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Generation of Tumor-Infiltrating Lymphocyte Cultures for Use in Adoptive Transfer Therapy for Melanoma Patients

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Summary

The generation of T lymphocytes with specific reactivity against tumor antigens is a prerequisite for effective adoptive transfer therapies. Melanoma-specific lymphocyte cultures can be established from tumor infiltrating lymphocytes (TILs) by in vitro culture in high levels of IL-2. We have optimized methods for generating melanoma-reactive TIL cultures from small resected tumor specimens. We report a retrospective analysis of 860 attempted TIL cultures from 90 sequential melanoma biopsy specimens from 62 HLA-A2^{+} patients. Multiple independent TIL derived from a single tumor often exhibited substantial functional and phenotypic variation. Tumor specific activity was detected in TIL from 29 (81%) of 36 patients screened. TIL cultures selected for high activity were generally capable of large numerical expansion using a single round of a rapid expansion protocol. Limited clonal T-cell populations in an oligoclonal TIL culture could confer specific tumor recognition in these highly selected, highly expanded TIL cultures. These methods were efficient at generating TILs suitable for adoptive transfer therapy.

Keywords

adoptive cell transfer; tumor-infiltrating lymphocyte; melanoma; tumor antigen; immunotherapy

Immunotherapy for the treatment of patients with cancer relies on the generation of lymphocytes that specifically recognize tumor cells and eliminate them in vivo. Some active vaccination strategies have been successful in increasing the number of tumor antigen reactive lymphocytes circulating in patients' peripheral blood, but only sporadic objective clinical responses have been reported following such vaccinations. Adoptive cell transfer (ACT) therapy is a treatment strategy that allows the activation and expansion of tumor-reactive T cells ex vivo for subsequent infusion back into the autologous host. ACT therapy theoretically allows for the selection of the most highly avid antitumor lymphocytes, their expansion in the absence of endogenous regulatory mechanisms, and the manipulation of the host immune environment in their absence. Although recent reports have documented the potential clinical efficacy of ACT,[1,2] the generation of large numbers of highly active anti-tumor T cells is a significant technical challenge and remains a serious impediment to the wider use of T-cell transfer as a standard cancer therapy.

In one study,[2] T-cell populations derived from tumor infiltrating lymphocytes (TILs) were reported to induce objective clinical responses in ACT therapy for melanoma patients. TIL cultures for adoptive transfer were initiated with a large number of cells (>1 × 10^{8} TIL) and

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expanded in a high concentration of IL-2 until the target cell number for transfer was reached. In a series of 86 patients treated with TIL generated by this method in combination with high-dose IL-2 therapy, 29 patients exhibited objective clinical responses although many were of short duration. In that clinical trial, TIL were administered based solely on their expansion to a target cell number, irrespective of their tumor recognition. Subsequent attempts to improve on ACT for melanoma patients have focused on more reliable methods of generating cultures that specifically recognize tumor cells.

One method that has been vigorously pursued is the use of T-cell clones from patients' peripheral blood. Transfer of T-cell clones was highly successful for prophylaxis of CMV- and EBV- associated diseases in the posttransplantation setting.[3,4] However, this approach has proven largely ineffective as a treatment of patients with metastatic melanoma. Although large numbers of highly avid, tumor-reactive cloned T cells were administered to patients alone, with IL-2, or following conditioning chemotherapy with or without IL-2, no objective clinical responses were observed.[5-7] This apparent lack of clinical efficacy of cloned lymphocytes could be the result of erosion of their proliferative potential by the repetitive stimulations needed to achieve target cell numbers for transfer. Alternately, effective ACT therapy could require the administration of heterogeneous cell populations that are eliminated by the cloning protocol.

We have investigated alternate strategies for generating TIL cultures suitable for ACT of melanoma patients. In this report we detail our attempts to optimize methods for generating, selecting, and expanding TILs to large numbers. We observed that the independent initiation and expansion of multiple cultures from even a small melanoma specimen enhanced the frequency of generating tumor-reactive cultures. Increase in cell numbers to therapeutic levels was readily achieved using a single round of a rapid expansion protocol. A single clonal population within an oligoclonal TIL often conferred the tumor specificity of such expanded lymphocyte cultures. These methods often led to the generation of multiple different antigen specificities from independently derived TIL from a single lesion, which were then combined for patient administration. In a previous report using these highly selected TIL in combination with non-myeloablative chemotherapy and high-dose IL-2 therapy to treat patients with metastatic melanoma,[1] we observed 6 objective responses in 13 patients treated, and 4 additional patients who demonstrated mixed or minor responses.

MATERIALS AND METHODS

Biologic Materials Derived from Patient and Normal Donors

Patients were entered into an Institutional Review Board–approved clinical protocol and signed an informed consent prior to initiation of lymphocyte cultures for possible therapeutic use. The results from all HLA-$A2^+$ patients between January 2001 and August 2002 who underwent an initial excisional biopsy with the intent to generate TIL for adoptive transfer therapy are included in this report. Some of these patients underwent subsequent resections to obtain materials for additional TIL generation or protocol analysis, but those results are not included in this report. Tumor specimens were excised aseptically, and tissue was processed under "good laboratory practice" conditions. All resected specimens were sampled for pathologic confirmation of the diagnosis of metastatic melanoma. "Feeder" lymphocytes were obtained by apheresis of normal donors in the Surgery Branch apheresis unit. All donors were required to undergo testing for infection with common blood-borne pathogens and viruses including RPR, HIV, LCMV, and HVC. Apheresis specimens from normal donors were purified on Ficoll-Hypaque step gradients (LSM Lymphocyte Separation Medium, ICN Biochemicals Inc., Aurora, OH) and cryopreserved. Human AB serum was purchased from several commercial sources (Valley Biomedical, Winchester VA; Gemini Bioproducts, Woodland, CA) after screening for optimal performance to promote the growth of lymphocyte clones.

