Development of increased potency tumor specific CD4 T cell products for cancer treatment

Immunotherapy, particularly immune checkpoint inhibitors (ICI), has transformed treatment of melanoma and multiple other types of cancer[1-3], but many patients do not benefit or develop resistance, possibly due to insufficient numbers of functional tumor-specific T cells or a non-permissive tumor microenvironment (TME).[4] Most studies have focused on the role of cytotoxic CD8+ T cells in tumor immunity, but "helper" CD4+ T cells are required for ICI efficacy in animal models,[5] and the adoptive transfer of tumor-specific $CD4^+$ T cells alone can promote remissions in cancer patients.[6,7] Most tumors are class II MHC negative[8] but $CD4^+$ T cells can be activated by class II^+ antigen presenting cells (APC) and contribute to tumor regression through indirect mechanisms.[9-12] We recently used targeted single cell RNA sequencing (scRNA seq) and matching of T cell receptor (TCR) sequences to identify the transcriptional signatures and functional correlates of tumor-specific $CD4^+$ T cells infiltrating human melanoma.[13] Tumor-specific $CD4^+$ T cells but not virus-specific $CD4^+$ T cells or $FoxP3^+$ regulatory T cells were defined by expression of CXCL13, and the $CXCL13^+$ $CD4^+$ T cells could be subdivided into clusters expressing memory markers, T follicular helper (T_{FH}) markers, or cytolytic markers and IFN-γ. Notably, the frequency of $CXCL13^+$ $CD4^+$ T cells correlated with numbers and activation of $CD8^+$ T cells, the chemokine signature of macrophages and maturation of B cells in the TME, and predicted patient survival (**Preliminary data**).[13] *These findings led us to hypothesize that a sufficient tumor specific $CD4^+$ T cell response is essential for a pro-immune TME and that the adoptive transfer of tumor specific $CD4^+$ T cells to augment this response could mediate antitumor activity and improve response to ICI.*

Adoptive transfer of ex-vivo expanded tumor infiltrating lymphocytes (TIL) has induced remissions in a broad variety of cancer types including breast cancer, GI cancers, and cervical cancer, and importantly has activity in ICI refractory melanoma and non-small cell lung cancer.[6,14-18] Therapeutic TIL products contain CD8+ as well as CD4+ T cells that recognize tumor antigens, and while it is not known which cell fraction mediates clinical responses, tumor antigen specific CD4+ T cells form significant fractions of cells in TIL products of responding patients, including a patient with cholangiocarcinoma who had a durable complete response following infusion of TIL composed entirely of CD4+ T cells.[6] TIL therapy has two critical shortcomings. The first is that it is expanded from mixtures of tumor reactive and tumor non-reactive T cells, and because the tumor reactive populations are functionally impaired, they are often lost or reduced in frequency during the expansion process[19] (**Preliminary data**). Our work has identified a phenotype that may enable selective enrichment tumor reactive CD4+ T cells away from tumor nonspecific bystander T cells prior to T cell expansion.[13] Similar characteristics have been identified in multiple other cancer types,[20] suggesting this strategy could be broadly applied to isolate tumor specific cells. The second shortcoming is the loss of T cell function during ex vivo culture, due to terminal differentiation to cell states with less therapeutic efficacy.[21] Previous work focused on CD8+ T cells has found that modulation of TCR signaling duration[22] and of metabolic states through inhibition of AKT signaling[23] or extracellular potassium concentrations[24] can preserve stemness of T cells during expansion. Little is known about the capacity of these interventions to preserve function in tumor specific CD4+ T cells. We hypothesize that enhanced CD4+ T cell products capable of modifying the tumor microenvironment and supporting $CD8^+$ T cell function can be derived from TIL through enrichment of tumor specific CD4+ T cell subsets prior to expansion and by preserving functionality of these cells during culture. The specific aims are:

Aim 1: Develop a workflow for enrichment and expansion of CXCL13+ CD4+ T cell subsets from melanoma patients. Populations of conventional CXCL13+ CD4+ T cell subsets that contain T cells specific for tumor neoantigens and self-antigens will be enriched by flow cytometry based on a constellation of markers and expanded using different cell culture approaches. Maintenance of the diversity of tumor reactive clonotypes will be monitored by TCRVb sequencing and compared to conventional TIL culture methods from the same samples.

