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Minimally cultured tumor-infiltrating lymphocytes display optimal characteristics for adoptive cell therapy

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Abstract

Adoptive cell therapy (ACT) with tumor reactive lymphocytes in patients with refractory melanoma can result in tumor regression and prolonged survival. Generating tumor reactive lymphocyte cultures is technically difficult and resource intensive; these limitations have restricted the widespread application of ACT. Tumor infiltrating lymphocytes (TIL) from melanoma contain tumor antigen reactive cells. The “standard” method for producing TIL cultures for clinical administration requires extended in vitro expansion in interleukin-2, then identification of tumor reactive cells by immunological assays. We show here that limitations in reagents and methods during screening under-represent the actual reactivity of TIL cultures. Furthermore, the extended culture times necessitated by the screening assays resulted in telomere shortening and reduced expression of CD27 and CD28 in the TIL cultures, properties that our prior studies showed are correlated within vivo persistence and clinical response. We have thus developed an alternative “young” TIL method that demonstrated superior in vitro attributes compared with standard TIL. This approach utilizes the entire resected tumor to rapidly expand TIL for administration without in vitro testing for tumor recognition. Our observations suggest that younger TIL can have an undetermined but high level of antigen reactivity, and other advantageous attributes such as long telomeres and high levels of CD27 and CD28. We suggest that minimally cultured, unselected lymphocytes represent an alternative strategy for generating TIL cultures suitable for use in ACT therapy that, if effective in vivo, may facilitate the widespread application of this approach to a broader population of patients with melanoma.

Keywords

tumor infiltrating lymphocyte; adoptive cell therapy; melanoma; telomere; interleukin-2

INTRODUCTION

Metastatic melanoma has a poor prognosis with a five-year survival of 15%.¹ The two FDA-approved treatments for metastatic melanoma are dacarbazine-based chemotherapy and interleukin-2 (IL2) with objective response rates below 20%.^{2,3} Adoptive cell therapy (ACT) using tumor reactive tumor-infiltrating lymphocytes (TIL) following host lymphodepletion can lead to objective response rates of 50-70% and durable responses in patients with refractory melanoma⁴⁻⁶. However, this therapy requires sophisticated cell processing and in

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vitro lymphocyte culturing for extended periods. These requirements have introduced technical, regulatory, and logistic challenges that have limited the use of antigen specific TIL as a biological therapy. Wider application of this promising therapy will require simplifying the production of TIL treatments while improving patient outcomes. No systematic studies have been undertaken to compare TIL produced by different methods for their suitability for ACT.

The first clinical trials of ACT for metastatic melanoma were first reported in 1988 and summarized in 1994.^{7;8} TIL cultures for these clinical studies were generated by sequential serial passage in 6000 IU/ml interleukin (IL)-2 without antigen stimulation until an average of more than 1×10^{11} lymphocytes was obtained; then autologous TIL were administered to 86 patients irrespective of recognition of tumor. Patients also received high-dose bolus interleukin 2 (IL-2), and 57 patients received a single dose of 25 mg/kg cyclophosphamide. An overall objective clinical response rate of 34% was observed in this trial. There were no significant differences between the response rate of patients who had received prior IL-2 vs. those who had not; and receiving prior cyclophosphamide had no apparent impact. However, several features of the administered TIL were significantly correlated with objective responses. Most significantly, “younger” TIL cultures which required the shortest time in culture prior to achieving sufficient cell numbers for patient administration, were correlated with objective responses ($P_2=0.0001$). Similarly, TIL which demonstrated autologous tumor lysis were significantly associated with objective clinical responses ($P_2=0.0008$). Subsequent clinical investigations of ACT have emphasized the generation of cells with specific tumor recognition.

