Tumor-Specific Effector CD8<sup>+</sup> T Cells That Can Establish Immunological Memory in Humans after Adoptive Transfer Are Marked by Expression of IL7 Receptor and c-myc


Abstract

The optimal T-cell attributes for adoptive cancer immunotherapy are unclear. Recent clinical trials of ex vivo–expanded tumor-infiltrating lymphocytes indicated that differentiated T effector cells can elicit durable antitumor responses in some patients with cancer, with their antitumor activity tightly correlated with their persistence in the host. Thus, there is great interest in the definition of intrinsic biomarkers that can predict the conversion of short-lived tumor antigen–specific T effector cells into long-lived T memory cells. Long-term persistence of ex vivo–expanded tumor-specific CD8<sup>+</sup> T effector clones has been reported in refractory metastatic melanoma patients after adoptive T-cell transfer. By using highly homogeneous clone populations from these preparations, we performed a comparative transcriptional profiling to define preinfusion molecular attributes that can be ascribed to an effector-to-memory transition. Through this route, we discovered that preinfusion T-cell clones that expressed the IL7 receptor (IL7R) and c-myc were more likely to persist longer after adoptive transfer to patients. The predictive value of these two biomarkers was strengthened by using IL7R protein, IL7–induced pSTAT5, and c-myc mRNA expression to prospectively identify human tumor-specific T effector clones capable of engraftment into immunodeficient mice. Overall, our findings reveal IL7R and c-myc expression as intrinsic biomarkers that can predict the fate of CD8<sup>+</sup> T effector cells after adoptive transfer. Cancer Res; 75(16); 3216–26. ©2015 AACR.

Introduction

A central pursuit in the adoptive immunotherapy of cancer and viral diseases is the repopulation of the host immune system with antigen-specific T cells that can mediate potent effector function, yet also establish long-term memory. Clinical trials transferring ex vivo–expanded autologous tumor-infiltrating lymphocytes have provided evidence that differentiated T effector cells can mediate objective tumor responses in patients with a variety of solid tumors (1–4). Retrospective analyses of these trials have associated the ability of the transferred cells to persist in vivo with their antitumor efficacy (3, 5, 6). Thus, in an effort to improve treatment outcomes, there has been active interest in identifying intrinsic markers that can predict whether antigen-specific T effector cells develop into long-lived memory cells versus undergoing programmed cell death after transfer into patients. However, a significant challenge in defining the precise cellular attributes associated with these dichotomous fates stems from the heterogeneity of the polyclonal cell products infused in prior human trials. A clinical strategy to address this problem involves the adoptive transfer of highly characterized monoclonal populations of tumor-specific T cells. We recently reported the long-term persistence of ex vivo–expanded melanocyte differentiation antigen (MDA)-specific CD8<sup>+</sup> T effector clones in patients with refractory metastatic melanoma after adoptive T-cell transfer (NCT00665470 and NCT01495572; refs. 7, 8). By virtue of the genetically unique T-cell receptor (TCR) sequences expressed in these individual clones, we were able to accurately track their in vivo engraftment and survival without the interpretable ambiguity associated with bulk polyclonal cell infusions. Interestingly, although all of the infused clones were highly differentiated, lytic CD8<sup>+</sup> T effector cells, several clones were able to establish long-term memory and repopulate the immune repertoire of patients (8). In the current study, we sought to define the cellular and molecular attributes associated with this effector-to-memory transition by comparative transcriptional profiling of CD8<sup>+</sup> T effector clones that could persist versus those that could not. Here, we report that the preinfusion clone expression levels of the IL7R and the proto-oncogene, c-myc, directly correlated with the level of persistence of these CD8<sup>+</sup> T effector cell clones after adoptive transfer in humans. These clinical observations were experimentally validated upon an independently isolated set of CD8<sup>+</sup> T effector cell clones by controlled adoptive transfer into highly immunodeficient mice. These findings support that IL7R and c-myc expression may serve as valuable cell intrinsic markers that can predict the fate of CD8<sup>+</sup> T effector cells after adoptive transfer.
Patients and Methods

Patients and clinical protocol

HLA-A2+ patients with metastatic melanoma were treated with either gp100-specific CD8+ T-cell clones or MART-1-specific CD8+ T-cell clones at the Surgery Branch, National Cancer Institute (NCI), between January 2009 and January 2013 on two consecutive phase II clinical protocols (NCT00665470 and NCT01495572; ref. 8) approved by the Institutional Review Board and FDA. 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 and have measurable metastatic melanoma that expressed gp100 or MART-1 and MHC-I by immunohistochemistry. 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 (3). One day after completion of their lymphodepleting regimen, patients received expanded CD8+ T-cell clones intravenously, either with or without high-dose IL2 (72,000 IU/kg) every 8 hours to tolerance.