Aim 2: Develop strategies for minimizing differentiation and exhaustion in expanded CD4+ T cell products. The clinical activity of T cell products used for adoptive therapy can be enhanced by preserving stem like properties in $CD8^+$ T cells, but this strategy has not been examined for tumor reactive CD4+ T cells. We will culture cells using modulators of T cell receptor signaling and metabolic states of T cells and monitor the maintenance of a stem like memory phenotype by flow cytometry, epigenetic profiling, and ability to proliferate in response to stimulation with tumor antigens.

Innovation

This project will leverage recent discoveries that identity novel phenotypic characteristics of tumor reactive T cells to isolate these therapeutically critical cells and manufacture a more potent clinical product. We will apply innovative approaches previously studied in CD8+ T cells to determine if stem like qualities can be preserved in tumor specific $CD4^+$ T cells following in vitro expansion. If successful, this project will enable clinical trials of tumor derived CD4+ T cell products alone and with $CD8^+$ T cells in ICI inhibitor refractory cancers.

B. Preliminary Data

a). Identification of neoantigen-specific $CD4^+$ T cell TCRVb clonotypes in melanoma TIL: Using targeted single cell RNA sequencing (scRNA seq) and matching of T cell receptor (TCR) sequences, we identified transcriptional signatures and functional correlates of tumor antigen-specific $CD4^+$ T cells infiltrating human melanoma. This analysis has been performed recently for tumor-specific and bystander $CD8^+$ T cells,[25-27] but not for $CD4^+$ T cells. We performed whole exome sequencing on tumor and normal cells and RNA sequencing of tumor from 4 patients to identify and rank prevalent nonsynonymous mutations that could serve as tumor neoantigens. We then isolated $CD4^+$ T cells from each of the patients that were specific for candidate neoantigens, tumor associated self-antigens and viral antigens. The specificity of neoantigen-specific and viral specific TCR clonotypes was confirmed by cloning of patient T cells or by gene transfer of TCR sequences.

b) A signature of tumor antigen specific $CD4^+$ T cells: Sorted $CD4^+$ T cells from TIL samples of 4 melanoma patients were stained with 53 oligonucleotide labelled antibodies and analyzed by targeted scRNA seq of 405 immune response genes and TCR VDJ rearrangements.[28] Unsupervised clustering of cells by mRNA expression defined distinct populations of $FoxP3^+$ regulatory T cells (T_{REG}), $CXCL13^+$ non-T_{REG} conventional T cells (T_{CONV}) that expressed surface PD-1, and $CXCL13^-$ T_{CONV} that expressed high levels of IL7R mRNA (**Fig. 1A**). 259 cells (17-146 per patient) expressed 40 different TCR clonotypes (2-18 per patient) specific for neoantigens, 108 cells expressed 14 TCR clonotypes specific for self-antigens, and 9 cells expressed 5 TCR clonotypes specific for viral antigens (**Fig. 1B**). Strikingly, alignment of TCRVb sequences placed 97% of neoantigen-specific and 99% of self-antigen specific T cells in $CXCL13^+$ clusters, and all 9 virus-specific T cells in the $CXCL13^-$ T_{CONV} cluster. Three distinct $CXCL13^+$ phenotypic clusters contained tumor antigen specific cells. One cluster had increased expression of memory genes (TCF7, IL7R) and genes (BCL6 and CD200) and surface markers (CXCR5) associated with T follicular helper (T_{FH}) cells. A second $TCF7^-$ "effector" population expressed co-inhibitory (Tim-3, LAG3), inflammatory (CCL3, CCL4, IFNG), cytolytic (GZMA/K, PRF1) markers. (**Fig. 1A**). This suggested that tumor reactive CD4+ T cells were heavily enriched in the CXCL13+ PD-1 high subset.

