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Tissue Harvesting for Adoptive Tumor Infiltrating Lymphocyte Therapy in Metastatic Melanoma

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Abstract. *Background/Aim: Adoptive transfer of tumor-infiltrating lymphocytes (TILs) combined with non-myeloablative chemotherapy (NMA) has been shown to prolong survival in patients with metastatic disease. Materials and Methods: Tissue harvesting was performed form a variety of sites. TILs were isolated, expanded and infused with bolus high-dose IL-2. Results: Between 2008 and 2018, 242 lesions were resected for TILs harvesting from a range of sites form 196 patients without mortality and with minimal morbidity. Of those harvested, 75 were unable to complete therapy because of clinical deterioration during the wait period. Of 121 evaluable treated patients, there was no effect of metastatic site biopsied on the mean fold TIL expansion. Those receiving prior ipilimumab had a higher TIL fold expansion but a lower TIL fold expansion than those exposed to anti-PD1 therapy. Conclusion: Harvesting may be safely performed with successful TIL expansion from most sites. Prior check point inhibitory immunotherapy may potentially influence TIL fold expansion.*

Until recently, metastatic melanoma had a poor prognosis with an average survival <1 year (1). There have been a range of recent developments in immunotherapy that offer new promise which include monoclonal antibody therapies directed toward checkpoint inhibitory molecules (*e.g.* anti-CTLA-4 – ipilmumab) and anti-PD-1 antibodies, (pembrolizumab, nivolumab). Both of these treatments are designed to interrupt signal pathways which inhibit antigen-restricted T cell activation (2, 3). Another approach uses a variety of adoptive T cell (ACT) treatments, which take advantage of the endogenously elicited immune response (4, 5). By comparison with older conventional therapies such as interleukin-2 (IL-2) and dacarbazine (DTIC) where objective tumor regression was possible in only 15-20% of patients (6), ACT has consistently shown high response rates with reported long-term sustained remissions and survival (7-9) that may be comparable to PD-1-based blockade (2). ACT has consistently shown high response rates with reported long-term sustained remissions and survival (7-9) in metastatic melanoma that may be comparable to PD-1-based blockade (2). In addition, ACT has shown promise in other malignancies as well (10-12). Adoptive transfer of autologous *ex vivo*-expanded tumor-infiltrating lymphocytes (TILs), originally pioneered by Rosenberg and colleagues, has specific advantages capitalizing on the proliferation of polyclonal populations of T cell tumor infiltrates capable of recognizing multiple shared melanoma tumor-associated antigens (TAAs) and mutation-derived neoantigens (5, 8, 13). An enhanced ACT success has been achieved by patient pre-conditioning using non-myeloablative chemotherapy (NMA) with cyclophosphamide and fludarabine with or without the addition of total body irradiation (TBI) following which there is an infusion of large numbers of autologous TILs (14). The TIL-ACT approach is supplemented with high-dose bolus IL-2 designed to prolong adoptive TIL survival with this combined therapeutic regimen showing a 50% response rate in metastatic melanoma patients (15-17). In this respect, Rosenberg and colleagues have shown increased long-term response when compared with historical controls treated with conventional pre-immunotherapy era therapies (8). We previously reported a 40% objective

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response rate in 57 patients with a 15.2 month median survival, (9) comparable to published response rates ranging between 40-60% which have been reported from the few centers worldwide that are using a combined lymphodepletion, ACT and high-dose IL-2 protocol (15, 18, 19).

There are a number of novel therapeutic ACT strategies under development, based on the isolation and cultivation of TAA-specific T cells derived from melanomas. These cells can be obtained from peripheral blood mononuclear cells (PBMC) or from T cells transduced *ex vivo* either with TAA-reactive T-cell receptor (TCR) genes or chimeric antigen receptors (CARs) specific for melanoma cell surface antigens (14, 20, 21). As all patients require fresh tumor tissue for the harvesting of TILs which then need to be cultured and expanded, the choice of the most appropriate metastatic site (or sites) where a harvest can be safely performed should be personalized for each patient. Our group has previously shown that certain tumor sites may generate TILs more successfully than others (22), where TILs with an increased *in vitro* anti-tumor reactivity have been derived from lung lesions as opposed to non-lung metastatic sites. These types of considerations must be weighed, however, against the morbidity and complexity of the surgery involved and the impact of a range of anti-melanoma pre-treatments which could potentially affect successful TIL culture and growth. We provide an update of our ongoing program from the Ella Lemelbaum Institute of Immuno Oncology at the Chaim Sheba Medical Center, Israel presenting surgical considerations relevant to TIL harvesting in ACT-eligible patients.

