

TIL therapy broadens the tumor-reactive CD8⁺ T cell compartment in melanoma patients

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Keywords: tumor immunology, TIL therapy, high throughput screening, pMHC multiplexing, T-cell reactivity

There is strong evidence that both adoptive T cell transfer and T cell checkpoint blockade can lead to regression of human melanoma. However, little data are available on the effect of these cancer therapies on the tumor-reactive T cell compartment. To address this issue we have profiled therapy-induced T cell reactivity against a panel of 145 melanoma-associated CD8⁺ T cell epitopes. Using this approach, we demonstrate that individual tumor-infiltrating lymphocyte cell products from melanoma patients contain unique patterns of reactivity against shared melanoma-associated antigens, and that the combined magnitude of these responses is surprisingly low. Importantly, TIL therapy increases the breadth of the tumor-reactive T cell compartment in vivo, and T cell reactivity observed post-therapy can almost in full be explained by the reactivity observed within the matched cell product. These results establish the value of high-throughput monitoring for the analysis of immuno-active therapeutics and suggest that the clinical efficacy of TIL therapy can be enhanced by the preparation of more defined tumor-reactive T cell products.

Introduction

Immunotherapeutic treatment of melanoma has seen major progress in recent years. As a first example, following work in mouse model systems that demonstrated that antibody-mediated blockade of T cell checkpoint molecules could be used to enhance tumor-specific T cell responses,¹ a number of such antibodies have been evaluated in clinical trials. Antibody-mediated blockade of the PD-1/PD-L1 axis has shown promise in early phase clinical trials.² More importantly, a recent randomized Phase III trial has demonstrated a survival benefit of anti-CTLA4 treatment in patients with metastatic disease.³ In patients that experience a clinical benefit of anti-CTLA4 treatment, an increase in blood CD8⁺ T-cell counts has been observed.⁴ Likewise, an increase in CD8⁺ T-cell frequencies has been observed in tumor lesions that regress upon anti-CTLA4 treatment.⁵

As a second strategy to enhance melanoma-specific T-cell reactivity, Stage IV melanoma patients have been treated by infusion of large numbers of ex vivo expanded tumor-infiltrating T cells (TIL). TIL therapy has led to a 50% RECIST response rate in clinical trials in 2 centers.^{6,7} Although unfractionated TIL products used for therapy generally contain both CD4⁺ and CD8⁺

T cells, cell therapy with CD8-enriched TIL products has also been shown to lead to clinical responses.⁸

Based on the above data it is reasonable to speculate that cancer regression upon T cell checkpoint blockade or TIL therapy is at least in part mediated by the activity of cytotoxic CD8⁺ T cells. However, our knowledge of the CD8⁺ T cell reactivity that is induced by either therapy is highly limited. This lack of information can be explained by a combination of two factors. First, the number of shared melanoma-associated epitopes to which CD8⁺ T-cell responses have been described is in the order of hundreds. Second, the amount of clinical material required for classical strategies for tumor-specific CD8⁺ T-cell monitoring only allow one to evaluate T-cell reactivity against at most a few of these epitopes.

To understand how immunotherapy of cancer influences the patterns of tumor-specific CD8⁺ T-cell reactivity we developed a platform that can be used to profile T cell responses against the full panel of known melanoma-associated CD8⁺ T cell epitopes. We have used this T-cell profiling platform to evaluate melanoma-specific CD8⁺ T-cell reactivity in patients treated with TIL therapy, focusing on three main questions: (1) Which antigen classes are predominantly recognized by CD8⁺ T cells in TIL

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Submitted: 11/22/11; Accepted: 11/23/11
<http://dx.doi.org/10.4161/onci.18851>

products? (2) Does TIL therapy increase the magnitude or breadth of the melanoma-specific CD8⁺ T cell response? (3) Is there evidence for epitope spreading upon TIL therapy, or is T-cell reactivity post-therapy restricted to the T-cell reactivity that is infused? The data obtained demonstrate how CD8⁺ T-cell profiling can be used to reveal how immunotherapeutic strategies influence T-cell reactivity upon cancer treatment and suggest avenues for the further development of TIL therapy for human melanoma.

Results

Profiling of antigen-specific CD8⁺ T-cell responses in melanoma. To allow the analysis of therapy-induced CD8⁺ T cell reactivity against any of the known shared melanoma-associated epitopes we compiled a list of all previously described HLA class I-associated epitopes (Andersen RS et al., manuscript submitted). Of the 215 melanoma-associated epitopes identified, a large majority (145) was restricted to the HLA-A2 allele, and for this reason we focused our analysis of TIL-therapy-induced T-cell reactivity on this allele. This set of 145 HLA-A2 restricted epitopes (Table S1) is composed of three major subgroups: melanocyte differentiation (MD) antigens such as MART-1/Melan A (18%), cancer/testis (CT) antigens such as the MAGE family and NY-ESO 1 (27%), and a large group of overexpressed (OE) antigens, such as Meloe-1 and survivin (45%). Using UV-induced peptide exchange, a high-throughput technology for MHC multimer generation,⁹ we generated HLA-A2 multimers for all 145 melanoma-associated epitopes. Each peptide-HLA multimer was then conjugated to 2 out of 8 fluorescent dyes to create two-color codes. Subsequently, panels that each contain peptide-MHC multimers for 25 different epitopes were prepared for T cell staining, such that every pMHC multimer within the panel was encoded by a unique two-color code¹⁰ (Fig. S1).

Melanoma-specific CD8⁺ T-cell populations in TIL infusion products. To assess which melanoma-specific CD8⁺ T-cell reactivities are present within TIL products used for therapy, we analyzed 22 HLA-A2⁺ “young TIL”¹¹ that were produced by *in vitro* expansion of T cells from resected tumor material (16 NIH TIL products, 6 Ella TIL products). Specifically, cell samples from each TIL infusion product were stained with the panel comprising the 145 melanoma-HLA-A2 pMHC multimers, and hits were identified using standardized cut-off (see Methods). All hits were subsequently validated in an independent experiment, examples are shown in Figure 1A.

