Published in final edited form as:

J Immunother. 2005 ; 28(3): 258–267.

Survival, Persistence, and Progressive Differentiation of Adoptively Transferred Tumor-Reactive T Cells Associated with Tumor Regression

Jianping Huang, Hung T. Khong, Mark E. Dudley, Mona El-Gamil, Yong F. Li, Steven A. Rosenberg, and Paul F. Robbins

From the Surgery Branch, National Cancer Institute, National Institute of Health, Bethesda, Maryland.

Summary

Objective clinical responses have been observed in approximately 50% of patients who received non-myeloablative chemotherapy prior to the adoptive transfer of autologous melanoma-reactive tumor-infiltrating lymphocytes (TILs). Recent studies carried out through the use of antibodies directed against T-cell-receptor beta chain variable region (TRBV) products, as well as by direct sequencing of the expressed TRBV gene products, indicated that clinical responses in this trial were associated with the level of persistence of adoptively transferred T cells. In an attempt to further characterize T cells that persist *in vivo* following adoptive transfer, five dominant T-cell clonotypes were identified in TIL 2035, an adoptively transferred TIL that was associated with the complete regression of multiple metastases. The most highly persistent clonotype, which expressed the BV1 TR gene product, recognized the MAGE-6 cancer/testis antigen in the context of HLA-A23. This clonotype was detected in peripheral blood for over 16 months following adoptive transfer, expressed relatively higher levels of the co-stimulatory markers CD28 and CD27, and possessed telomeres that were long relative to other clonotypes present in TIL 2035 that showed only short-term persistence. The long-term persistent BV1 clonotype appeared to differentiate more slowly toward an end-stage effector *in vivo* than short-term persistent clonotypes, as manifested by the downregulation of CD28, CD27, and CD45RO and upregulation of CD57 and CD45RA expression on these T cells. These results indicated that the differentiation stage and replicative history of individual TIL clonotypes might be associated with their ability to survive and to persist *in vivo*, and progressive differentiation of the persistent clonotypes occurred following adoptive transfer.

Keywords

cell differentiation; human; T-cell receptors; T cells; tumor immunity

Recent clinical studies have provided a unique opportunity to study the factors involved in the *in vivo* survival of tumor-reactive lymphocytes in humans. In one study, clinical responses to adoptive transfer of tumor-reactive tumor-infiltrating lymphocytes (TILs) appeared to be enhanced by prior treatment of patients with a non-myeloablative chemotherapy regimen.¹ The *in vivo* persistence of T cells following adoptive transfer was also correlated with clinical response in a study carried out on a panel of 25 patients.² These observations suggested that the ability of T cells to persist following adoptive immunotherapy was one of the factors that limited response to this therapy, and provided an impetus to develop a better understanding of the characteristics of T cells that are associated with *in vivo* persistence.

Multiple populations of effector memory cells have been identified based on their expression of the co-stimulatory markers CD27 and CD28. The expression of CD27 and CD28 is associated with the proliferative potential of T cells as well as the ability of T cells to survive following activation.^{3–5} A lack of telomerase activity and the consequent shortening of chromosomal telomeres is also associated with the differentiation of T cells to an end-stage effector cell with limited proliferative capacity.^{6,7}

Chronic stimulation of T cells also appears to result in the induction of several markers that appear to be associated with a senescent phenotype. Upregulation of CD57 expression has been observed in chronically stimulated T cells that possess a limited proliferative capacity,⁷ and expression of the killer cell lectin-like receptor G1 (KLRG1) molecule has been observed on terminally differentiated T cells.⁸ The process of T-cell differentiation is also associated with a shift in the expression of the alternative CD45 isoforms CD 45RA and CD 45RO.^{9,10}

In this study, we examined the antigen specificity, persistence, and phenotype of T-cell clonotypes derived from an autologous TIL that was associated with complete tumor regression following adoptive transfer into patient 2035. The results suggest that the stage of differentiation of tumor-reactive T-cell clonotypes within populations of TIL 2035 may be associated with their ability to persist in vivo following adoptive transfer and mediate tumor regression.

MATERIALS AND METHODS

Cell Lines

Samples of PBMC were obtained from a 37-year-old male patient 2035 with metastatic melanoma before and after the administration of tumor-reactive TILs in a clinical protocol approved by the Institutional Review Board of the National Cancer Institute.¹ The 2035 melanoma cell line (mel) was established from a metastatic lesion that was excised from patient 2035 in June 2002. The TIL 2035 in vitro cultured cell lines used to treat patient 2035 were established from three tumor fragments, designated F3, F6/8, and F13, by culturing dissociated cells in 6,000 IU/mL recombinant IL-2,¹¹ and then expanded using OKT3 stimulation in the presence of PBMC feeder cells.¹² Patient 2035 received a mixed culture comprising 30% of the F3, 50% of the F6/8, and 20% of the F13 culture.

Antibodies and FACS Analysis

For the antibody blocking assay, 2.5×10^4 tumor target cells were incubated with 25 μ g/mL of either anti-HLA-A2 (SB02) or anti-HLA-A23 for 30 minutes at 37°C, followed by the addition of 2.5×10^4 TIL 2035 cells and measurement of IFN γ release. Characterization of the expression of T-cell receptor beta chain variable region (TRBV) expression on clones as well as TILs was carried out using a panel of antibodies obtained from Beckman/Coulter (Miami, FL) and Pierce/Endogen (Rockford, IL) that recognize approximately 50% of the germline BV genes. Anti-CD28-FITC, anti-CD27-FITC, anti-CD107a-PE, anti-CD57-FITC, anti-CD8–PerCP, anti-CD45RO-FITC/PE, and anti-CD45RA-FITC/PE were purchased from Becton Dickinson Biosciences (San Diego, CA). An IFN γ capture assay (Miltenyi Biotec, Auburn, CA) that detects cells that have secreted IFN γ was carried out according to the manufacturer's directions. FACS analysis was performed by using CELLQuest software (Becton Dickinson).