Human AB or A serum was also collected from blood donated to the Department of Transfusion Medicine, National Institutes of Health.

Initiation and Expansion of TIL Microcultures from Tumor Fragments

Each tumor specimen was dissected free of surrounding normal tissue and necrotic areas. Small chunks of tumor (usually 8–16) measuring about 1 to 2 mm in each dimension were cut with a sharp scalpel from different areas around the tumor specimen. A single tumor fragment was placed in each well of a 24-well tissue culture plate with 2 mL of complete medium (CM) plus 6000 IU per mL of rhIL-2 (Chiron Corp., Emeryville, CA). CM consisted of RPMI 1640, 25 mmol/L HEPES pH 7.2, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L-glutamine, and 5.5×10^{-5} mol/L β-mercaptoethanol, supplemented with 10% human serum. The plates were placed in a humidified 37°C incubator with 5% CO_2 and cultured until lymphocyte growth was evident. Each fragment was inspected about every other day using a low-power inverted microscope to monitor the extrusion and proliferation of lymphocytes. Whether or not lymphocyte growth was visible, half of the medium was replaced in all wells no later than 1 week after culture initiation. Typically, about 1 to 2 weeks after culture initiation, a dense lymphocytic carpet would cover a portion of the plate surrounding each fragment. When any well became almost confluent, the contents were mixed vigorously, split into two daughter wells and filled to 2 mL per well with CM plus 6000 IU/mL IL-2. Subsequently, the cultures were split to maintain a cell density of $0.8–1.6 \times 10^6$ cells/mL, or half of the media was replaced at least twice weekly. Each initial well was considered to be an independent TIL culture and maintained separately from the others.

TIL Cultures Derived from Single-Cell Digests

The generation of bulk TIL cultures by enzymatic digestion of tumor explants has been described in detail.[2] Briefly, each solid tumor specimen was carefully dissected free of surrounding normal tissue and necrotic areas. The tumor was sliced with a sharp scalpel into small pieces (approximately 2mm on each side). The tumor fragments were immersed in a mixture of collagenase, hyaluronidase, and DNAse in serum-free RPMI 1640, and incubated overnight with gentle agitation. The single-cell slurry was passed through sterile wire mesh to remove undigested tissue chunks. The digested single-cell suspensions were washed twice in HBSS, viable cells were purified on a single step Ficoll gradient, and cells were resuspended for plating. Multiple wells of a 24-well plate were seeded with 1×10^6 viable cells in 2 mL CM with 6000 IU/mL IL-2. The plates were placed in a humidified 37°C incubator with 5% CO_2. Whether or not lymphocyte growth was visible, half of the medium was replaced in all wells no later than 1 week after culture initiation. When any well became nearly confluent, the contents were mixed vigorously, split into two daughter wells, and filled to 2 mL per well with CM plus 6000 IU/mL IL-2. Subsequently, half the media was replaced at least twice weekly, or the cultures were split to maintain a cell density of 0.8 to 1.6×10^6 cells/mL. Some of the TIL from digests were derived from multiple original wells that were regularly mixed and eventually pooled for assessment of activity. Other TIL from digests were derived from individual wells of a 24-well plate. For these cultures, all progeny cells from any individual well were treated as an independent TIL culture and were maintained separately from the descendants of any other original well. In this way, multiple cultures were obtained from the same initial single-cell suspension.

TIL Cultures and Tumor Cell Lines Derived by Physical Disaggregation of Tumor Samples

Some TIL were derived by a method of physical disaggregation of melanoma fragments using a device called a Medimachine (Becton Dickenson) with 50 μm “medicon” chambers, which are mini sterile and disposable homogenizers. Fragments of tumor about 2 mm per side were prepared by dissection of biopsy specimens free from normal and necrotic tissue. Several

fragments at a time were physically disaggregated by a 30-second Medimachine treatment, which disaggregated the tumor chunks using mechanical shear provided by a rotating disk that forced the tumor chunks across a small grater inside the medicon. The resulting slurry of single cells and small cell aggregates was washed once, and resuspended in CM. The cell suspension was layered onto a two-step gradient with a lower step of 100% Ficoll, and a middle step of 75% Ficoll and 25% CM. After 20 minutes' centrifugation at 2000 rpm (about 1100*g*), the interfaces were collected. The lower interface containing the lymphocyte-enriched fraction was processed separately from the upper interface containing the melanoma-enriched cells. Each fraction was washed twice. The lower, TIL-enriched fraction was plated in 24-well plates, and individual TIL cultures were generated exactly as for the single-cell suspensions derived by enzymatic degradation. The upper, tumor-cell-enriched fraction was plated at approximately 2×10^5 cells/mL in RPMI-based media containing 10% "defined" fetal calf serum (Hyclone, Logan, UT) without IL-2. In our experience, the Medimachine method was highly efficient at generating tumor cell lines, and about 50% of tumors processed by this approach were successfully converted to long-term cell lines (unpublished results).

Cytokine Release Assays

TIL activity and specificity were determined by analysis of cytokine secretion. TIL and control T-cell lines were washed twice prior to coculture assay to remove IL-2. TIL cells (1×10^5) were plated per well of a 96-well flat-bottom plate with 1×10^5 stimulator cells. TIL cultures were generally stimulated with at least two HLA-$A2^-$ melanoma cell lines (888mel and 938mel) and at least two HLA-$A2^+$ tumor cell lines (526mel and 624mel). When available, TIL were also stimulated with an autologous tumor cell line or a thawed aliquot of cryopreserved single-cell tumor digests ("fresh tumor"). For some TIL, the TAP-deficient T2 cell line was pulsed with melanoma antigen peptides including MART-1:27-35 (referred to as MART) or gp100:209-217 (referred to as g209). After overnight coculture, supernatants were harvested and IFN-γ secretion was quantified by ELISA (Pierce/Endogen, Woburn, MA). All cytokine release assays were routinely controlled with the JB2F4 T-cell clone specific for the MART-1:27-35 antigen and the CK3H6 T cell clone specific for the gp100:209-217 antigen.