Media and cell culture

Human cultured cell lines, including T2 cells (HLA-A2+ peptide transporter–associated protein deficient T-B hybrid) and melanoma tumor lines, 526mel (HLA-A2+/gp100+/MART-1+), and 888mel (HLA-A2+/gp100+/MART-1+), were routinely cultured in complete medium as previously described (8). The melanoma cell lines, 526mel and 888mel, were obtained from the cell production facility in the Surgery Branch, NCI. The tumor cells had been characterized to confirm tumor morphology, antigen, and HLA expression by immunohistochemistry; they were obtained and used within 6 months of testing. Human peripheral blood mononuclear cell (PBMC) used in this study were obtained by leukapheresis from HLA-A2+ metastatic melanoma patients evaluated on Institutional Review Board-approved protocols at the Surgery Branch, NCI (NIH, Bethesda, MD) and cultured in medium with 10% heat-inactivated human AB serum (Gemini Bio-Products).

Microarray and gene expression analysis of T-cell clones

Total RNA from preinfusion CD8+ T effector cell clones was isolated using an RNeasy Kit (Qiagen) as per the manufacturer’s instructions and quality was validated with Agilent Bioanalyzer. About 100 ng of total RNA samples were reverse transcribed and labeled by biotin. Biotin-labeled cDNA was hybridized to Affymetrix Human Genome U133 Plus 2.0 Array according to manufacturer’s instructions. Washing, staining, and scanning of the microarray was carried out under strictly controlled conditions with the Affymetrix Fluidics Station and Scanner. Raw data from the generated cell intensity files (.cel extension) were imported into Partek Genomics Suite with normalization performed by robust multichip analysis (RMA) algorithm. Genes with differences in expression between persisting and nonpersisting clones were identified by 2-way ANOVA (Partek) with a primary stringent P < 0.01. Genes with differences in expression were filtered by the Benjamini–Hochberg false discovery rate procedure (P < 0.05) and a between-group "fold-change" criterion of more than 2.0 (P < 0.05). The microarray data have been deposited in the National Center for Biotechnology Information under accession number GSE65627. The Ingenuity Pathway Analysis online software was used for pathway analysis of the list of differentially expressed genes. Relative mRNA quantitation for selected genes was determined by qRT-PCR validation of microarray findings was performed using Taqman primer/probe sets (Applied Biosystems). Results are presented relative to β-actin expression.

Adaptive transfer into NSG mice

NSG (NOD.Cg-Prkdcsdcid Il2rgtm1Wjl/SzJ) mice were purchased from Jackson Laboratories and housed at the animal facility at the NCI in pathogen-free conditions. Mouse experiments were approved by the NCI Animal Care and Use Committee and performed in accordance with NIH guidelines. Cohorts of NSG mice were adoptively transferred with equal numbers of CD8+ T-cell clones (5e6 cells per mouse) via tail vein injection. At the time of adoptive transfer, mice also received concomitant intraperitoneal administration of human recombinant IL15 (1 μg) and every alternate day for the duration of the experiment. At day 21 after transfer, mice were euthanized by CO2 asphyxiation and spleens were harvested to quantitate the persistence of the transferred clones by flow cytometry.

Additional methods have been described in the Supplementary section.

Results

Tumor-specific CD8+ T effector clones have highly variable levels of engraftment after adoptive transfer in humans

We analyzed samples of ten unique MDA-specific CD8+ T-cell clones that had been cryopreserved immediately before infusion into nine HLA-A*0201+ patients with refractory metastatic melanoma. All patients had undergone a nonmyeloablative lymphodepleting conditioning regimen as previously described (8). The characteristics of the analyzed cohort of patients and their clone therapy are shown in Table 1. The first four patients were treated with CD8+ T-cell clones specific for the gp100154-162 epitope and the next five patients received CD8+ T-cell clones specific for the...
Heterogeneous engraftment of CD8⁺ T effector clones after adoptive transfer in humans. A, dichotomous long-term engraftment of M1 and M4 MART-specific CD8⁺ T effector clones as determined by the absolute number of CD8⁺Tetramer⁺Vβ⁺ T cells per microliter of blood at time points relative to infusion in respective patients with melanoma. Arrow, the day of clone infusion. B, heterogeneous persistence of infused effector clones (n = 10 clones infused into nine patients) as assessed by % CD8⁺Tetramer⁺Vβ⁺ cells identified in PBMC at day 30 after infusion. NP clones were defined to have engraftment levels of ≤0.1% of CD8⁺ T cells. P clones had engraftment levels >0.1% of CD8⁺ T cells. C, preinfusion phenotype of NP (n = 4) and P (n = 6) effector clones as determined by FACS expression of the indicated differentiation markers (% of cells). Cognate peptide avidity (D) and reactivity (E) against 526 Mel (A2⁺) tumor cell line for NP (n = 4) and P (n = 5) effector clones. Shown are supernatant IFNγ levels after subtracting background reactivity against either control peptide or 888 Mel (A2⁻) tumor cell line. Statistical comparison performed by the unpaired t test. ns, nonsignificant. Bar on graphs represents mean.