Evaluation of Antigen Reactivity

Multiple assays were carried out to evaluate the antigen reactivity of T cells from TIL 2035. Bulk cytokine release was determined by incubating T cells overnight with autologous (HLA-A2, A23) or allogeneic melanoma cells 888mel (HLA-A1, A24), followed by the measurement of IFN γ release, determined by ELISA using a monoclonal antibody pair (Pierce/Endogen). A

recently described assay that measures the induction of cell surface expression of the LAMP-1 or CD107a protein following antigen stimulation was performed as previously described.^{13, 14} Briefly, target cells were co-cultured with effector cells in 24-well plate with an E/T ratio of 1:1 for 6 hours or overnight. The co-cultured cells were then washed twice with PBS containing 0.5% BSA and 0.1 mM EDTA (PBS-BE), and the cell pellets were stained with CD107a conjugated to FITC-PE followed by FACS analysis. The MACS IFN γ capture assay (Miltenyi Biotec) was also used to directly detect populations of T cells responding to specific antigen stimulation and was carried out according to the manufacturer's instructions using an anti-IFN γ antibody conjugated with allophycocyanin (APC) to detect cytokine-secreting cells. To evaluate reactivity directed against a variety of known tumor antigens, 293 cells that were stably transfected with HLA-A2 were transiently transfected with constructs encoding MART-1, gp100, tyrosinase, TRP-1, TRP-2, MAGE-1, and MAGE-3 assayed for their ability to stimulate the release of IFN γ from TIL 2035 T cells, as previously described.¹⁵

Isolation of HLA-A23 and Screening of 2035mel cDNA Library

The HLA-A23 gene product was isolated from TIL 2035 obtained from patient 2035 by carrying out RT-PCR with the Platinum Taq DNA Polymerase High Fidelity kit (Invitrogen, Carlsbad, CA) using the forward primer EX1-A6 (5'CAGACGCCGAGGATG GCC) and the reverse primer A-EX8-1 (5'CACACAAGGCAGCTGTCTCACA),¹⁶ which were designed to amplify HLA-A locus gene products. The RT reaction was carried out for 1 hour at 50°C and 5 minutes at 85°C, followed by a PCR consisting of a 4-minute incubation at 94°C, followed by 35 cycles comprising incubations of 1 minute at 94°C, 1 minute at 58°C, and 1 minute 30 seconds at 72°C, and a final incubation at 72°C for 10 minutes. The RT-PCR products were then cloned into pcDNA3.1/V5-His expression vector (Invitrogen). Sequence reactions were carried out using the Big Dye Terminator V 1.1 Kit (Applied Biosystems) using a standard T7 forward primer or a BGH reverse primer and were analyzed using the ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). A cDNA expression library was constructed according to the manufacturer's procedures (Invitrogen) using 5 μ g poly (A)⁺ RNA that was isolated from 2035mel by using the PolyATtract mRNA Isolation Systems Kit (Promega, Madison, WI). The cDNA library was divided in pools of approximately 100 bacterial colonies per well, and 0.3 μ g DNA was co-transfected with 0.3 μ g of the pcDNA3.1-HLA-A23 gene construct into 90,000 293 cells in 96-well plates by using LipofectAMINE 2000 (Invitrogen). Eighteen hours after transfection, 5 $\times$ 10⁴ TIL 2035 F6/8 cells were added to each well. Supernatants from each well were harvested 18 hours later and tested for IFN γ by ELISA. Individual cDNAs isolated from positive wells were tested and sequenced as above.

T-Cell-Receptor β Chain Analysis

Amplification of the TRBV region sequences expressed by TIL 2035 T cells was carried out using the SMART RACE cDNA Amplification Kit (Becton Dickinson) as previously described.¹⁷ Briefly, first-strand cDNA was synthesized from 0.1 to 1 μ g total RNA using an oligo-dT primer, and amplification was carried out using a primer complementary to the TCR beta chain constant region 5'-CTCTTGACCATGGCCATC and an anchor primer according to the manufacturer's instructions. Following amplification, the 5' RACE products were cloned into the pcDNA3.1 TA cloning vector (Invitrogen) and sequenced as described above.

Telomere FISH

The FISH assay was carried out as previously described.^{18,19} Briefly, TIL 2035 was stained with PE-conjugated antibodies directed against individual TRBV products or directed against TRBV families, followed by the addition of MicroBeads that had been conjugated with an anti-PE antibody (Miltenyi Biotec) and sorting of the positive cells using a magnetic separation column (Miltenyi Biotec). Purified cells then were permeabilized for 30 minutes in the dark

and washed twice with PBS-BE and once with 1 mL hybridization buffer (70% deionized formamide, 20 mM Tris, pH 7.0, and 1% BSA). Cells were then incubated for 20 minutes in the dark with 0.3 µg/mL of a telomeric peptide nucleic acid (PNA) probe, Flu-OO-CCC-TAA-CCC-TAA-CCC-TAA, or a control probe, Flu-OO-CCC-ATA-ACT-AAA-CAC. Samples were then heated at 83°C for 10 minutes and incubated at room temperature in the dark for 2 hours. FACS analysis was performed after washing the samples twice with buffer (70% formamide, 10 mM Tris [pH 7.0], 0.1% BSA, and 0.1% Tween 20).

