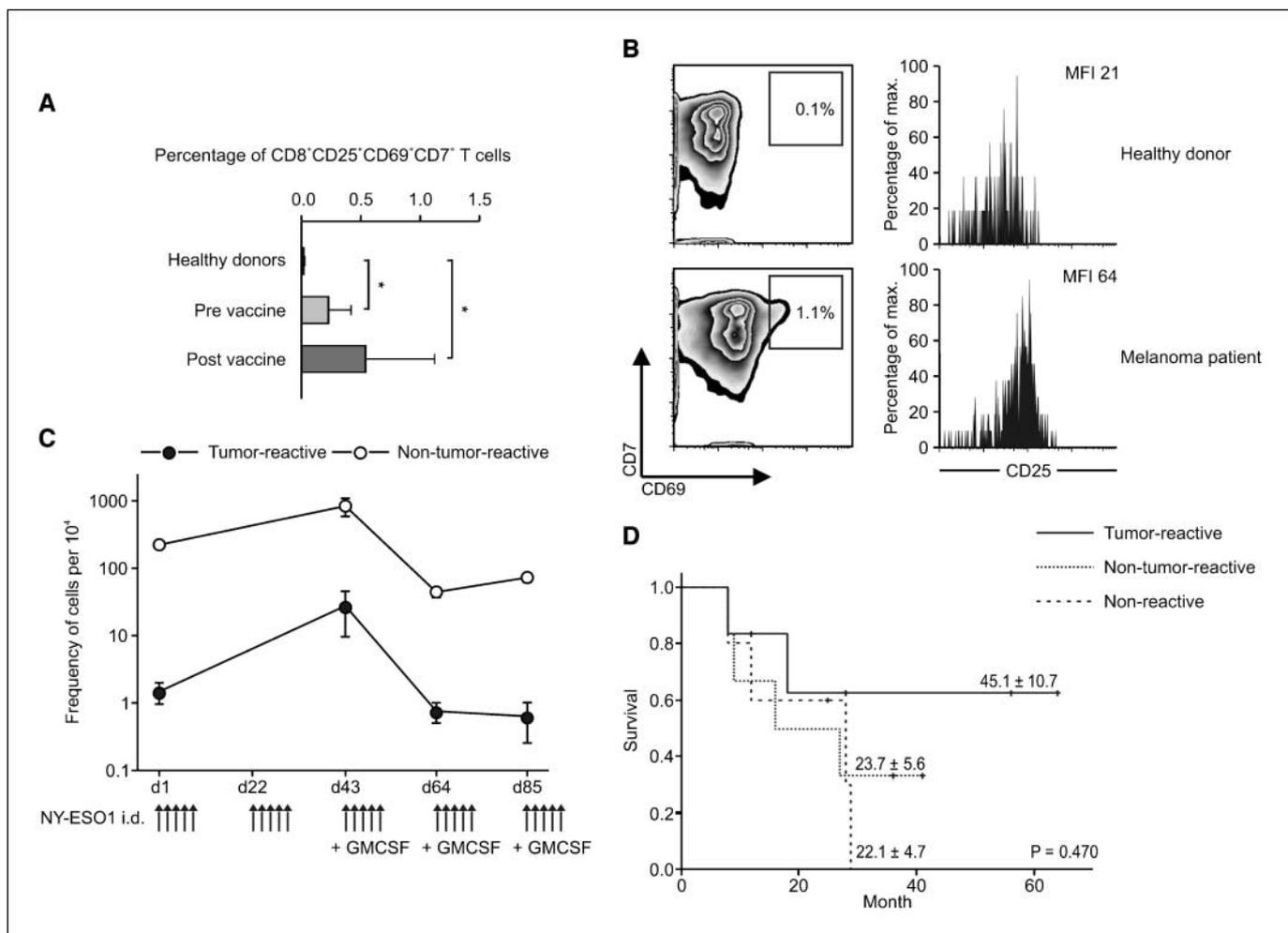


Figure 6. Detection of non-tumor-reactive CD8⁺ T-cell clones *in vivo* and assessment of patient survival. **A**, flow cytometric assessment of frequencies of CD8⁺CD25⁺CD69⁺CD7⁺ T cells in healthy donors ($n = 10$) and melanoma patients before ($n = 7$) and after ($n = 7$) vaccination. Mean \pm SD expression. *, $P < 0.05$. **B**, assessment of CD25 expression on the CD8⁺CD69⁺CD7⁺ T-cell subpopulation for one healthy donor and one melanoma patient after vaccination. **C**, frequencies of the non-tumor-reactive and tumor-reactive CD8⁺ T-cell clone determined by quantitative PCR for patient NW1789. PCR was normalized to CD8 DNA to determine absolute frequencies. Mean frequencies determined from at least five independent experiments. **D**, to visualize patient survival, Kaplan-Meier curves were calculated for overall survival of 17 cancer patients. Samples were divided into three groups according to CD8⁺ T-cell reactivity.