This screen resulted in the identification of 60 melanoma antigen-reactive T-cell populations that targeted 19 different epitopes (Fig. 1B). Notably, while these T-cell products were expanded from tumor-infiltrating lymphocytes, the magnitude of the HLA-A2-restricted T-cell responses that were detected was strikingly low (median magnitude of individual epitope-specific T-cell responses: 0.12% of CD8⁺ T-cells, range 0.006–4.5%; median combined magnitude of HLA-A2-restricted T-cell responses per patient product: 0.59% of CD8⁺ cells, range 0–4.75%). Even taking into account that HLA-A2 forms only one out of six HLA class I alleles that can be expressed by an

individual patient, these data indicate that the frequency of T cells specific for shared melanoma antigens that is detected in “young TIL” is very low.

Antigen-specific T-cell populations exist at low frequencies. Clinical trials of TIL therapy have both been performed with “young TIL” cell products and with “selected TIL” cell products. In the latter case, TIL cultures that produce IFN γ upon incubation with autologous or partially HLA-matched melanoma are selected from a series of parallel cultures. While this ELISA-based screening system establishes that tumor-reactive T cells are present within these cultures, the magnitude of these responses is not revealed. To determine whether the selection for tumor-reactive TIL or the extended *in vitro* culture that is required to produce these “selected TIL” could yield cell products with enhanced frequencies of T cells reactive against shared melanoma-associated antigens, we screened a total of 12 HLA-A2⁺ “selected TIL” samples (9 NIH/3 Ella institute). Notably, also in these cell products, T cells reactive with shared HLA-A2-restricted antigens made up a surprisingly small fraction of the total CD8⁺ T-cell pool (median 0.159%, range 0.006–10.295% of CD8⁺ cells; Fig. 2).

To address whether the low level T-cell responses detected in TIL reflected true antigen-driven T-cell expansion, for a small set of antigens we assessed whether the presence of an antigen-specific T-cell response correlated with the expression of the respective antigen in fresh tumor tissue from the same patient. Comparison of these two independently generated data sets indicated that T-cell reactivity and antigen expression were strongly correlated (30 out of 32 comparisons matched, Fig. 3A). Second, for 4 TIL (3 pre-REP TIL, one infusion product), we evaluated which proportion of reactivity against autologous tumor can be attributed to T-cell populations detected by MHC multimer staining. On average, T-cell reactivity as measured by MHC multimer staining corresponded to approximately one-sixth (18%) of that detected by functional screening (Fig. 3B). Although the IFN γ -based assay can obviously only reveal functionally active cells, this ratio fits well with the fact that in the former case reactivity is only measured for one out of six possible HLA alleles. As a final validation of our assay system, we analyzed the cell product from a tumor lesion of a patient that had previously been treated by infusion of MART-1 TCR-modified T cells.¹² Notably, in this TIL cell product a very prominent (> 80% of CD8⁺ T cells) MART-1-reactive T-cell population was detected (Fig. 3C). Thus, in a case in which a high magnitude tumor-reactive T cell response is present, such a response is readily detected.

To address whether TIL cell products also contain T-cell populations specific for non-melanoma antigens, we analyzed 13 TIL products for the presence of T cells reactive with a panel of eight epitopes from common human pathogens (CMV, EBV, influenza A). Interestingly, reactivity against viral epitopes was detected in 6 out of 13 TIL products screened, although the magnitude of these virus-specific T cell responses (average 0.036, n = 13) was below that seen in peripheral blood in those cases in which matched samples were available (average 0.87, n = 4) (Fig. 3D). In conclusion, both young and selected TIL products