RESULTS

Patient Characteristics

Patient 2035 was a 37-year-old male with metastatic melanoma that had spread to multiple subcutaneous sites, the adrenal gland, and the brain. The brain lesion was excised, but progressive disease was observed at other sites despite treatment with IL-2 and chemotherapy. He was subsequently treated with lymphodepleting chemotherapy, TIL cell transfer, and IL-2 as previously described.¹ This treatment resulted in a partial response, and following the resection of a single residual lesion, he remains disease-free 31 months later.

Dominant T-Cell Clonotypes in TIL 2035 and PBMC Samples

Five predominant CD8⁺ T-cell populations that express the BV1, BV2, BV5a, BV14, and BV17 TR-BV gene products were initially identified in TIL 2035 as well as in PBMCs obtained 6 days after transfer using a panel of antibodies that specifically recognized individual TRBV gene families (Table 1). Two populations expressing BV1 and BV14 were highly represented in PBMCs obtained 26 days after adoptive transfer, indicating that these T cells may have persisted following adoptive transfer, whereas only a relatively small percentage of T cells expressing the BV2, BV5a, and BV17 markers were detected at this time (Table 1). Approximately 50% of the BV gene products cannot be detected using commercially available antibodies, however, and distinct T-cell clones that express the same BV gene product cannot be distinguished from one another. To identify individual T-cell clonotypes, the TRBV gene products expressed in samples of TIL 2035 and PBMCs were amplified using a TR constant region primer, and between 64 and 81 cDNA clones were sequenced from each of the TIL 2035 and PBMC samples. Sequence analysis indicated that a single dominant BV1⁺ T-cell clonotype comprising 2% of the sequences detected in the administered TIL 2035 expanded relative to the other dominant TIL clonotypes in vivo following adoptive transfer; it represented 9% to 12% of the sequences detected 7, 19, and 61 days after transfer (Table 2). The dominant BV2⁺ and BV5a⁺ clonotypes were not detected in PBMCs at any time following transfer, and the dominant BV17⁺ clonotype that represented 31% of the sequences in TIL 2035 declined rapidly following adoptive transfer to a level of only 1% of the total PBMCs 2 months after transfer. Two dominant BV14⁺ clonotypes, designated BV14a and BV14b, were detected in the transferred TIL 2035, and 1% and 18% of the sequences amplified from PBMCs obtained 7 days after transfer corresponded to the BV14a and BV14b clonotypes, respectively. Sixty-one days after transfer, the BV14b sequence was not detected, and only 1% of the sequences amplified from peripheral blood corresponded to the BV14a clonotype.

Antigen Reactivity of Dominant T-Cell Clonotypes in TIL 2035

The ability of the dominant T-cell clonotypes present in TIL 2035 to recognize autologous tumor cells was initially evaluated using a recently described assay using flow cytometric analysis of cell surface CD107a expression.¹⁴ The CD107a molecule is an integral membrane protein present in cytolytic granules that is expressed on the cell surface following antigen stimulation as a result of degranulation. TIL 2035 T cells were cultured with either autologous 2035 mel cells or a control mel line 888 that does not share expression of HLA class I alleles with 2035. Cell surface expression of CD107a was observed on each of the dominant BV1⁺,

BV2⁺, BV14⁺, and BV17⁺ cell populations following stimulation with autologous 2035 mel, but not with a melanoma lines that failed to share any HLA alleles with 2035, 888 mel (Fig. 1A), suggesting that TIL 2035 contains multiple tumor-reactive T-cell clones. The reactivity directed against the autologous 2035 mel was partially inhibited by incubation with the anti-HLA-A2 antibody but was strongly inhibited by the anti-HLA-A23 antibody, indicating that one or more of the dominant clonotypes present in TIL 2035 recognized a tumor antigen in the context of HLA-A23, and also suggesting the recognition of tumor via multiple antigens and multiple HLA restriction elements. Recognition of an HLA-A2 restricted antigen was confirmed by using antibody blocking of TIL 2035. F6/F8 reactivity against an allogeneic HLA-A2⁺ melanoma line, 624mel, was completely blocked by incubation with an anti-HLA-A2 antibody but was unaffected by incubation with an antibody directed against HLA-A23 (Fig. 1B). A cDNA library was then constructed from autologous mel cells, and cDNA pools were screened for their ability to stimulate TIL 2035 following co-transfection with HLA-A23 into highly transfectable 293 target cells. Screening assays resulted in the isolation of a partial cDNA encoding amino acids 135 to 314 of the MAGE-6 protein, a cancer/testis antigen previously shown to be recognized by tumor-reactive T cells in the context of the HLA-A34 class I restriction element.²⁰ The results of an IFN γ cytokine secretion assay performed with 293 cells co-transfected with the MAGE-6 and HLA-A23 constructs confirmed that recognition of this antigen was mediated primarily or exclusively by BV1⁺ T cells (Fig. 1C). To further characterize the antigen reactivity of TIL 2035, HLA-A2-positive 293 cells were transfected with genes that encoded multiple HLA-A2-restricted tumor antigens. Target cells transfected with a construct encoding NY-ESO-1, but not the other antigens, were recognized by TIL 2035 T cells (data not shown). The dominant BV17 clonotype recognized autologous EBV-B cells that were pulsed with the previously described HLA-A2-restricted NY-ESO 1:157-165 peptide²¹ (Fig. 1C). In addition, one or more of the dominant BV14⁺ clonotypes appeared to recognize a tumor antigen in the context of HLA-B or C (data not shown).