MART27-35 epitope. The TCR clonotype for each clone was defined by complete molecular sequencing of the β-chain variable region (Vβ). Each patient received a single clonotype except for patient M2 who received 2 unique clonotypes. To evaluate the in vivo persistence of the transferred clones, peripheral blood samples, obtained before and 1 month after cell infusion, were compared by flow cytometry for the percentage of CD8⁺ T cells that were tetramer positive. Furthermore, each CD8⁺tetramer⁺ population was FACS sorted to more than 99% purity to allow complete TCR Vβ molecular sequencing to determine the antigen-specific clonotypes that were present in the peripheral blood before and after clone infusion. The presence of the infused clonotype after infusion, but not before infusion, was defined as clonal persistence. The percentage of this clonotype among total circulating CD8⁺ T cells was used to define the persistence frequency. Using this stringent criterion, we detected highly variable levels of clonal engraftment ranging from undetectable (<0.1%) to 7.8% of all circulating CD8⁺ T cells at 1 month after infusion (Table 1). To evaluate the long-term fate of these clones, we obtained extended peripheral blood samples from patients and demonstrated the sustained persistence of some clones beyond 120 days (e.g., clone M4) whereas other clones could never be detected in the circulating peripheral blood lymphocytes (PBls; e.g., clone M1; Fig. 1A). These persisting clones were additionally found to be functionally capable of re-responding to cognate antigen stimulation immediately ex vivo without culturing or cytokine support (7, 8). Next, to better study the dichotomous fate of the transferred clones, we categorized the infused clones as "persisting (P)" if they demonstrated peripheral blood levels of >0.1% of circulating CD8⁺ T cells at 1 month after transfer and "nonpersisting (NP)" clones had engraftment levels of ≤0.1% of CD8⁺ T cells at 1 month after transfer (Fig. 1B). The preinfusion phenotype of both the NP and P set of clones demonstrated uniformly high expression of CD45RO and low expression of CD62L, consistent with their differentiated effector status (Fig. 1C). Furthermore, preinfusion functional assays demonstrated no difference between NP and P clones in their avidity for cognate peptide pulsed on T2 target cells (Fig. 1D) and naturally presented peptide on an allogeneic HLA-A2⁺ melanoma tumor cell line (526 Mel; Fig. 1E) by IFNγ cytokine release assay. CUMULatively, these data suggested that the clinically administered CD8⁺ T effector cell clones had similar phenotypic and functional attributes but possessed very different abilities to engraft in the host.

Comparative gene expression profiling reveals preinfusion transcriptional differences between persisting and nonpersisting CD8⁺ T effector clones

Because conventional phenotypic and functional profiling failed to reveal significant preinfusion differences between P and
NP clones, we next sought to determine whether there was a distinct molecular signature that was associated with the eventual engraftment of the effector clones. We performed comparative microarray gene expression profiling on P (n = 6) and NP (n = 4) clones, which were procured immediately before their adoptive transfer. Of the 54,675 transcripts analyzed, there were 112 unique genes that were differentially expressed (P < 0.05 and fold difference >2) between the P and NP clones (Fig. 2A and

IL7R and c-myc Identify Effector T Cells That Can Establish Memory

Figure 2.
Comparative gene expression profiling reveals preinfusion transcriptional differences between persisting and nonpersisting CD8+ T effector clones. A, Affymetrix cDNA Microarray comparison of NP (n = 4) and P (n = 6) clones. Volcano plot highlights selected overexpressed transcripts (P < 0.05 and fold difference >2) in NP and P effector clones. B, Ingenuity Pathway Analysis of the 112 differentially expressed transcripts between NP and P effector clones. Shown are functional categories ranked by statistical significance. C, relative mRNA quantitation by RMA normalized intensity of selected genes expressed in NP (n = 4) and P (n = 6) effector clones. Each dot represents an individual infused CD8+ T-cell clone. *, P < 0.05; **, P < 0.01; *** , P < 0.001; unpaired t test. Bar on graphs represents mean.
Preinfusion expression levels of IL7R and c-myc by CD8+ T effector clones correlate with their level of persistence after adoptive transfer in humans

