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The Stoichiometric Production of IL-2 and IFN- γ mRNA Defines Memory T Cells That Can Self-Renew After Adoptive Transfer in Humans

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Abstract

Adoptive immunotherapy using ex vivo-expanded tumor-reactive lymphocytes can mediate durable cancer regression in selected melanoma patients. Analyses of these trials have associated the in vivo engraftment ability of the transferred cells with their antitumor efficacy. Thus, there is intensive clinical interest in the prospective isolation of tumor-specific T cells that can reliably persist after transfer. Animal studies have suggested that central memory CD8⁺ T cells (T_{CM}) have divergent capabilities including effector differentiation to target antigen and stem cell-like self-renewal that enable long-term survival after adoptive transfer. We sought to isolate human melanoma-specific T_{CM} to define their in vivo fate and function after autologous therapeutic transfer to metastatic patients. To facilitate the high-throughput identification of these rare cells from patients, we report that T_{CM} have a defined stoichiometric production of interleukin-2 (IL-2) and interferon- γ (IFN- γ) mRNA after antigen stimulation. Melanoma-specific T cells screened for high relative IL-2 production had a T_{CM} phenotype and superior in vitro proliferative capacity compared to cells with low IL-2 production. To investigate in vivo effector function and self-renewal capability, we allowed melanoma-specific T_{CM} to undergo in vitro expansion and differentiation into lytic effector clones and then adoptively transferred them back into their hosts. These clones targeted skin melanocytes in all five patients and persisted long term and reacquired parental T_{CM} attributes in four patients after transfer. These findings demonstrate the favorable engraftment fitness for human T_{CM}-derived clones, but further efforts to improve their antitumor efficacy are still necessary.

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Competing interests: A provisional patent application has been filed by U.S.K. (PCT/US2011/47719): Methods of identifying central memory T cells and obtaining antigen-specific T cell populations. All other authors declare that they have no competing interests.

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INTRODUCTION

The adoptive transfer of ex vivo–expanded autologous tumor-infiltrating lymphocytes in conjunction with lymphodepleting conditioning regimens can mediate durable complete tumor regression in selected patients with metastatic melanoma (1–4). Retrospective analyses of these trials have associated the ability of the transferred cells to persist in vivo with their antitumor efficacy (4–6). Thus, in an effort to improve treatment outcomes, there is active interest in the identification of tumor-specific T cells that can reliably engraft and survive long term after transfer. Memory CD8⁺ T cells (T_M) have long been considered to play an important role in the immune response against viral pathogens on the basis of their ability to rapidly expand to massive cell numbers, differentiate into potent effectors, and eventually self-renew and persist for the lifetime of the host (7–10). Given these special properties, there has been recent interest in the use of T_M for the adoptive immunotherapy of cancer. The pool of CD8⁺ T_M can be divided into effector memory T cells (T_{EM}), which acquire potent lytic function to protect the host, and central memory T cells (T_{CM}), which have the lymph node–homing molecules CD62L and CCR7 and are capable of robust proliferation and stem cell–like self-renewal (11). Animal studies comparing the adoptive transfer of CD8⁺ T cell subsets with these distinct memory properties have suggested that they have quite different in vivo fates and therapeutic abilities. In the *pme1* murine model, naïve T cell receptor (TCR) transgenic CD8⁺ T cells, which had undergone limited in vitro differentiation to attain T_{CM} properties, demonstrated superior ability to persist and mediate melanoma tumor regression when compared to more extensively cultured cells with T_{EM} properties (12–14). With prolonged in vitro culturing, CD8⁺ T cells were found to progressively lose in vivo proliferative potential and subsequently become senescent and undergo apoptosis (13). These observations led to the prevailing theory that in vitro effector cell differentiation was the dominant explanation for the poor persistence and limited antitumor efficacy of extensively expanded CD8⁺ T effector (T_E) clones administered in previous adoptive transfer clinical trials (15–19).

However, recent observations have challenged the belief that all ex vivo–expanded T_E clones have poor survival fates after adoptive transfer. In an intriguing study involving the administration of autologous cytomegalovirus (CMV)–specific CD8⁺ T cells in a nonhuman primate model (20), highly differentiated and extensively expanded T_E clones derived from T_{CM}, but not those derived from T_{EM}, established persistent memory in the host animals. Similar findings were reported for human T_{CM}-derived CMV-specific cells transferred into immunodeficient mice (21). The authors from these studies suggested that despite acquiring potent effector function, the T_E that were derived from T_{CM} precursors retained intrinsic properties related to their memory lineage, which enabled them to persist after adoptive transfer and revert back to the memory cell pool. Although recent human studies have also noted selective long-term persistence of differentiated effector T cells after adoptive transfer, the parental memory properties for these persisting cells were not assessed, and thus, direct evidence for T cell self-renewal after transfer is still lacking (1–4, 22, 23). To provide critical insight into the role of human T_{CM} for cancer immunotherapy, we sought to prospectively generate highly differentiated T_E clones exclusively from autologous tumor-specific T_{CM} to

precisely define their in vivo effector function, persistence, and self-renewal capacity after therapeutic transfer to patients with metastatic melanoma.

The prohibitive obstacles in conducting human studies with tumor-specific T_{CM} have been the lack of efficient in vitro methods and clinically approved reagents to isolate these rare cells for therapeutic use. To overcome these challenges, we developed a high-throughput isolation method based on the cytokine profile of memory T cells. We reveal here that T_{CM} and T_{EM} have distinct stoichiometric production of interleukin-2 (IL-2) and interferon- γ (IFN- γ) mRNA after antigen exposure, which can facilitate their differential isolation from peripheral blood. Using this strategy, we isolated low-frequency tumor-specific T cells with prototypic T_{CM} characteristics from short-term cultures. To investigate in vivo effector function and self-renewal capability, we allowed gp100-specific T_{CM} isolated from metastatic melanoma patients to undergo ex vivo differentiation into potent effector clones and then adoptively transferred them back into their hosts. We demonstrate that T_{CM}-derived gp100-specific effector clones could effectively target skin melanocytes, persist long term, and also reacquire parental T_{CM} phenotypic and functional attributes after adoptive transfer.