Rapid Expansion Protocol and Preparation of Cells for Infusion

Active TIL cultures were expanded to treatment levels using a rapid expansion protocol (REP) as previously described.[3,8] Briefly, the REP used OKT3 (anti-CD3) antibody (Ortho Biotech, Bridgewater, NJ) and IL-2 in the presence of irradiated, allogeneic feeder cells at a 200:1 ratio of feeder cells to responding TIL cells. PBMC feeder cells obtained from normal volunteers by apheresis were thawed, washed, resuspended in CM, and irradiated (50 Gy). PBMC (2×10^8), OKT3 antibody (30 ng/mL), CM (75 mL), AIM V media (GIBCO/BRL, 75mL), and TIL effector cells (1×10^6) were combined, mixed, and aliquoted to a 175 cm^2 tissue culture flask. Flasks were incubated upright at 37°C in 5% CO_2. IL-2 was added to 6000 IU/mL on day 2. On day 5, 120 mL of culture supernatant was removed by aspiration (cells are retained on the bottom of the flask) and media was replaced with a 1:1 mixture of CM/AIM V containing 6000 IU/mL IL-2. On day 6 and every day thereafter, cell concentration was determined and cells were split into additional flasks or transferred to Baxter 3-L culture bags with additional medium containing 6000 CU/mL IL-2 as needed to maintain cell densities around 1×10^6 cells/mL. About 14 days after initiation of the REP, cells were harvested from culture bags and prepared for patient treatment. Harvesting was accomplished using a Baxter/Fenwal continuous centrifuge cell harvester system. The cells were then washed in 0.9% sodium chloride and resuspended in 45 to 150 mL of 0.9% sodium chloride with 2.5% human albumin. Samples were removed from the infusion product for QC testing, aliquots were cryopreserved for future experimental analysis, and the remaining cells were infused into the patient by intravenous administration over 30 minutes.

TCR CDR3 Size Pattern Analysis

The T-cell receptor (TCR) complementarity determining region (CDR)3 of TIL and PBMC was investigated using the Immunoscope approach as previously described.[9] Briefly, total RNA was extracted from pretreatment PBMC or TIL using RNAeasy columns (Qiagen, Valencia, CA), and reverse transcribed into cDNA using oligo-dT primers and AMV (Roche, Mannheim, Germany). cDNA was amplified using BV and BC specific primers, and the unlabeled PCR product was copied in 5-cycle run-off reactions using a nested fluorescent BC primer. Aliquots were analyzed on an Applied Biosystems 377 DNA sequencer and size patterns obtained with the aid of the Immunoscope software.[9]

FACS Analysis

T cells were washed and resuspended at 1×10^7 cells/mL in FACS buffer consisting of PBS + 5% fetal calf serum. Staining with anti-CD8 antibody and a panel of TCR Vb specific antibodies (Beckman/Coulter/Immunotech) or HLA-A2/MART-1:26-55(27L) iTAG tetramer complexes (Beckman/Coulter/Immunomics) was carried out according to the manufacturer's recommendations. Cells were washed twice in FACS buffer and analyzed using a FACSCaliber (BD Biosciences) with live/dead cell gating based on propidium iodide exclusion. FACS results were analyzed with Cellquest software (Becton Dickenson, San Jose, CA).

RESULTS

Multiple TIL Cultures Were Readily Established from Excisional Tumor Biopsies

Between January 2001 and August 2002, 90 melanoma biopsies from 62 consecutive HLA-$A2^+$ patients were received, and initiation of multiple independent TIL cultures was attempted from these specimens (Table 1). The lymphocytes in some cultures did not expand to sufficient cell numbers to merit functional assessment, and these cultures were considered to have "no growth." Cultures that on microscopic inspection exhibited elimination of tumor cells and growth of lymphocytes were expanded to several wells of a 24-well plate. Such cultures generally contained more than 5×10^6 lymphocytes, and they were either subjected to functional analysis directly, or cryopreserved for potential later analysis (depending on the clinical status of the patient from whom they were derived).

Fragments were set up from all 90 tumors. Of 710 individual fragment-derived cultures (range 4 to 24 cultures per patient), 496 (69.9%) expanded sufficiently to characterize their activity. Enzymatic digestion was performed on tumors from only 33 patients, resulting in the initiation of 119 individual cultures (range 1 to 8 per patient), of which 112 (94.1%) grew sufficiently to characterize. Medimachine preparations from 17 patients yielded 31 individual cultures (range: 1 to 6 cultures per patient), of which 28 (90.3%) grew. When analyzed by patient, evaluable cultures were obtained from tumor fragments in 59 of 62 patients (95%), from digests in 32 of 33 patients (97%) and from Medimachine in 14 of 17 patients (82%).

Different TIL Cultures Derived from a Single Tumor Specimen Often Demonstrated Different Predominant Antigenic Specificities

We investigated the activity and antigen specificity of TIL cultures by analyzing their specific cytokine secretion when stimulated with HLA-A2-matched tumor cell lines or with autologous tumor cells (when available). Multiple independent TILs from 36 patients were screened for activity (Table 1); these were derived from fragments (302 cultures) or enzymatic digestion (67 cultures). Only six patients (32 independent TIL from nine tumors) yielded cultures with no detectable specific activity against HLA-A2 matched or autologous tumor cells. Independent TIL cultures derived from a single tumor demonstrated qualitatively different patterns of antigen recognition in 25 of the 36 patients.