ACT with tumor antigen specific cells can be a highly effective and potentially curative therapy for patients with some metastatic cancers. Autologous^{9;10} or allogeneic¹¹ cell lines specific for Epstein-Barr Virus (EBV)-derived antigens have proven effective for treatment of refractory post transplant lymphoproliferative disease. EBV antigen specific lines can also mediate regression of nasopharyngeal cancers of viral origin in some patients¹². ACT for melanoma patients with antigen specific cells generated by TCR genetic engineering¹³ or in vitro restimulation approaches^{14;15} can also occasionally cause objective clinical regressions. The most effective ACT treatments of patients with melanoma have utilized tumor reactive TIL, administered following host lymphodepleting preparative conditioning⁴. TIL grown from microcultures and selected for tumor-specific reactivity were rapidly expanded to large numbers for patient treatment in three sequential clinical trials with increasing intensity lymphodepleting preparative regimens⁶. Overall, 52 of 93 patients (56%) exhibited an objective response to following TIL administration, including 10 patients who had a complete response. These examples suggest that ACT with antigen specific cells can be therapeutically beneficial for selected patients.

While tumor-antigen specificity is known to be a prerequisite for successful anti-tumor therapy, other attributes of T cells which may contribute to in vivo effectiveness have not been completely defined. Substantial preclinical evidence and some clinical anecdotes support an effort to use “younger” lymphocytes in ACT protocols. In a murine model of ACT, the number of restimulations in vitro was inversely correlated with the therapeutic efficacy of the transferred lymphocytes^{17;18}. The decreased in vivo efficacy of the extensively cultured T cells paralleled their differentiation in culture, with loss of expression of surface markers associated with traffic to lymph nodes (CCR7, CD62L) and expression of costimulation receptors (CD27, CD28). An indirect link has also been established between the “age” of the TIL used for ACT clinical trials and their efficacy. First, TIL with shorter telomeres were associated with reduced clinical responses and in vivo persistence^{19;20}. Second, CD27 and CD28 expression were associated with proliferation and survival of adoptively transferred TIL^{21;22}.

In this paper we examined the relationship between the time in culture and the phenotypic and functional characteristics of TIL. Surprisingly, we found that minimally cultured TIL, referred to here as “young” TIL, demonstrated specific anti-tumor reactivity comparable to standard TIL despite having a significantly different composition of lymphocytes. Additionally, young TIL expressed attributes that were more closely matched with persistent cells and those that generated clinical responses than TIL generated by standard methods. These results suggest that generating young TIL without screening for specific tumor recognition significantly simplifies the production of TIL with improved phenotypic attributes for ACT therapy, and support the evaluation of a new clinical approach that may be more generally applied for patients with metastatic melanoma.

Materials and Methods

Generation of TIL

Patients were entered into clinical protocols and signed informed consents that were approved by the Institutional Review Board of the National Cancer Institute prior to tumor resection. TIL were prepared as previously described in detail²³. Briefly, multiple independent TIL cultures were set up using enzymatic digests and tumor fragments (1 mm³) procured by sharp dissection. TIL from tumor digests were generated by culturing single-cell suspensions (5×10^5 /ml) obtained by overnight enzymatic digestion of tumor fragments in media containing collagenase, hyaluronidase, and DNase. Fragments and digests were initiated in 2ml wells of complete medium (CM) and IL2 (6000 IU/ml, Chiron Corp., Emeryville, CA) in a humidified 37°C incubator with 5% CO₂. CM consisted of RPMI1640 with glutamine, plus 10% human AB serum, 25 mM HEPES, 10 ug/ml gentamicin, and 5.5×10^{-5} M 2-mercaptoethanol. Five days after initiation, one half of the media was aspirated from the wells and replaced with fresh CM and IL-2, and media was replaced every two to three days thereafter as needed. Under these conditions, lymphocytes will first lyse the adherent cells in the well, and then begin to multiply and grow.

Young TIL were defined as cultures which had just expanded to confluent growth of the original 2-ml well and eliminated adherent tumor cells, typically about 10-18 days after initiation. In practice, this was about 2×10^6 lymphocytes from each original tumor fragment or digest well. By pooling all the wells in a single 24 well plate, approximately 5×10^7 young TIL cells would be obtained.

Standard TIL were propagated by splitting an individual confluent well into two daughter wells, and maintaining each initial fragment or well of digest as an independent culture. Each standard TIL culture was split multiple times until it comprised confluent growth of 24 daughter wells generated from one original 2-ml well. A standard TIL culture would typically generate 5×10^7 lymphocytes from each original well of tumor fragment or digest after 21-36 days, and TIL were assayed for activity at that time.