Patients and Methods

Patients. Permission was provided by the Institutional Review Board for the conduct and analysis of the data, which are maintained in a dedicated prospective database. Participating patients signed an informed consent which had been approved by the Israeli Ministry of Health (approval No. 3518/2004) in accordance with the conduct of a prior registered clinical trial - NCT 00287131 (clinicaltrials.gov). The Ella Lemelbaum Institute for Immuno Oncology, within the Chaim Sheba Medical Center, is the principal tertiary referral center for melanoma and immunotherapy treatment in Israel. Inclusion criteria permitted analysis of stage IV melanoma patients with an ECOG performance status of 0-2, measurable disease and a life expectancy of at least 3 months. Exclusion criteria were: age of patients <18 years, HIV-positivity and Hepatitis B and/or C-positivity.

Tissue resection and TILs harvesting. Patients underwent tissue harvesting prior to initiating the ACT protocol with the production of young TILs as has been previously described (9, 23). In brief, preliminary surgical consultation was made for determining the most appropriate site for tumor excision and TIL harvesting. This decision was based upon tumor mass, size, location, Positron emission tomography (PET) avidity (as a qualitative measure of cellular infiltrate) and ease of access. Following harvest, after a combined collagenase and DNase digestion of the tumor (either for 2-3 h at 37˚C or overnight at room temperature) the tissue was broken up into 1-3 mm^3 fragments and then transferred to 24-well plates. Cells were cultured in medium with 3,000 IU/ml rhIL-2 (Proleukin, Chiron B.V.) for 2 to 4 weeks. A cut-off for established cultures was defined as 80×10^6 T cells. Unselected TIL cultures were further expanded *ex vivo* to treatment levels in a 14-day rapid expansion procedure (REP) using anti-CD3 antibody (Orthoclone OKT3, Janssen-Cilag: Johnson and Johnson, New Brunswick,NJ USA 3,000 IU/mL rhIL-2 and irradiated feeder cells as previously described (24, 25). On the day of infusion, reduction of the culture medium volume was performed using a COBE Spectra apheresis machine (COBE Laboratories Inc. Lakewood, CO, USA) (26).

Prior to NMA conditioning, granulocyte colony-stimulating factor (GCSF)-mobilized stem cells were obtained with leukapheresis as a safety net for those cases unable to reconstitute their hematopoietic system.

Patient treatments. Up until January 1st 2018, patients received an NMA conditioning regimen of cyclophosphamide (60 mg/kg for 2 days) plus fludarabine (25 mg/m^2 for 5 days) prior to cell infusion. Because of 3 mortalities potentially attributable to cyclophosphamide-induced cardiomyopathy, this NMA protocol was changed to fludarabine (25 mg/m^2 for 3 days) and Total Body Irradiation (TBI) (200 cGy for one day). Young TILs were administered intravenously followed by bolus high-dose IL-2 (720,000 IU/kg) every 8 h to tolerance (with a maximum of 15 doses). Following TIL infusion, patients commenced preventative therapy with GCSF (10 mg/kg daily) plus fluconazole and acyclovir until the absolute neutrophil count reached 1,000/μl. Platelets were transfused when the platelet count was <20,000/μl. Patients also received sulphamethoxazole /trimethoprim twice weekly for at least 6 months (where possible) until the CD4 counts reached 200/μl.

Statistical analysis. Clinical data were analyzed as part of the ongoing prospective clinical trial. Data were entered and analyzed in SPSS version 23.0. (Version 23.0; SPSS Inc., Chicago, IL, USA). Descriptive statistics were produced using frequencies (N, %) for categorical variables (*e.g.* gender) and means and standard deviations or medians and ranges for continuous variables (*e.g.* age). The cohort was divided by source of tissue harvest in order to explore the fold expansion means differences using the one-way ANOVA test. To predict primary outcome (High fold expansion), multivariate analysis (logistic regression) was conducted to obtain Odds-Ratios (OR) with 95% confidence intervals (CI). p-Values <0.05 were considered significant.

Results

Between 2008 and 2018, 242 lesions were surgically resected for TIL harvesting from 196 patients eligible for the ACT protocol. This cohort included some patients who underwent tissue harvest from more than one source. There were 75 females and 121 males (overall median age 53 years, range=18-70 years). The most common sources of harvested tumor tissue were subcutaneous nodules (93/242; 38.43%), lymph nodes (58/242; 24%), lung nodule excisions (47/242; 19.42%), and other sites, which included visceral organs such as the liver, spleen, gall bladder, adrenal, small bowel and colon, (32/242; 13.22%). There were other miscellaneous sites (12/242; 5%) which included the CNS,

Table I. *List of sites for TIL harvesting (n=242).*

Tissue source	Number (%)
Subcutaneous	93 (38.4)
Lymph node	58 (24)
Lung	47 (19.4)
Visceral	32 (13.2)
CNS	5 (2.1)
Muscle	4 (1.7)
Bone	2 (0.8)
Breast	1 (0.4)