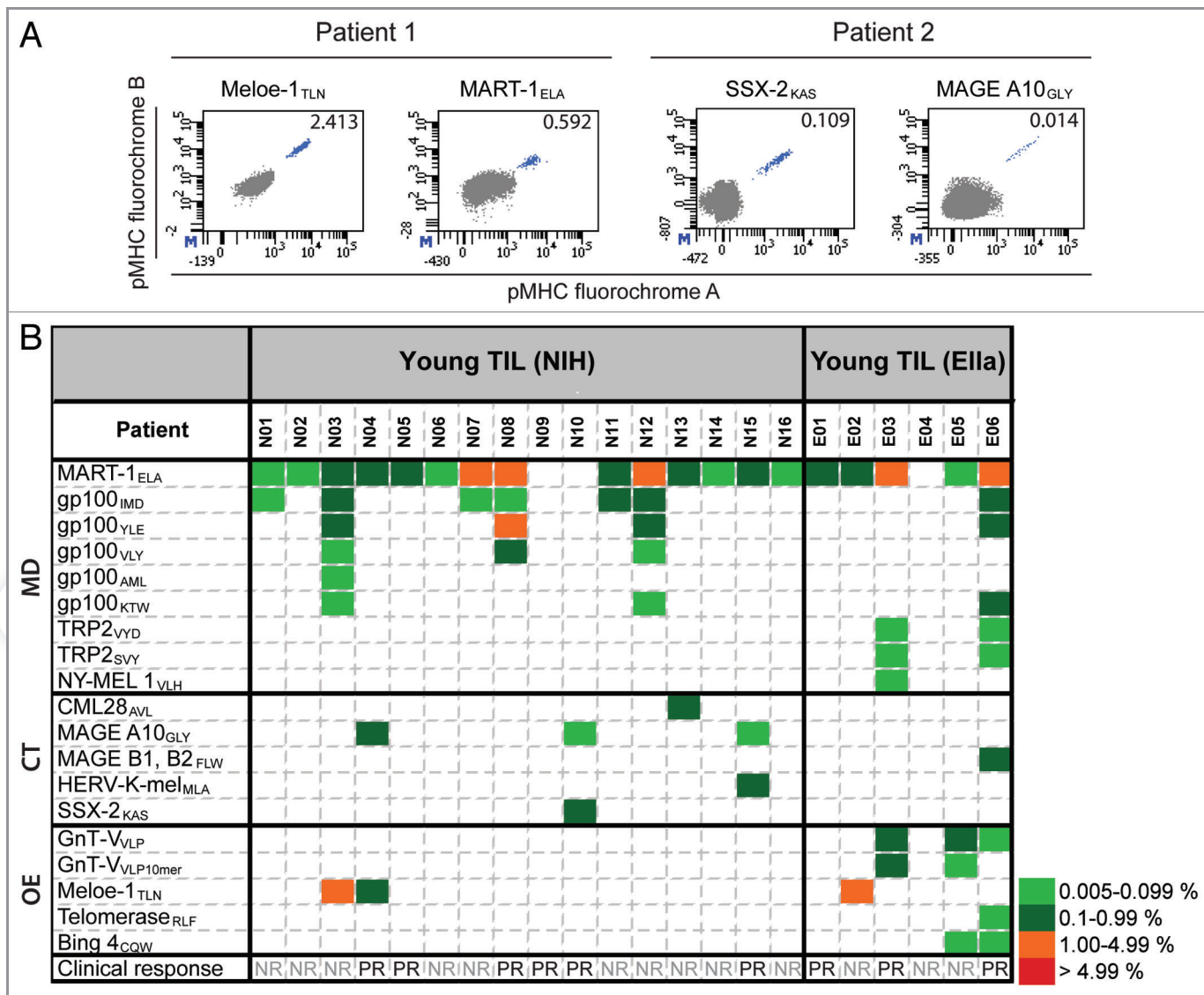


Figure 1. Melanoma-specific CD8⁺ T cell reactivities within TIL infusion products. (A) Examples of flow cytometry plots displaying fluorescence intensity for Meloe-1_{TLN}, MART-1_{ELA}, SSX-2_{KAS} and MAGE A10_{GLY} pMHC multimer-reactive cells in TIL samples from two patients. Dot plots were gated on approximately 500,000 CD8⁺ lymphocytes. Grey dots represent CD8⁺ T cells with no pMHC multimer binding, blue dots represent pMHC multimer-reactive CD8⁺ T cells. Plots are shown with bi-exponential axes. Values indicate the % of antigen-specific T cells out of total CD8⁺ T cells. (B) Summary of antigen-specific T-cell populations identified in HLA-A2⁺ NIH and Ella TIL infusion products. The presence of antigen-specific T cell populations is indicated by the colored boxes, with the different colors reflecting antigen-specific CD8⁺ T-cell response magnitude. Only those epitopes are shown for which T-cell reactivity was detected in at least one patient sample. Patient numbers on top row, clinical responses on bottom row. PR, partial response; NR, no response.

contain relatively low frequencies of T cells that are reactive with shared HLA-A2 restricted melanoma-associated antigens and TIL infusion products do contain T cells that are reactive with non-melanoma antigens.

When comparing the relative contribution of T cell responses against the MD, CT and OE antigens, it is apparent that reactivity patterns against these antigen classes are different. Specifically, within the 34 TIL infusion products analyzed, reactivity against OE antigens is found against less than 10% of the epitopes that have been described within this antigen class. Interestingly, four out of five OE epitopes against which reactivity is observed are derived

from cryptic open reading frames or alternative splicing events, suggesting that T-cell recognition is mostly observed for those OE epitopes for which thymic tolerance can be expected to be less strict. The percentage of CT antigen-derived epitopes to which T cell responses are observed is higher (22.5%). This CT reactivity is diverse rather than being dominated by the frequent recognition of the same epitopes, as shown by the fact that the number of T cell responses goes up in an almost linear fashion with the number of patients screened (Fig. S2). Reactivity against the melanocyte differentiation antigens is observed against an even higher percentage of the epitopes within this class, but in this case, reactivity against

| | | Selected TIL | | | | | | | | | | | |
|-------------------|---------------------------|--------------|-------|-------|-------|-------|-------|-------|-------|-----|-----|-----|-----|
| Patient | | E07 | E08 | E09 | N17 | N18 | N19 | N20 | N21 | N22 | N23 | N24 | N25 |
| MD | MART-1 _{ELA} | | | | | | | | | | | | |
| | gp100 _{IMD} | | | | | | | | | | | | |
| | gp100 _{YLE} | | | | | | | | | | | | |
| | gp100 _{KTW} | | | | | | | | | | | | |
| CT | MAGE A10 _{GLY} | | | | | | | | | | | | |
| | MAGE A4 _{GYY} | | | | | | | | | | | | |
| | MAGE C2 _{LLF} | | | | | | | | | | | | |
| | MAGE C2 _{ALK} | | | | | | | | | | | | |
| | NY-ESO 1 _{SLL} | | | | | | | | | | | | |
| OE | GnT-V _{VLP10mer} | | | | | | | | | | | | |
| | Bing 4 _{CQW} | | | | | | | | | | | | |
| Clinical response | | CR,NR | NR,NR | PR,PR | PR,PR | CR,PR | PR,PR | NR,NR | CR,NR | | | | |

Figure 2. Selection for tumor-reactive TIL does not lead to enhanced frequencies of shared melanoma-antigen reactive CD8⁺ T cells. Summary of antigen-specific T cell populations identified in HLA-A2* NIH and Ella “selected TIL” infusion products. TIL were selected on the basis of IFN γ production in the supernatant upon culturing with autologous or shared melanoma lines. The presence of antigen-specific T-cell populations is indicated by the colored boxes, with the different colors reflecting antigen-specific CD8⁺ T cell response magnitude (see Figure 1B for key). Only those epitopes are shown for which T cell reactivity was detected in at least one patient sample. Clinical responses are summarized in the last row. CR, complete response; PR, partial response; NR, no response.

some epitopes (e.g., MART-1_{ELA}, gp100_{IMD}, gp100_{YLE}) is clearly more frequent than that against others (Fig. 4; Fig. S2).

Finally, in one TIL product that was prepared for research purposes, we identified a T-cell response against a mutated epitope of CDK4 (CDK4_{ACD}; Fig. 3E and F), and the presence of a CDK4_{R24L} mutation in the tumor (but not lymphocytes) of this patient could subsequently be demonstrated. The magnitude of this mutant CDK4-specific T-cell response (0.68% of CD8⁺ cells) was in the same range as that observed for non-mutated antigens, indicating that the magnitude of T-cell responses that are directed against neo-antigens can also be low.

Melanoma-specific T-cell reactivity identified in TIL products predicts T-cell reactivity post-therapy. Having analyzed the composition of TIL products used for infusion, we determined whether TIL therapy alters the tumor-reactive T-cell repertoire in melanoma patients. To this purpose we profiled antigen-specific T-cell reactivity in one-month post-infusion PBMC samples from 10 NIH patients and 9 Ella patients. Within these 19 post-infusion PBMC samples a total of 32 antigen-specific T-cell populations targeting 13 distinct epitopes were identified (Fig. 5A). Comparison of the T-cell responses in the matched TIL products indicated that a large fraction of the T-cell responses that was infused was also encountered in post-infusion PBMC (31 out of 56, 55%), and likelihood of engraftment was correlated with the magnitude of the TIL T-cell response (data not shown). Importantly, nearly all (31 of 32, 97%) of the melanoma-reactive T-cell responses that were observed in post-therapy PBMC were also detected in the TIL product that was used for infusion.