Rapid expansions of young TIL and Standard TIL cultures was performed using the Rapid Expansion Protocol (REP) as previously described^{23;24}. Briefly, TIL cells were cultured in T25 flasks with a 200 fold excess of irradiated (40 Gy) allogeneic peripheral blood mononuclear “feeder” cells in complete medium (CM) with 30 ng/ml anti-CD3 antibody and 6000 IU/ml IL-2. Half of the media was exchanged on day 5 using CM with 6000 IU/ml IL-2, and cells were split as needed thereafter. Cell activity was assessed on day 14 of the rapid expansion (TIL expanded an average of more than 3000 fold).

Specific Reactivity of Standard TIL

Specific reactivity of TIL was assessed by interferon- γ (IFN- γ) release assay. TIL were washed prior to use to remove IL-2, then cultured overnight with autologous, HLA-matched, or HLA-mismatched tumor cells at a ratio of 1:1. Single cell suspensions of fresh tumor digests were prepared as targets from autologous or allogeneic melanoma specimens by overnight digestion of macerated tumor fragments in media containing collagenase, hyaluronidase, and DNase. The single cell suspension was washed twice with HBSS and aliquots were cryopreserved. Targets were thawed on the day of coculture, and viable tumor cells were assessed by trypan blue exclusion. The supernatant from each coculture was then assayed for IFN- γ by ELISA (Peirce/Endogen) according to the manufacturer recommendations. A TIL culture was defined as possessing specific reactivity if IFN- γ release was twice background (coculture of TIL with HLA-mismatched tumors) and at least 200 pg/mL unless otherwise noted.

Specific Reactivity of Young TIL

When cultures designated for young TIL generation expanded to confluence in 2-ml wells, they were tested for specific reactivity. Because the young TIL were set up in large numbers (typically groups of 24 per tumor) it was not feasible to count each TIL culture individually. The young TIL specificity assay measures activity per volume rather than activity per cell. Each well was mixed thoroughly, and exactly one hundred microliters of lymphocytes (estimated 1×10^5 cells) was washed and cocultured with 1×10^5 autologous or HLA-mismatched tumor cells overnight. IFN- γ release was then measured with ELISA and reactivity defined as above.

Differentiation Phenotype of Young TIL

Expression of CD3, CD4, CD8, CD27, CD28, CD56, CD62L, and CCR7 were measured by Flow cytometry with antibodies from BD Biosciences (San Jose, CA) using a FACSCanto II Flow Cytometer (BD Biosciences). Significant differences between populations were calculated with the student's t-test with a two tailed $P < 0.05$ considered significant.

Telomere Lengths of TIL in Culture

To determine the relationship between telomere length and time in culture, we measured telomere lengths of TIL cultures generated from 48 consecutive patients by quantitative fluorescent in-situ hybridization (QFISH)²⁰. Briefly, cells were incubated in a solution containing FITC-labeled (CCCTAA)₃ telomere probes (Applied Biosystems, Foster City, CA) and washed. Intensity of telomere binding was measured using a FACSCalibur (BD Biosciences, San Jose, CA). Fluorescent units were converted to physical lengths using a standard curve generated from standard samples. Linear correlation was calculated with Pearson's correlation using Excel.

Results

Melanoma TIL cultures often exhibit multiple antigen specificities

When multiple independent TIL cultures are generated from a single melanoma tumor and screened for tumor recognition they often exhibit multiple patterns of reactivity. A representative example from an HLA-A2+ patient is shown in Table 1. In this example, six independent TIL cultures were generated from tumor fragments (F1-F6) and four independent TIL cultures were generated from enzymatic single cell digests (D1-D4) of one melanoma tumor. All cultures were the same age and were evaluated in the same coculture assay. When stimulated with HLA-A2+ and HLA-negative melanoma lines, five of the ten independent TIL cultures (50%) had specific reactivity by objective criteria (IFN γ release that was greater than 200 pg/ml and more than twice the highest HLA-mismatched control). No autologous tumor

cell line was available from this patient, but when the TIL cultures were stimulated with uncultured autologous tumor cells and HLA-mismatched tumor cell controls, seven of ten independent TIL cultures (70%) had specific reactivity. Some TIL cultures recognized one source of tumor antigen but not the other source, suggesting the presence of multiple antigen reactive lymphocyte populations in these TIL cultures.