Tumor 2035, derived from a subcutaneous lesion resected from the thigh of a 36-year-old male patient, was an example of the diverse reactivities arising from a single tumor source. Multiple fragments from this resected tumor specimen were used to generate independent TIL cultures. At least four patterns of reactivity were observed (Table 2). TIL F8 exhibited no recognition of any tested target. TIL F6, DF3, and DF13 exhibited recognition all three HLA-A2$^+$ tumors tested. This activity was later determined to be mediated by T cells recognizing the HLA-A2-restricted MART-1:27-35 antigen.[10] TIL DF6 and DF8 recognized the HLA-A2$^+$ tumor cell lines 624mel and 1938mel but not the HLA-A2$^+$ 526mel or non-HLA-A2$^+$ melanoma cell lines. This pattern was later determined to be mediated by lymphocytes specific for an HLA-A2 restricted epitope derived from the NY-ESO-1 antigen[11], which is not expressed by the 526mel line. Finally, several lines recognized exclusively the autologous melanoma line 1938mel that was established from a previously resected melanoma lesion of the same patient. TIL DF3, DF6, DF8, and DF13 were subjected to a single round of rapid expansion for therapeutic infusion into the autologous patient, and maintained their diverse specificities after more than 1000-fold cell expansion.

A diversity of reactivities was similarly observed among multiple TIL derived from 5 different biopsies from a single patient. At least one TIL derived from all 5 tumors exhibited no specific cytokine release when stimulated by any of the targets. Four of the 5 biopsies produced at least 1 TIL that specifically recognized both of the HLA-A2$^+$ melanoma cell lines tested, mel526 and mel624 (Table 3). The absolute level of IFN-γ secretion on stimulation with melanoma cell lines varied widely among the TIL cultures. For this patient, the heterogeneity of activity exhibited by the TIL from any single lesion was as great as the heterogeneity in activity between fragments derived from different lesions. The three TIL lines with the highest activity (1931-2 F4, 1932-1 F5, and 1941 F1) were further expanded and used for adoptive transfer into the autologous patient.

These examples are representative of the diversity of antigen specificities that was often exhibited by independent TIL established from fragments of a resected melanoma lesion. Independent TIL derived from single cell suspensions of enzymatically digested tumor were also capable of exhibiting quantitatively diverse patterns of reactivity. Six independent TIL cultures were established from tumor 2009 and were assayed for tumor recognition by cytokine release (Table 4). All cultures exhibited qualitatively similar but quantitatively distinct patterns of recognition of different targets. The six independent TIL demonstrated more than 100-fold difference in IFN-γ secretion when stimulated by T2 cells pulsed with the MART-1:27-35 peptide epitope, 10-fold differences in recognition of HLA-A2$^+$ 624mel, and greater than 5-fold differences in recognition of the autologous 2009 tumor cells, thus suggesting that several different antigens were recognized by the different TIL cultures. Subsequent specificity analysis demonstrated recognition by some of these cultures of the HLA-A2 restricted TRP-2:180-188 antigen[12] (data not shown).

TIL Cultures Derived from a Single Tumor Specimen Were Comprised of Phenotypically Diverse Populations

The diversity of independent cultures derived from single lesions was further investigated using FACS analysis for expression of CD4 or CD8 by the cultured lymphocytes (Table 5). Multiple independent TIL cultures from tumor biopsy 1921 recognized HLA-A2$^+$ melanoma cell lines as assessed by cytokine secretion. However, these functionally similar cultures contained different CD4$^+$ T cells frequencies, ranging from 19% to 70% of the total cells. Additional active TIL cultures from this patient (biopsies 1941-1 and 1941-3) showed a similarly diverse proportion of CD4$^+$ and CD8$^+$ cells.

Generation of Multiple Independent TIL from Fragments Increased the Likelihood of Identifying Cultures that Recognized Shared HLA-A2 Restricted Antigens

Autologous tumor cells are not always available for screening TIL activity. Thus, a method for increasing the frequency of TILs that recognize shared HLA-A2 restricted melanoma antigens would be valuable. We compared TIL obtained from fragments with TIL obtained from digests for recognition of HLA-A2 matched allogeneic melanoma cells. The percentage of individual cultures that recognized HLA-A2-restricted shared antigens was similar between fragment derived TIL (87 of 302, 29%) and digest-derived TIL (16 of 67, 24%). Because more independent TIL cultures were typically generated from fragments for each patient than from digests, the fragments proved to be better on a per patient basis. Thus, for the group of 20 patients who had evaluable TIL from both fragments and digests, 10 of 20 patients produced at least one active fragment-derived TIL compared with 3 of 20 patients with at least one active TIL from digests. Thus, the number of independent cultures derived from each tumor may be more significant than the method of generating the cultures.

One Cycle of Rapid Expansion Generated Large TIL Cell Numbers With High Functional Activity Appropriate for Patient Treatment

When tumor-reactive TIL cultures were identified, they were expanded in complete media with 6000 IU/mL IL-2 until a cell number greater than 3×10^7 was reached. These lymphocyte cultures constituted the progenitor cell populations for patient treatments, and each TIL was expanded once using a rapid expansion protocol (REP) to generate large cell numbers for therapeutic administration. Between July 2001 and September 2002, 13 TIL cultures from 8 patients discussed above were expanded to treatment levels (Table 6). Starting with an average of 3.4×10^7 TIL preselected for high activity and diversity of antigen recognition, the cultures expanded during the 14-day protocol to an average of 4.1×10^{10} cells on the day of infusion. These cell numbers represent an average of 1320-fold expansion for each culture (range: 181–2623-fold) and correspond to 7 to 12 cell doublings in 14 days. All TIL cultures shown in Table 6 exhibited similar antigen specificity and activity before and after large numerical expansion by the REP.

Melanoma TIL Derived from Small Fragments that Are Rapidly Expanded May Comprise Only a Few Predominant Clones

Two of the TIL cultures expanded for treatment from TIL 1931-2 exhibited strong recognition of the HLA-A2-restricted MART-1 antigen (Table 3). The cellular basis of this antigen recognition was investigated with FACS analysis using HLA-A2/MART-1:26-35(27L) tetrameric complexes and a panel of TCR β-chain specific antibodies. Interestingly, both TIL contained a majority of $CD8^+$ cells that bound the A2/MART-1 tetramer (Fig. 1). The two MART-1 reactive TILs expressed different TCR Vβ chains; TIL F4 was composed predominantly of a $CD8^+$ Vβ12+ clone, whereas TIL F5 was predominantly a $CD8^+$ clone with expression of a different TCR (one that was not stained with the anti-Vβ12 antibody nor any antibody in the panel).