Phenotypic Markers Associated with Survival and Persistence of Tumor-Specific T Cells Following Adoptive Transfer

Additional studies were then carried out to examine the phenotype of the dominant tumor-reactive BV1⁺, BV2⁺, BV14⁺, and BV17⁺ T-cell clonotypes present in TIL 2035, as well as the phenotype of the corresponding clonotypes that persisted in vivo following adoptive transfer. Nearly 90% of the BV1⁺ T cells present in TIL 2035 expressed CD28, whereas less than 50% of the cells corresponding to the BV2⁺, BV14⁺, and BV17⁺ clonotypes appeared to express significant levels of CD28 (Fig. 2A). The BV1⁺ T cells also expressed CD28 at levels that were higher than those observed on the additional clonotypes present in TIL 2035, as determined by comparison of the mean fluorescent intensity (MFI) of CD28 expression on those cells. The analysis of PBMCs obtained 7 days after transfer, however, revealed that the majority of the persistent clonotypes expressed relatively high levels of CD28 (Fig. 2A). Nineteen days after transfer, more than 80% of BV1⁺ T cells in peripheral blood expressed CD28, whereas only 48% of the BV14⁺ T cells expressed CD28 at this time (Fig. 2A), indicating that the BV14⁺ TIL clonotypes may represent cells that are at a later stage of differentiation than the BV1⁺ clonotype. The observation that the BV1⁺ clonotype persisted to a similar level 61 days following transfer, whereas the two BV14⁺ clonotypes were nearly absent at this time point (Table 2), indicates that the stage of differentiation may influence the long-term persistence of adoptively transferred effector T cells.

Analysis of the expression of the CD27 co-stimulatory molecule indicated that nearly 80% of the BV1⁺ T cells present in the in vitro cultured TIL 2035 expressed this marker (Fig. 2B). In addition, the level of CD27 expression on BV1⁺ T cells from TIL 2035 was significantly higher than the CD27 levels observed on the BV2⁺, BV14⁺, and BV17⁺ T cells present in TIL 2035. The levels of CD27 expression observed on BV1⁺ and BV14⁺ T cells analyzed 7 days after

adoptive transfer appeared to be significantly lower than that those found on the corresponding populations of T cells from TIL 2035, as fewer than 50% of each of these populations expressed this marker at this time point. Over 80% of the BV1⁺ and BV14⁺ T cells expressed CD27 19 days after transfer, however, indicating that this marker is re-expressed at a later time point or that there is a selective survival of CD27⁺ T cells.

Analysis of the co-expression of the CD28 and CD27 markers on the BV1⁺, BV2⁺, BV14⁺, and BV17⁺ cells present in TIL 2035 indicated that 75% of the BV1⁺ T cells were CD28⁺CD27⁺, 42% of the BV14⁺ T cells were CD28⁺CD27⁺, and less than 30% of the BV2⁺ and BV17⁺ clonotypes were CD28⁺CD27⁺ (Fig. 2C). These results indicated that co-expression of CD27 and CD28 on TIL 2035 was also correlated with the degree of in vivo persistence.

The expression of CD57 and CD45RA, markers that are associated with the state of T-cell differentiation, was then examined on TIL 2035 as well as PBMCs obtained following adoptive transfer. The CD45RO marker was expressed at high levels on all of the cells present in TIL 2035, whereas expression of the CD57 and CD45RA markers was not detected on these T cells (data not shown). Approximately 50% of the BV14⁺ and BV17⁺ T cells present in the peripheral blood of patient 2035 expressed CD57 12 days after transfer, whereas approximately 10% of the BV1⁺ T cells expressed CD57 at this time (Fig. 3A). Within each of the dominant BV populations, greater than 70% of CD28⁺ cells expressed CD57, although upregulation of CD57 expression was not associated with the loss of CD27 expression at this time (Fig. 3B). Examination of PBMCs from patient 2035 revealed that the BV14⁺ T cells expressed higher levels of CD45RA 12 days after transfer than BV1⁺ T cells, while the levels of CD45RA observed on the BV1⁺ T cells 61 days after transfer were comparable to those found on the BV14⁺ T cells 12 days after transfer (Fig. 3C).

Proliferative history also appears to be related to the stage of T-cell differentiation and can be evaluated by measurement of telomere length.^{22,23} The lengths of telomeres present in populations of T cells isolated from TIL 2035 were therefore evaluated by carrying out FISH analysis with a specific oligonucleotide probe. Consistent with the previous observations that the BV1⁺ T-cell clonotype appeared to represent a less terminally differentiated population of T cells, the BV1⁺ T cells from TIL 2035 possessed significantly longer telomeres than the BV14⁺ T cells (Fig. 4). In addition, the BV2⁺ and BV17⁺ T cells in TIL 2035 also possessed relatively short telomeres (data not shown). Taken together, these results indicate that the dominant BV1⁺ T-cell clonotype from TIL 2035 represents a less differentiated population of cells than the dominant BV2⁺, BV14⁺, and BV17⁺ T cells present in this TIL 2035, and suggest that the differentiation stage of tumor-specific T-cell populations in TIL 2035 may be associated with their ability to persist.