CD8⁺ T cells can be detected *in vivo*. First, we assessed the overall frequency of CD8⁺ T cells coexpressing the cell surface molecules CD7, CD25, and CD69 in healthy donors yet were unable to detect such a subpopulation in healthy individuals (Fig. 6A and B). In contrast, a significant number of CD8⁺ T cells coexpressing these markers were identified in NY-ESO-1⁺ cancer patients before (mean, 0.23%) and after (mean, 0.54%) vaccination. The further expansion observed in cancer patients after vaccination, however, did not reach statistical significance. These data suggested an expansion of non-tumor-reactive CD8⁺ T cells *in vivo*. In one patient, we were able to develop a highly concise quantitative PCR approach for clone-specific genomic TCR DNA to test expansion of the CD8⁺ T-cell clones. TCR quantities were calibrated on genomic CD8 DNA to determine the absolute frequencies of each CD8⁺ T-cell clone. For patient NW1798, primers specific for the CDR3 region in combination with PCR-specific probes could be designed for both tumor-reactive and non-tumor-reactive T-cell clones (Supplementary Fig. S11). To show specificity of the PCR, a series of experiments was done. First, the two primer pairs specifically recognized the corresponding T-cell clone, whereas using primer

pairs matching the other clone always resulted in no detectable PCR product (Supplementary Fig. S11). As negative control, primers specific for a known CDR3 sequence were used (data not shown; ref. 15). Similarly, when using polyclonal CD8⁺ T cells isolated from peripheral blood of healthy individuals ($n = 3$), no PCR product was detected (data not shown). This genomic PCR was then applied to the patient's samples. Similar to a recent report by Germeau and colleagues (36), tumor-reactive and non-tumor-reactive TCR sequences were already detectable before vaccination (Fig. 6C). Furthermore, the frequency of the non-tumor-reactive CD8⁺ T-cell clone was significantly higher (day 1, Fig. 6C) compared with the tumor-reactive CD8⁺ T-cell clone. After the second cycle of vaccination, the frequency of the non-tumor-reactive CD8⁺ T-cell clone was further increased (4-fold, day 43). However, after addition of GM-CSF to the vaccine formulation, the frequency of the non-tumor-reactive CD8⁺ T-cell clone declined to a level below the start of vaccination (day 64) and remained steady for another vaccination cycle (day 85). Albeit the base frequency of the tumor-reactive CD8⁺ T-cell clone was significantly lower on day 1, there was an 18-fold increase after two cycles of NY-ESO-1 peptide

vaccination. Compared with the non-tumor-reactive CD8⁺ T-cell clone, however, following this expansion, a contraction occurred during later vaccine cycles.

Assessment of patient survival in context of tumor reactivity. Within a clinical phase I vaccination trial, we assessed tumor reactivity of CD8⁺ T cells in context of survival in 17 NY-ESO-1⁺ cancer patients. In 5 patients, CD8⁺ T cells recognizing NY-ESO-1 could not be detected ("nonreactive"; Fig. 6D). In 6 patients, only non-tumor-reactive peptide-specific CD8⁺ T cells were detectable, whereas tumor-reactive CD8⁺ T cells were present in the remaining 6 patients. Although this classic phase I vaccination trial is not powered to estimate survival benefits, we were able to observe the following trend. Four of 6 patients harboring tumor-reactive CD8⁺ T cells are still alive (mean survival time, 45.1 ± 10.7 months), whereas 4 of 5 patients with no T-cell reactivity deceased (mean survival time, 22.1 ± 4.7 months). Mean survival time (23.7 ± 5.6 months) of patients with non-tumor-reactive CD8⁺ T cells was more similar to the group of patients with no T-cell reactivity. These results did not reach statistical significance as assessed by the log-rank test; still, the trend observed in this limited number of patients analyzed in this phase I clinical trials warrants future investigations.

