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Enrichment of CD8⁺ Cells From Melanoma Tumor-infiltrating Lymphocyte Cultures Reveals Tumor Reactivity for Use in Adoptive Cell Therapy

Peter A. Prieto, Katherine H. Durflinger, John R. Wunderlich, Steven A. Rosenberg, and Mark E. Dudley

Surgery Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD.

Summary:

Adoptive cell therapy (ACT) using tumor-infiltrating lymphocytes (TIL) for metastatic melanoma has shown objective response rates as high as 72%. The successful application of this therapy requires the selection of unique tumor-reactive lymphocyte cultures for each patient. This is a technically and logistically difficult undertaking, and patients who do not have tumor-reactive TIL are not considered eligible for treatment. To simplify the methods of TIL generation and extend TIL-based immunotherapy to additional patients, methods were developed to use unselected, minimally cultured (“young”) TIL. Young TIL cultures contain a variable number of CD8⁺, CD4⁺, and CD3[−] CD56⁺ natural killer cells. In this study we retrospectively investigated a role for these subsets in the clinical outcome of patients treated with TIL derived from selected microcultures. This analysis demonstrated a suggestive but nonsignificant association between the number of CD8⁺ cells administered and tumor regression. We therefore investigated the feasibility of selecting CD8⁺ cells from young TIL cultures for ACT therapy. The available methods for clinical scale CD8⁺ enrichment proved inadequate for TIL, so an optimized CD8⁺ enrichment method was developed and is reported here. We observed that CD8⁺ enrichment of some TIL cultures revealed in vitro tumor recognition that was not evident in bulk culture, and an improved in vitro recognition of tumor in other TIL cultures. In addition, the enriched CD8⁺ young TIL expanded more reliably and predictably in rapid expansions than the bulk TIL. Thus, optimized CD8⁺ selection combines the benefits of antigen-selected TIL and young TIL for generating lymphocyte cultures for ACT, and should be evaluated in cell transfer therapy protocols.

Keywords

tumor-infiltrating lymphocytes (TIL); minimally cultured lymphocytes (young TIL); immunotherapy, CD8⁺enrichment, CliniMACS

Metastatic melanoma is an aggressive malignancy with limited treatment options and a poor prognosis. US Food and Drug Administration approved therapies for treatment of metastatic melanoma patients including dacarbazine chemotherapy and high-dose interleukin (IL)-2

Reprints: Peter A. Prieto, Surgery Branch, National Cancer Institute, National Institutes of Health, CRC 3W-5809 10 Center Drive, Bethesda, MD 20892-1201, (prietop@mail.nih.gov).

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therapy and experimental immunotherapies such as ipilimumab (anti-cytotoxic T lymphocyte-associated-4 antibody) have objective response rates of 10% to 20%.^{1–5}

Adoptive cell therapy (ACT) is an experimental approach that has proven effective for some patients with refractory metastatic melanoma.^{6,7} ACT consists of the administration of tumor-reactive lymphocyte cultures after ex-vivo expansion and activation. Methods for generation of tumor-reactive lymphocytes from peripheral blood have included limited dilution cloning,^{8–11} culture establishment by in vitro restimulations,^{12–15} and genetic engineering.^{16,17} Response rates after administration of these cell products to patients have varied from 0% to 30%. When melanoma-reactive cultures can be established from tumor-infiltrating lymphocytes (TIL) instead of peripheral blood, the response rates to treatment have been substantially higher. We recently reported a series of 93 highly pretreated patients with refractory melanoma who received tumor-reactive TIL [based on secretion of interferon- γ (IFN- γ)] microcultures that were rapidly expanded in vitro to an average cell dose of 50 billion cells. All patients received 1 of 3 lymphodepleting regimens and high dose IL-2. The overall response rate was 56%, with 13 complete responders.⁶

The clinical results with ACT using TIL cultures combined with lymphodepletion are encouraging. However, the challenges to establishing a unique, autologous antigen-reactive TIL microculture for each patient are substantial and limit the population of patients who are eligible for treatment. For instance, some TIL cultures expanded in vitro but did not have suitable tumor “targets” for screening purposes, and were therefore not used for treatment because tumor reactivity of the TIL cultures could not be demonstrated. Other patients required an extended time for the expansion and screening of the microcultures to identify tumor reactivity, and they experienced disease progression during this interval that excluded their enrollment for clinical reasons (for instance development of new brain metastases). Post hoc evaluation of treatment frequency after surgical resection at our institution suggests an actual rate of treatment around 27% (Dudley and Yang, personal communications).

To simplify the TIL manufacturing process and potentially improve the attributes of the administered TIL cultures, we described a minimally cultured TIL or “young” TIL protocol.¹⁸ This protocol described the use of minimally cultured TIL from bulk cultures. TIL from many microcultures can be pooled together in bulk, without screening for tumor reactivity, then rapidly expanded and administered after lymphodepletion. We and others¹⁹ have initiated clinical trials using this young TIL approach. Young TIL have some notable differences from selected microculture TIL. We have previously reported that young TIL have longer telomeres and the young TIL CD8⁺ cells have high expression of cell surface markers associated with a less differentiated phenotype including CD27 and CD28. Also, the fraction of CD4⁺ cells in TIL decreases with time in culture, so young TIL that are cultured for shorter times have a disproportionately high fraction of CD4⁺ cells.