Progressive Differentiation of Long-Term Persistent T Cells

The observation that approximately 12% of the T cells present in the peripheral blood of patient 2035 16 months after transfer expressed BV1, 75% of which corresponded to the sequence of the dominant BV1⁺ clonotype, provided an opportunity to evaluate changes in the phenotype of these T cells that occurred over more than a year after adoptive transfer. Expression of the CD27 and CD28 co-stimulatory molecules on the BV1⁺ clonotype appeared to gradually decline over the course of 16 months, with the exception of a sharp decline in CD27 expression that was observed 12 days after transfer in conjunction with a relatively small increase in CD28 expression (Fig. 5A, 5B). Increased expression of CD45RA (Fig. 3C), coupled with a progressive loss of CD45RO expression (Fig. 5C), was observed on the BV1⁺ T cells following adoptive transfer. In addition, expression of CD57 on BV1⁺ T cells gradually increased from undetectable levels in TIL 2035 to approximately 60% of the BV1⁺ T cells in peripheral blood 16 months following adoptive transfer (Fig. 5D), at which time 95% of the BV1⁺CD57⁺ cells

expressed low or undetectable levels of CD28 (data not shown). Taken together, these observations suggest that the adoptively transferred T cells undergo progressive differentiation *in vivo* following adoptive transfer, and in addition suggest that the degree of persistence of the transferred cells may be related to their stage of differentiation.

DISCUSSION

Results of prior studies of 13 cancer patients indicated that the administration of non-myeloablative chemotherapy enhanced clinical responses to adoptively transferred tumor-reactive T cells, but in addition appeared to enhance the *in vivo* persistence of T cells following transfer.¹ In a recent study of 25 patients treated with this regimen, adoptively transferred T cells were found to persist at relatively high levels in the peripheral blood of some of the treated patients, and the degree of persistence was associated with clinical responses.² The observation that the levels of persistence of individual T-cell clonotypes in peripheral blood samples obtained from patients in this trial were highly variable has led to efforts to identify characteristics of T cells that are associated with *in vivo* T-cell persistence.

This study shows that five dominant CD8⁺ tumor-reactive T-cell clonotypes present in a polyclonal population of *in vitro* cultured TIL, TIL 2035, persisted to varying degrees in peripheral blood following adoptive transfer. A dominant T-cell clonotype in this TIL that expressed the BV1 T-cell receptor persisted at relatively high levels for over 1 year after transfer. In contrast, the dominant BV2⁺ and BV17⁺ clonotypes, which represented 9% and 31%, respectively, of the T cells that were initially present in TIL 2035, were present at low or undetectable levels as early as 1 week after transfer. A dominant BV14⁺ T-cell clonotype that showed an intermediate degree of persistence was detected in the peripheral blood of patient 2035 1 week after transfer at a level that was comparable to that observed in the *in vitro* cultured TIL 2035, but it was not detected in peripheral blood 2 months after transfer.

Studies carried out to evaluate the antigen reactivity of the dominant clonotypes present in TIL 2035 showed that the BV17⁺ clonotype recognized a previously described HLA-A2 restricted epitope of the NY-ESO-1 protein. Screening assays carried out by transfection of HLA-A23 expressing target cells with a cDNA library resulted in the isolation of MAGE-6, which was recognized by the dominant and long-term-persistent BV1⁺ clonotype. The BV14⁺ and BV2⁺ clonotypes also recognized autologous melanoma cells, but the antigenic targets of these T cells have not been identified.

Naive T cells express high levels of the CCR7, CD62L, CD27, and CD28 molecules, and a linear differentiation pathway has been proposed wherein antigen stimulation results in the initial loss of CCR7 and CD62L expression.^{24,25} Chronic antigen stimulation can then result in a progression to end-stage effector cells that have lost expression of CD28 and CD27.²⁴ Expression of CCR7 and CD62L was not observed on a significant percentage of TIL 2035 T cells (data not shown), but the results presented in this report indicate that the expression of CD27 and CD28 varied among the dominant TIL 2035 clonotypes. Furthermore, the *in vivo* persistence of these cells appeared to be correlated with the relative levels of expression of CD27 and CD28 on the dominant TIL 2035 TRBV clonotypes. The CD27 and CD28 markers were also co-expressed on approximately 75% of the BV1⁺ T cells in the *in vitro* cultured TIL 2035, but on only 14% of the cultured BV17⁺ T cells. The BV14⁺ T cells, which persisted at relatively high levels for only 1 week after transfer, possessed an intermediate phenotype, as 42% of the T cells present in TIL 2035 that expressed this BV gene product co-expressed CD27 and CD28. These results indicate that memory T cells in TIL 2035 that express CD27 and CD28 may selectively survive and persist following adoptive cell transfer. The observation that persistent T cells from patient 2035 expressed relatively high levels of CD27 and CD28 following adoptive transfer is consistent with results reported in a recent study of multiple

melanoma patients enrolled in the same clinical trial who received adoptive immunotherapy following non-myeloablative chemotherapy.²⁶

Several molecules that are expressed on natural killer (NK) cells, including member of the NK inhibitory receptor family of molecules, have also been shown to be expressed on subsets of human T cells; however, the effects of these molecules on T-cell function have not been fully elucidated. Previous studies have suggested that one member of this family, KLRG1, represents a marker for memory T cells that have undergone extensive cell division.^{14,27–29} Mouse studies have indicated that one function of NK inhibitory receptors may be to raise the activation threshold for T cells and contribute to the regulation of the memory T-cell pool.³⁰ Another NK marker, CD57, also appears to be associated with end-stage T-cell differentiation.^{7,28} Elevated expression of CD57 has been observed on tumor-specific T cells and may be the consequence of persistent chronic antigen stimulation that results in the accumulation of highly cytolytic effector cells that are capable of rapidly secreting cytokines but that lack proliferative capacity.³¹ The *in vitro* cultured TIL 2035 did not express significant levels of CD57, and 12 days after adoptive transfer, relatively low levels of expression were observed on the persistent BV1⁺ clonotype. In contrast, relatively high levels were observed on the BV14⁺ T cells in PBMCs 12 days after adoptive transfer. These findings again suggest that the BV1⁺ clonotype in TIL 2035 represented a less differentiated cell than the BV14⁺ clonotype.