To evaluate whether this close link between T-cell reactivity in infusion products and post-infusion PBMC reflected the effect of TIL therapy, or simply reflected the presence of pre-existing peripheral blood T-cell responses against these antigens we analyzed pre-therapy PBMC samples from seven patients for whom material was available. In these pre-treatment PBMC samples only two melanoma-specific T-cell responses could be detected in total, whereas the matched TIL product and post-therapy PBMC samples contained 20 and 16 detectable T-cell responses, respectively (Fig. 5B). These data indicate that TIL therapy results in a substantial broadening of the detectable tumor-specific CD8⁺ T-cell response in melanoma patients ($p = 0.006$).

To examine the role of stochastic processes in determining the reactivities observed in TIL cultures, we generated enzymatic digests from tumors of two patients. Subsequently, six TIL cultures of each tumor digest were prepared in parallel. After two weeks of culture, individual TIL cultures were screened using the 145 epitope panel. Even though these TIL cultures were derived from the same starting cell population, the composition of individual TIL cultures varied substantially (Fig. 6A–B; Fig. S3). For instance, T-cell populations reactive against MAGE-C2_{LLF} in patient NKI1 were either among the strongest detected or were below the level of detection. In patient NKI2, a MART-1_{ELA} specific T-cell response was detected in all six cultures but at frequencies that varied over 100-fold. These data indicate that even a stochastic variation between TIL cultures prepared from the same starting material can lead to marked variation in the T-cell reactivity that emerges.

Discussion

Despite the fact that cytotoxic T-cell activity is considered to be responsible for at least part of the clinical effects of recently developed cancer immunotherapies, our knowledge of therapy-induced CD8⁺ T-cell activity is still very modest. Here we have utilized a strategy for antigen-specific CD8⁺ T-cell profiling to dissect melanoma-specific T-cell reactivity in TIL therapy. Even though analysis was performed for only a single HLA allele, melanoma-specific CD8⁺ T-cell responses were observed in 30 out of 34 TIL products that were analyzed, and in most TIL products this involved reactivity against multiple epitopes. These data demonstrate that the recognition of shared melanoma antigens by TIL products is a very frequent event. Nevertheless, the most striking finding of our analyses is the relatively low magnitude of the T-cell responses against this panel of 145 epitopes. Only a minor fraction of the antigen-specific T-cell responses detected exceeded 1% of CD8⁺ T cells and in fact in most TIL products the combined magnitude of T-cell reactivity against these 145 epitopes explained less than 1% of CD8⁺ TIL. For those TIL products/ antigens for which this was analyzed, the presence of T-cell reactivity correlated tightly with antigen expression, underlining the reliability of our T-cell assays even for low-level responses. As T-cell reactivity against the large panel of known shared melanoma-associated antigens explains only a small fraction of CD8⁺ TIL, what is the antigen reactivity of the remaining cells? First, our data demonstrate that TIL do contain