RESULTS

Memory CD8⁺ T cells have distinct stoichiometric production of IL-2 and IFN- γ mRNA

We previously reported that functional screening of polyclonal T cell cultures by measuring the peak quantitative production of IFN- γ mRNA after a 3-hour antigen exposure could detect a single antigen-specific CD8⁺ T cell spiked into 100,000 background cells (24, 25). This sensitive screening assay could be performed in a high-throughput manner and facilitate the rapid cloning of a variety of rare tumor-specific CD8⁺ T cells from human peripheral blood for use in adoptive transfer clinical trials (24). Building on this experience, we sought to adapt this methodology to isolate tumor-specific CD8⁺ T cells with distinct memory attributes. Human T_{CM} have been reported to preferentially produce greater quantities of IL-2 and less IFN- γ than their T_{EM} counterparts (11, 26, 27). Thus, we hypothesized that functional profiling for the relative production of these cytokines after T cell stimulation might be both a sensitive strategy to identify rare antigen-specific CD8⁺ T cells and a specific strategy to elucidate their memory status. To explore the feasibility of this approach, we first defined the relative production of IL-2 and IFN- γ mRNA from sort-purified bulk populations of naïve, T_{CM}, and T_{EM} CD8⁺ T cells that were defined by the coexpression of CD45RO and CD62L from the peripheral blood mononuclear cells (PBMCs) of three healthy donors (Fig. 1A). After a 3-hour stimulation with anti-CD3/CD28 beads, we found that the T_{CM} subset produced the highest copy number of IL-2 mRNA, the T_{EM} subset produced the highest copy number of IFN- γ mRNA, and the naïve cells remained quiescent with the least production of both cytokines. To integrate the stoichiometric production of these two cytokines into a single reference value, we divided absolute copies of IL-2 mRNA by absolute copies of IFN- γ mRNA. This quotient was multiplied by a constant of 1000 to derive a normalized value for each T cell subset called the *IL-2:IFN* index. From fresh PBMCs, the mean *IL-2:IFN* indices for the naïve, T_{CM}, and T_{EM} subsets were found to be statistically distinct, with the T_{CM} subset having a significantly higher *IL-2:IFN* index when compared to the other two populations ($P < 0.05$).

We next sought to determine whether prospective screening for differences in the *IL-2:IFN* index could be used to identify low-frequency antigen-specific T cells with distinct memory properties from short-term peripheral blood cultures. Fresh PBMCs from a human leukocyte antigen (HLA)-A201⁺ metastatic melanoma patient were used to establish multiple oligoclonal microcultures ($n = 192$), which were subjected to in vitro sensitization with an immunodominant peptide from the gp100 tumor differentiation antigen (gp100₁₅₄₋₁₆₂). After 14 days, the cultures were screened for the production of IFN- γ and IL-2 mRNA in response to cognate antigen reexposure. Consistent with a low endogenous T cell frequency, we identified only 9 cultures of the 192 (4.7%) with gp100₁₅₄₋₁₆₂ reactivity based on the production of either cytokine (fig. S1). The copies of IFN- γ and IL-2 mRNA produced by these nine cultures varied considerably (Fig. 1B), and there was no correlation between the cytokine transcripts [$r^2 = 0.03$, $P =$ not significant (n.s.)]. To better understand the observed heterogeneity in cytokine mRNA, we performed fluorescence-activated cell sorting (FACS) analysis to determine the frequency and memory phenotype of the gp100-specific T cells within these cultures. Tetramer staining revealed the presence of gp100-specific T cells in all nine selected cultures with frequencies ranging from 1 to 23% of CD8⁺ cells (Fig. 1C). The copies of IFN- γ mRNA produced after antigen exposure strongly correlated with the percentage of tetramer⁺CD8⁺ cells in the cultures ($r^2 = 0.94$, $P < 0.0001$); however, there was no association between tetramer frequency and IL-2 mRNA production ($r^2 = 0.003$, $P =$ n.s.) (Fig. 1D). We next calculated the *IL-2:IFN* index for the nine cultures. Microcultures 1 to 6 each had an *IL-2:IFN* index < 5 , whereas microcultures 7 to 9 had an index > 100 (Fig. 1E). The memory phenotype of the tetramer⁺ cells within each of these microcultures was assessed by performing surface staining for CD45RO and CD62L (Fig. 1F). The tetramer⁺ cells in the microcultures with an *IL-2:IFN* index < 5 uniformly had low CD62L expression ($6.3 \pm 0.6\%$), consistent with a T_{EM} phenotype. In contrast, the tetramer⁺ cells in the microcultures with an index > 100 had high CD62L expression ($83.7 \pm 0.9\%$), consistent with a T_{CM} phenotype. There was no significant correlation between memory phenotype with either the absolute levels of IL-2 or IFN- γ mRNA produced by the individual cultures ($P =$ n.s.). However, when the *IL-2:IFN* index for these nine microcultures was compared in a regression analysis to the percent of gp100 tetramer⁺CD8⁺ cells with a T_{CM} phenotype, we found a strong linear correlation ($r^2 = 0.96$, $P < 0.0001$) between the parameters (Fig. 1G). When additional cell surface markers were compared (fig. S2), we found that the tetramer⁺CD8⁺ cells from the high-index microcultures demonstrated a less differentiated phenotype with greater expression of CD45RA, CD62L, and CD27 while having lower expression of CD45RO when compared to cells from sister microcultures with a low index. Cumulatively, these data suggested that the magnitude of IFN- γ mRNA produced by a sensitized culture correlated with the general frequency of antigen-specific CD8⁺ T cells, but the stoichiometric ratio of IL-2 to IFN- γ correlated with the specific memory phenotype of those T cells.

High-throughput profiling for the *IL-2:IFN* index identifies melanoma-specific T_{CM} from the peripheral blood of patients

To determine whether functional profiling for the *IL-2:IFN* index could serve as an efficient strategy to isolate low-frequency melanoma-specific T_{CM} for clinical use, we next prospectively screened early PBMC microcultures from six HLA-A201⁺ metastatic