The expression of alternative isoforms of the CD45 molecule has also been associated with T-cell differentiation,²⁴ as naive T cells express CD45RA, while activated T cells downregulate CD45RA expression but upregulate CD45RO expression. Continued stimulation results in the generation of late-stage effector CD8⁺ T cells with a reduced proliferative potential that upregulate expression of CD45RA and down-regulate expression of CD45RO, CD27, and CD28.^{24,32} Examination of TIL 2035 as well as PBMCs obtained 12 and 61 days after transfer showed that there was a loss of CD45RO expression and an increase in CD45RA expression on BV1⁺ cells following adoptive transfer. The observation that the BV14⁺ T cells expressed higher levels of CD45RA than the BV1⁺ T cells 12 days after transfer provides a further indication that the BV14⁺ clonotype may have differentiated to an end-stage effector cell at this time point, when the majority of the BV1 clonotype corresponded to a less differentiated phenotype.

The relatively long persistence of the BV1⁺ clonotype allowed the expression of differentiation markers to be evaluated on these cells over a period of 16 months following adoptive transfer. A portion of the BV1⁺ cells appeared to differentiate from a CD45RO⁺CD45RA[−]CD27⁺CD28⁺CD57[−] memory cell to a late-stage CD45RO[−]CD45RA⁺CD27[−]CD28[−]CD57⁺ effector phenotype over this time period. Over 95% of the CD28[−] T cells, which represented approximately 50% of the BV1⁺ T cells at this time, expressed CD57 16 months after transfer. Similar phenotypic changes were also observed in the BV14⁺ clonotypes; this process, however, appeared to take place over a significantly shorter time period than that required for changes in the BV1⁺ clonotype.

Telomere length represents a marker that is associated with the replicative *in vivo* history or life span, and thus may represent one of the factors that may influence T-cell persistence following adoptive transfer. Previous studies have suggested that antigen stimulation resulted in upregulation of telomerase activity, which may interfere with telomere erosion.³ Telomerase activity does not appear to be sustained indefinitely in memory T cells, however, and the consequent shortening of telomeres may play a role in differentiation to an end-stage effector with limited replicative capacity.^{22,23} Consistent with these observations, the telomere length of the highly persistent BV1⁺ clonotype was long relative to the telomere lengths observed for the less persistent clonotypes.

Recent results have established a link between T-cell persistence and tumor regression following cell transfer therapy.² The results presented here suggest that the differentiation stage and replicative history of T-cell clonotypes present in highly heterogeneous TIL 2035 may be associated with their ability to persist in vivo following adoptive transfer. Furthermore, these results suggest that it may be possible to identify tumor-specific T cells with a greater capacity to persist following adoptive transfer, and thereby potentially enhance the efficacy of adoptive immunotherapies.

Acknowledgements

The authors thank Mr. Arnold Mixon and Mr. Shawn Farid for their assistance with fluorescent cell analysis.

References

1. Dudley ME, Wunderlich JR, Robbins PF, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 2002;298:850–854. [PubMed: 12242449]
2. Robbins PF, Dudley ME, Wunderlich JR, et al. Persistence of transferred lymphocyte clonotypes correlates with cancer regression in patients receiving cell transfer therapy. *J Immunol* 2004;173:7125–7130. [PubMed: 15585832]
3. Rufer N, Zippelius A, Batard P, et al. Ex vivo characterization of human CD8+ T subsets with distinct replicative history and partial effector functions. *Blood* 2003;102:1779–1787. [PubMed: 12750165]
4. Hendriks J, Xiao Y, Borst J. CD27 promotes survival of activated T cells and complements CD28 in generation and establishment of the effector T cell pool. *J Exp Med* 2003;198:1369–1380. [PubMed: 14581610]
5. Jones RG, Elford AR, Parsons MJ, et al. CD28-dependent activation of protein kinase B/Akt blocks Fas-mediated apoptosis by preventing death-inducing signaling complex assembly. *J Exp Med* 2002;196:335–348. [PubMed: 12163562]
6. Effros RB. Loss of CD28 expression on T lymphocytes: a marker of replicative senescence. *Dev Comp Immunol* 1997;21:471–478. [PubMed: 9463780]
7. Brenchley JM, Karandikar NJ, Betts MR, et al. Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells. *Blood* 2003;101:2711–2720. [PubMed: 12433688]
8. Voehringer D, Koschella M, Pircher H. Lack of proliferative capacity of human effector and memory T cells expressing killer cell lectin-like receptor G1 (KLRG1). *Blood* 2002;100:3698–3702. [PubMed: 12393723]
9. Champagne P, Ogg GS, King AS, et al. Skewed maturation of memory HIV-specific CD8 T lymphocytes. *Nature* 2001;410:106–111. [PubMed: 11242051]
10. Michie CA, McLean A, Alcock C, et al. Lifespan of human lymphocyte subsets defined by CD45 isoforms. *Nature* 1992;360:264–265. [PubMed: 1436108]
11. Dudley ME, Wunderlich J, Nishimura MI, et al. Adoptive transfer of cloned melanoma-reactive T lymphocytes for the treatment of patients with metastatic melanoma. *J Immunother* 2001;24:363–373. [PubMed: 11565838]
12. Walter EA, Greenberg PD, Gilbert MJ, et al. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *N Engl J Med* 1995;333:1038–1044. [PubMed: 7675046]
13. Rubio V, Stuge TB, Singh N, et al. Ex vivo identification, isolation and analysis of tumor-cytolytic T cells. *Nat Med* 2003;9:1377–1382. [PubMed: 14528297]
14. Betts MR, Brenchley JM, Price DA, et al. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *J Immunol Methods* 2003;281:65–78. [PubMed: 14580882]
15. Robbins PF, El-Gamil M, Li YF, et al. A mutated β -catenin gene encodes a melanoma-specific antigen recognized by tumor infiltrating lymphocytes. *J Exp Med* 1996;183:1185–1192. [PubMed: 8642260]