Table II. *Multivariate analysis comparing the predicted TIL fold expansion according to age, gender, BRAF mutation status or prior treatment with the anti CTLA-4 antibody ipilimumab or anti-PD1 antibody therapy (pembrolizumab/nivolumab). Data are shown as Odds Ratios (OR) + 95% Confidence Intervals (CI).*

Parameter	OR	*p*-Value	95%CI
Age (>53 years)	0.646	0.343	0.261, 1.594
Female gender	0.447	0.092	0.1751, 0.142
BRAF mutation (positive)	1.018	0.976	0.325, 3.191
Prior BRAF inhibitor therapy	1.000	1.000	0.259, 3.866
Prior ipilimumab therapy	2.850	0.083	0.871, 9.329
Prior anti-PD1 antibody therapy	0.529	0.311	0.154, 1.813

Multivariate regression: R^2=0.01, χ^2 (6)=7.02, p=0.32.

muscle, bone and breast tissue (Table I). Of these 242 operations, 162 (66.9%) were performed under general anesthesia and 80 (33.1%) under local anesthesia.

Mortality with severe surgical morbidity (defined as any complication that resulted in delay in initiation of the NMA lymphodepletion protocol) was limited to three cases. In one, there was a complete dehiscence of the laparotomy wound with evisceration which required urgent reoperation and repair. In another case, there was a persistent pancreatic leak following splenectomy which required long term drainage. The third case presented with persistent pleural effusion which required ongoing thoracocentesis.

There were 75 patients (38.2%) who underwent harvesting surgery but who did not complete the treatment plan. The majority of them had clinical deterioration before TILs could be infused. Within this group, there were 3 chemotherapy-related deaths (all of which were cyclophosphamide-induced cardiomyopathy) as well as those patients whose TIL cultures could not be generated successfully. Overall, 105 patients were able to undergo the complete treatment protocol with infusion of both autologous TILs and rhIL-2 following NMA lymphodepletion. Another 16 patients are currently awaiting treatment resulting in a total of 121 patients whose TILs will be successfully cultured and expanded ready for infusion. The mean fold expansion after REP was 1,083±499 (median 1,062, range=207-2667). After excluding groups such as the CNS and others with small sample sizes, one-way ANOVA with *post-hoc* testing showed no significant difference in the average fold expansion between the main sites (F (3.99)=0.559, p=0.64).

Groups were separated according to the median fold expansion value (≤ or >1062) and the median age (≤ or >53 years) and the impact of gender, BRAF mutation status, prior treatment with BRAF inhibitors and previous treatment with ipilimumab, pembrolizumab or nivolumab on predictably larger TIL fold expansion was analyzed by logistic regression (Table II). Although overall no statistically significant differences were noted which might predict for a greater or lesser TIL fold expansion (p=0.32), patients who had received ipilimumab therapy prior to tissue harvesting were more likely to show a higher fold TIL expansion (OR=2.85, 95%CI=0.87-9.33; p=0.083) whereas prior anti PD-1 treatment had a negative impact on TIL fold expansion (OR=0.53, 95%CI=0.15-1.81; p=0.3). Older age and female gender were also associated with a lower TIL fold expansion although without significance.

Discussion

In this study, of 242 lesions resected from a range of sites for TIL harvesting for the purpose of treating patients with metastatic melanoma, no differences were noted in TIL expansion between the sites of biopsy. A higher fold TIL expansion was, however, found in those cases previously receiving the anti-CTLA-4 antibody ipilimumab whereas less TIL fold expansion was observed in patients previously treated with anti-PD-1 monoclonal antibody therapy (pembrolizumab or nivolumab).

Tumor-infiltrating lymphocyte (TIL) therapy has both theoretical and practical advantages in the management of stage IV melanoma. Large populations of TILs can be selected *in vitro* for the adoptive transfer of cells (ACT) which are designed to overcome tumor tolerance. This approach is combined with a pretreatment regimen of lymphodepletion capable of modifying the host and stimulating an optimal anti-tumor microenvironment by potentially eliminating immunoregulatory (Treg) cells and myeloid-derived suppressor cells (27) as well as by reducing the competition for cytokines normally promoting lymphocyte growth (28). It has been suggested that there is a better anti-tumor responsiveness when, after infusion, there are persistent TILs with multiple tumor-reactive T cell clonotypes detectable both in the peripheral blood and within tumor deposits (29, 30).