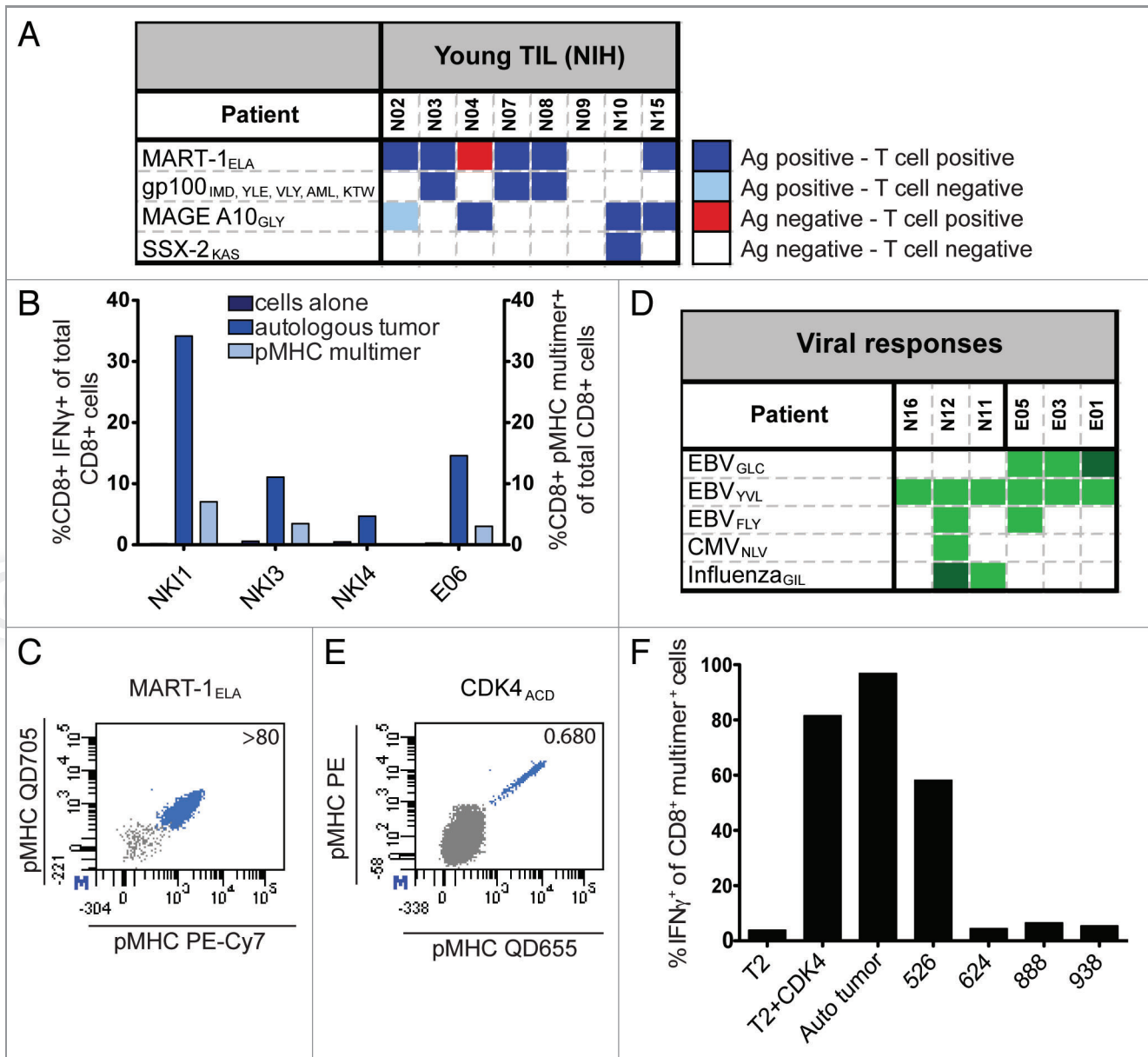


Figure 3. Validation of low-frequency T-cell populations detected in TIL. (A) MART-1, gp100, MAGE A10, SSX-2 expression by fresh tumor cells was measured by quantitative RT-PCR and correlated with the antigen-reactivities detected in matched TIL. Dark blue: tumor cells express the antigen and antigen-specific T cell population detected in the TIL infusion product. Light blue: tumor cells express the antigen, but no antigen-specific T cell population detected in the TIL infusion product. Red: tumor cells do not express the antigen, but antigen-specific T-cell population detected in the TIL infusion product. White: tumor cells do not express the antigen and antigen-specific T-cell population not detected in the TIL infusion product. (B) Intracellular IFN γ staining assay after co-culture of TIL with autologous melanoma cell lines (left axis) in comparison with the total frequency of CD8⁺ pMHC multimer⁺ identified (right axis). (C) Dot plot of MART-1_{ELA} specific T-cell population in TIL of a recipient of MART-1 TCR-modified T cells. Value indicates the percentage of antigen-specific T cells out of total CD8⁺ T cells. (D) Summary of virus-specific T cell populations identified in six HLA-A2⁺ NIH and E1a TIL infusion products (see **Figure 1B** for key). (E) Dot plot of CDK4_{ACD}-specific T-cell population detected in TIL. Value indicates the percentage of antigen-specific T cells out of total CD8⁺ T cells. (F) Intracellular IFN γ staining assay performed with FACS-sorted CDK4_{ACD}-reactive T cells, with the cells being incubated either with T2 cells pulsed with or without the CDK4_{ACD} peptide or an autologous tumor line (HLA-A2⁺). Melanoma cell lines 526 (HLA-A2⁺, CDK4_{R24C}), 624 (HLA-A2⁺, CDK4_{WT}), and 888 and 938 (HLA-A2⁺) were used as controls.

T cells that are reactive against non-melanoma antigens, as shown by the presence of T-cells specific for EBV, influenza and CMV antigens. Second, TIL can contain T cells reactive against neo-antigens, as shown by the presence of CDK4_{R24L}-specific T cells in the TIL of a patient with a CDK4-mutant melanoma. At

present the relative contribution of these two types of reactivity has not been established. However, if the number of neo-antigens per HLA allele is around 7–10, as based on prior predictions,¹³ individual neo-antigen-specific T-cell responses would have to be of a much larger magnitude than the shared antigen-specific T-cell

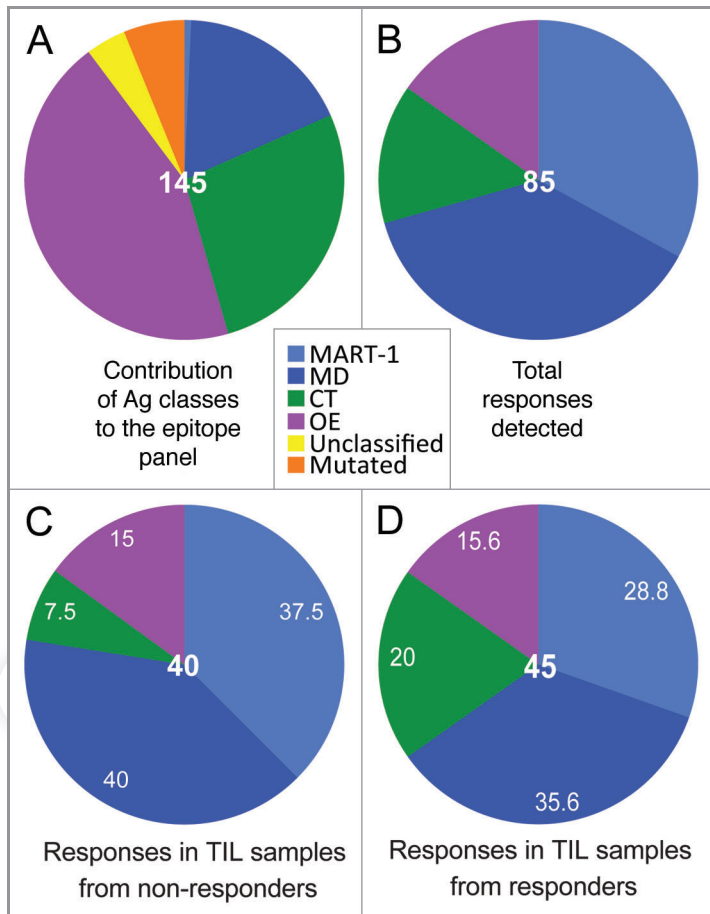


Figure 4. Contribution of antigen classes to T cell reactivity in TIL. (A) Contribution of the indicated antigen classes to the epitope panel. MD, melanoma differentiation antigens; CT, cancer/testis antigens; OE, overexpressed antigens; unclassified, antigens that cannot be designated to a specific class based on available data; mutated, mutated antigens. (B) Contribution of antigen classes to the antigen-reactive T-cell populations detected in both non-selected and selected TIL infusion products. (C and D) Contribution of antigen classes to the antigen-reactive T-cell populations detected in TIL infusion samples from clinical non-responders (C) and responders (D). The numbers in the center of the pie charts represent the total number of T-cell responses detected. The numbers within the individual pie sections indicate the percentage of T-cell responses in each category of all T-cell responses detected.

responses studied here to collectively make up a large part of the TIL T-cell population.