The role of CD4⁺ cells in tumor immunity is complex and the implications of significantly more CD4⁺ cells in an infused young TIL product has important implications for in vivo cell function. CD4⁺ T-cell function and immunologic impact depend on their specificity, polarization, traffic, and other attributes. The administration of tumor-reactive CD4⁺ Th17 polarized cells can cause the complete regression of a transplanted B16 tumor in syngeneic

C57Bl/6 mice,²⁰ whereas the administration of CD4⁺ T_{reg} cells have the opposite effect and abrogate the therapeutic efficacy of transferred CD8⁺.²¹ CD4⁺ T cells in humans demonstrate a similarly complex spectrum of CD4⁺ functions in vitro. Direct evidence for CD4⁺ T-cell involvement in human tumor regression is rare, but one case study linked the administration of a CD4⁺ T-cell clone with therapeutic effectiveness.²² Other reports suggest a correlation between CD4⁺ T_{reg} infiltration in tumors and poor clinical prognosis.^{23–26} These data in mouse and man suggest a complex and patient-specific interaction between CD4⁺ T cells and growing tumors. In many patients CD4⁺ cells may have a net negative on antigen-specific CD8⁺ lymphocytes, yet in other patients there is a clear antitumor advantage to this CD4⁺ subset.

In this report we investigated a strategy for selection of a minimally cultured T-cell product. Initially we evaluated the antigen-specific TIL cultures administered to patients in previous protocols and detected a trend toward improved response with fewer CD4⁺ cells. We therefore optimized the enrichment of CD8⁺ cells from young TIL before rapid expansion. The CD8⁺-enriched cultures demonstrated tumor reactivity that was sometimes disproportionate to the fractional enrichment. In addition, elimination of natural killer (NK; CD56⁺CD3[−]) cells improved the reliability of the rapid expansion. This use of CD8⁺ enrichment of young TIL produces a T-cell product with enhanced characteristics for clinical use.

MATERIALS AND METHODS

Established Cell Lines and Patient Material

Melanoma cell lines were grown in Roswell Park Memorial Institution 1640 supplemented with glutamine, penicillin, streptomycin and gentamycin, and 10% fetal calf serum (Hyclone, Logan UT). Cell lines 888mel and 938mel are human leukocyte antigen (HLA)-A2[−] whereas 526mel and 624mel are HLA-A2⁺. The TIL line DMF4 has been described previously.^{16,27,28} The DMF4 TIL line is predominated by a single HLA-A2 restricted, MART-1:26 to 35 reactive T-cell clone that is CD8 dependent.¹⁶ All human tissues used in this study were collected as part of a clinically approved protocol after formal (Clinical Research Center Institutional Review Board approved) consent of the patient.

Generation of TIL

TIL were procured and expanded ex-vivo by methods previously described.^{18,29,30} Briefly TIL generation followed 1 of 2 pathways. Antigen-specific TIL generation involved the initiation of multiple independent cultures in media containing 6000 IU/mL IL-2 in complete media in separate wells of a 24-well plate, starting with multiple individual tumor fragments or multiple wells of a homogeneous cell suspension. These separate cultures were grown independently, to several million lymphocytes each, and screened for tumor recognition by IFN- γ release assay. Tumor-reactive cultures were subsequently activated and expanded approximately 1000-fold further using a rapid expansion protocol (REP) with OKT3 (anti-CD3) antibody, IL-2, and irradiated peripheral blood mononuclear feeder cells. If more than one cell culture line was pooled for a particular TIL it was carried out at this point (few TIL cultures were pooled that differed by more than 2 d time in culture and

exclusion of these few patients did not alter the outcome of the analysis). This entire process typically took an average of 45 days. The second pathway involved minimally cultured young TIL. This required the plating multiple wells of a homogeneous cell suspension in a 6000 IU/mL IL-2 and monitoring the cultures until the lymphocytes had eliminated all adherent cells, expanded, and become confluent. This was followed immediately by pooling of the cultures and rapid expansion without performing tumor specificity assays. This latter process took on average 30 days.

CD8⁺ Enrichment of TIL

CD8⁺ enrichment of TIL was initially performed using a prior established standard operating procedure (SOP) developed for lymphocyte harvest from peripheral blood leukapheresis samples. In this SOP CD8⁺CD4⁻ lymphocytes were selected by positive selection with immunomagnetic beads by CliniMACS good manufacturing practice isolation strategies as recommended by the manufacturer (Miltenyi Biotec, Bergish-Gladbach GDR). The TIL product was transferred from a 250 mL conical to a sterile 600 mL transfer pack then diluted 1:3 with CliniMACS buffer (phosphate buffered-saline/ ethylenediaminetetraacetic acid/human serum albumin)+ 25% human albumin. The cell suspension was centrifuged at 300g for 15 minutes (room temperature), without brake. The supernatant was removed and the cells were resuspended in 95 mL of buffer. Immunomagnetic labeling was accomplished with 7.5 mL of CliniMACS CD8 reagent, a murine monoclonal CD8 antibody in phosphate buffered-saline stabilized with 0.03% of poloxamer 188 conjugated to an iron-dextran colloid. After incubating (room temperature) the cells on a rocking platform for 30 minutes the suspension was brought to a volume of 600 mL with buffer and once again washed and centrifuged at 300g for 15 minutes, without brake. The labeled cells were then brought to a final loading volume of 100 mL and placed on the CliniMACS automated separation device. Enrichment program 1.1 was initiated per manufacturer guidelines. Once separation was complete the positive fraction (CD8⁺), negative fraction (CD8-depleted), and waste isolates were all counted to determine recovery and viability was assessed by trypan blue exclusion. After separation, CD8⁺ enriched and CD8-depleted TIL fractions were resuspended at a concentration of 1×10^6 /mL and assayed immediately or cultured for several days before functional and phenotypic evaluation.