A similarly restricted TCR use was evident in the treatment TIL from another patient with MART-1-reactive cells. In this TIL, 89% of the $CD8^+$ cells expressed a Vβ3 TCR, and stained with HLA-A2/MART-1:26-35(27L) tetramer (not shown). However, minor populations expressing other TCR Vβ were identified in the TIL and each comprised 1% to 7% of the total $CD8^+$ cells. The TCR diversity within these populations was further assessed using TRBV and TRBC primers to generate fluorescent PCR products for CDR3 size pattern/Immunoscope analysis.[9,13] Representative results (Fig. 2) showed that in this TIL, each Vβ subfamily consisted of one or very few predominant clones, in contrast to the heterogeneous mixture of TCR β chains present in the patient's pretreatment PBL. Sequence analysis demonstrated that the BV3 subfamily (comprising 87% of the $CD8^+$ cells) consisted of a single clone (data not

shown). The antigen specificity (ie, tumor reactivity) of each of the minor clonal populations was not investigated, and more work is needed to determine whether each clone had a selective advantage during in vitro culture conferred by tumor reactivity, or simply represents a persistent "passenger" population.

DISCUSSION

The use of autologous T cells as a therapeutic modality is still in its infancy. Initial clinical results using adoptive transfer of TIL in combination with nonmyeloablative lymphodepletion for the treatment of patients with metastatic melanoma have shown some promise.[1] Adoptive cell transfer therapies depend on reliable and reproducible methods for the generation of tumor-reactive T lymphocytes. In this report, we summarize our experience generating tumor-reactive TIL from melanoma excisional biopsies, and present an improved protocol for establishing tumor-reactive cultures from small biopsy samples. This strategy involves the initiation of multiple independent TIL cultures, screening each TIL for antitumor activity, and rapid expansion of the most active cultures. This strategy proved to be a robust method for the generation of tumor-reactive TIL. By initiating multiple independent cultures from a single lesion, it was often possible to generate qualitatively and quantitatively different antigen-specific activities in different cultures. Subsequent growth of the most active cultures in IL-2-containing medium without additional TCR stimulation usually resulted in the expansion of the cells to moderate "pretreatment" cell numbers, that is, greater than 3×10^7 cells. These cultures were highly responsive to anti-CD3-mediated rapid expansion, and proliferated in a single expansion cycle to the target number for treatment while maintaining their antigen specificities. The culture manipulations from resection to cell administration could be completed in as little as 5 weeks, but typically required 6 to 8 weeks for generation of sufficient cells for patient administration. The resultant cultures could reach absolute cell numbers around 5×10^{10} cells, and often contained one or a small number of highly expanded clones that was responsible for the tumor recognition within an oligoclonal TIL population.

The observed diversity of independent TIL cultures derived from a single melanoma biopsy is an interesting and somewhat unexpected finding. Local heterogeneity of T cells could account for much of the variable activities observed among TIL derived from different fragments of a single tumor. Anecdotally, we also observed instances of different tumor nodules from a single patient that generated markedly different TIL populations. The existence of significant heterogeneity of T-cell precursors in different microenvironments within a single tumor nodule or in different nodules from a single patient is a reasonable explanation for these observations. However, a qualitatively similar variation in TIL culture activities was also observed among independent TIL cultures that were initiated from a homogeneous single-cell suspension of tumor. Any explanation of variability between these cultures requires hypothesizing an intrinsically unpredictable or sporadic advantage for individual cells at the time of culture initiation, resulting in the emergence of different oligoclonal populations from a homogeneous mix.

Although the underlying mechanism for the establishment of different antigen specificities among independently derived TIL is not fully understood, it has at least two practical benefits. First, the segregation of different functional activities between cultures allowed the frequent identification of an active TIL culture or multiple different TIL specificities suitable for additional rapid expansion and patient infusion. In this set of consecutive biopsies from HLA-$A2^+$ patients for potential ACT therapy, at least one reactive TIL was obtained from 29 of 36 patients (81%). Furthermore, 24 of the 36 patients screened (67%) generated independent TIL cultures with qualitatively different characteristics. As reported previously,[14] many TIL cultures derived from melanoma biopsies preferentially recognized their autologous tumors. We observed specific cytokine secretion stimulated by autologous tumor cells in at least 1 TIL

from 78% of patients (21 of 27) and 59% of 301 TIL cultures. Current efforts in our laboratory and others seek to identify the antigenic basis of this recognition, including the restricting MHC antigens and the molecular identification of the immunogenic peptide epitopes uniquely expressed by different tumors.

Second, the ability to identify and expand TIL cultures that recognize shared antigens is important to investigating combinations of ACT therapy and immunization strategies. Pursuing such strategies has been difficult because TIL cultures that originated from large pools of cells only rarely recognized shared tumor antigens.[14,15] For instance, in one report less than than 13% of TIL initiated from about 5×10^7 cells mediated lysis of MHC-matched tumor targets,[14] and in the currently study none of 11 TIL that were initiated with more than 1×10^6 cells exhibited specific cytokine secretion to HLA-A2-matched tumor cells. However, the strategy of initiating multiple cultures with a small number of cells enabled the frequent generation of TIL cultures with specific recognition of HLA-A2-restricted, shared epitopes, such as the MART-1:27-35 antigen. Thus, we report here that 28% of 370 individual TIL cultures exhibited HLA-$A2^+$ tumor recognition, and at least one TIL specifically recognized HLA-$A2^+$ tumors in 64% (23 of 36) of patients. Thus, for many patients it was apparently possible to separate a rapidly growing population of cells from a minor population of shared antigen-specific cells to generate shared antigen-reactive TIL cultures. Such cultures may be useful for clinical trials investigating potential synergies between antitumor immunization and ACT therapy.