melanoma patients for gp100_{154–162} or MART_{27–35} reactivity. From each of these patients, we identified paired reactive microcultures but with dichotomous *IL-2:IFN* index values. The antigen-specific cells in each of the sister cultures were then assessed by FACS for their memory phenotype and frequency. Among the pairs, the cultures with the higher *IL-2:IFN* index consistently demonstrated a greater frequency of tetramer⁺ cells that expressed CD62L⁺ when compared to their counterpart cultures with a lower *IL-2:IFN* index (Fig. 2A). When the *IL-2:IFN* index for each of the microcultures was compared in a regression analysis to the percent of tetramer⁺CD8⁺ cells with a T_{CM} phenotype, we again observed a strong correlation ($r^2 = 0.90$) between the parameters; however, the relationship was logarithmic rather than linear (Fig. 2B). Cultures with an *IL-2:IFN* index above 50 appeared to cluster together and contain uniformly high frequencies of antigen-specific T_{CM}, $84 \pm 2\%$ (mean $\pm$ SEM). Conversely, cultures with an *IL-2:IFN* index <10 contained only $16 \pm 5\%$ T_{CM}. The categorical stratification of the *IL-2:IFN* index as either <10 or >50 was highly statistically associated ($P < 0.0001$) with the presence of antigen-specific T_{CM} in the respective cultures (Fig. 2C). There was no association ($P = 0.359$) between the *IL-2:IFN* index of these same cultures and the frequency of tetramer⁺ cells (Fig. 2D), which varied between 2 and 88% of total CD8⁺ cells. Thus, the ability of the *IL-2:IFN* index to distinguish the memory phenotype was not limited by the frequency of antigen-specific T cells in the culture. On the basis of these results, we concluded that high-throughput functional profiling of T cell cultures for their *IL-2:IFN* index could serve as an effective means to identify tumor-specific CD8⁺ cells with a T_{CM} memory phenotype from the peripheral blood of melanoma patients.

Melanoma-specific CD8⁺ T cells with a high *IL-2:IFN* index demonstrate superior in vitro proliferation and responsiveness to IL-2

A hallmark of T_{CM}, compared to T_{EM}, is their ability to undergo considerable proliferation in response to secondary antigen exposure (26). We thus sought to determine whether melanoma-specific CD8⁺ cells selected by a high *IL-2:IFN* index had not only a T_{CM} phenotype but also greater proliferative capacity upon stimulation when compared to cells with a low *IL-2:IFN* index. Synchronous pairs of gp100-reactive microcultures with dichotomous *IL-2:IFN* indices [high (>50) and low (<10)] were identified from the PBMCs of four metastatic melanoma patients. These paired cultures were exposed to anti-CD3 antibody, IL-2, and autologous irradiated PBMCs to induce a rapid polyclonal expansion. A representative expansion is shown in Fig. 3A. After 12 days, the high-index culture showed relative maintenance of the tetramer⁺CD8⁺ frequency, whereas the low-index culture showed a substantial decrease in frequency. Absolute cell counts from these expanded cultures demonstrated that the tetramer⁺CD8⁺ cells in the high-index culture expanded 1350-fold, whereas those in the low-index culture expanded only 32-fold (Fig. 3B). To confirm that this difference in proliferation was not due to experimental variability between the two cultures, we analyzed the proliferation of the tetramer⁺CD8⁺ populations from both cultures and found that they expanded nearly identically (1777- and 1649-fold, respectively). The proliferation of paired cultures from four independent patients (Fig. 3C) confirmed that the tetramer⁺CD8⁺ cells from cultures with a high *IL-2:IFN* index demonstrated a significantly greater fold expansion compared to the tetramer⁺CD8⁺ cells in the sister cultures with a low *IL-2:IFN* index (mean fold expansion, 656 versus 17; $P < 0.05$). As an internal control, the

tetramer⁺CD8⁺ populations from both sets expanded nearly identically (mean fold expansion, 1127 versus 1183; $P = \text{n.s.}$).

To determine whether the superior expansion of the high-index T cells was due to their intrinsic proliferative capacity rather than cell death or apoptosis of the low-index cells, we compared carboxyfluorescein diacetate succinimidyl ester (CFSE) dye dilution in paired gp100-reactive microcultures with dichotomous *IL-2:IFN* indices (>50 and <10) (Fig. 3D). Five days after stimulation with anti-CD3/CD28 beads, 51% of the tetramer⁺ cells in the low-index microculture diluted their CFSE compared to 97% of the tetramer⁺ cells in the high-index microcultures, demonstrating that a greater percentage of the high-index cells had undergone cell division. Furthermore, the magnitude of CFSE fluorescence [mean fluorescence intensity (MFI)] in the tetramer⁺ cells was considerably lower in the high-index cells after stimulation, indicating that they had undergone a greater number of cell divisions compared to the tetramer⁺ cell with a low index. As an internal control, the tetramer⁺ populations from both cultures demonstrated similar percent CFSE dilution (low index: 88% versus high index: 92%) and MFI changes.

We next asked whether the inferior proliferative response of the low-index cells could be remedied by supplementing the cultures with increasing concentrations of exogenous IL-2. To answer this question, we generated four independent pairs of gp100-reactive microcultures with dichotomous *IL-2:IFN* indices from two melanoma patients and evaluated their proliferation in response to titrated amounts of IL-2 ranging from suboptimal (5 IU/ml) to saturating concentrations (500 IU/ml) in the absence of TCR stimulation (Fig. 3E). After 7 days, we found that the tetramer⁺ and tetramer⁺ cells in both the high- and the low-index cultures demonstrated IL-2 dose-related increases in proliferation as measured by CFSE dilution. However, the tetramer⁺ cells in the high-index cultures demonstrated enhanced responsiveness across all IL-2 concentrations as evident by greater percent CFSE dilution compared to the tetramer⁺ cell in the low-index cultures [two-way analysis of variance (ANOVA), $P < 0.001$] (Fig. 3F). Further, the proliferative response for the low-index cells at the highest IL-2 concentration (500 IU/ml) was still lower than the proliferative response of the high-index cells at their lowest concentration (5 IU/ml). As an internal control for these observed differences, we found that the tetramer⁺ cells from these cultures showed similar proliferation. To quantify the relative IL-2 dose responsiveness between high- and low-index cells, we calculated the EC_{50} , the dose required to reach 50% of the maximum proliferative response. The EC_{50} for the high-index cells was 25 IU/ml, whereas the low-index cells had such poor proliferation that the estimated EC_{50} was >90 IU/ml. Thus, the proliferation of the low-index cells could not be improved by providing saturating amounts of IL-2. Cumulatively, we concluded that microcultures with a high *IL-2:IFN* index were composed of memory T cells with an intrinsically superior proliferative capacity and greater responsiveness to exogenous IL-2 when compared to the cells from low-index cultures.