Optimization of CD8⁺ Enrichment of TIL

To minimize centrifugation times and improve cell recovery for small process products, the 600 mL transfer packs were eliminated from the SOP and all TIL processing steps were adapted to 250 mL conical tubes throughout the enrichment process. To maintain sterility in the cell product, all processing steps were performed in a laminar flow tissue culture hood in the Surgery Branch Cell Production Facility. Immunomagnetic labeling was optimized empirically and bead/labeling volume ratios are discussed in the results section. These modifications were necessary to accommodate small cell numbers in the cell product (ranged from 4.1×10^7 to 3.14×10^8) as well as minimizing contamination from unwanted cell populations with low CD8 expression (including cultured CD4⁺ and NK cells). Other aspects of the enrichment process remained as recommended by the manufacturer.

Fluorescence-activated Cell Sorting

Phenotype was determined by 6 color flow cytometric analysis using fluorescence-activated cell sorting (FACS) Canto II Flow Cytometer (BD Bioscience, San Jose, CA). Antibodies for analysis included CD3, CD4, CD8, and CD56 (BD Biosciences). NK cells were defined as CD56⁺CD3⁻ lymphocytes. At least 10,000 cells were counted for each analysis.

IFN- γ Release

Specificity assays were initiated as early as day +1 after enrichment. Coculture assays were collected as previously described.²⁹ Briefly, assays were performed in 96-well flat bottom plates with 1×10^5 responder TIL cocultured with 1×10^5 autologous or HLA-matched/mismatched tumor cells overnight. Supernatant was quantified for IFN- γ release by enzymes linked-immunosorbent assay technique according to manufacturers guidelines (Invitrogen). For each enrichment cohort, cocultures included bulk (unseparated), CD8⁺ enriched (positive fraction), and CD8⁺ depleted (negative fraction or CD4⁺) populations for tumor-specific reactivity. Reactivity was defined as IFN- γ release of at least 200 pg/mL and greater than twice the background released by coculture with HLA-mismatched controls.

Ex-vivo Expansion

Experimental populations underwent “small scale” ex-vivo expansion [test-Rapid expansion (tREP)] to determine the ability of enriched fractions to maintain purity and reactivity if present through an expansion. Cells (3×10^5) were placed in T75 flasks with 200-fold excess of irradiated (40 Gy) donor peripheral blood mononuclear “feeder” cells, complete media (Gemini) +AIM V, 1 mg/mL anti-CD3, and 500 CU/mL of IL-2 (Novartis). Media was changed at day 5 and cells counted and split thereafter. Cell purity and reactivity were assayed by FACS, coculture, and enzymes linked-immunosorbent assay on or after day +12 after the REP. To determine if the time interval between enrichment and expansion had any significant effect on fold expansion of newly enriched TIL a series of tREP's were sequentially begun on days 0, +1, +2, and +7 (t_0 , 1, 2, and 7) after enrichment of experimental TIL populations 6 and 7. These results are summarized in Figure 3.

RESULTS

Low CD4⁺ Lymphocyte Numbers and Time in Culture Were Evaluated for Improved Clinical Outcome for Antigen Specific, Microculture Generated TIL

We previously published a clinical and immunologic analysis of 3 sequential clinical trials in which 92 patients received tumor-reactive TIL after 1 of 3 different lympho-depleting regimens.⁶ TIL for patient infusion in these trials were initiated in 24-well plates, screened for tumor recognition, and then expanded to large numbers for infusion.²⁹ In this report, we retrospectively analyzed the infusion TIL from this group of patients to evaluate whether the length of time in culture and the lymphocyte subset composition of the TIL cultures correlated with the outcome of treatment. The results of this analysis are shown in Table 1. Shorter time in culture significantly correlated with better outcome when all patients are combined across all 3 clinical trials. However, these results should be interpreted with caution as many confounding factor are present when multiple trials are combined. For

instance, later clinical trials resulted in consistently “younger” TIL cultures for patient infusion than the first clinical trial. Whether this was simply a result of experience in the laboratory and refined methods or whether investigator prejudice biased the TIL culture selection as clinical trials progressed is not known.