16. Bettinotti MP, Hadzikadic L, Ruppe E, et al. New HLA-A, -B, and -C locus-specific primers for PCR amplification from cDNA: application in clinical immunology. *J Immunol Methods* 2003;279:143–148. [PubMed: 12969555]
17. Huang J, El-Gamil M, Dudley ME, et al. T cells associated with tumor regression recognize frameshifted products of the CDKN2A tumor suppressor gene locus and a mutated HLA class I gene product. *J Immunol* 2004;172:6057–6064. [PubMed: 15128789]
18. Rufer N, Brummendorf TH, Kolvraa S, et al. Telomere fluorescence measurements in granulocytes and T lymphocyte subsets point to a high turnover of hematopoietic stem cells and memory T cells in early childhood. *J Exp Med* 1999;190:157–167. [PubMed: 10432279]
19. Rufer N, Dragowska W, Thornbury G, et al. Telomere length dynamics in human lymphocyte subpopulations measured by flow cytometry. *Nat Biotechnol* 1998;16:743–747. [PubMed: 9702772]
20. Zorn E, Hercend T. A MAGE-6-encoded peptide is recognized by expanded lymphocytes infiltrating a spontaneously regressing human primary melanoma lesion. *Eur J Immunol* 1999;29:602–607. [PubMed: 10064076]
21. Jager E, Chen YT, Drijfhout JW, et al. Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. *J Exp Med* 1998;187:265–270. [PubMed: 9432985]
22. Effros RB, Dagarag M, Valenzuela HF. in vitro senescence of immune cells. *Exp Gerontol* 2003;38:1243–1249. [PubMed: 14698803]
23. Reed JR, Vukmanovic-Stejic M, Fletcher JM, et al. Telomere erosion in memory T cells induced by telomerase inhibition at the site of antigenic challenge in vivo. *J Exp Med* 2004;199:1433–1443. [PubMed: 15148341]
24. Appay V, Dunbar PR, Callan M, et al. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. *Nat Med* 2002;8:379–385. [PubMed: 11927944]
25. Hamann D, Roos MT, van Lier RA. Faces and phases of human CD8 T-cell development. *Immunol Today* 1999;20:177–180. [PubMed: 10203715]
26. Powell DJ Jr, Dudley ME, Robbins PF, et al. Transition of late stage effector T cells to CD27+ CD28+ tumor-reactive effector memory T cells in humans after adoptive cell transfer therapy. *Blood* 2005;105:241–250. [PubMed: 15345595]
27. Voehringer D, Blaser C, Brawand P, et al. Viral infections induce abundant numbers of senescent CD8 T cells. *J Immunol* 2001;167:4838–4843. [PubMed: 11673487]
28. d'Angeac AD, Monier S, Pilling D, et al. CD57+ T lymphocytes are derived from CD57-precursors by differentiation occurring in late immune responses. *Eur J Immunol* 1994;24:1503–1511. [PubMed: 7517872]
29. Kern F, Khatamzas E, Surel I, et al. Distribution of human CMV-specific memory T cells among the CD8pos. subsets defined by CD57, CD27, and CD45 isoforms. *Eur J Immunol* 1999;29:2908–2915. [PubMed: 10508265]
30. Ugolini S, Arpin C, Anfossi N, et al. Involvement of inhibitory NKRs in the survival of a subset of memory-phenotype CD8+ T cells. *Nat Immunol* 2001;2:430–435. [PubMed: 11323697]
31. Tarazona R, DelaRosa O, Alonso C, et al. Increased expression of NK cell markers on T lymphocytes in aging and chronic activation of the immune system reflects the accumulation of effector/senescent T cells. *Mech Ageing Dev* 2000;121:77–88. [PubMed: 11164462]
32. Hamann D, Baars PA, Rep MH, et al. Phenotypic and functional separation of memory and effector human CD8+ T cells. *J Exp Med* 1997;186:1407–1418. [PubMed: 9348298]

FIGURE 1.

Characterization of the antigen reactivity of TIL 2035 T cells. A, TIL 2035 cells were co-cultured with either autologous melanoma line 2035mel (HLA-A2, A23) or HLA-mismatched control melanoma line 888mel (HLA-A1, A24) for 6 hours, followed by incubation with an anti-CD107a monoclonal antibody and FACS analysis. Stimulations carried out with 2035 mel are indicated by the bold lines, and those carried out with control 888 mel are indicated by the filled histograms. Gating was carried out on the cell populations that expressed the indicated BV gene products. B, The autologous melanoma line and HLA-A2-positive melanoma cell line, 624 mel, were incubated with either no antibody (Control), anti-HLA-A*02 antibody (Anti-A2) or anti-HLA-A23 antibody (Anti-A23). TIL 2035-F6/8 was then added, and 18 hours later IFN γ release was measured by ELISA. C, Autologous EBVB cells were pulsed with either a control peptide (upper left) or the HLA-A2-restricted NY-ESO-1: 157-165 peptide (upper right) for 2 hours, and then co-cultured with TIL 2035-F6/8 for 2 hours. An IFN γ capture assay was then carried out, cells were co-stained with an anti-BV17 antibody, and FACS analysis was performed. Two hundred ninety-three cells were co-transfected HLA-A23 with either a control vector (lower left) or a vector encoding MAGE-6 (lower right) for 24 hours, followed by the addition of TIL 2035-F6/8. Eighteen hours later, an IFN γ capture assay was carried out; cells were co-stained with an anti-BV1 antibody and analyzed using FACS analysis. The data represent one of three individual experiments.