The patterns of T-cell reactivity that were observed for the three major classes of shared melanoma antigens varied substantially. Reactivity against overexpressed antigens was rare, in particular when taking into account that this antigen class comprises a substantial fraction of the T cell epitopes that have been described for melanoma. On the contrary, consistent with prior data on T cell reactivity in melanoma,^{14,15} reactivity against the melanocyte differentiation antigen MART-1 was very common, and reactivity against multiple gp100 epitopes was also frequently observed. However, the presence of MART-1 or gp100-specific T cell responses did not correlate with a favorable clinical outcome, and in this cohort, reactivity against MD antigens made up a slightly

larger fraction of the T cell responses detected in non-responding patients (77.5%) than in patients that showed cancer regression upon TIL infusion (64.4%) (Fig. 4C and D). CD8⁺ T-cell responses against CT antigens, the third major class of antigens, were substantially less common than T-cell responses against MD antigens. Interestingly though, there was a trend ($p = 0.12$, n.s.) toward the preferential detection of T-cell responses against CT antigens in patients that showed a clinical response to TIL therapy (six of eight patients with CT reactivity vs. 10 of 26 without CT reactivity). By the same token, reactivity against CT antigens made up a larger fraction of the T-cell responses detected in responding (20%) than in non-responding patients (7.5%). The hypothesis that recognition of CT antigens may be associated with a favorable clinical outcome upon TIL therapy is consistent with the clinical responses of recipients of NY-ESO TCR-modified T cells,¹⁶ but analysis of a larger cohort of TIL-treated patients will obviously be essential to test this hypothesis.

To address whether TIL therapy influences the melanoma-specific CD8⁺ T-cell repertoire and to what extent such an effect could be predicted by the composition of the TIL product we compared CD8⁺ T-cell responses against the 145 epitope set in pre-treatment PBMC, the TIL product, and post-treatment PBMC. From the comparison of pre- and post-treatment PBMC it is evident that TIL therapy leads to a substantial broadening of the detectable melanoma-specific CD8⁺ T-cell repertoire ($p = 0.006$). Importantly, virtually all the CD8⁺ T-cell responses that were observed at 1 mo post-therapy were already detectable within the matched infusion product. This indicates that within this timeframe, no detectable new T-cell responses arise from either systemic T cells that survived the conditioning regimen, or from low frequency T cells contained within the $\sim 10^{10}$ T cells infused. Thus, the epitope spreading for which there is evidence in some vaccination studies^{17,18} does not play a detectable role in TIL therapy: T-cell reactivity at 1 mo post-therapy—when tumor regression is often already clinically evident—is determined by the composition of the cell product.

What do the current data mean for the further development of TIL therapy? The observation that all the antigen-specific T-cell responses that are detected in TIL infusion products are low-level, including those T-cell responses that are directed against antigens for which tolerance is presumed to be less stringent (CT antigens) or non-existent (mutant CDK4) is unexpected and at first glance disappointing. The fact that these responses are of such a low magnitude however opens the possibility that strategies to steer T-cell reactivity to some of these antigens could further increase the efficacy of TIL therapy. Specifically, as melanoma-specific T-cell reactivity post-therapy is essentially fully explained by the composition of the cell product, the use of T-cell populations that are enriched for reactivity against one or multiple antigens of interest, either by MHC multimer enrichment^{19,20} or cytokine capture^{21,22} is likely to lead to a more profound reactivity against these antigens post-therapy. Based on the current data, infusion of T cells enriched for reactivity against CT antigens could be considered. Furthermore,