T_E clones generated from high and low *IL-2:IFN* index precursors acquire similar cytolytic effector characteristics but differ in the expression of cell survival genes

We next compared the function, phenotype, and gene expression profiles of T_E clones that were differentiated from antigen-specific cells with high and low *IL-2:IFN* indices. Melanoma-specific memory cells were isolated from peripheral blood cultures of each of five HLA-A2⁺ melanoma patients and categorized into two groups on the basis of their antigen-induced *IL-2:IFN* index and expression of CD62L. The high-index group included T cells with an *IL-2:IFN* index >50 and a T_{CM} phenotype (high CD62L expression). In contrast, the T cells in the low-index group had an *IL-2:IFN* index <10 and a T_{EM} phenotype (low CD62L expression). A representative comparison is shown in Fig. 4A. From each of these parental populations, a single antigen-specific T_E clone was generated with a standardized rapid T cell cloning methodology (24). The average total time to generate the CD8⁺ T cell clones was 28 ± 5 days. All T cell clones underwent large-scale expansion and were uniformly analyzed at an in vitro age of 56 ± 9 days. Despite initial differences in the CD62L expression and *IL-2:IFN* index of the parental cells, all of the derived clones acquired similar effector phenotype (loss of CD62L) and potent in vitro antitumor lytic reactivity (Fig. 4B). We next performed microarray analysis to compare the resting transcriptional profile of the high- and low-index derived T_E clones. We identified 141 genes that were differentially expressed ($P < 0.01$) between the two clone groups (Fig. 4C and table S1). Ingenuity Pathway Analysis revealed that the three dominant categorical functions for this gene set were related to cell survival (cell death, cellular development, and cellular growth and proliferation) (Fig. 4D and fig. S3). The most statistically up-regulated gene expressed in the high-index clones was *CD40 ligand* (*CD40L*) ($P = 0.0075$; mean fold increase, 6.2), and the most down-regulated gene was the proapoptotic molecule *Bcl2-modifying factor* (*BMF*) ($P = 0.0073$; mean fold decrease, 1.8). To validate the microarray findings, we performed TaqMan analysis to quantify the *CD40L* and *BMF* mRNA expression in an expanded cohort of clones. The normalized expression of *CD40L* in the high-index clones was 507 ± 78 copies compared to 15 ± 7 copies found in the low-index clones. Conversely, the normalized expression of *BMF* in the high-index clones was 10 ± 1 copies compared to 39 ± 12 copies found in the low-index clones. Thus, there was an inverse pattern of expression of *CD40L* and *BMF* in T_E clones derived from low- and high-index precursors (Fig. 4E).

Cumulatively, these data demonstrated that T_E clones with similar functional and phenotypic characteristics have significant transcriptional differences related to the *IL-2:IFN* index and memory status of their parental cells. The dominant functions of these differentially expressed genes were involved in cell survival, and thus, they may play an important role in governing the in vivo fate of these T cell clones.

gp100-specific T_E clones derived from high *IL-2:IFN* index precursors induce autoimmune dermatitis in metastatic melanoma patients after adoptive transfer

Given the prototypic T_{CM} properties observed for T cells with a high *IL-2:IFN* index, we next asked whether melanoma-specific T_E clones derived specifically from these parental cells could effectively target antigen in vivo and establish long-term persistent memory after adoptive transfer in metastatic melanoma patients. In past clinical efforts, the adoptive

transfer of cloned lymphocytes rarely resulted in detectable numbers of the transferred cells in the peripheral blood of patients beyond 2 weeks, even when administered in conjunction with a lymphodepleting preparative regimen (17). To readdress this issue, we enrolled five consecutive HLA-A2⁺ metastatic melanoma patients on a pilot clinical trial to determine the in vivo fate and therapeutic efficacy of expanded gp100_{154–162}-specific T_E clones that were derived from high *IL-2:IFN* index precursors. All patients received a lymphodepleting preparative regimen followed by infusion of the T cell clones and intravenous high-dose IL-2. Table 1 shows the patient demographics, T cell characteristics, and treatment details. The patients had advanced metastatic melanoma (M1b or M1c) originating from a variety of primary sites (ocular, cutaneous, or mucosal). High-throughput screening of the PBMCs from each patient successfully identified gp100_{154–162}-specific CD8⁺ T cells with a high *IL-2:IFN* index (range, 51 to 111). The starting CD8⁺ cells from patients 3 to 5 were also found to have high expression of CD62L, consistent with a T_{CM} phenotype. Parental cells from patients 1 and 2 were not available for phenotypic analysis. Single-cell limiting dilution was performed on each parental population to derive antigen-specific CD8⁺ clones for patient administration. A consistent finding was the identification of a single clonotype from each patient as assessed by TCR variable-chain sequencing. The isolated clones underwent large-scale ex vivo expansion for patient therapy, with the average infused cell number being 23.9×10^9 cells (range, 0.4×10^9 to 45.1×10^9 cells). Not surprisingly, at the time of infusion, all clones had the functional and phenotypic characteristics of differentiated T_E. Each clone demonstrated high avidity and specificity against the gp100 antigen by secreting large amounts of IFN- γ in response to naturally presented peptide on HLA-A2⁺ tumors and peptidepulsed T2 target cells (table S2). Analysis of the clones by intracellular FACS for IL-2 and IFN- γ production after antigen stimulation revealed an effector cytokine profile with $73 \pm 5\%$ of the reactive cells producing only IFN- γ , $26 \pm 5\%$ of cells producing both IFN- γ and IL-2, and a barely detectable population of cells producing only IL-2 ($1 \pm 1\%$) (Fig. 5, A and B). Further, the clones were found to be highly lytic with $78 \pm 10\%$ specific lysis of HLA-matched melanoma tumor lines at a 30:1 effector/target ratio. Phenotypically, each of the clones demonstrated high expression of CD45RO and CD95 and no detectable expression of CD62L, CD127, and CD28, consistent with effector cell differentiation (fig. S4). The only atypical feature of the derived clones was the variable, persistent expression of CD27, which is usually absent on the surface of extensively expanded T cell clones.

Within 5 days of infusing gp100-specific T_E clones derived from the high-index precursors, there was evidence of in vivo trafficking and targeting of gp100-expressing melanocytes in skin. All five patients developed a diffuse erythematous skin rash with biopsies revealing histologic changes of autoimmune dermatitis, such as intraepidermal spongiosis, CD8⁺ lymphocyte infiltration, and loss of intraepidermal melanocytes (Fig. 5C). These observations, noted at a time when the endogenous lymphocytes had been depleted, strongly suggested that the epidermal melanocytes were the targets of immune attack by the transferred clones.

gp100-specific T_E clones derived from high *IL-2*/*IFN* index precursors demonstrate in vivo persistence and self-renewal after adoptive transfer

To evaluate the in vivo survival of the transferred clones, we compared peripheral blood samples, obtained before and 1 month after cell infusion, by FACS for the frequency of gp100 tetramer⁺CD8⁺ cells (Fig. 6A). None of the five treated patients had detectable gp100 tetramer⁺CD8⁺ cells in their peripheral blood before treatment; however, 1 month after clone transfer, we observed that the first four patients had considerable circulating frequencies of these cells ranging from 1.2 to 12% of all CD8⁺ T cells. Patient 5 was the only patient in this pilot cohort who did not show evidence of peripheral blood persistence. To evaluate the long-term fate of the persisting clones, we obtained extended peripheral blood samples from patient 3, which demonstrated the presence of circulating clones at a frequency of 1% beyond 100 days (Fig. 6B). As a control, we found no change in the frequency of MART-specific T cells in the peripheral blood of these patients after treatment.