Identification of active TIL depends on the availability of appropriate target tumor cells

Some TIL cultures do not specifically recognize HLA-matched melanoma cell lines, but do recognize autologous tumor cells (such as F3, D2, and D4 in Table 1). A dramatic example of how the availability of appropriate antigen source can influence the clinical use of TIL cultures is demonstrated in Figure 1. The initial results from a cytokine release assay for several TIL cultures from a patient with a large, inoperable scalp lesion (Figure 1B) and multiple lung metastases showed no reactivity against HLA-matched tumor lines as targets. In this assay no autologous tumor cells were available. Since no reactive TIL were available, the patient was discharged from the protocol and the TIL cultures were cryopreserved. After several additional weeks, an autologous tumor cell line was established. When the TIL were thawed and the autologous tumor was used as a target, it revealed significant tumor specific recognition by several cultures, including TIL F7 (Figure 1A). The patient was contacted and consented to receive the F7 TIL cells with high dose IL-2 following a non-myeloablative lymphodepleting preparative chemotherapy on the experimental clinical protocol⁵. He experienced a dramatic regression of tumor at all sites and has an ongoing objective response now more than three years after initial TIL treatment (Figure 1C). Without the appropriate autologous tumor target cells, the F7 TIL culture could not have been used for treatment in this protocol, which requires evidence of tumor recognition as part of the certificate of release for the cell product.

To quantify the potential clinical impact of autologous tumor target availability, we undertook a retrospective analysis of sequential melanoma samples received between October 2006 and August (Table 2). During this ten month period, 142 tumors were processed from 83 patients. TIL failed to grow from 26 tumors, resulting in 11 patients who had no TIL to screen for tumor reactivity. TIL cultures were successfully screened by coculture assay and IFN γ ELISA from one or more independent TIL culture from the remaining 116 tumors from 72 patients, including 49 HLA-A2 patients. Forty tumors (26%) from 30 different patients (36%) exhibited specific recognition of HLA-matched melanoma tumor lines, including 24 HLA-A2+ patients and six HLA-A2 negative patients. Additional specific tumor recognition was revealed for 21 tumors (15%) from 11 patients (13%) by using autologous tumor cells as stimulator cells in cytokine release assays. These data emphasize that tests for in vitro reactivity may underestimate the number of tumor-reactive TIL cultures unless ideal tumor target cells are available. Based in part on these results, we initiated an investigation of alternative strategies for the production of TIL cultures for use in ACT trials, including the use of early TIL cultures with untested antigen reactivities.

TIL Cultures Evolve Tumor Antigen Specificity Early During Expansion

The kinetics of the development of tumor reactivity in TIL cultures was investigated by testing individual cultures derived from a single tumor for activity and specificity at two times during culture progression. TIL were initially tested at the earliest time after all tumor cells were lysed, when lymphocytes had recently become confluent in their initial wells. TIL cultures were tested a second time approximately 13 days later after each culture had been passaged and expanded extensively in vitro. Eighteen of 34 consecutive tumor specimens received in the Cell Processing Facility Between May and October, 2006, were sufficiently large to establish both young TIL and standard TIL cultures from dissected tumor fragments (Table 3). Specificity testing by IFN- γ release assay demonstrated that seven young TIL from fragments had specific reactivity (39%) and seven standard TIL from fragments also had specific reactivity (39%).

Six tumor specimens (33%) had specific reactivity for both young and standard TIL. At the time of testing for specificity, young TIL had a mean age of 12 days and standard TIL had a mean age of 25 days.

Sixteen out of 34 consecutive tumors had enough tissue to generate both standard and young TIL by the enzymatic digestion method (Table 4). The same trend of reactivity observed for tumor fragments was seen for enzymatic digests of tumor. Testing by IFN- γ release assay demonstrated that seven young TIL (44%) had specific reactivity and six standard TIL (38%) had specific reactivity. Five specimens (31%) had specific reactivity for both young and standard TIL.