Although the microarray screening had revealed several genes that were differentially expressed between P and NP CD8+ T effector clones, we noted that the level of overexpression of each of these genes was highly variable with a significant range of expression among the individual clones (Fig. 2C). Furthermore, we also appreciated that the levels of persistence after adoptive transfer were equally variable among these same clones (Table 1). On the basis of these observations, we postulated that our initial categorization of clones as either P or NP was insufficient to accurately identify the individual genes that were most strongly associated with clonal persistence. Thus, to better account for the observed heterogeneity, we next analyzed the relationship between the quantitative expression levels of each gene transcript profile in the microarray analysis and the precise level of persistence for each individual clone. Normalized mRNA transcript levels in the preinfusion clone samples were statistically correlated with the absolute level of persistence for that clone at 1 month after transfer. Pearson correlation coefficients (r) were derived for each of the correlation analyses and ranked to identify the genes with the strongest positive and negative correlation with in vivo persistence (P < 0.05, Table 2). We found that preinfusion mRNA expression levels of ATF3 (r = 0.791) and TNEM64 (r = 0.741) were most negatively correlated with clonal persistence. Conversely, the genes most positively correlated with clonal persistence was IL7R (r = +0.747) followed by MYC (r = +0.717). Prior reports from murine models had suggested that CD8+ T effector cells, which selectively expressed cell surface IL7 receptor (IL7R), could give rise to long-lived memory cells after adoptive transfer (9). However, these findings had not been clearly demonstrated in human adoptive transfer studies. Thus, we next sought to determine whether preinfusion IL7R cell surface protein expression and the phosphorylation of its downstream signal transduction molecule, STAT5, were associated with human clonal persistence. IL7R expression was measured by antibody staining and flow cytometric quantitation after the infused clones were rested for 24 hours in cytokine-free media. Regression analysis found that IL7R expression [mean fluorescence intensity (MFI) and % expressing cells] on the preinfusion clones was strongly and linearly correlated with the eventual persistence of those clones after adoptive transfer (IL7R MFI: R² = 0.73, P = 0.003; % IL7R: R² = 0.71, P = 0.004; Fig. 3A and

Table 2. Correlation between gene expression and level of in vivo clonal persistence

<table>
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<tr>
<th>Gene symbol</th>
<th>Gene title</th>
<th>Pearson correlation coefficient (r)</th>
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<tbody>
<tr>
<td>ATF3</td>
<td>Activating transcription factor 3</td>
<td>−0.791</td>
</tr>
<tr>
<td>TNEM64</td>
<td>Transmembrane protein 64</td>
<td>−0.741</td>
</tr>
<tr>
<td>PTGDR</td>
<td>Prostaglandin D2 receptor (OP)</td>
<td>−0.710</td>
</tr>
<tr>
<td>TNFAIP9</td>
<td>Tumor necrosis factor, alpha-induced protein 3</td>
<td>−0.698</td>
</tr>
<tr>
<td>ATPOD</td>
<td>ATPase, class V, type 10D</td>
<td>−0.668</td>
</tr>
<tr>
<td>GADD45A</td>
<td>Growth arrest and DNA damage-inducible, alpha</td>
<td>−0.666</td>
</tr>
<tr>
<td>ZMAT3</td>
<td>Zinc finger, matrin-type 3</td>
<td>−0.656</td>
</tr>
<tr>
<td>MCC</td>
<td>Mutated in colorectal cancers</td>
<td>−0.650</td>
</tr>
<tr>
<td>TNFSF9</td>
<td>Tumor necrosis factor (ligand) superfamily, member 9</td>
<td>−0.635</td>
</tr>
<tr>
<td>PHLDA3</td>
<td>Pleckstrin homology-like domain, family A, member 3</td>
<td>−0.632</td>
</tr>
<tr>
<td>CLEC1</td>
<td>C-type lectin-like 1</td>
<td>−0.626</td>
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Positive correlation with clone persistence