The infused TIL number and subset composition was also examined (Table 1). There was no trend or pattern to the absolute number of TIL infused across the 3 clinical trials or between responding and nonresponding patients. In contrast, there was a consistent pattern of lymphocyte subsets observed. Nonresponders consistently received more CD4⁺ cells than responders in all 3 clinical trials (Table 1), as well as a higher percentage of CD4⁺ cells and fewer CD8⁺ cells (not shown). Because of small patient numbers and a large variation in absolute CD4⁺ numbers infused, the results do not approach significance for any individual trial. Interestingly, when the data from all 3 trials are aggregated, the difference between responding and nonresponding patients trends to statistical significance (Fig. 1, $P = 0.090$). This may reflect a small and varied sample population or it may indicate an inconsistent role of CD4⁺ cells in tumor immunity, with a net negative impact of CD4⁺ cells on a majority of TIL infusions, but a positive impact in a fraction of TIL infusions where tumor-reactive CD4 clones are present (NEJM¹¹). The trend toward better response in patients receiving fewer CD4⁺ cells provided an incentive for us to attempt to manipulate lymphocyte subsets in young TIL.

CD8⁺ Enrichment of TIL by Positive Selection Under GMP Conditions Required Optimization

Methods and reagents for enrichment of CD8⁺ lymphocytes from peripheral blood under GMP conditions have been established (Miltenyi Biotec). The manufacturer recommended SOP was followed for experimental enrichments of young TIL. Initial enrichments yielded both poor recovery and purity (Fig. 2A, Table 2).

Several properties of young TIL may have resulted in poor CD8⁺ cell enrichment from these cultures. These factors include the level of CD8 expression by target and nontarget cells, TIL susceptibility to serum withdrawal, and total cell number available for enrichment. We therefore optimized a number of steps in the purification process to maximize CD8⁺ TIL yield, purity, and viability under GMP conditions. Table 2 shows results from experiments with progressive titration of the CD8⁺ bead:labeling volume, starting at 7.5 mL beads in 100 mL labeling volume, progressing through 3.5 mL beads in 200 mL, and finally attempting 1.0 mL beads in 200 mL. Results of these and similar experiments resulted in an optimal labeling method of 1.0 mL beads in 400 mL labeling buffer that yielded a highly enriched CD8⁺ cell product with good recovery and excellent viability (Fig. 2B, Table 3).

CD8⁺ Enrichment Under Optimal Conditions Yielded a Highly Purified Product Suitable for Clinical Use

To further characterize the cell product obtained from the optimized CD8⁺ enrichment protocol, multiple young TIL were subjected to CD8⁺ purification. The resulting products were analyzed for viability, phenotype, and sterility. The results of 6 sequential enrichments at optimized conditions are summarized in Table 3. The mean number of cells before CD8⁺

enrichment was 1.21×10^8 (range: 0.41 to 3.14×10^8) characteristic, of young TIL (approximately 14 to 21 days old) such as would be used in a clinical protocol. The mean absolute number of CD8⁺ cells after enrichment was 6.66×10^7 (range: 1.75 to 10.1×10^7), suitable for initiation of rapid expansions and patient treatment in all cases. Viability of enriched CD8⁺ cells, determined by trypan blue exclusion, remained over 94%. The mean purity of 6 consecutive enriched CD8⁺ CD4⁻ cell populations was 96%. The purified TIL products were subjected to microbiologic contamination testing identical to those used for cell products for patient infusion in our facility and no microbial contamination was detected. Furthermore, the optimized protocol used cell therapy practices that are readily adapted to GMP quality for clinical protocols.

CD8⁺ Purification of TIL Had no Impact on Functionality nor Ability to Expand After Selection

The separation process, including the impact of the residual immunomagnetic beads bound to the CD8⁺ cells, was investigated for its potential impact on the ability of the enriched TIL to expand. Purified cells were washed and plated in media with IL-2, then initiated in expansions with feeder cells, OKT-3, and IL-2 beginning on days 0, +1, +2, and +7 (t₀, t₁, t₂ and t₇) after enrichment. This was carried out for all 3 TIL cohorts [unseparated, CD8⁺ enriched, and depleted (CD4⁺)]. These small scale expansions (tREP) were followed for 13 days by counting viable cell number and calculating the fold expansion. CD8⁺ lymphocytes were capable of reliably undergoing rapid expansion immediately after magnetic separation or after resting for up to a week (Fig. 3). Comparing fold expansion between unseparated and CD8⁺-enriched TIL no significant difference was seen in any of the tREP cohorts (t₀P = 0.60, t₁P = 0.40, t₂P = 0.75, t₇P = 0.98, data not shown). Because CD56⁺ CD3⁻ NK cells expand poorly under OKT3 (anti-CD3) stimulated REP conditions, the reliability of the REP was further enhanced by elimination of NK cells. Unseparated TIL had a mean of 14.8% NK cells (range: 5%–40%) whereas CD8⁺-enriched TIL had a mean of 2.2% NK cells (range: 0%–4%).

In an effort to determine the effect of the CliniMACS immunomagnetic beads on newly enriched TIL effector function, a small scale positive selection column (MidiMACS, Miltenyi Biotech Inc) was run according to manufacturer's recommendations to enrich a known CD8⁺ restricted TIL clone, DMF4. There was no significant impairment observed on the ability of newly enriched (immunomagnetically labeled) to secrete IFN- γ (results not shown).