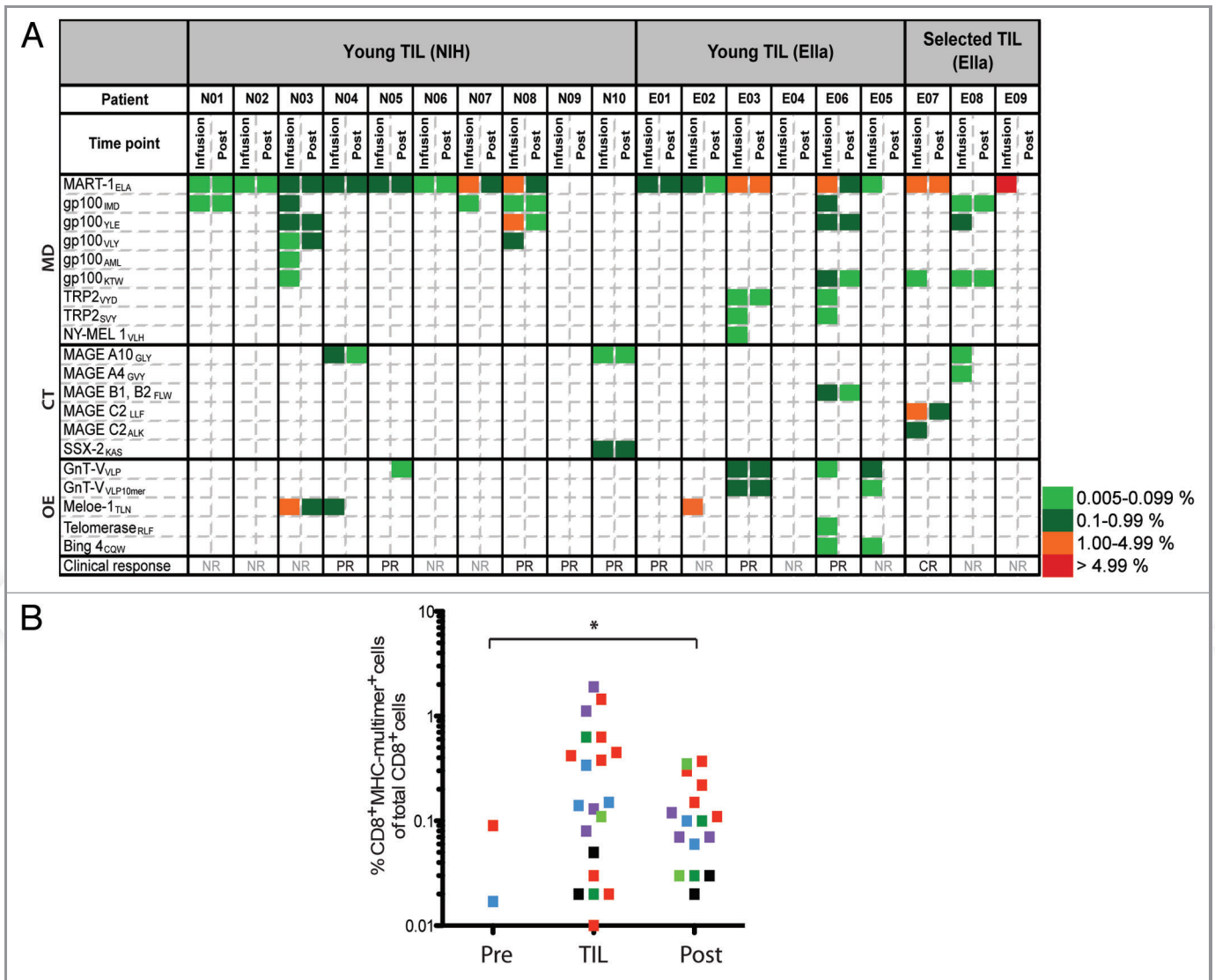


Figure 5. Melanoma-specific T cell reactivity identified in TIL products predicts T-cell reactivity post-therapy. (A) Summary of antigen-specific T-cell populations identified in one-month post-infusion PBMC samples (post) from NIH and Ella patients depicted together with the T-cell reactivities identified in the matched TIL infusion product (infusion). The presence of antigen-specific T-cell populations is indicated by the colored boxes, with the different colors reflecting antigen-specific CD8⁺ T-cell response magnitude. Only those epitopes are shown for which T-cell reactivity was detected in at least one patient sample. Patient numbers on top row, clinical responses on bottom row. CR, complete response; PR, partial response; NR, no response. (B) Relationship between melanoma-specific T-cell responses (% of total CD8⁺ T cells) detected in pre-therapy PBMC (pre), TIL infusion samples, and post-infusion PBMC (post) from seven NIH patients. Each square represents an antigen-specific T-cell population, each color represents T-cell responses detected within one patient (* p < 0.05)

with the development of technology to evaluate the patient-specific repertoire of mutated epitopes^{23,24} it may well become possible to expand such a selection to T-cell populations that are reactive against patient-specific neo-antigens.

Finally, while we here have used CD8⁺ T-cell profiling to evaluate the effects of TIL therapy, the technology should also be useful to address a series of other issues. Ongoing experiments suggest that it will be feasible to address to what extent Ipilimumab treatment³ influences T-cell reactivity against the shared melanoma antigens in melanoma patients and to what extent this correlates with clinical course. As a second example, the targeting of mutant B-RAF by Vemurafenib has shown a very

high response rate in patients with metastatic melanoma,²⁵ and the combination of anti-CTLA4 treatment and B-RAF inhibition forms a logical next step. It will be of interest to determine to what extent tumor cell death induced by B-RAF inhibition can by itself enhance melanoma-specific T cell responses, or whether B-RAF inhibition can enhance the effects of anti-CTLA4 treatment on the tumor-specific T-cell repertoire in melanoma.

Materials and Methods

Generation of pMHC complexes. Melanoma-associated peptides were purchased from Pepsican (Pepsican Presto BV) and UV

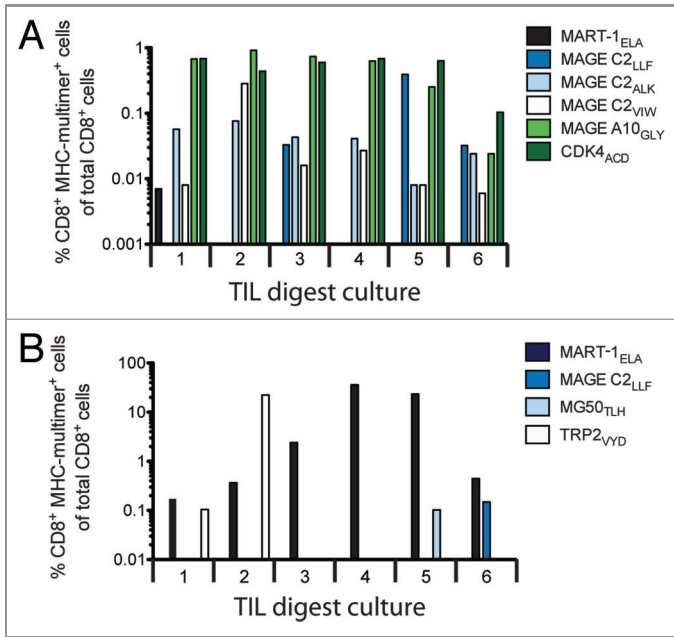


Figure 6. Random variability in TIL composition during in vitro culture. Magnitude of antigen-specific T-cell populations (percent of total CD8⁺ T cells) in six different T-cell cultures originating from the same tumor digest of (A) patient NK1 and (B) patient NK2. T-cell cultures were initiated in separate wells (1–6) and cultured for approximately 2 weeks prior to analysis of T-cell reactivity.

cleavable peptides were synthesized in-house as described previously.⁹ Recombinant HLA-A2 heavy chains and human β 2 m light chain were produced in *Escherichia coli* and isolated from inclusion bodies. MHC class I refolding reactions and purification by gel-filtration HPLC was performed as described previously.²⁶ Specific peptide-MHC complexes were generated by UV-induced ligand exchange⁹ in a 96 well format. In brief, pMHC complexes loaded with UV-sensitive peptide (100 μ g ml⁻¹) were subjected to 366 nm UV light (Camag) for 1 h at 4°C in the presence of rescue peptides (200 μ M).¹⁰

Generation of pMHC multimers. pMHC multimers were generated using a total of eight different fluorescent streptavidin (SA) conjugates (Invitrogen). For each 10 μ l of pMHC monomer (100 μ g ml⁻¹), the following amount of SA-conjugates was added: 1.5 μ l SA-QD605 (Q10101MP), 1.0 μ l SA-QD625 (A10196), 1.5 μ l SA-QD655 (Q10121MP), 1.5 μ l SA-QD705 (Q10161MP), 1.0 μ l SA-QD800 (Q10171MP), 1.1 μ l SA-PE (1 mg μ l⁻¹, SA1004–4), 1.1 μ l SA-PE-Cy7 (1 mg μ l⁻¹, SA1012) and 0.6 μ l SA-APC (1 mg μ l⁻¹, SA1005). For each pMHC monomer, conjugation was performed with two of these fluorochromes, as detailed in Figure S1. Mixtures were incubated 30 min on ice. NaN₃ (0.02% wt/vol) was added and an excess of D-biotin (26.4 mM, Sigma) was added to block residual binding sites.