We next assessed the ability of the persisting clones from the first four patients to re-respond to antigenic stimulation. Without culturing or addition of exogenous cytokines, peripheral blood samples obtained 1 month after cell infusion were assayed against T2 cells pulsed with gp100 peptide or a control peptide (HIVpol). Intracellular FACS for IL-2 and IFN- γ production revealed that the persisting clones from each of the patients maintained robust functional reactivity against gp100 targets (Fig. 6C). However, we noted that the persisting cells had a different cytokine profile when compared to the infused clones. Of the reactive cells, $65 \pm 4\%$ produced only IFN- γ , $22 \pm 3\%$ produced both IFN- γ and IL-2, and $13 \pm 5\%$ produced exclusively IL-2 (Fig. 6D). The appearance of this distinct population of IL-2-producing gp100-reactive T cells demonstrated that a subset of the infused T_E clones regained a memory cytokine profile characterized by high IL-2 and absent IFN- γ production. Further, when the phenotype of the persisting gp100-specific clones was analyzed, we found a distinct subset of cells that reacquired T_{CM} phenotypic properties. Compared to the infused clones, the persisting cells showed greater percent cell surface expression of CD27, CD28, CD45RA, and CD62L while showing a decrease in CD45RO (Fig. 6E). We also compared the expression of the negative regulatory molecules CTLA-4, PD-1, and TIM-3 on the clones at the time of infusion and after their engraftment. Before adoptive transfer, the clones demonstrated uniform high coexpression of CTLA-4, PD-1, and TIM-3 (Fig. 7A), a phenotype consistent with highly exhausted T cells (28–30). However, among the persisting cells from each of the four patients were a small but statistically significant percentage of cells that had lost expression of these negative regulatory markers (Fig. 7B). Further, low Ki-67 staining of the persisting cells suggested that after engraftment, the cells had reverted back to a quiescent state similar to circulating memory cells. Collectively, these findings indicate that despite undergoing extensive ex vivo expansion and effector differentiation toward an exhausted state, a subset of infused T_E clones could engraft, persist long term, and repopulate the memory T cell pool after adoptive transfer.

gp100-specific T_E clones demonstrate weak antitumor efficacy after adoptive transfer

Despite evidence of melanocyte targeting in all of the patients and clone persistence in four of the five patients, none of these initially treated patients demonstrated an overall tumor response by standard oncologic Response Evaluation Criteria in Solid Tumors (RECIST)

criteria. We did observe mixed and minor biologic activity related to the T cell clone transfer with radiographic shrinkage of individual tumors in two of the patients. In patient 1, a mediastinal lymph node demonstrated late regression 8 months after clone transfer without intervening therapy (fig. S5A). Patient 5 showed complete regression of a hepatic metastasis after therapy (fig. S5B). However, in both of these patients, other measurable lesions demonstrated progression during the same evaluation period. Thus, these limited antitumor responses did not appear to provide sufficient clinical benefit for these patients.

DISCUSSION

Here, we prospectively isolated human melanoma-specific T_{CM} to define their in vivo effector function and fate after ex vivo expansion and autologous adoptive transfer to metastatic cancer patients. We found that highly differentiated and expanded T_E clones that were specifically derived from gp100-specific T_{CM} could effectively target skin melanocytes, persist long term in treated patients, and reacquire parental memory properties after therapeutic transfer. To our knowledge, this represents the first human adoptive transfer trial in which effector clones derived from a defined memory subset have been administered as a primary therapy. The infusion of antigen-specific T cell clones, rather than a polyclonal product, allowed us to precisely monitor the in vivo phenotypic and functional changes of a single genetically identical population of T cells, without the interpretive ambiguity of selective survival of a subpopulation. These findings support recent reports of long-term T cell persistence in nonhuman primates of viral-specific T_E clones derived from T_{CM} precursors (20), and also further challenge the dogmatic belief that human effector cells with an extensive replicative history are destined for engraftment failure after adoptive transfer. Our detailed comparative analysis from individual patients of their isolated parental T_{CM} populations, their derived T_E clones, and their engrafted cells provides evidence for plasticity in the fate of a subset of human effector cells that has not been described in previous adoptive transfer clinical trials.

This study addresses a critical challenge in the field of adoptive immunotherapy: the prospective identification of antigen-specific T cells that have the ability to engraft and survive long term after transfer. Previous animal studies have suggested that less differentiated memory populations have superior ability to persist and mediate tumor regression after adoptive transfer when compared to more differentiated cells (12–14, 31). However, the translation of these findings to human cancer therapy trials has been difficult given the ex vivo expansion and consequent cellular differentiation that is required to generate a T cell product for adoptive immunotherapy. Thus, the persistence in this study of effector clones derived from parental T_{CM} suggests a potential strategy that would allow for significant ex vivo cellular expansion and differentiation, but not at the expense of in vivo cell survival after transfer.

The innovation that enabled this study was our approach in isolating rare tumor-specific T_{CM} from cancer patients. Previous T_{CM} isolation strategies have relied on flow cytometric sorting or magnetic beads to perform phenotypic segregation based on differences in the cell surface expression of the lymph node-homing molecules CD62L and CCR7 (11, 26). However, applying these approaches to the isolation of human tumor-specific T_{CM} has been

difficult because of the low frequency of these cells and the lack of clinically approved reagents to perform such separations. To overcome this major obstacle, we developed a high-throughput isolation method based on the differential production of IL-2 and IFN- γ mRNA by memory cells after antigen stimulation. Several previous reports have described that T_{CM} characteristically produce greater quantities of IL-2 and less IFN- γ than T_{EM} (11, 26, 27), but this functional attribute has not been described previously as a means to isolate these populations. We found that screening short-term cultures for their stoichiometric production of these cytokines could efficiently identify and discriminate rare antigen-specific CD8⁺ T cells with either T_{CM} or T_{EM} properties. The T cells isolated with a high *IL-2:IFN* index had not only a T_{CM} phenotype but also greater proliferative capacity and responsiveness to exogenous IL-2 when compared to cells with low autocrine IL-2 production. Further, the progeny of the high-index cells demonstrated a distinct gene profile associated with cell survival. On the basis of these favorable in vitro properties, we therapeutically transferred clones exclusively derived from high *IL-2:IFN* index precursors to a pilot cohort of metastatic melanoma patients. The robust establishment of T cell persistence in these patients parallels the findings of recent murine studies that have reported that autocrine IL-2 production is essential for the development of CD8⁺ T cell memory (32, 33).