Tumor Reactivity of Young TIL is Persistent during large numerical expansions

To test the stability of the tumor recognition during large numerical expansions, four young TIL with autologous tumor reactivity (Table 5) were identified. The TIL were expanded to approximately 2×10^6 cells at which time the mean age of these TIL was 12 days. The TIL cultures were then aliquoted into three groups: The first group was immediately cryopreserved. The second group was slowly expanded in culture as standard TIL (without further antigen stimulation) by adding media and splitting wells as needed. The third group was rapidly expanded. The second and third group cells were cryopreserved on the same day, then all groups were thawed at once and tested for tumor recognition by cytokine release assay. The cultures that were expanded as standard TIL exhibited an average of 19 fold growth over the 14 day assay period. Three of the four cultures retained specific recognition of autologous tumor after standard expansion. The rapidly expanded TIL increased in number by an average of 3116 fold over the same 14-day period. The three TIL cultures which showed specificity with standard expansion also demonstrated specificity after rapid expansion (Table 5).

Young TIL cultures have a different lymphocyte composition than standard TIL

To investigate the cellular composition of TIL cultures over time, aliquots of 14 TIL cultures were sampled and cryopreserved as soon as the lymphocytes became confluent and tumor cells were eliminated from wells. Then cultures were maintained by standard methods for 14 additional days and another sample was cryopreserved. Then both samples were thawed and analyzed by FACS simultaneously. Analysis of lymphocyte subsets in the TIL demonstrated that the young TIL had a higher frequency of CD4+ lymphocytes than standard TIL (Figure 2A, $p=0.02$). However, there were no significant differences in the frequency of CD8+ cells between young and standard TIL (Figure 2B). The frequency of CD3-CD56+ natural killer (NK) cells was lower in young TIL ($p = 0.02$, Figure 2C).

Young TIL Express High Levels of Co-stimulatory Molecules and Have Long Telomeres

FACS analysis of gated CD8+ cells from the TIL populations demonstrated a strong relationship between culture age and the expression of CD27 and CD28 (Figure 3). Strikingly, the percentage of CD8+ cells co-expressing CD27 and CD28 was higher in all 14 samples of younger TIL than standard TIL. There were no significant differences in the expression of CD62L and CCR7 by young and standard TIL (data not shown).

To investigate the impact of culture time on the telomere lengths of the TIL, 495 independent TIL cultures from 48 consecutive specimens were tested (Figure 4). Although the telomere lengths varied widely at any given TIL age, there was an inverse correlation between time in culture and the mean telomere length of TIL ($p < 0.001$).

Discussion

Adoptive cell therapy with tumor reactive TIL can be an effective therapy for eligible patients with metastatic melanoma. TIL transfer in ACT clinical trials exhibit a high objective response rate not achieved with the administration of melanoma reactive peripheral blood lymphocytes or PBL clones^{13-15;25;26}. TIL cultures from tumor histologies other than melanoma have exhibited only rare or sporadic examples of antigen specificity in vitro^{27;28}. Understanding the unique features of melanoma tumors that enable TIL cultures to be propagated in vitro and used effectively as therapeutic agents in vivo has implications for a wide range of immunotherapy approaches. However, the logistical and technical barriers to growing TIL cultures have prevented the widespread application of this therapy.

One of the regulatory requirements for TIL therapy in recent clinical practice has been the demonstration of tumor reactivity of the transferred cells. Tumor antigen recognition is central to the tumor rejection paradigm; however, a systematic review of our data suggested that the available in vitro assays were underreporting tumor recognition by melanoma TIL cultures. We observed that 50% of resected melanoma tumors at the Surgery Branch from May 2004 to May 2006 generated tumor-reactive TIL. This figure is consistent with our experiences at the NCI over the past five years. However, this figure is likely to be an underestimate because measures of tumor-reactivity depend on the availability and quality of appropriate tumor targets. We published previously that for 27 tumors that had autologous tumor cell lines available for screening, 21 (78%) generated TIL with detectable tumor specificity, a much higher proportion than detected in clinical studies where autologous tumor cell lines are not routinely available.