CD8⁺ Enrichment of TIL Resulted in Specific IFN- γ Release From Previously Nonreactive TIL

To investigate the relative function of young TIL before and after CD8⁺ enrichment, TIL cultures were subjected to clinical scale CD8⁺ enrichment, then rested overnight in complete medium containing IL-2. Unseparated TIL and the CD8-depleted fraction were cultured in complete media with IL-2 under similar conditions. All cultures were then compared for their ability to secrete the inflammatory cytokine, IFN- γ , when cocultured with autologous (n = 5), HLA-matched (n = 8), or HLA-mismatched tumors (no autologous tumor or significantly HLA-matched tumor cell lines were available for patient 2, whose TIL were

not screened). Tumor recognition was considered to be significant and specific when absolute IFN- γ release was at least 200 pg/mL and more than twice the level released from the highest “negative control” in the assay (non-matched HLA tumor used for stimulation or no stimulation).

Five samples were evaluated where autologous tumor was available to use as stimulation in the coculture assay (Table 4). One sample (patient 7) showed robust IFN- γ release when unseparated TIL was stimulated with auto-logous tumor; and the level of IFN- γ release did not increase substantially after CD8⁺ enrichment from 49% initially to 93% (Tables 3, 4). Two patients (patients 5 and 9) demonstrated recognition of autologous tumor by the unseparated TIL, and recognition was improved after CD8⁺ enrichment. Interestingly, for patient 5 the enrichment of CD8⁺ cells from 83% to 96% (Table 3) increased specific IFN- γ release more than 7-fold, suggesting that the elimination of the CD4⁺ cells was also important for the increase in IFN- γ release. Two samples (patients 1 and 6) did not demonstrate specific release of IFN- γ by the bulk TIL to autologous tumor stimulation, but after CD8⁺ enrichment, specific recognition of autologous tumor was revealed. For patient 1 where the protocol used for enrichment of CD8⁺ cells was suboptimal and no CD8⁺ enrichment was observed, this effect may be due to reduction of CD4⁺ cells.

Five patients' TIL samples showed recognition of HLA Class I allele-matched tumor cell lines (Table 4). All 5 TIL samples showed improved recognition after CD8⁺ enrichment. Three patients (patients 3, 6, and 8) demonstrated recognition of tumor in the original nonselected TIL that was enhanced by CD8⁺ enrichment. Interestingly, for patient 8 the amount of IFN- γ released after enrichment (3.7-fold) was disproportionately large compared with the CD8⁺ fractional enrichment (1.2-fold, Fig. 4). This may indicate that the removal of CD4⁺ cells or another type of cell also impacted tumor recognition. For patients 1 and 5, the unselected TIL cultures did not demonstrate specific tumor recognition of HLA-matched tumor cell lines, but specific recognition was revealed by CD8⁺ enrichment.

Overall, 7 of the 8 CD8⁺-enriched TIL that were evaluable (all except patient 4) showed recognition of HLA-matched or autologous tumor measured by specific cytokine release (Table 4). When all examples of tumor recognition were compared between unseparated and CD8⁺-enriched TIL, the IFN- γ release from CD8⁺ TIL was significantly higher ($P = 0.03$, Student t test). Both CD8⁺ lymphocyte enrichment and CD4⁺ lymphocyte depletion may have augmented the function of the CD8⁺-enriched TIL cultures. IFN- γ release was decreased or absent in the depleted fraction of all 7 reactive TIL, suggesting that CD8⁺ lymphocytes express majority of the functional activity of young TIL cultures (Table 4) and that specifically reactive CD8⁺ cells are necessary for tumor recognition observed in the TIL investigated here. The elimination of CD4⁺ cells or other suppressor cell may have impacted specific cytokine release in some TIL cultures. The ratio of IFN- γ release of CD8⁺ enriched compared with unseparated TIL was substantially higher than the ratio of CD8⁺ cells in CD8⁺ enriched compared with unseparated TIL (Fig. 4).

A further 33 patients had their TIL procured, cultured, enriched, and assayed for cytokine release in the same manner as the first 9 patients described. Among the 33 additional patients evaluated, 21 (64%) had reactivity as determined by IFN- γ release. Interestingly, of

the 21 patients with measured reactivity, 8 (38%) showed activity that was again only revealed after CD8⁺ enrichment. These further coculture results, although not shown, are part of a larger data set being compiled to analyze our current clinical trial examining the clinical application of CD8⁺ enrichment of TIL.

DISCUSSION

Cell transfer therapy can be an effective treatment for patients with refractory melanoma. The attributes of TIL associated with tumor regression include specificity for tumor antigens and a minimal time in culture, probably associated with a highly proliferative, relatively undifferentiated phenotype. The generation of an autologous culture for each patient with these optimal characteristics has proven to be technically challenging and has limited the widespread application of cell transfer therapy despite its potential clinical efficacy. One strategy to simplify the generation of autologous TIL cultures suitable for patient therapy involves the use of young, unselected cultures.¹⁸ These young TIL have some desirable attributes compared with antigen-selected TIL derived from micro-cultures, including a less differentiated phenotype and a higher proliferative potential. However, young TIL are not selected for antigen specificity, and also contain a high proportion of CD4⁺ cells compared with previously administered, antigen-specific TIL, with a variable and unpredictable fraction of NK cells. Young TIL may be easier to apply in a clinical setting than antigen-selected TIL from microcultures and a preliminary report suggests that young TIL can be clinically effective, as 3 of 7 evaluable patients responded to treatment with young TIL.¹⁹ These characteristics of young TIL make them an encouraging prospect for further clinical investigation, but additional improvements and simplifications may also be attainable. The lymphocyte composition of young TIL cultures is a characteristic that can be manipulated quickly and efficiently, and can have important consequences on the reliability of treatment generation and the ultimate treatment efficacy.