A critical paradox in our study was the observation that despite clone persistence and autoimmune targeting of normal melanocytes, there was only a modest impact on tumor growth with no objective tumor responses in this initial cohort of patients. Immune-mediated tumor regression likely represents a complex integration of several host, tumor, and T cell variables. We believe that our current adoptive transfer study addressed several of these factors in a systematic fashion. All of the administered clones had high avidity recognition of a target antigen that was confirmed to be highly expressed in the patients' tumors. The transferred clones demonstrated potent in vitro lytic capability against gp100-expressing melanoma tumor cell lines, and there was clear evidence of in vivo targeting of normal melanocytes in all of the patients. Finally, these clones could consistently engraft and survive long term in the host after transfer. Despite these findings, we did not observe clinically meaningful tumor regression. Our analyses of the clones have suggested potential explanations for their limited efficacy. The in vivo phenotypic monitoring of the infused clones revealed that a considerable percentage of the persisting clones continued to express the negative regulatory molecules CTLA-4, PD-1, and TIM-3, with only a small population of the cells losing expression of these markers. Further, the persisting cells appeared to become quiescent in vivo with limited Ki-67 expression at late time points. On the basis of these findings, we hypothesize that the transferred cells in this study persisted in a state of functional anergy incapable of mediating sustained tumor antigen targeting. Given the availability of clinically available reagents to block PD-1 and CTLA-4 on the surface of T cells, the combination of T cell transfer with antibodies against these negative regulatory molecules may help improve in vivo efficacy.

Another potential therapeutic obstacle is the presence of immunosuppressive regulatory T (T_{reg}) cells and myeloid-derived suppressor cells, which have been shown to preferentially exist at high levels in the tumor microenvironment (34–37). The recent analysis of four clinical trials using nonmyeloablative chemotherapy with or without total body irradiation

before adoptive T cell transfer revealed that the percentage and number of reconstituting CD4⁺FoxP3⁺ T_{reg} cells observed in the peripheral blood was higher in nonresponders than in responders (38). These observations suggest that endogenous CD4⁺ T_{reg} cells have a negative impact on cancer therapy, and that strategies, such as the use of increased intensity preparative regimens (3) to more effectively reduce T_{reg} cell levels, may provide clinical benefit to patients.

To systematically evaluate adjuvant therapies (such as cytokines, vaccines, checkpoint blockade, and host conditioning) that may improve the efficacy of T cell transfer, a population of antigen-specific T cells that can reliably engraft and persist will be necessary. This report describes a high-throughput means to isolate such cells based on their quantitative production of IL-2 and IFN- γ mRNA.

MATERIALS AND METHODS

Additional experimental methods are available in the Supplementary Materials.

In vitro sensitization of PBMC microcultures

PBMCs from HLA-A2⁺ melanoma patients underwent depletion of CD4⁺ lymphocytes by magnetic bead separation (Miltenyi) and were plated as individual microcultures in 96-well flat-bottomed plates at $\sim 1 \times 10^5$ cells per well. The cells were stimulated for 14 days in the presence of the cognate peptide (1 μ g/ml) and IL-2 (90 IU/ml) as previously described (24).

High-throughput functional screening of microcultures for IFN- γ and IL-2 mRNA after antigen stimulation

A sample of each sensitized microculture was screened for antigen-specific reactivity with a rapid 3-hour quantitative polymerase chain reaction (qPCR) assay for IFN- γ and IL-2 mRNA production after coculture with T2 cells pulsed with the cognate peptide versus a control peptide, as previously described (24). Briefly, RNA isolation was performed in a 96-well format with the RNeasy 96 BioRobot 8000 kit (Qiagen). Total RNA for each sample was transcribed into complementary DNA with TaqMan Reverse Transcription Reagents (Applied Biosystems). Quantitative real-time PCR was performed with plasmid DNA standards to determine the absolute copy number for IFN- γ and IL-2 mRNA in each stimulated sample with the ABI 7500 Fast Real-Time PCR System (Applied Biosystems), as described previously (25, 39). Reactivity for each microculture was calculated as mRNA copies produced in response to cognate peptide minus the background reactivity in response to control peptide. Cultures with reactivity twice the background and copy number >1000 were considered as having significant specific peptide reactivity.

Limiting dilution cloning and expansion of antigen-specific CD8⁺ T cell clones

Individual microcultures that exhibited specific peptide reactivity by the qPCR screening assay were selected for limiting dilution cloning. Briefly, between 1 and 3 T cells were plated in each well of a 96-well U-bottomed plate in 0.2 ml of conditioned medium containing orthoanti-CD3 (50 ng/ml) (Ortho-Biotech) and IL-2 (300 IU/ml) with 5×10^4 autologous irradiated (40 Gy) PBMCs. On day 5 and every 3 to 4 days thereafter, half of the

medium in each well was replaced with fresh medium containing IL-2. Growth-positive culture rate was typically ~10 to 12%. About 10 to 14 days after culture initiation, wells in which cell growth was visibly apparent were screened in a microcytotoxicity assay to identify clones with cytolytic activity against peptidepulsed T2 cells. Further characterization of clone function was performed with enzyme-linked immunosorbent assay to quantify IFN- γ secretion in response to limiting concentrations of peptide pulsed onto T2 cells and antigen-positive tumor lines. Selected clones were subsequently expanded with ortho-anti-CD3 (50 ng/ml), IL-2 (300 IU/ml), and 5×10^6 irradiated allogeneic PBMCs in upright 25-cm² flasks.