These theoretical considerations motivated us to investigate several features of the development and propagation of TIL cultures in vitro. We initially investigated the origin and stability of anti-tumor reactivity in TIL cultures. We hypothesized that most young TIL cultures would start with low specific anti-tumor activity due to a low proportion of antigen-reactive cells in the initial TIL population. In this model, older TIL cultures would have more robust anti-tumor reactivity due to clonal expansion of tumor reactive cells in vitro. Our results with IFN γ release do not support this model. Instead, we found that the frequency of tumor reactive young TIL cultures was similar to that of older TIL cultures. We observed that 41% of 18 consecutive melanoma tumors generated young TIL with specific reactivity while 38% of the same tumors generated standard TIL with specific reactivity. These results are more consistent with a large proportion of antigen-reactive lymphocytes in TIL that may be anergic in situ, but can rapidly establish tumor reactivity in vitro.

A consistently higher frequency of CD4 cells was present in minimally cultured TIL than in aged, antigen-selected TIL cultures. The antigen specificity and clinical significance of these CD4 cells for ACT in patients is not known, and this issue is further complicated by lack of knowledge about the expression of HLA Class II molecules on melanoma in situ. However, it is intriguing to consider that CD4 enhancement of CD8 recall immune responses are well documented^{29;30}. If CD4 cells in young TIL exhibit significant tumor recognition in parallel to CD8 cells, then the transfer of increased numbers of CD4 cells could sustain the tumor reactive CD8 cells' persistence in vivo and improve patient responses. Further characterization of these CD4 TIL populations may be warranted.

Comparison of the CD8+ cells from minimally cultured TIL and aged (selected) TIL revealed significantly higher expressions of CD27 and CD28 by the younger cells. Previous studies in our laboratory linked the expression of these costimulatory receptors with persistence and survival of individual TIL clones after transfer in melanoma patients who responded to ACT treatment²¹, and with TIL populations that mediated objective clinical responses³¹. We also

reported a strong correlation between telomere length and time in culture for melanoma TIL. These experiments allow a prediction of an estimated loss in telomere length of 0.8 kb per week for TIL in culture. Taken together, these studies predict that the young TIL method would result in a lymphocyte population for infusion with telomeres that are about 1.4kb longer than TIL currently administered, as well as higher expression levels of CD27 and CD28.

Prior clinical approaches have optimized the growth of TIL to obtain tumor reactive cultures, and subjugated other potentially significant features of TIL efficacy. The experiments reported here question the assumption that antigen selection in vitro is a requirement for producing highly antigen reactive TIL cultures from melanoma. We show that many TIL have a high proportion of tumor reactive lymphocytes after minimum in vitro culture, and these are suitable for rapid expansion and clinical use. This approach optimizes the speed and simplicity of TIL generation and minimizes the in vitro expansion of TIL cultures. These minimally cultured TIL have features associated with in vivo persistence, proliferation, and antitumor activity, including a high proportion of CD4 cells, long telomere length and high expression of CD27 and CD28.

Based on observations reported in this paper, we have initiated a clinical trial (NCI-007-C-0176) to examine the safety and efficacy of administering young TIL following a non-myeloablative lymphodepletion in the treatment of patients with metastatic melanoma. Accrual to prior clinical trials was complicated by the long lead times required to establish, test and expand TIL cultures (4-6 weeks), and because only about 50% of TIL demonstrated tumor reactivity. In contrast, it is anticipated that most patients enrolled on a young TIL protocol will be treated, since bulk TIL are readily generated from most melanoma tumors, and the time to generation of a TIL product is considerably shortened. These simplifications and improvements may change the qualities of the administered cells and improve the impact of TIL in ACT trials. Additionally, the simplified approach to generating TIL may accelerate the use of this therapeutic approach in clinical practice.

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Figure 1.