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene title</th>
<th>Pearson correlation coefficient (r)</th>
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<tbody>
<tr>
<td>IL7R</td>
<td>Interleukin 7 receptor</td>
<td>+0.747</td>
</tr>
<tr>
<td>MYC</td>
<td>v-myc myelocytomatosis viral oncogene homolog (avian)</td>
<td>+0.717</td>
</tr>
<tr>
<td>SIK1</td>
<td>Salt-inducible kinase 1</td>
<td>+0.695</td>
</tr>
<tr>
<td>CASP4</td>
<td>Caspase-4, apoptosis-related cysteine peptidase</td>
<td>+0.692</td>
</tr>
<tr>
<td>GSK3</td>
<td>Oncostatin M</td>
<td>+0.682</td>
</tr>
<tr>
<td>AIH1</td>
<td>Abelson helper integration site 1</td>
<td>+0.645</td>
</tr>
<tr>
<td>CD2B</td>
<td>CD2B molecule</td>
<td>+0.637</td>
</tr>
<tr>
<td>MYB</td>
<td>v-myb myeloblastosis viral oncogene homolog (avian)</td>
<td>+0.634</td>
</tr>
<tr>
<td>IRA4</td>
<td>Interferon regulatory factor 4</td>
<td>+0.630</td>
</tr>
<tr>
<td>CHD7</td>
<td>Chromodomain helicase DNA-binding protein 7</td>
<td>+0.630</td>
</tr>
<tr>
<td>SYCP2</td>
<td>Synaptonemal complex protein 2</td>
<td>+0.628</td>
</tr>
<tr>
<td>EGR1</td>
<td>Early growth response 1</td>
<td>+0.625</td>
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Complete gene list in Supplementary Table S1). Ingenuity Pathway Analysis revealed that the dominant functional role for this gene set was related to cell death and survival (Fig. 2B). The profiling specifically identified 34 genes that were overexpressed in P clones (Fig. 2A), including IL7R, BCL-6, CD28, MYC, and MYB, which are known to be involved in T-cell survival and proliferation (9–13). To more accurately define the expression pattern of these genes in the individual infused clones, we quantified the relative mRNA levels of the selected genes by RNA-sequencing. We observed that IL7R, BCL-6, CD28, MYC, and MYB mRNA expression were uniformly low in the NP clones but significantly higher in the P clones (Fig. 2C). Furthermore, there were 78 genes identified to be overexpressed in NP clones (Fig. 2A), including ATF3, BAX, CDKN2B, TNFSF4, and ZMAT3, which are involved in cell-cycle arrest, stress, terminal effector function, and apoptotic cell death (14–18). Relative mRNA quantitation of these selected genes demonstrated uniformly high expression in P clones but significantly higher expression in the NP clones (Fig. 2C). From these findings, we concluded that persisting CD8+ T effector clones have higher preinfusion expression of genes associated with cell survival and proliferation, whereas nonpersisting clones overexpressed genes associated with cell death.