Although our group has previously shown that lung-derived TILs tend to have a higher rate of culture success where larger cell numbers with an enhanced anti-tumor reactivity are

generated (22), the present analysis indicates that tissue source per se is not a guarantee of a larger fold expansion of TILs. Although the lungs comprise between 15-35% of melanoma metastases, viable TILs have been able to grow from the majority of patients and from most sites of resection (31). Therefore, in contrast to the previous study, the data in this study suggest that biopsy site is less important in fold expansion of harvested TILs and the selection by prior immunotherapy of likely immuno-stimulatory TIL subpopulations is noteworthy. As in our case, however, there will be patients who undergo TIL harvesting but are not treated largely because of clinical deterioration, a status which has partly been obviated by the use of TILs with a shorter growth cycle, termed "young TILs" (7, 25, 32, 33). In this setting, as the NMA commences 7 days before TIL infusion, patients selected for therapy should be fit enough to undergo treatment about 3 weeks post-surgery and the predicted risk of surgical complications associated with larger biopsy procedures should be deemed minimal. Specifically, the morbidity of any proposed procedure needs to account for the patient being fit for treatment in a timely fashion. Our cohort included 3 patients whose treatment was delayed because of their post-surgical morbidity, which can have important oncological consequences. The typical patient chosen for ACT has already progressed through a range of conventional therapies. The surgeon's role is critical in patients with a limited life span or with extensive tumor burden so that the patient is correctly selected for ACT as is the surgical approach likely to result in a high TIL yield with minimal morbidity. There is also the option of cryopreservation of cells for delayed treatment. In general, although tumor size does not correlate with TIL efficacy, lesions generally >2 cm in diameter should be chosen so as to ensure adequate tissue processing, avoiding those tumors with potentially necrotic or infective elements (34).

There was an enhanced TIL expansion in patients who received ipilimumab and conversely a reduced TIL expansion if there was prior anti-PD-1 therapy. Anti-tumor T cell responses are regulated *via* a complicated balance of inhibitory and activating signals by which some tumors escape immune surveillance. Checkpoint inhibitory molecules are designed to rescue exhausted anti-tumor T cell populations and restore TCR activation through different, but complementary mechanisms (35). The anti-CTLA-4 monoclonal antibody ipilimumab inhibits the interaction of CD80/86 on antigen presenting cells with CTLA-4 on T cells *via* CD28, blocking the inhibition of T cell co-stimulation and reversing an expected suppression of T cell activation (36). Ipilimumab, which has shown an improvement in overall survival of patients with metastatic melanoma in two phase III randomized controlled trials (37, 38) enhances anti-tumor activity and TIL persistence and maintains a broad repertoire of TCR specificities lowering the threshold for TCR activation and permitting clonal expansion (39).

By contrast, PD-1 inhibitors are implicated in reverting peripheral tolerance by blocking the binding of PD-1 ligands (PD-L1 and PD-L2) and restoring the immune capacity of T cells in the periphery which have been previously chronically activated. Both pembrolizumab and nivolumab also affect the suppressive function of Tregs and other dampening effector T cells, normally present in ongoing sites of inflammation within tumors, which utilize the PD-1 axis as a shield from immune-mediated destruction. The effects of anti-PD-1 therapy on the signaling of B cells and antigen presenting cells will also result in T cell-independent immune stimulation (40). The association, however, between the inflammatory tumor infiltrate and the use of checkpoint inhibitory immunotherapy is complicated since infused TILs can trigger their own inhibition by secreting cytokines which drive the PD-1 axis and its production of inhibitory ligands. Some of these paradoxical effects allow TILs to actually thwart their immunostimulatory impact by producing inflammatory cytokines which can induce tumor-derived PD-1 expression and secondarily promote T cell apoptosis (41, 42).

The nature of the immune tumor microenvironment affects melanoma survival and may precede anti-PD-1 therapy (43). Despite there being an association between the outcome in advanced melanoma and the degree of inflammatory infiltrate into the tumor (44), geographic localization of infiltrate with PD-L1 expression at the edge of tumor can cause adaptive immune resistance. This is likely in response to prior immunotherapeutic treatments (45).

In this context, the positive correlation between the presence of an inflamed tumor microenvironment and the response to agents such as ipilimumab would imply that prior immunotherapies can protect or potentiate ongoing rather than *de novo* anti-tumor immunity (46). For different melanomas, the persistence of TILs is important in the anti-tumor response, however, this may also reflect a selective feedback mechanism of immune resistance during immunotherapy which is induced by the tumor infiltrate itself. The nature of this interaction between TILs, the induced cytokine profile and the local checkpoint inhibitory ligand expression needs further assessment along with the correlation between these parameters and the composition of the infiltrate, the persistence of inflammation and the type of immunotherapy used.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

Study Concept and Design: DZ, JS MJB; Data Acquisition: OFE, SR, DH, GS; Statistical Analysis: GS; Data Analysis and

Interpretation: DZ, GM, OI, MJB; Manuscript preparation: DZ; Manuscript editing and review: DZ, GM, AN, MJB.

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