In vitro assessment of TIL 2427 initially failed to demonstrate recognition of shared tumor antigens, but eventually revealed significant recognition of autologous tumor targets. A) IFN γ ELISA results (pg/ml) after overnight coculture of TIL and tumor targets. Each melanoma cell line is shown above its HLA-A loci. AK1700-3 and JKF6 are “control” TIL with known specificities. F1, F2, F3, F4, and F7 are independent TIL derived from tumor 2427. Values that are twice background and >200pg/ml are indicated in bold font. B) Inoperable scalp lesion of patient 2427 prior to ACT treatment. C) Scalp area of patient 2427 after two courses of ACT treatment consisting of non-myeloablative chemotherapy, TIL 2427 F7 and high dose IL-2 therapy.

Figure 2.

Composition of TIL cultures changed significantly over time. Each line connects the frequency of marker expression for young TIL (left) with the frequency of marker expression for standard TIL (right) generated from the same tumor. Filled circles represent TIL generated from enzymatic digests of tumor and open squares represent TIL from tumor fragments. Mean age was 15 days for young TIL and 31 days for standard TIL. A. Young TIL contained significantly more CD4+ cells than standard TIL. B. There were no significant differences in CD8+ cells between young and standard cells. C. Young TIL contained significantly more CD3-CD56+ cells than standard TIL.

Figure 3.

The phenotype of CD8+ lymphocytes changed over time as TIL were maintained in culture. Each line connects the frequency of marker expression for young TIL (left) with the frequency of marker expression for standard TIL (right). Filled circles represent TIL generated from enzymatic digests of tumor and open squares represent TIL from tumor fragments. Young TIL were in culture for a mean of 15 days while standard TIL were in culture for a mean of 31 days. A. CD8+ young TIL expressed significantly more CD27 than standard TIL. B. CD8+ young TIL expressed significantly more CD28 than standard TIL. C. CD8+ young TIL expressed significantly more CD27 and CD28 than standard TIL.

Figure 4.

Telomere length decreased over time as TIL were maintained in culture. Telomere lengths of 495 TIL from 48 consecutive patients were evaluated by quantitative fluorescent in-situ hybridization. Mean telomere lengths calculated from 10 or more independent samples are indicated by large circles, and from less than 10 samples are indicated by small circles. Error bars indicate standard errors. The trend line was calculated from all 495 individual data points.

Table 1
Independent TIL cultures from a single tumor demonstrated diverse patterns of tumor recognition.

TIL* A2+	Melanoma Cell Line				Fresh Tumor		
	888 A2-	938 A2-	526 A2+	624 A2+	Reactivity [†]	IFNg (pg/ml)	Autologous Reactivity
F1	49	63	73	120	-	73	377
F2	83	69	1470	1676	+	63	360
F3	40	62	71	95	-	55	45
F4	51	44	122	230	+	81	268
F5	35	35	84	248	+	48	94
F6	13	16	19	22	-	16	162
D1	66	44	394	395	+	175	3340
D2	77	52	140	140	-	257	5380
D3	280	224	1561	1420	+	528	4870
D4	168	145	200	269	-	350	3110
Total positive (%)					5 (50)		7 (70)

* Results for ten independent TIL cultures derived from a single tumor from an HLA-A*2+ patient are shown. Six cultures prefixed by an "F" were derived from tumor fragments and four cultures prefixed by a "D" were derived from enzymatically-prepared digests.

[†] Bold numbers indicate a positive test defined as at least 200 pg/mL and greater than twice IFN- γ released by coculture with HLA-mismatched controls. The TIL culture was considered reactive if either HLA-matched target was positive.

Table 2

Detection of active TIL cultures depends on the availability of HLA-matched and autologous tumor cell targets.

	Patients (% of total)	Tumors (% of total)
Tissue received in Cell Processing Facility ¹	83 (100%)	142 (100%)
One or more independent TIL culture growth to > 10 million cells ²	72 (87%)	116 (82%)
Shared melanoma Ag recognition (HLA matched tumor cell line recognition ³)	30 (36%)	40 (26%)
Unique Ag recognition (autologous cryopreserved tumor recognition)	11 (13%)	21 (15%)
Total TIL available for treatment	41 (49%)	61 (43%)

¹ From October 2006 to August 2007 all consecutive melanoma tissue received with intent to generate TIL for possible patient infusion.