Some of the cultures generated by the techniques described here were rapidly expanded and safely administered to patients. Importantly, some TIL proved to be clinically effective in mediating tumor regression in immunodepleted melanoma patients. This clinical efficacy contrasts with our previously reported results[5,6] and those of Yee et al.[7] demonstrating that cloned $CD8^+$ T cells are not capable of mediating objective clinical responses in melanoma patients. There are several differences between the cloned T cells grown for therapy and the TIL cultures described here that might account for the difference in their clinical efficacy. First, although TIL may be dominated by a few T-cell clones, they remain a heterogeneous mixture of cells and virtually all TIL cultures contained significant numbers of $CD4^+$ lymphocytes. The presence of this CD4 population may have important effects on the $CD8^+$ cells either in vitro during their expansion or in vivo during tumor regression. Alternately, cloned T cells must be stimulated through their T-cell receptor in repetitive rapid expansion cycles to achieve therapeutic numbers of cells. The effects of this repetitive stimulation on the proliferative potential and differentiation status of the clones are not well understood. These features of T-cell function are under investigation, and their resolution may augment the efficacy and patient range of adoptive cell transfer therapy.

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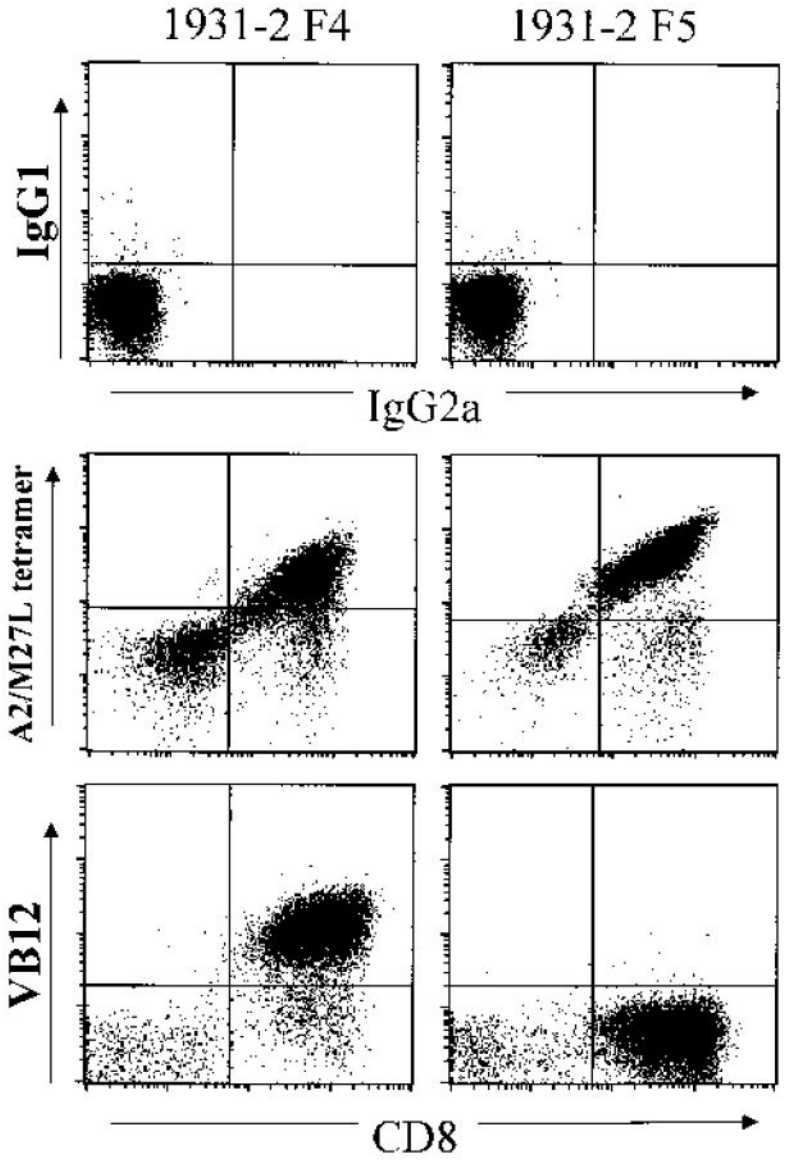

FIGURE 1.

Two independent TIL cultures from tumor 1931-2 have similar functional activities but comprised different clones. **Top:** TIL F4 (left) and F5 (right) exhibit low background staining. **Middle:** TIL F4 (left) and F5 (right) comprised predominantly cells that are doubled labeled by HLA-A2/MART-1:26-35(27L) tetramer and anti-CD8 antibody. **Bottom:** TIL F4 comprises predominantly T cells expressing Vβ12, whereas TIL F5 has no $V\beta12^{+}$ cells.