Patients and clinical protocol

Five consecutive HLA-A2⁺ patients with metastatic melanoma were treated with gp100-specific CD8⁺ T cell clones at the Surgery Branch, National Cancer Institute (NCI), between January 2009 and February 2010 on a clinical protocol (registry no. NCT00665470 at <http://www.clinicaltrials.gov>) approved by the Institutional Review Board and U.S. Food and Drug Administration. All patients gave informed consent for treatment in accordance with the Declaration of Helsinki. The patients were required to be 18 years of age or older, have measurable metastatic melanoma expressing gp100 and major histocompatibility complex class I by immunohistochemistry, and have Eastern Cooperative Oncology Group status 0 or 1. Before clone infusion, patients were transiently lymphoablated with a nonmyeloablative lymphodepleting regimen including intravenous administration of cyclophosphamide (60 mg/kg) for 2 days followed by fludarabine (25 mg/m²) for 5 days, as previously described (1–3). One day after completion of their lymphodepleting regimen, patients received expanded T cell clones infused intravenously followed by high-dose IL-2 (720,000 IU/kg) (aldesleukin; Novartis) every 8 hours to tolerance. Patients received baseline computed tomography (CT) and/or magnetic resonance imaging (MRI) before and after treatment. Tumor size was evaluated monthly by CT and MRI or documented with photography for cutaneous/subcutaneous lesions. Tumor measurements and patient response were determined according to RECIST (40). For evaluation of in vivo T cell trafficking and activity, skin biopsies were obtained and immunohistochemically stained for the presence of CD8⁺, CD4⁺, CD3⁺ T cells. Melanocyte density was evaluated with Melan-A staining. All interpretations were performed by a single dermatopathologist in the Clinical Pathology Department, National Institutes of Health. The peripheral blood frequency, phenotype, and function of persisting clones were performed by obtaining pre- and posttreatment PBMCs and analyzed with methods described in the Supplementary Materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Defining the stoichiometric production of IL-2 and IFN- γ mRNA by memory CD8⁺ T cells. (A) (Left) Representative PBMC sorting for defined memory subsets. (Middle) Expression of IFN- γ (black bars) and IL-2 (gray bars) mRNA from sort-purified memory subsets after 3-hour stimulation with anti-CD3/CD28 beads. (Right) Comparison of the *IL-2:IFN* index. Data are means $\pm$ SEM of three independent patients. * $P < 0.05$, paired t test. (B) Quantitative comparison of antigen-induced IFN- γ and IL-2 mRNA by gp100-reactive microcultures ($n = 9$). (C) FACS analysis of the percent of gp100 tetramer⁺CD8⁺ cells (black dots) found in microcultures. Numbers in dot plot denote percent of CD8⁺ cells that are gp100 tetramer⁺. (D) Correlation between the percent of gp100 tetramer⁺CD8⁺ cells in microcultures and IFN- γ mRNA (top) or IL-2 mRNA (bottom). (E) Calculated *IL-2:IFN* index for microcultures. (F) FACS analysis of the memory phenotype of gp100 tetramer⁺CD8⁺ cells found in microcultures. Overlaid dot plots represent all cells within the lymphocyte gate; the gp100 tetramer⁺CD8⁺ T cells are shown in black dots and all other lymphocytes are shown in gray dots. Numbers in dot plot denote percent of gp100 tetramer⁺CD8⁺ cells that have a T_{CM} phenotype. (G) Correlation between the percent of gp100 tetramer⁺CD8⁺ T cells with a T_{CM} phenotype and their *IL-2:IFN* index.

Fig. 2. Identification of melanoma-specific T_{CM} and T_{EM} by profiling for the *IL-2:IFN* index. **(A)** Comparison of cytokine mRNA production, *IL-2:IFN* index, and memory phenotype from paired antigen-sensitized microcultures from HLA-A2⁺ melanoma patients ($n = 6$). Numbers in FACS dot plots correspond to percent of tetramer⁺ cells that are CD62L⁺. **(B)** Correlation between the percent of tetramer⁺CD8⁺ cells with a T_{CM} phenotype and the *IL-2:IFN* index of the culture ($n = 12$). **(C)** Paired comparison of the percent of tetramer⁺CD8⁺ cells with a T_{CM} phenotype in cultures with an *IL-2:IFN* index <10 versus >50. **(D)** Paired comparison of the percent of total CD8⁺ T cells that are tetramer⁺ in cultures with *IL-2:IFN* index <10 versus >50. Microculture pairs from six individual melanoma patients are denoted by an individual symbol. Statistical comparison was performed with paired *t* test. Bar represents mean.

Fig. 3.

Melanoma-specific CD8⁺ T cells with a high *IL-2:IFN* index demonstrate superior in vitro proliferation and responsiveness to IL-2 exposure. (A) Representative FACS comparing the percent of gp100 tetramer⁺CD8⁺ T cells found in paired microcultures with high *IL-2:IFN* index (>50) and low *IL-2:IFN* index (<10) before and after polyclonal expansion with anti-CD3 antibody, IL-2 (300 IU/ml), and irradiated PBMC feeder cells. Numbers in dot plot denote percent of CD8⁺ cells that are gp100 tetramer⁺. (B) Fold expansion of tetramer⁺CD8⁺ and tetramer⁻CD8⁺ cells described in (A) at day 12. (C) Summary of day 12 proliferation cell counts from paired microcultures (high index versus low index) from four melanoma patients. Paired microcultures from individual patients are denoted by an individual symbol. Statistical comparison was performed with paired *t* test. Bars and numbers represent means. (D) Comparative CFSE dilution in tetramer⁺CD8⁺ and tetramer⁻CD8⁺ T cells from paired microcultures with high or low *IL-2:IFN* indices after 5-day exposure to anti-CD3/CD28 stimulation beads. Numbers in dot plots corresponding to percent of total CD8⁺ T cells that are tetramer⁺. Tetramer⁺CD8⁺ cells are shown in black

dots and tetramer⁻CD8⁺ cells are in gray dots. Overlaid histograms demonstrate CFSE expression of tetramer⁺CD8⁺ cells (shaded black) and tetramer⁻CD8⁺ cells (shaded gray). Histogram gating reflects percent of tetramer⁺CD8⁺ cells with diluted CFSE compared to baseline. MFI values reflect fluorescence of CFSE in tetramer⁺CD8⁺ cells. Analysis is representative of four independent experiments performed. (E) Representative comparison of CFSE dilution in tetramer⁺CD8⁺ and tetramer⁻CD8⁺ T cells from paired microcultures with high and low *IL-2:IFN* indices after 7-day exposure to varying concentrations of IL-2. Numbers in dot plots corresponding to percent of tetramer⁺ (red) and tetramer⁻ cells (black) with diluted CFSE compared to baseline. (F) Dose-response relationship between supplemented exogenous IL-2 and percent of tetramer⁺CD8⁺ and tetramer⁻CD8⁺ cells with diluted CFSE. Data are means $\pm$ SEM of four independent pairs of high-versus low-index cultures from two patients. * $P < 0.05$, paired t test; *** $P < 0.001$, two-way ANOVA. EC₅₀ refers to the concentration of IL-2 (IU/ml).