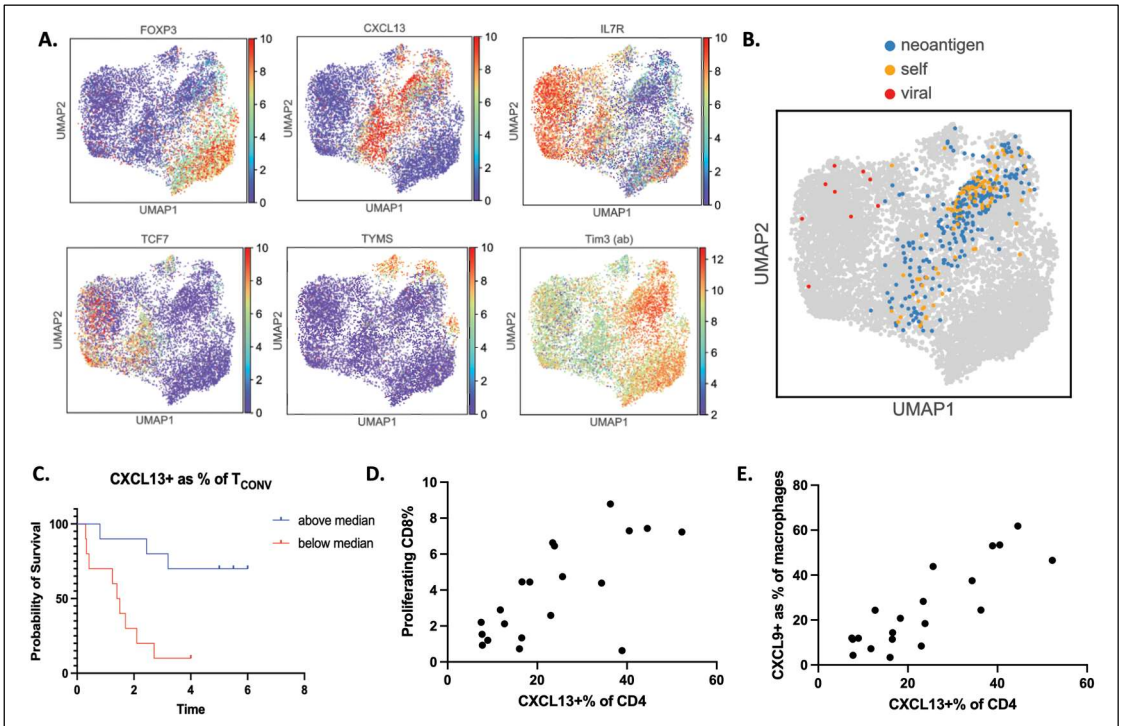

Figure 1. Phenotype of tumor antigen specific conventional CD4+ T cells in melanoma A. 10,186 CD4+ T cells from 4 primary melanoma samples were sequenced and visualized by UMAP, with expression of the indicated genes. B. Cells from (A) with tumor and viral antigen specific TCR sequences are shown. C. survival of 20 melanoma patients by fraction of conventional T cells that were CXCL13+. D. Proliferating CD8+ T cells as a fraction of CD45+ cells and E. CXCL9+ cells as a fraction of macrophages were correlated to CXCL13+ CD4+ cells as a fraction of CD4+ T cells.

c) CXCL13^{+} CD4^{+} T cells are predictive of survival and activation of the immune tumor microenvironment (TME) in melanoma: We then conducted single cell analysis of CD45^{+} immune cells from a cohort of 20 melanoma patients. The fraction of CXCL13^{+} CD4^{+} T_{CONV} varied between 7% and 55% of tumor infiltrating CD4^{+} T cells and those patients with greater than the median of CXCL13^{+} CD4^{+} T cells as a fraction of CD4^{+} T_{CONV} cells exhibited improved survival (**Fig. 1C).** The frequency of CXCL13+ CD4+ T cells correlated with several different measures of TME activation, including the number of proliferating CD8+ T cells present (R=0.65, p= .005), and the activation of macrophages as measured by expression of the lymphocyte recruiting chemokine CXCL9 (R=0.83, p<0.0001, **Fig. 1D-E**).

d) Tumor antigen specific CD4+ T cell clones are lost during standard TIL therapeutic product generation. Cellular products were generated from 3 patients by standard TIL tumor fragment cultures from unselected cells in tumors, and TCR sequencing of the initial tumor and of the therapeutic product illustrated that expanded clones from within the CXCL13+ CD4+ clusters were lost both in terms of total fraction of the T cells (from 3-15% of T cells to less than 0.5%) and in terms of number of unique clones (**Fig. 2A**).