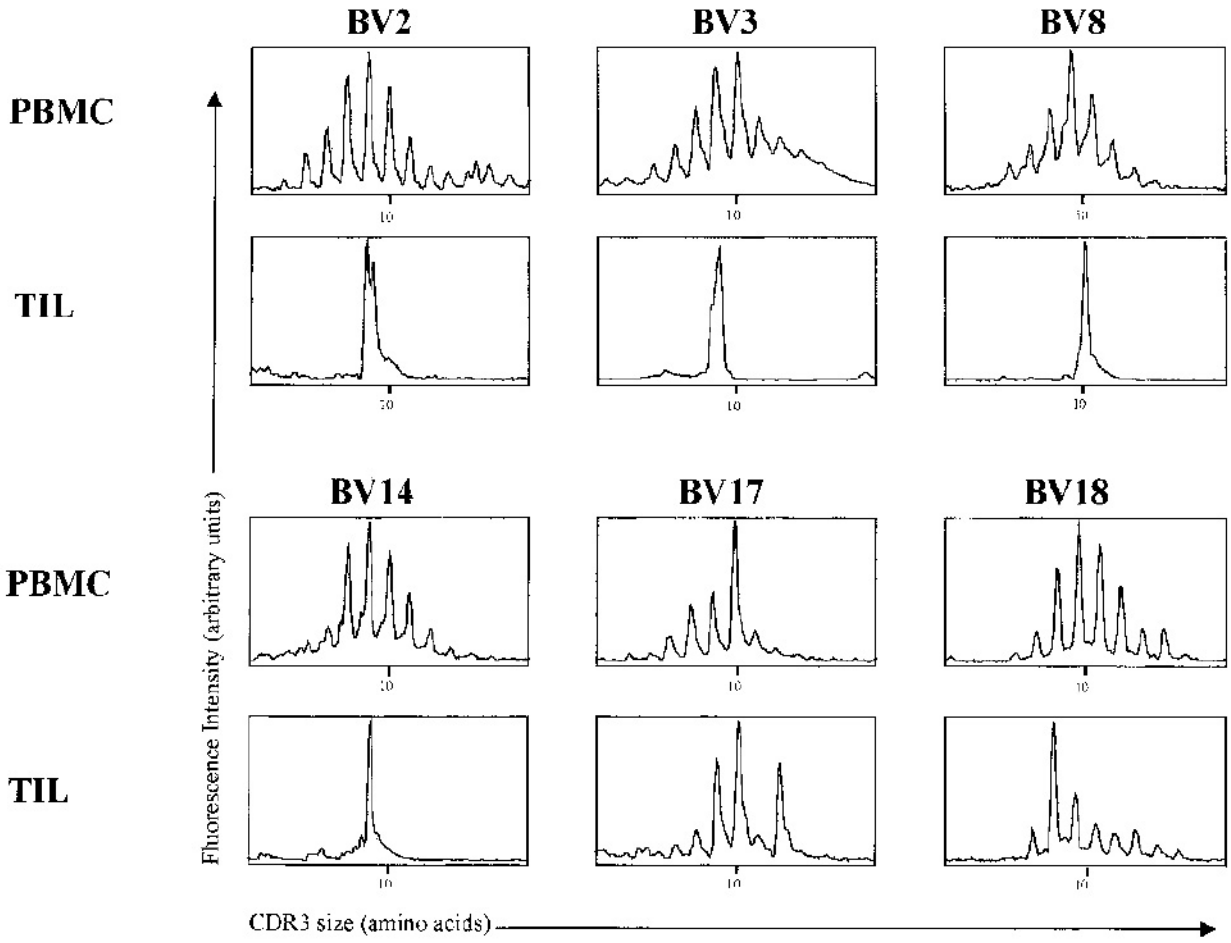

FIGURE 2.

Typical TCR β-chain transcript CDR3 size patterns from normal PBMC **(upper)** and a highly selected, highly in vitro expanded TIL **(lower)**. Fluorescent TRBV-BC PCR products were analyzed on an automated sequencer using Immunoscope software. The fluorescence intensity is expressed in arbitrary units. BV nomenclature is as previously described[16] and also as used by the anti Vβ antibody manufacturer. TRBV3 in this system corresponds to TRBV28 in the IMGT nomenclature.[17] Peripheral blood lymphocytes generate a characteristically wide distribution for each BV family, representing a diversity of expressed TCR. The in vitro selected, REPed TIL exhibits a highly reduced repertoire with 0, 1, or 2 dominant clones in each BV family.

TABLE 1

Melanoma excisional biopsies processed to establish TIL for potential adoptive cell transfer therapy

	Total initiated	"+" growth[†]	Cryopreserved[‡]	Active TIL[*]		REPed for treatment[§]
				Allogeneic HLA-A2$^+$ (no. screened)	Autologous (no. screened)	
Fragments						
Patients	62	59	23	23 (36)	21 (27)	6
Tumors	90	83	33	34 (50)	34 (44)	7
Cultures	710	496	194	87 (302)	151 (247)	9
Digests						
Patients	33	32	11	3 (21)	10 (17)	3
Tumors	42	41	16	3 (25)	11 (21)	3
Cultures	119	112	44	16 (68)	26 (54)	4
Medimachine						
Patients	17	14	5	4 (9)	3 (8)	0
Tumors	26	20	8	7 (12)	5 (11)	0
Cultures	31	28	5	9 (23)	13 (21)	0

[*] At least 1 TIL culture demonstrated specific IFN-γ release when stimulated with indicated tumor cells (defined as IFN-γ release greater than 100 pg/mL and at least twice the value of any A2$^-$ cell lines).

[†] At least 1 culture expanded sufficiently for screening or cryopreservation (more than approximately 5×10^6 cells).

[‡] No TIL were screened, but at least one culture was cryopreserved at sufficient cell numbers for screening.

[§] Eight of the 62 patients included in this data set were treated with the REPed cultures included in this table. Eight additional patients had an initial biopsy that let to cultures included in this data set, but were ultimately treated with cells derived from later biopsies or other sources.

TABLE 2

Multiple patterns of tumor cell line recognition were displayed by TIL cultures generated independently from fragments of tumor 2035

	Melanoma cell lines*					
	888 A2⁻	**938 A2⁻**	**526 A2⁺**	**624 A2⁺**	**1938 Autologous**	**Ag†**
TIL 2035						
F6	80	64	*415*	*559*	*618*	MART-1
F8	99	378	24	192	22	None detected
F15	13	1	19	14	*770*	Unknown
F16	396	125	377	499	457	None detected
F17	24	2	148	18	*424*	Unknown
DF3	48	7	*695*	*584*	*1939*	MART-1
DF4	4	5	12	42	*1668*	Unknown
DF6	1	0	11	*2214*	*1473*	NY-ESO-1
DF8	21	4	20	*2810*	*1947*	NY-ESO-1
DF13	27	6	*739*	*704*	*1481*	MART-1
DF17	203	32	57	86	*1785*	Unknown

*Values indicate IFN-γ release (pg/mL) by TIL when stimulated overnight with the indicated melanoma cell line. Specific recognition of 526mel, 624mel, or 1938mel was defined as IFn-γ release greater than 100 pg/mL and at least twice the greater value of the HLA-A2⁻ cell lines, and is indicated by values that are italic.