Although the microarray screening had revealed several genes that were differentially expressed between P and NP CD8+ T effector clones, we noted that the level of overexpression of each of these genes was highly variable with a significant range of expression among the individual clones (Fig. 2C). Furthermore, we also appreciated that the levels of persistence after adoptive transfer were equally variable among these same clones (Table 1). On the basis of these observations, we postulated that our initial categorization of clones as either P or NP was insufficient to accurately identify the individual genes that were most strongly associated with clonal persistence. Thus, to better account for the observed heterogeneity, we next analyzed the relationship between the quantitative expression levels of each gene transcript profile in the microarray analysis and the precise level of persistence for each individual clone. Normalized mRNA transcript levels in the preinfusion clone samples were statistically correlated with the absolute level of persistence for that clone at 1 month after transfer. Pearson correlation coefficients (r) were derived for each of the correlation analyses and ranked to identify the genes with the strongest positive and negative correlation with in vivo persistence (P < 0.05, Table 2). We found that preinfusion mRNA expression levels of ATF3 (r = 0.791) and TNEM64 (r = 0.741) were most negatively correlated with clonal persistence. Conversely, the genes most positively correlated with clonal persistence was IL7R (r = +0.747) followed by MYC (r = +0.717). Prior reports from murine models had suggested that CD8+ T effector cells, which selectively expressed cell surface IL7 receptor (IL7R), could give rise to long-lived memory cells after adoptive transfer (9). However, these findings had not been clearly demonstrated in human adoptive transfer studies. Thus, we next sought to determine whether preinfusion IL7R cell surface protein expression and the phosphorylation of its downstream signal transduction molecule, STAT5, were associated with human clonal persistence. IL7R expression was measured by antibody staining and flow cytometric quantitation after the infused clones were rested for 24 hours in cytokine-free media. Regression analysis found that IL7R expression [mean fluorescence intensity (MFI) and % expressing cells] on the preinfusion clones was strongly and linearly correlated with the eventual persistence of those clones after adoptive transfer (IL7R MFI: R² = 0.73, P = 0.003; % IL7R: R² = 0.71, P = 0.004; Fig. 3A and
Supplementary Fig. S1). Interestingly, the expression of IL7Rα was detected on a fraction of cells ranging from 0% to 12.4% of the total infused clone population. To better understand the differentiation status of the IL7Rα+ cells, we performed comparative phenotypic profiling of the IL7Rα+ and IL7Rα− cells from the preinfusion clone products. Flow cytometric analysis of the IL7Rα+ subsets demonstrated these cells to have effector memory differentiation based upon high expression of CD45RO and low expression of CD62L. This expression was similar to the IL7R− cells within the respective clones. Additional markers, including cytokine receptors, costimulatory molecules, activation, and inhibitory markers were performed and showed no difference between the IL7Rα+ and IL7Rα− cells from the preinfusion cloned products. A representative phenotype profiling of clone M4 is shown in Supplementary Fig. S2. Next, we sought to determine whether the detected IL7R on these cells could specifically and functionally respond to its cognate ligand, IL7. We measured the phosphorylation of STAT5 (pSTAT5) by flow cytometry after independently exposing each of the infused T-cell clones to a panel of common γ-chain cytokines, including IL7, IL2, and IL15. An illustrative signal transduction assay performed upon two control clones with known high (99% of cells) and low (<1% of cells) IL7R expression is shown in Fig. 3B. In response to rIL2 and rIL15 exposure, both clones demonstrated a uniform increase in pSTAT5 expression (shift in 99% of cells) when compared with baseline levels in the absence of cytokine. These findings suggested that the clones possessed comparable levels of the IL2 and IL15 receptors, which could functionally respond to their respective γ-chain cytokines. In contrast, when the same clones were exposed to rIL7, the clone with high IL7R expression demonstrated a significant increase in pSTAT5 (95% of cells), whereas the clone with low IL7R expression demonstrated negligible pSTAT5 (5% of cells). When this assay was performed on the clinically administered CD8+ T effector clones (n = 7), we found that rIL2 and rIL15 induced marked pSTAT5 increases in all of the clones (range: 60%–99% of cells) and these levels did not correlate with clonal persistence (R² = 0.06 and −0.06, respectively; Fig. 3C). However, when these same clones were exposed to rIL7, we observed highly variable levels of pSTAT5 expression (range: 4%–60% of cells), which did strongly correlate with the persistence of these clones after adoptive transfer (R² = 0.81, P = 0.006). Next, we sought to further evaluate the other gene found by
microarray screening to be associated with clonal persistence, the c-myc proto-oncogene. Quantitative mRNA analysis was performed on preinfusion samples of the administered clones (n = 9). Regression analysis revealed a significant linear correlation between normalized c-myc mRNA levels and the eventual persistence of those clones (R² = 0.51, P = 0.02; Fig. 3D). Collectively, these findings demonstrated that preinfusion cell surface expression of IL7R (MFI and %), the level of IL7-induced pSTAT5, and c-myc mRNA expression by CD8⁺ T effector clones were all strongly associated with the engraftment capability of these cells after adoptive transfer in humans.

Preinfusion expression levels of IL7R and c-myc by CD8⁺ T effector clones predict their level of persistence after adoptive transfer in NSG mice