Cells and T cell staining. TIL infusion products and PBMC samples were obtained from individuals with Stage IV melanoma in accordance with local guidelines, with informed consent. The median number of CD8⁺ cells infused was 31.7×10^9 (range 0.72–55.8 $\times 10^9$) for 15 products for which data was available.

Pre-treatment PBMC samples were collected with a median of 36 d (standard deviation 66 d) prior to TIL infusion. Post-treatment PBMC samples were collected 27 d (median, standard deviation 3 d) subsequent to TIL infusion. All samples were cryopreserved in FCS with 10% DMSO and stored in liquid N₂ before shipment to NKI. Samples were shipped on dried ice from NIH and Ella institute, and again stored in liquid N₂ until used. Cells were thawed on the day of analysis and cell numbers were determined using trypan blue to exclude dead cells.

For T cell staining, the following amounts of fluorescently labeled pMHC complexes were pooled together for combinatorial coding, or used separately for confirmations: 1 μ l of PE-pMHC, 2 μ l of APC-pMHC, 3 μ l of QD605-pMHC, 2 μ l of QD625-pMHC, 2 μ l of QD655-pMHC, 4 μ l of QD705-pMHC, 4 μ l of QD800-pMHC, 3 μ l of PE-Cy7-pMHC. Final staining volume was 128 μ l and cells were incubated at 37°C for 15 min. For combinatorial coding on PBMCs, 2 μ l anti-CD8-FITC (BD 345772), 1 μ l anti-CD4-AF700 (Invitrogen MHCD0429), 1 μ l anti-CD14-AF700 (Invitrogen MHCD1429), 1 μ l anti-CD16-AF700 (Invitrogen MHCD1629), 3 μ l anti-CD19-AF700 (Invitrogen MHCD1929) and 0.5 μ l LIVE/DEAD[®] Fixable IR Dead Cell Stain Kit (invitrogen L10119) was then added. Antibody stainings were followed by incubation on ice for 30 min. Before flow cytometry analysis, cells were washed twice.

Flow cytometry. Data acquisition was performed on a LSR-II flow cytometer (Becton Dickinson) with FACS Diva software. The following 11 color instrument setting was used for combinatorial coding analyses: UV laser (355 nm): QD605, 595LP, 605/12; QD705, 685LP, 710/50; QD800, 750LP, 780/60. Violet laser (405 nm): QD625, 610LP, 625/20; QD655, 635LP, 655/8. Blue laser (488 nm): FITC, 505LP, 525/50. Yellow-green laser (561 nm): PE, 585/15; PE-Cy7, 750LP, 780/60. Red laser (640 nm): APC, 670/14; AF700, 685LP, 710/50; IR-Dye, 750LP, 780/60. To identify antigen-specific T cells, the following gating strategy was used. (1) Selection of live (IR-dye negative) single cell lymphocytes (FSC-W/H low, SSC-W/H low, FSC/SSC-A). (2) Selection of anti-CD8-FITC⁺ and “dump” (anti-CD4, -CD14, -CD16, -CD19) negative cells. (3) Selection of CD8⁺ T cells that were positive in two and only two MHC multimer channels. Cut off values for the definition of positive responses were $\geq 0.005\%$ of total CD8⁺ cells and ≥ 10 events. 500,000 CD8⁺ T cells were recorded per sample when possible and a minimum of 50,000 CD8⁺ T cells was acquired.

IFN γ release assay. To address reactivity of mutant CDK4 epitope-specific T cells, tetramer-positive were sorted and cultured as described previously.¹⁰ T2 cells were loaded with the indicated peptides for 1 h and washed once. Subsequently, 1×10^5 T cells (79.5% MHC-multimer⁺) were incubated with 1×10^5 T2 cells for 4 h at 37°C in RPMI with 10% human serum and protein transport inhibitor (BD GolgiPlug, 555029). Cells were stained with PerCP-Cy5.5-conjugated anti-CD8 (BD, 341051) for 20 min at 4°C, fixed and permeabilized (BD Cytofix/Cytoperm kit, 555028), and stained with APC-conjugated anti-IFN γ (BD, 340452) for 30 min at 4°C. Samples were analyzed by flow cytometry (Calibur, Becton Dickinson), data analysis was performed using FlowJo software.

To address reactivity of TIL against autologous tumor cell lines, TIL cultures (1×10^5 , pre-rapid expansion cultures for the NKI samples and TIL infusion product for the TIL sample from Ella Institute) were incubated with 1×10^5 autologous tumor cells for 4 h at 37°C in RPMI with 10% human serum and protein transport inhibitor (BD GolgiPlug, 555029). Staining and analysis of the cells were conducted as described above.

Tumor antigen analysis. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, 74104). First strand cDNA was generated from 0.5 mg total RNA using the ThermoScript RT-PCR System (Invitrogen, Inc., 11146-016). Quantitative RT-PCR assays analyzing tumor antigen gene expression were performed for 40 cycles with an annealing temperature of 60°C using TaqMan Gene Expression Assays (Applied Biosystems, Inc.) and were normalized using GAPDH.

Statistical analyses. The difference in the number of T-cell responses in pre- and post-treatment PBMC samples was assessed by two-tailed, paired Student's t-test. The correlation between clinical outcome and the presence of CT specific T-cell reactivities in TIL products was assessed by Fisher's exact test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acknowledgments

We would like to thank Anita Pfauth and Frank van Diepen for flow cytometry support and Willeke van de Kastele for technical assistance. This research was supported by grants from the Melanoma Research Alliance (105636), CTMM (04I-301) and The Dutch Cancer Society (2009-4282).

Author Contributions

C.J.S., P.K. and B.H. designed and performed experiments, interpreted data and co-wrote the paper, M.F., M.T., N.v.R., C.L., M.v.B. and J.H.M.U. performed experiments and interpreted data, C.A.T. designed the epitope library, J.B.B. analyzed data, P.t.S. and G.G.K. co-supervised parts of the study, Y.F.L. and P.F.R. contributed qPCR data, M.J.B., J.S., M.E.D., S.A.R. and J.B.A.G.H. contributed patient samples and interpreted data, S.R.H. supervised the design of the epitope library and contributed to study design, T.N.M.S. designed the study, interpreted data and co-wrote the paper.

Supplemental Material

Supplemental materials may be found here:

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