A dramatic difference between young TIL cultures and previously administered TIL is the proportion of CD4⁺ cells in the cultures. The role of CD4⁺ lymphocytes in tumor immunity is complex. Tumor-reactive CD4⁺ cells may contribute to antitumor responses.²² However, our analysis of antigen specificity of CD8 depleted cultures resulted in only 1 sample of 5 tested that showed IFN- γ release when stimulated with autologous tumor whereas all 5 showed specific tumor recognition by the CD8⁺-enriched fractions. This small sample suggests that CD4⁺ tumor recognition is relatively infrequent, although a more systematic analysis needs to be undertaken.

More consistently, CD4⁺ cells are associated with functions that are detrimental to tumor therapy. For instance, CD4⁺ cells have been implicated in autoimmune inflammation and disease,³¹ and the administration of highly activated “nonspecific” CD4⁺ cells from young TIL could result in inflammatory toxicities. Alternately, the CD4⁺CD25⁺ T_{reg} cell subset can result in the active suppression of antitumor immunity in mouse models of ACT and similar cells have been reported in human melanoma lesions.³² The prevalence and activity of CD4⁺ T_{reg} cells in cultured TIL is not known, but administration of T_{reg} cells with active specific CD8⁺ TIL could potentially limit their therapeutic efficacy. The retrospective analysis of ACT with antigen-reactive TIL cultures reported here is also consistent with a complex role

for CD4⁺ cells in tumor immunity and suggests that there is a link between administration of fewer CD4⁺ lymphocytes and objective clinical response, although the data did not reach statistical significance ($P = 0.068$). This data is consistent with a generally inhibitory impact of CD4⁺ cells on tumor responses in vivo.

In this report we describe an optimized method for the enrichment of CD8⁺ lymphocytes from young TIL cultures for use in ACT. Therapeutic cell products derived from magnetic fractionation have typically started with minimally manipulated (uncultured) cell mixtures. Protocols for enriching rare cells such as CD34⁺ or CD133⁺ hematopoietic progenitor cells from pheresis products have been thoroughly vetted at clinical scale on the CliniMACS platform and are in common use. Other experimental procedures for depleting lymphocyte subsets such as CD25⁺ T_{reg} cells have also been clinically employed,^{33–35} and virus-specific cells from the peripheral blood lymphocyte pool have been selected for clinical use through IFN- γ secretion after antigen stimulation.^{36,37} In contrast, the protocol for generating CD8⁺-enriched TIL reported here involves an initial time for the TIL to develop in cell culture, followed by enrichment of CD8⁺ cells. The use of a cultured cell product presented some unique challenges, and the CD8⁺ enrichment process required optimization to accommodate them. The small number of total starting cells in some young TIL populations, the prior adaptation of cultured cells to serum and IL-2, and the low level of expression of the target antigen (CD8) by nontarget cells (cultured CD4⁺ and NK cells) were all issues that were addressed. The optimized selection process minimized the processing time to reduce the consequences of serum and IL-2 withdrawal. Importantly, the purity of the CD8⁺ fraction was optimized, to minimize contamination from CD4⁺ and NK cells which express low levels of CD8 after in vitro culture. The optimized protocol reported here yields a consistently pure CD8⁺ cell product suitable for clinical expansion for patient administration.

Functional evaluation of optimally enriched CD8⁺ young TIL cultures suggests that CD4⁺ cells are actively suppressing CD8⁺ tumor reactivity in some TIL cultures. Bulk unseparated TIL often demonstrated tumor recognition, and the tumor recognition always partitioned with the CD8⁺ lymphocyte fraction. Interestingly, enrichment of CD8⁺ cells was associated with a significant increase in cytokine release to autologous or HLA-matched tumor ($P = 0.03$). Furthermore, there were 4 patients out of 7 with at least one HLA-matched or autologous tumor target that failed to elicit specific recognition by the unseparated TIL, but the CD8⁺-enriched TIL demonstrated specific recognition of the same tumor target. Finally, multiple examples were observed of TIL cultures that underwent a disproportionate increase in cytokine release upon CD8⁺ enrichment. The increase in absolute cytokine level was not explained solely by the increase in the number of CD8⁺ cells in the assay based on the fractional enrichment. For these cultures, the increase in IFN- γ release was apparently caused by removal of CD4⁺ cells, possibly by removing active regulatory or inhibitory mechanisms. Further examination of TIL reactivity by intracellular cytokine release or enzyme-linked immunospot assay may prove beneficial in examining this phenomenon. A recent report examining complex FACS profiles from TIL cultures predicted that the depletion of “marker” genes of unknown function from TIL could enhance IFN- γ secretion in lymphocyte cultures in vitro.³⁸ The results we report may reflect a similar phenomenon, in that a few cells within the CD4⁺ population may have a disproportionate

impact on the culture function and their removal may improve the systematic function of the culture.