The development of T-cell memory responses in patients undergoing adoptive transfer therapy may be confounded by the inherent heterogeneity among recipient host immune systems. Patient-specific variables that might have influenced clonal engraftment in our clinical trials include varying levels of homeostatic cytokines after cell transfer, differences in antigen load and presentation, presence of immunoregulatory cells, and other unique host factors. Thus, to more precisely define the intrinsic cellular attributes associated with the engraftment of CD8⁺ T effector cells, we sought to experimentally eliminate host heterogeneity by studying the fate of human effector clones after highly controlled adoptive transfer into immunodeficient NSG mice. We prospectively isolated an independent cohort of MART-specific CD8⁺ T effector clones by high-throughput in vitro sensitization and limiting dilution cloning (7, 8, 19) from a single patient with metastatic melanoma. Each of the expanded ‘sister’ clones (n = 7) demonstrated monoclonal antigen specificity by anti-CD8 and MART MHc tetramer staining (Fig. 4A). Furthermore, all of the clones possessed the typical phenotypic appearance of highly differentiated antigen experienced CD8⁺ T effector cells with uniform high expression of CD45RO and low expression of CD62L (Fig. 4A). The replicative histories of the clones were also highly similar based upon telomere length assessment, which ranged from 5.4 to 6.6 kb (Fig. 4B). However, when we assessed the cell surface expression of IL7R on these clones, we found significant heterogeneity with MFI values ranging from 0 to 1038 (Fig. 4C, left). Furthermore, these same clones demonstrated highly variable c-myc mRNA transcript levels (range, 344–1,582 copies per 10⁵ β-actin copies; Fig. 4C, right). Linear regression analysis found that the c-myc mRNA levels were highly correlated with the IL7R expression in these clones (R² = 0.78, P = 0.018; Supplementary Fig. S3). Next, to evaluate the functionality of the IL7R, we analyzed pSTAT5 expression after exposure of the clones to γ-chain cytokines (Fig. 4D). We found that rIL2 and rIL15 induced uniform pSTAT5 expression (99% of cells) in each of the clones, suggesting no difference in the expression of their respective receptors. However, when these same clones were exposed to rIL7, we observed highly variable levels of pSTAT5 expression (range, 3%–57% of cells; Fig. 4D), which strongly and linearly correlated with their respective IL7R MFI expression (R² = 0.86, P = 0.0025; Supplementary Fig. S4). On the basis of our prior correlation studies involving the patient infused clones (Fig. 3), we hypothesized that these independently isolated effector clones would also have differing engraftment capabilities that could be predicted on the basis of their IL7R and c-myc expression. Thus, we next evaluated the persistence of these sister clones after controlled prospective adoptive transfer into NSG mice. In this NSG xenograft model, we and others have found that human CD8⁺ T cells require human cytokines to support their engraftment (20, 21). In multiple independent experiments comparing the efficacy of IL2, IL7, and IL15 to support the engraftment of human cells into NSG mice, we found engraftment only in the presence of administered rIL15 (data unpublished). Therefore, our experimental model included repeated administration of human rIL15 cytokine after the transfer of the individual effector clones into the NSG mice (n = 3 for each clone; Fig. 5A). The persistence of each of the infused clones was evaluated at day 21 after transfer by determining the frequencies of human CD3⁺CD8⁺ T cells present in the spleens of replicate mice (Fig. 5B). Notably, there was no detectable engraftment of the CD8⁺ T effector clones that had low preinfusion expression of IL7R (MFI < 96), low IL7-induced pSTAT5 (<10% of cells), and low c-myc expression (<434 copies per 10⁵ β-actin copies). In contrast, the transfer of the effector clones with higher expression of these factors resulted in reproducible engraftment in the animals. When the mean level of persistence was determined for each clone, we found a strong predictive correlation with the clone’s preinfusion IL7R MFI expression (R² = 0.95, P = 0.0002), IL7-induced pSTAT5 expression (R² = 0.94, P = 0.0003), and c-myc mRNA expression (R² = 0.89, P = 0.005; Fig. 5C). These results strongly paralleled our retrospective clinical trial findings in humans and further supported that preinfusion expression level of IL7R and c-myc could be used to predict the level of persistence of CD8⁺ T effector clones after adoptive transfer.

Discussion

The optimal T-cell attributes for the adoptive immunotherapy of cancer and viral diseases are currently unclear. Murine models of adoptive transfer have suggested that less differentiated memory populations have superior ability to persist and mediate tumor regression after infusion when compared with more differentiated effector cells (20, 22, 23). With prolonged in vitro culturing, CD8⁺ T cells were found to progressively lose in vivo proliferative potential and subsequently become senescent and undergo apoptosis (24, 25). These observations led to the prevailing theory that in vitro effector cell differentiation was the dominant explanation for the poor long-term persistence and limited antitumor efficacy of extensively expanded CD8⁺ T effector cells administered in prior adoptive transfer clinical trials. However, this hypothesis has been difficult to reconcile with human clinical trial data, which have demonstrated that differentiated T effector cells can mediate complete and durable tumor responses in patients with metastatic melanoma (3). Furthermore, the administration of less differentiated T cells in human cancer therapy trials has been technically challenging given the obligatory in vitro expansion and consequent cellular differentiation that occurs with the generation of a T-cell product for adoptive transfer. A potential solution to this problem is the identification and administration of differentiated T cells that have potent effector function but also possess intrinsic properties that can facilitate their long-term survival. We previously reported that selected tumor-specific CD8⁺ T effector clones could engraft and persist for prolonged durations in patients with metastatic melanoma, despite these cells having undergone massive ex vivo expansion and differentiation (8). In the
In the current study, we compared the preinfusion transcriptional profile of effector clones with varying engraftment capabilities to help identify intrinsic cellular attributes that may predict cellular persistence in future adoptive transfer clinical efforts. We found the preinfusion clone mRNA expression levels of IL7R and the proto-oncogene, c-myc, directly correlated with the level of persistence of these clones after adoptive transfer in humans. The predictive value of these markers was confirmed by using IL7R protein, IL7-induced pSTAT5, and c-myc mRNA expression to prospectively identify human tumor-specific effector clones that could engraft after adoptive transfer into NSG mice.
The mechanistic role of IL7R and c-myc in effector to memory development is still unclear. Although the requirement of IL7 for the survival of naïve and memory cells has been well described (26–28), its importance in differentiated effector cells has been more difficult to elucidate. In previously reported murine studies, selective expression of IL7R identified a subset of antigen-experienced differentiated effector cells that could preferentially survive and establish long-term immunological memory (9). Furthermore, adoptive transfer of this IL7R expressing population in cytokine knockout mouse models suggested that IL7 was functionally required for their in vivo survival (9). However, in follow-up studies, the constitutive expression of IL7R was unable to alter the fate and rescue effector cells from activation-induced cell death, suggesting a more permissive rather than instructive role for the IL7/IL7R axis in memory cell development (29). Furthermore, multiple lines of evidence have recently supported the role of IL15, in the absence of IL7, in promoting effector-to-memory cell development (30, 31). These findings are consistent with our observation that IL7 cytokine support was not required for the persistence of effector clones in our NSG model. On the basis of these findings, we hypothesize that IL7R expression in effector clones may represent a correlative marker for other more direct prosurvival pathways.