Fig. 4.

Melanoma-specific CD8⁺ T_E clones generated from high and low *IL-2:IFN* index precursors acquire similar cytolytic effector characteristics but differ in the expression of cell survival genes. (A) Representative comparison of parental melanoma-specific CD8⁺ memory T cells with dichotomous *IL-2:IFN* index and CD62L expression by FACS. Numbers in dot plots correspond to percent of gp100 tetramer⁺ cells that are CD62L⁺. (B) (Left) Phenotype of derived CD8⁺ T cell clones. Overlaid histograms reflect tetramergated cells; isotype staining is shown in black and specific antibody staining in gray. Values in FACS histograms correspond to percent shift from isotype control. (Right) Reactivity of derived CD8⁺ T cell clones. Intracellular cytokine FACS for IL-2 and IFN-γ after stimulation with T2 cells pulsed with gp100 peptide (gray dots) versus HIVpol control peptide (black dots). Numbers in dot plots represent percent of gp100-reactive cells found in respective gates. Tumor cytotoxicity was determined by coculture against 526 mel (HLA-A2⁺/gp100⁺) (black boxes) and 888 mel (HLA-A2⁻/gp100⁺) (white boxes). Plots demonstrate means ± SEM of triplicate samples. Analysis is representative of five independent patient clones that were examined. (C) Heat map of differentially expressed genes ($P < 0.01$) between resting clone groups (high *IL-2:IFN* index derived versus low *IL-2:IFN* index derived). (D) Ingenuity

Pathway Analysis of differentially expressed genes; shown are cell functions ranked by the number of identified genes associated with the function. (E) TaqMan analysis for the expression of *CD40L* and *BMF* in derived clones. Values are reported as mRNA copies relative to 10^4 copies of β -actin.

Fig. 5.

In vitro and in vivo reactivity of adoptively transferred gp100-specific CD8⁺ T cell clones. (A) Intracellular cytokine FACS for IL-2 and IFN- γ production performed on infused gp100-specific clones. Clones were stimulated with T2 cells pulsed with gp100 peptide (gray dots) versus HIVpol control peptide (black dots). Numbers in dot plots represent percent of gp100-reactive cells in respective gates after CD3 gating. (B) Pie chart showing the percent of reactive cells producing the denoted cytokine (mean values from five patients). (C) (Top left) Representative photograph of skin rash seen on the torso of patient 1 on day 5 after clone infusion. (Top right) Close-up view of rash. (Bottom) Histologic comparison of pre- and post-infusion (day +5) skin biopsies (original magnification, $\times 20$) demonstrating treatment-induced intraepidermal spongiosis (black arrows) seen on hematoxylin and eosin (H&E) staining, CD8⁺ lymphocyte infiltration (yellow arrows), and loss of melanocytes (red arrows) seen with Melan-A staining. Analysis is representative of biopsies from five independent patients.

Fig. 6.

In vivo persistence and self-renewal of gp100-specific CD8⁺ T cell clones after adoptive transfer. **(A)** FACS for the percent of gp100 tetramer⁺CD8⁺ T cells found in pre-infusion peripheral blood lymphocytes (PBLs), infusion product, and post-infusion (day +30) PBL samples. Numbers in dot plot denote percent of CD8⁺ cells that are gp100 tetramer⁺. Infusion product contained >99% gp100 tetramer⁺CD8⁺ T cells with clonality verified by TCR gene sequencing. **(B)** Absolute number of gp100 tetramer⁺CD8⁺ T cells per microliter of blood (top) and FACS analysis for the percent of gp100 tetramer⁺CD8⁺ T cells found in PBLs at specified time points for patient 3 (bottom). Arrow denotes day of infusion. **(C)** Reactivity of gp100⁺CD8⁺ T cells persisting in PBLs. Day 30 PBMC samples were thawed, stimulated with T2 cells pulsed with gp100 peptide or HIVpol control peptide, and analyzed by intracellular cytokine FACS for IL-2 and IFN-γ. FACS dot plots represent CD3-gated cells. Color coding of cytokine-producing cells is based on isotype gating. **(D)** Pie chart

showing the percent of gp100-reactive cells producing the denoted cytokines (mean values from four patients). (E) Phenotypic comparison of the gp100⁺CD8⁺ T cell clones that were infused (Infu) versus those cells that persisted (Pers). Red squares denote mean values and error bars denote SEM of four independent patients.

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Fig. 7. Expression of negative regulatory molecules on infused and persisting clones. **(A)** Representative FACS profiling from patient 3 for negative regulatory molecules (CTLA-4, PD-1, and TIM-3) and proliferation marker (Ki-67) on the gp100-specific clones that were infused and the clones that persisted in peripheral blood after adoptive transfer. Numbers indicate the percent of tetramer⁺ and tetramer⁻ cells with expression of indicated marker. Gating was based on isotype controls. **(B)** Mean expression of indicated markers for patients 1 to 4. Error bars denote SEM ($n = 4$). * $P < 0.05$; ** $P < 0.01$, two-tailed paired t test.

Table 1.

Patient demographics, T cell characteristics, and treatment details. N/A, not available for analysis; LN, lymph node; SQ, subcutaneous.

Patient characteristics			Parental T cell population			Derived CD8 ⁺ T cell clone characteristics			Treatment details	
Patient no.	Age/ gender	Melanoma origin	Stage and disease sites at study entry	IL-2/IFN index	Memory phenotype	Antigen specificity	TCR clonotype	CD40L mRNA [*]	BMF mRNA [*]	No. of clones infused (x10 ⁶)
1	56/M	Cutaneous	M1B: lung, LN	50 (high)	N/A	gp100	Vα5/Vβ6.5	562	9	45.1
2	60/M	Ocular	M1c: liver	91 (high)	N/A	gp100	Vα12.2/Vβ7.6	530	13	0.4
3	56/M	Cutaneous	M1c: LN	71 (high)	T _{CM}	gp100	Vα35/Vβ7.6	253	9	33.1
4	51/M	Ocular	M1c: liver	83 (high)	T _{CM}	gp100	Vα35/Vβ7.6	453	8	22.2
5	55/F	Mucosal	M1c: lung, liver, SQ, LN	111 (high)	T _{CM}	gp100	Vα5/Vβ29.1	735	12	18.8

*Copies per 10⁴ β-actin.