FIGURE 2.

Phenotypic analysis of dominant T-cell populations in the infusion TIL 2035 and in peripheral blood. The infusion TIL 2035 as well as peripheral blood samples obtained 8 days or 20 days following adoptive transfer were gated on CD8⁺ cells and analyzed for expression of CD28 (A) and CD27 (B). The MFI of CD28 expression for the gated populations was 29 (BV1), 9 (BV2), 12 (BV14), and 6 (BV17). C, Co-expression of CD27 and CD28 was analyzed on TIL 2035 populations that were gated using the indicated TRBV antibodies. Quadrants were drawn based on staining obtained with IgG control antibodies. The data represent one of three individual experiments.

FIGURE 3.

Expression of differentiation markers on PBMCs obtained following adoptive transfer. Co-expression of CD28 and CD57 (A) and CD27 and CD57 (B) was analyzed on peripheral blood samples obtained 12 days after adoptive transfer that were gated using the indicated BV antibody. Quadrants were drawn based on staining obtained with IgG control antibodies. C, Expression of CD45RA was evaluated for populations of PBMCs obtained 13 or 61 days following transfer that were gated using the indicated BV antibodies. Data represent one of three individual experiments.

FIGURE 4.

Telomere fluorescence intensity of dominant clonotypes present in TIL 2035. BV1⁺ and BV14⁺ T cells were isolated from TIL 2035, and FISH was carried out using a telomeric peptide nucleic acid (PNA) probe, or a control PNA probe for each of the separated populations, followed by FACS analysis. The control staining corresponds to that observed for BV1⁺ T cells. Data represent one of two individual experiments.

FIGURE 5.

Alterations in the phenotype BV1⁺ T cells in peripheral blood following adoptive transfer. Gated populations of BV1⁺ T cells present in peripheral blood samples 13 days, 61 days, 280 days, and 480 days after the adoptive transfer were analyzed for expression of (A) CD27, (B) CD28, (C) CD45RO, or (D) CD57. The day 0 sample represents TIL 2035. Data represent one of three individual experiments.

TABLE 1
Dominant TCR-BVs in the Infusion TILs and PBMCs

BV Family	Percentage of BVs in TILs and PBMCs*			
	PBMC (– Day17) [†]	TIL (Day 0)	PBMC (Day 6)	PBMC (Day 26)
	51.5% CD8 ⁺	98.4% CD8 ⁺	52.5% CD8 ⁺	69.6% CD8 ⁺
1	2.3	2.8	10.8	12.5
2	2.3	22.6	1.9	1.4
3	0.3	0.0	0.0	0.0
5a	1.6	5.8	0.2	1.5
5c	1.5	0.8	0.2	0.8
6.7	0.1	0.1	0.2	0.1
7	2.5	1.1	0.5	2.2
8	1.4	0.1	0.4	0.5
9	1.0	0.1	0.0	0.2
11	0.4	0.1	0.0	0.2
12	0.7	0.1	1.4	0.9
13	1.8	0.6	0.3	1.0
14	3.2	24.4	29.7	9.7
16	0.6	0.3	0.0	0.2
17	3.9	28.6	3.4	1.5
18	0.3	0.7	0.0	0.2
20	1.2	0.4	0.2	6.0
21.3	1.2	0.7	0.3	0.3
22	2.8	1.1	0.5	0.4
23	0.8	0.4	0.1	0.2

* TIL and PBMC samples were stained with CD8 and BV specific antibodies, and % of each BV⁺ in the TIL and PBMC samples was analyzed by FACS.

[†] The day of the TIL infusion is considered day 0, and the PBMC sample was obtained 17 days before the infusion.

TABLE 2
Clonal Analysis of Dominant T-Cell Populations in TIL 2035 and Posttransfer PBMCs

Dominant TCR-BV Clonotype Present in TIL 2035*	TIL (Day 0)		PBMC (Day 7)†		PBMC (Day 19)		PBMC (Day 61)	
	% of Total Sequences	% of Indicated TRBV Family‡	% of Total Sequences	% of Indicated TRBV Family	% of Total Sequences	% of Indicated TRBV Family	% of Total Sequences	% of Indicated TRBV Family
1	2	100	12	90	9	78	12	77
2	9	67	<1	—§	<1	—	<1	—
14a	6	18	1	5	1	8	1	12
14b	28	82	18	65	3	17	<1	—
17	31	100	3	50	<1	63¶	1	33

* A total of 64 to 81 TRBV gene transcripts were analyzed for TIL or PBMC samples, and the percentage of each of the sequences amplified from individual samples is indicated. The numbers in this column correspond to the TR-BV gene product that was detected. The two BV-14 sequences that were detected in TIL 2035 were noted as 14a and 14b. Sequences that were detected at a level of 2% or greater in the TIL 2035 were analyzed.

† PBMC samples were obtained at the indicated number of days following adoptive transfer.

‡ The percentage reflects the ratio of sequences corresponding to a particular clonotype to all sequences encoded by the same TRBV gene that were amplified from a given TIL or PBMC sample.

§ A dash indicates that the percentage of sequences from an individual TRBV gene family that corresponded to the particular clonotype was not evaluated.

¶ The frequency of T cells corresponding to the dominant TRBV17 clonotype was evaluated by carrying out an RT-PCR using a TRBV17-specific primer and a TR beta chain constant region primer to amplify sequences corresponding to this family and determining the % of amplified sequences corresponding to this clonotype.