†Antigen reactivity was determined subsequently. The HLA-A2 restricted peptide epitopes from MART-1 and NY-ESO-1 have been described previously,[10] and additional unknown antigen recognition by some TIL is not excluded.[18] The molecular identity of the unknown antigen is currently being investigated.

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TABLE 3

Multiple patterns of tumor cell line recognition were displayed by independent TIL cultures generated from fragments of 5 tumor specimens resected from a single patient; the variation in activity among independent TIL derived from a single lesion is as great as the variation in activity among TIL derived from different lesions

		Tumor cell line stimulator*				
			HLA-A2$^-$		HLA-A2$^+$	
Biopsy	**TIL line**	**None**	**888**	**938**	**526**	**624**
1	1926-1 F7	9	32	6	*120*	*357*
	1926-1 F8	10	270	54	359	*662*
	1926-1 F9	3	194	50	68	149
	1926-1 F10	0	126	48	74	170
2	1926-2 F2	1	0	0	19	19
	1926-2 F4	6	231	184	*832*	*2042*
	1926-2 F5	16	97	65	92	137
	1926-2 F6	1	43	14	*753*	*862*
	1926-2 F10	5	1	0	28	40
3	1931-1 F1	138	181	92	76	113
	1931-1 F6	61	52	20	13	19
4	1931-2 F2	28	1378	200	870	1440
	1931-2 F3	43	319	48	175	301
	1931-2 F4	14	114	38	*1473*	*2327*
	1931-2 F5	72	198	53	*2016*	*2744*
	1931-2 F6	11	39	13	57	126
	1931-2 F11	21	821	211	117	409
5	1942 F1	29	57	12	*3183*	*3132*
	1942 F2	56	29	22	*284*	*399*
	1942 F3	28	25	23	20	58
	1942 F4	11	3	3	*418*	*364*
	1942 F5	128	64	20	90	138
	1942 F6	38	232	42	55	99

*Values indicate IFN-γ release (pg/mL) by TIL when stimulated overnight with the indicated melanoma cell line. Specific recognition determined as in Table 2.

TABLE 4

Quantitatively different patterns of peptide antigen specificity and tumor cell recognition were displayed by TIL cultures generated from a single-cell suspension of tumor 2009

		T2/peptide*		Melanoma line†		Tumor digest‡	
	Media	MART	g209	888	624	1866	2009
Controls§							
Media	0	68	0	3	2	0	0
CK3H6	3	5	*2785*	17	*2210*	179	*878*
JB2F4	3	*18,500*	5	6	*6290*	75	*420*
TIL 2009							
W1	4	*4470*	34	250	*7190*	220	*4730*
W2	2	*8790*	25	35	*3910*	27	*811*
W3	0	*7390*	26	14	*2180*	33	*1860*
W4	0	*116*	19	71	*904*	63	*829*
W5	6	*14,200*	77	14	*7610*	52	*1300*
W6	5	*405*	60	11	*>10,910*	182	*4330*

*Values indicate IFN-γ release (pg/mL) by TIL when stimulated with the indicated melanoma cell line. Specific recognition determined as in Table 2. The HLA-A2 binding peptide epitopes MART (MART-1:27-35) and g209 (gp100:209-217) were pulsed at 1.0 μmol/L concentration onto T2 cells, then used as stimulators.

†Melanoma cell line 888 was HLA-$A2^-$ and line 526 was HLA-$A2^+$.

‡Tumor digests were cryopreserved immediately after preparation, and thawed for use as stimulators on the day of the coculture assay. Number 1866 was from an HLA-$A2^-$ patient and number 2009 was from the autologous patient. Strong recognition of tumor number 2009 by some TIL was later determined to be mediated by TRP-2 reactive T cells.

§CK3H6 and JB2F4 are previously characterized cloned T cells with HLA-A2-restricted specificity for the peptide epitopes shown.

TABLE 5

TIL cultures derived from a single patient demonstrate substantial variation in their CD4+ and CD8+ cell compositions

	Cytokine release*				FACS analysis†	
TIL culture	**888**	**938**	**526**	**1824**	**CD4+**	**CD8+**
1921 F4	17	4	*1168*	*1516*	70.0	37.2
1921 F5	14	30	*711*	*749*	36.8	54.6
1921 F6	22	15	*382*	*569*	46.0	54.8
1921 F11	271	236	*1188*	*1099*	19.2	42.6
1941-1 F4	0	0	*2590*	*2529*	0.3	97.6
1941-1 F6	21	5	*2334*	*2363*	2.1	96.3
1941-3 F4	72	41	*889*	*1283*	46.0	53.7

*Values indicate IFNγ release (pg/mL) by TIL when stimulated with the indicated melanoma cell line. Specific recognition determined as in Table 2.

†Value indicates the percentage of viable lymphocytes in each TIL culture expressing the indicated phenotype. Values may be different from 100% due to double positive or double negative cells in the TIL culture.

TABLE 6

A single rapid expansion of TIL cultures from fragments generated large cell numbers suitable for patient infusion

Patient (culture)	Starting TIL ($\times 10^6$)	Expanded TIL ($\times 10^9$)	Fold expansion
1	48	29	604
2a	36	7	181
2b	36	10	289
3a	12	18	1517
4a	48	100	2083
4b	18	38	2106
5a	30	44	1467
5b	6	4	667
6	60	17	277
7a	38	94	2484
7b	13	34	2623
7c	37	32	851
8	54	109	2019
Average	34	41	1320