² Growth positive TIL was considered 10 million cells from any single independent culture. Typically 20 to 50 million cells were obtained in 3 to 4 weeks.

³ Tumor recognition was defined by IFNg release assay with specific release being greater than 200 pg/ml and twice background.

Table 3
TIL from fragments: tumor reactive cells were detected at equal frequencies in young TIL cultures and standard TIL cultures generated from TIL fragments

Specimen ID [*]	Young		Standard	
	Setup [†]	Reactive(%) [‡]	Age [§]	Age
4	12	10 (83)	12	27
5	24	21 (88)	17	26
6	28	27 (96)	14	25
8	24	0 (0)	10	27
9	32	32 (100)	7	14
10	24	1 (4)	18	NG [¶]
13	16	2 (13)	10	21
16	24	0 (0)	17	33
18	16	0 (0)	14	28
22	12	0 (0)	7	21
23	12	9 (75)	7	27
24	8	2 (25)	14	20
25	12	0 (0)	12	27
34	12	12 (100)	12	26
Mean age	Total positive (%)		12	25
	7 (39)			7 (39)

^{*} Eighteen out of 34 consecutive specimens had tumor fragments that were used to generate young and standard TIL. Four specimens did not expand and are not listed.

[†] Number of fragments set up for TIL from each tumor specimen.

[‡] Number (and percent) of TIL which expanded and had specific reactivity. Specimens which had 25% or more TIL cultures with specific reactivity as defined by at least 200 pg/mL and greater than twice IFN- γ released by coculture with HLA-mismatched controls were considered positive (bold).

[§] Age (days) at which TIL were tested for reactivity.

[¶] TIL did not expand adequately for screening.

^{||} Total number and frequency of positive specimens for all 18 specimens.

Table 4

TIL from single cell enzymatic digests: tumor reactive cells were detected at equal frequencies in young TIL cultures and standard TIL cultures generated from TIL digests

Specimen ID *	Young			Standard
	Setup [†]	Reactive(%) [‡]	Age [§]	
1	96	18(19)	14	NG [¶]
5	96	72(75)	17	26
6	24	19(79)	16	3(100)
8	24	0(0)	9	24
9	48	41(85)	9	46
10	48	1(2)	4	0(0)
16	48	0(0)	11	0(0)
18	48	0(0)	16	25
22	24	13(54)	13	0(0)
23	24	15(63)	13	NG
24	48	48(100)	13	27
32	24	24(100)	12	20
Mean age			13	26
Total positive (%)				6(38)

* Sixteen out of consecutive 34 specimens had enzymatic digests that were used to generate young and standard TTL. Four specimens did not expand and are not listed.

[†]Number of single-cell suspensions set up for TIL from each tumor specimen.

‡. Number and percent of TTL which expanded and had specific reactivity. Specimens which had 25% or more TTL cultures with specific reactivity as defined by at least 200 pg/mL and greater than twice the N-γ released by coculture with HLA-mismatched controls were considered positive (bold).

§ Age (days) at which TIL were tested for reactivity.

TIL did not expand adequately for screening.

¹Total number and frequency of positive specimens for all 16 specimens.

Table 5
Tumor reactivity of young TIL was maintained during in vitro expansion

Specimen	Young TIL*		Standard Expansion†		Rapid Expansion‡	
	Autol§	Contr¶	Autol	Contr	Autol	Contr
1	381	31	1448	111	1634	650
2	565	107	983	160	1053	523
3	103	0	201	33	1599	513
4	691	116	96	52	424	1021
						1733

* TIL generated from four enzymatically digested tumor samples were tested for specific reactivity when each culture expanded to 2×10^6 lymphocytes (mean = 12 days).

† Young TIL were expanded using standard methods for 14 more days and then tested.

‡ Young TIL were rapidly expanded for 14 more days and tested.

§ Values indicate IFN- γ release (pg/mL) when cocultured with autologous tumor. Bold numbers indicate a tumor-specific reactive test defined as at least 100 pg/mL and greater than twice IFN- γ released by coculture with HLA-mismatched controls.

¶ Values indicate IFN- γ release (pg/mL) when cocultured with HLA-mismatched control tumors.