Successful immunotherapy should ideally combine simplicity of administration with adequate potency. The generation of a unique cell product for each patient is still relatively complex compared with a “one size fits all” approach to patient treatment. However, the ability to simply and reliably generate a tumor-reactive TIL culture for almost every patient would substantially improve the utility of this approach. CD8⁺-enriched young TIL relies on the use of a simple magnetic selection during the culture process instead of technically challenging and time consuming culture manipulations to improve the culture characteristics. The highly enriched CD8⁺ product shows attributes that combine the benefits of selected standard culture TIL with those of minimally cultured young TIL. These cultures are currently being tested in the clinic for toxicity and efficacy in patients with metastatic melanoma.

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FIGURE 1.

Nonresponding patients generally received TIL with a higher percentage of CD4⁺ cells than responding patients. Ninety-two samples of tumor-reactive TIL that were administered to patients in Surgery Branch clinical protocols⁶ were evaluated for phenotype of lymphocyte composition by FACS. Percent of CD4⁺ cells is graphed for nonresponding patients and responding patients. Mean for each population is shown by the black bar. $P = 0.090$ (Student t test with allowance for unequal variance). FACS includes fluorescence-activated cell sorting; TIL, tumor-infiltrating lymphocytes.

FIGURE 2.

Process optimization was required for acceptable levels of magnetic enrichment of CD8⁺ cells. A, FACS profiles using nonoptimized CD8⁺ enrichment process. Upper left: starting TIL culture from patient 1. Upper middle: CD8⁺ fraction from nonoptimized protocol with 7.35% CD4⁺ cells. Upper right: CD8 depleted fraction from nonoptimized enrichment process. B, FACS profiles using optimized CD8⁺ enrichment process. Lower left: starting TIL from a patient enrolled in a current Surgery Branch clinical protocol using optimized CD8⁺ enrichment protocol. Lower middle: CD8⁺ enriched fraction from optimized protocol with 0.94% CD4⁺ cells. Lower right: CD8 depleted fraction from optimized process. FACS includes fluorescence-activated cell sorting; TIL, tumor-infiltrating lymphocytes.

FIGURE 3.

CD8⁺ enrichment using magnetic beads does not impair capacity for rapid expansion. To evaluate the ability of cells to undergo a REP at various times after magnetic enrichment, CD8⁺-enriched TIL cells were plated in complete media with IL-2 after purification. Immediately after purification (day 0) or at the indicated times, an aliquot of 1×10^5 purified TIL was removed and added to a complete REP mix. At the indicated times after the initiation of the REP, cells were counted. These results are representative of 3 independent experiments. IL indicates interleukin; REP, rapid expansion protocol; TIL, tumor-infiltrating lymphocytes.

FIGURE 4.

IFN- γ release increases disproportionately to CD8⁺ T cell number after CD8⁺ enrichment. The label below the x-axis describes a patient and a tumor target from Table 4. Each pair of bars represents 2 ratios. The left (dark) bar of each pair (CD8⁺) represents the ratio of the number of CD8⁺ cells in a coculture assay after CD8⁺ enrichment compared with the number before enrichment. The right bar (light) of each pair (IFN- γ) represents the ratio of the IFN- γ release after enrichment compared with the IFN- γ release before enrichment. The numerical value of each of these ratios is listed below each respective patient. For 4 samples of 5 shown, the increase in IFN- γ release cannot be explained simply by an increase in the number of CD8⁺ cells in the coculture assay after CD8⁺ enrichment. IFN- γ indicates interferon- γ .

TABLE 1.

Age and CD4⁺ Composition of TIL Infused for Treatment Varied Between Responding and Nonresponding Patients in Prior Surgery Branch Protocols

	Age of TIL (d) [*]	Lymphocytes Infused (×10 ⁹)	
		Total	CD4 ⁺
NMA [‡]			
NR (n = 22)	47.7 ± 2.1	58.7 ± 10.0	12.3 ± 3.3
PR + CR (n = 21)	42.4 ± 3.5	57.3 ± 6.8	7.9 ± 2.0
200 TBI			
NR (n = 11)	37.1 ± 1.6	58.4 ± 8.7	15.1 ± 4.5
PR + CR (n = 13)	37.3 ± 1.0	64.4 ± 6.2	11.0 ± 2.5
1200 TBI			
NR (n = 7)	39.6 ± 0.9	48.1 ± 11.6	5.0 ± 2.4
PR + CR (n = 18)	36.1 ± 0.9	68.7 ± 7.1	4.4 ± 0.9
Total			
NR (n = 40)	43.4 ± 1.5	56.7 ± 6.3	11.8 ± 2.3
PR + CR (n = 52)	38.9 ± 1.5	63.0 ± 4.0	7.5 ± 1.1
	<i>P</i> = 0.042 [‡]	<i>P</i> = 0.400 [‡]	<i>P</i> = 0.400 ^{‡§}

^{*} The total age of the youngest TIL culture used for each patient from tumor resection to reinfusion was calculated including time in bulk culture plus time during rapid expansion. If multiple cultures were combined for treatment, the age of the youngest culture was used.