Discussion

Vaccination with tumor antigen-derived peptides leads to the induction of tumor-reactive but also non-tumor-reactive peptide-specific CD8⁺ T cells (5). Here, we report for the first time that these two types of CD8⁺ T cells are of different origin and that their differential function is not temporary or interchangeable but rather based on distinct molecular programs induced in context of antigenic stimulation most likely by the tumor and potentiated by vaccination. In non-tumor-reactive CD8⁺ T cells, tumors not simply induce a suboptimal activation program with reduced function toward antigen and tumor cells in CD8⁺ T cells but rather leads to a molecular program that is most closely related to what has been described previously as "division arrest anergy" (27, 28, 37). Division arrest is characterized by expression of early activation markers such as CD25 and CD69. Increased levels of p27^{kip1} and limited amounts of cyclin D2 prohibit progression from G₁ to S phase of the cell-cycle inducing cell cycle arrest (37). Although peptide vaccination leads to amplification of non-tumor-reactive CD8⁺ T cells, we can show that these cells already exist *in vivo* before vaccination. These rather surprising results might reflect a general mechanism of the CD8⁺ T-cell repertoire in response to stimulation with tumor antigens or even any self-antigens (Supplementary Discussion).

The observed unresponsiveness of non-tumor-reactive CD8⁺ T cells toward tumor cells seems to be antigen-specific and can be reversed by polyclonal stimulation. Most likely, these cells have a higher avidity for yet unknown antigens. Reversibility and antigen specificity of the observed inhibitory mechanisms in CD8⁺ T cells with low to intermediate avidity TCR for the inducing antigen seem to be very useful mechanisms because these cells still could be recruited for immune responses toward antigens for which their TCR show higher avidity.

Non-tumor-reactive CD8⁺ T-cell clones stably expressed CD7, CD25, and CD69, and CD8⁺ T cells with this phenotype were also observed in cancer patients *in vivo*. Because the non-tumor-reactive CD8⁺ T-cell clones did not bind tetramer, alternative approaches to quantify them had to be developed. We quantified

the frequency of non-tumor-reactive CD8⁺ T cells on a clonal level applying a sensitive PCR technology for the detection of the clone-specific TCR. For tumor patients, our data indicate that an increase of these cells takes place already before vaccination most likely in context of tumor development. This accumulation might be supported by insufficient priming conditions and an immunosuppressive tumor microenvironment. One could postulate that non-tumor-reactive CD8⁺ T cells compete with tumor-reactive CD8⁺ T cells for peptide-loaded antigen-presenting cells or niches for proliferation, subsequently deviating a potentially successful immune response and leading to immunosuppression.

Although not intended, peptide vaccination leads to a further expansion of the non-tumor-reactive CD8⁺ T-cell clone. Currently, we cannot exclude the possibility that the observed differences stem from homeostatic variations. However, recent reports favor a relatively stable level of CD8⁺ T-cell clones over time (38). As an alternative and clinically more applicable approach, we assessed antigen-specific CD8⁺ T-cell activation in multiple bulk cultures from single patients in parallel and determined frequency of reactive cultures. This approach allowed us to distinguish three groups of patients: patients showing no reactivity to NY-ESO-1, patients with non-tumor-reactive CD8⁺ T cells, and patients with tumor-reactive CD8⁺ T cells. Assessment of these three groups in context of survival suggested that existence of non-tumor-reactive T cells is associated with a worse overall survival, similarly to patients showing no tumor reactivity. This would suggest that existence of non-tumor-reactive CD8⁺ T cells either as a consequence of competition for limited resources (e.g., antigen-presenting cells) or as a prerequisite might have effect on the clinical outcome after peptide vaccination.

Further clinical studies are necessary to determine whether other means of vaccination (longer CTL peptides, recombinant proteins, viral vectors, and RNA and DNA vaccines) would reduce the frequencies of non-tumor-reactive CD8⁺ T cells post-vaccination (39). Monitoring of T-cell responses with special focus on non-tumor-reactive CD8⁺ T cells might have to be complemented by the assessment of anergy-associated RNA fingerprints, cell surface molecules such as CD69, CD25, and CD7, or elevated expression of p27^{kip1} and lck^{p505} to optimize cancer vaccine development in the future, as non-tumor-reactive CD8⁺ T cells might affect immune monitoring.

We anticipate that existence and expansion of antigen-specific yet target nonreactive CD8⁺ T cells is not restricted to tumor-host interactions but also manifest in chronic infections and autoimmune diseases where antigen is also accessible over an extended period. The overall goal for an optimal vaccine would be to minimize the frequency of anergic, non-target-reactive T cells while at the same time enhancing the expansion of fully functional T cells with high avidity TCR.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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