Our finding that the levels of c-myc mRNA expression also correlated with the engraftment fate of human T effector cells has not been previously described. The transcription factor Myc is known to be involved in regulating expression of 15% of all genes, including several that control cell cycle, growth, proliferation, and differentiation (32). Furthermore, in recent years, the contributing role of Myc in directing pluripotent cell fates has been exploited to reprogram differentiated cells (33, 34). Deregulation of Myc occurs through several mechanisms and is one of the most common oncogenic events in human malignancies (35, 36). In the setting of hematologic malignancies, a clinically aggressive

![Diagram](image_url)

**Figure 5.** Preinfusion expression levels of IL7R and c-myc by CD8⁺ T effector clones predict their level of persistence after adoptive transfer in NSG mice. A, experimental xenograft model to evaluate the persistence of human CD8⁺ T effector cell clones after adoptive transfer into immunodeficient NSG mice. B, persistence in NSG mice of human MART-specific CD8⁺ T effector clones with varying preinfusion expression levels of IL7R, induced pSTAT5, and c-myc. FACs dot plots show the percentage of huCD3⁺ huCD8⁺ cells within the harvested spleens of mice (n = 3 mice per clone group; shown in columns) at day 21 after transfer. Each FACS plot represents splenocytes harvested from an individual replicate animal. C, respective correlation analyses between preinfusion IL7R MFI expression, IL7-induced pSTAT5 expression, and c-myc mRNA (per 10⁵ β-actin copies) expression in infused CD8⁺ T effector clones and mean clonal persistence (% huCD3⁺ huCD8⁺) at day 21 after adoptive transfer into NSG mice (n = 3). Linear regression plots demonstrate mean clonal persistence ± SEM (error bars). Experiment is representative of two independently performed adoptive transfer experiments.
subset of B-cell lymphomas can result from chromosomal breakpoint translocations of the loci encoding for c-Myc and either of the antiapoptotic proteins, BCL2 or BCL6. These genetic alterations result in constitutive activation of these genes in the so-called “double and triple hit” lymphomas (37). Interestingly, in our comparative transcriptional profiling of CD8+ T effector cells, we found both c-myc and BCL6 mRNA to be overexpressed in “non-malignant” T cells that could survive and persist after adoptive transfer (Fig. 2). Recent studies of primary T cells have suggested a robust physiologic role for Myc in global transcriptional amplification (12), proliferation (38), and reprogramming of the cellular metabolic state upon T-cell activation (39) but paradoxically also increased apoptosis (40). We hypothesize that the overexpression of Myc in our CD8+ effector clones may be balanced by the antiapoptotic and prosurvival effects of BCL6 and/or IL7R (41) to direct the memory fate of effector cells. However, further mechanistic studies are necessary to define this interaction. In sum, on the basis of our current analysis, we can conclude that IL7R and c-myc have a strong predictive association with T effector cell persistence after adoptive transfer. These findings have direct implications on the selection of CD8+ T cells for future adoptive immunotherapy studies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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29. Hand TW, Morre M, Kaech SM. Expression of IL-7 receptor alpha is necessary but not sufficient for the formation of memory CD8 T cells during viral infection. Proc Natl Acad Sci U S A 2007;104:11730–5.


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