[‡]Three lymphodepleting condition regimens are analyzed independently, following the protocol that patients were enrolled on and the convention established by Dudley et al.⁶ With minor improvements and optimizations, similar techniques were used to generate TIL for all 3 patient cohorts.

[‡]Student *t* test was used with 2 tails assuming unequal variance for analyzing data from the total patients.

[§]Mann-Whitney nonparametric, distribution-free test was used with 2 tails for analyzing data from the total patients. *P* values for the NMA, 200 TBI, and 1200 TBI patients were 0.857, 0.749, and 0.976, respectively.

NR indicates nonresponders by RECIST criteria; NMA, non-myeloid ablation; PR+CR, objective partial responders and objective complete responders by RECIST criteria; RECIST, Response Evaluation Criteria in Solid Tumors; TBI, total body irradiation; TIL, tumor-infiltrating lymphocytes.

TABLE 2.

Process Optimization Resulted in Improved Yield and Purity of the CD8⁺ TIL

ID	Starting Cell No. (×10 ⁶)	Bead:Buffer (mL)	Recovered Cells (%)	Initial TIL (%)			CD8 ⁺ Fraction (%) [*]	
				CD8 ⁺	CD4 ⁺	CD8 ⁺	CD8 ⁺	CD4 ⁺
1 [‡]	3.14	7.5:100	36	80	14	78	7	7
2	0.85	3.5:200	72	75	23	87	7	7
3	0.63	1.0:200	62	60	7	82	1	1

^{*} CD8⁺ and CD4⁺ fractions do not sum to 100% because of CD8[−] CD4[−] cells including CD3[−] CD56⁺ natural killer cells.

[‡] Sequential clinical scale enrichments were performed with the conditions shown. Only enrichment number 1 was performed with centrifugations in flexible bags. Other enrichments were performed in 250 mL coming centrifuge tubes.

TIL indicates tumor-infiltrating lymphocytes.

TABLE 3.

Highly Purified CD8⁺ TIL Were Routinely Obtained With Sufficient Starting Sample and Use of the Optimized Enrichment Process

ID	Starting Cell No. ($\times 10^6$)	Bead:Buffer (mL)	Recovered Cells (%)	Initial TIL (%)			CD8 ⁺ Fraction (%) [*]	
				CD8 ⁺	CD4 ⁺	CD8 ⁺	CD8 ⁺	CD4 ⁺
4	0.41	1.0:400	44	60	20	97	97	1
5	0.76	1.0:400	62	83	6	96	96	2
6	0.91	1.0:400	79	21	15	95	95	1
7	1.06	1.0:400	94	49	31	93	93	1
8	1.10	1.0:400	82	78	4	94	94	0
9	1.17	1.0:400	87	78	16	99	99	1

* CD8⁺ and CD4⁺ fractions do not sum to 100% because of CD8⁺CD4⁺ cells including CD3⁺CD56⁺ natural killer cells.

TIL indicates tumor-infiltrating lymphocytes.

TABLE 4.

Tumor Recognition Was Improved by CD8⁺ Enrichment of TIL

Patient (HLA-A2)	Interferon- γ (pg/mL)					Autologous
	None	888 mel	938 mel	526 mel	624 mel	
Patient 1 (A2 -)						
Unseparated TIL	34	20	85	19	45	96
CD8 ⁺ enriched	62	119	315	48	73	246
Depleted (CD4 ⁺)	52	34	30	36	43	47
Patient 3 (A2 -)						
Unseparated TIL	17	7460	116	21	316	
CD8 ⁺ enriched	35	9020	72	101	256	ND
Depleted (CD4 ⁺)	127	87	101	62	113	
Patient 4 (A2 -)						
Unseparated TIL	102	49	103	59	118	
CD8 ⁺ enriched	67	105	121	62	163	ND
Depleted (CD4 ⁺)	130	85	127	124	141	
Patient 5 (A2 +)						
Unseparated TIL	265	136	345	436	662	781
CD8 ⁺ enriched	335	272	477	562	1009	5920
Depleted (CD4 ⁺)	280	311	401	445	474	653
Patient 6 (A2 +)						
Unseparated TIL	188	55	130	381	1550	338
CD8 ⁺ enriched	65	115	100	794	2810	530
Depleted (CD4 ⁺)	200	114	123	132	310	209
Patient 7 (A2 -)						
Unseparated TIL	166	48	138	29	79	2380
CD8 ⁺ enriched	77	105	203	62	102	2440
Depleted (CD4 ⁺)	61	36	43	59	60	582
Patient 8 (A2 -)						
Unseparated TIL	91	1610	259	136	193	

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Patient (HLA-A2)	Interferon- γ (pg/mL)					Autologous
	HLA-A2 –		HLA-A2+			
	None	888 mel	938 mel	526 mel	624 mel	
CD8 ⁺ enriched	185	5950	473	182	368	ND
Depleted (CD4 ⁺)	120	161	136	213	185	
Patient 9 (A2 –)						
Unseparated TIL	257	100	193	138	551	4060
CD8 ⁺ enriched	181	232	337	159	517	5570
Depleted (CD4 ⁺)	883	449	459	557	380	711

HLA indicates human leukocyte antigen; TIL, tumor-infiltrating lymphocytes.