e) Isolation of CXCL13+ CD4+ T cells by flow cytometry. In order to isolate tumor antigen specific CXCL13+ CD4+ T cells from tumors, we used our sequencing data to develop a flow cytometry strategy that would allow enrichment of these cells. CD3+ CD4+ CD25- T_{CONV} were sorted by FACS into PD-1^{LOW} CD127^{HIGH} "bystander", PD-1^{HIGH} CD200^{HIGH} CXCR6^{-} TFH cells and PD-1^{HIGH} CD200^{HIGH} CXCR6^{HIGH} "effector" cell populations. This sorting strategy reliably separated cells into CXCL13- bystanders, CXCL13+ TCF7+ T_{FH} cells and CXCL13+ TCF7- effector cells as measured by RNA sequencing after FACS sorting (**Fig. 2B**). Sorted T_{FH} cells showed increased proliferation after anti CD3/CD28 bead stimulation relative to TCF7- "effector" cells (**Fig 2C**), consistent with them being a more stem-like population.

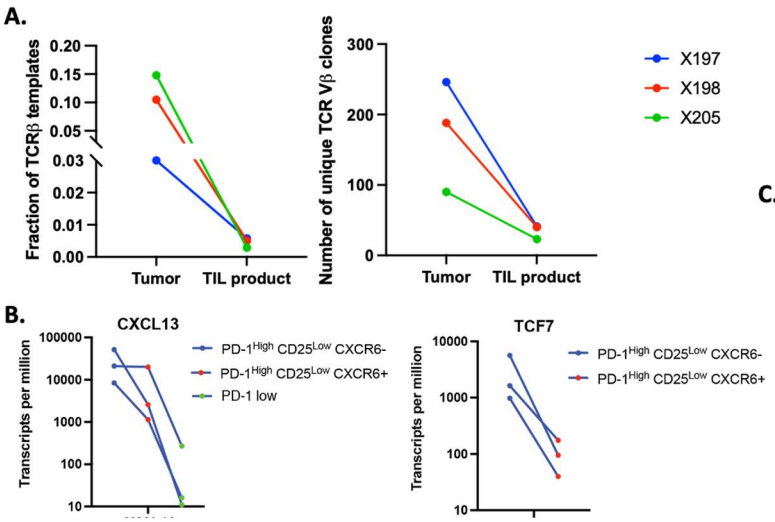

Figure 2. Tumor antigen specific CD4+ T cell clonotypes are lost during standard TIL culture, and isolation of CXCL3+ CD4+ subsets by flow cytometry. A. TCR sequences from expanded clones in CXCL13+ CD4+ T cell clusters from 3 melanoma patients were quantitated in primary tumor and conventional expanded TIL product by TCRVB sequencing. B. The indicated phenotype of cells from 3 melanoma patients were sorted by FACS and the expression of CXCL13 and TCF7 were quantitated by RNA sequencing. C. The indicated fractions of CD4+ T cells were sorted from 4 melanoma cases and stimulated with anti CD3/CD28 beads and proliferation was measured 4 days

Experimental design and methods

Aim #1: Develop a workflow for enrichment and expansion of CXCL13+ CD4+ T cell subsets from melanoma patients

It is possible that tumor reactive CD4+ T cells have a proliferative disadvantage relative to non-tumor reactive bystander T cells in standard TIL tumor fragment cultures, and that isolation of tumor reactive populations prior to expansion can generate therapeutic products with increased representation of tumor reactive cells. We have previously successfully expanded PD-1 high CD4+ T cell populations from melanoma tumors using a culture protocol comprised of high dose IL-2, irradiated allogeneic feeder cells, and stimulation with an anti-CD3 antibody.[13,29,30] We will now determine whether this maintains the diversity of TCRs represented in the tumor reactive subset in TIL better than traditional tumor fragment cultures used to make TIL products.

We will utilize a cohort of 10 patients with metastatic melanoma enrolled in a trial of therapeutic TIL treatment at the [redacted]. Cryopreserved tumor single cell suspensions will be stained with fluorescently labelled antibodies against CD3, CD4, CD25, CD127, CD200, PD-1, and CXCR6. The two cell populations containing tumor antigen specific cells, CD4+ CD127- CD25- $PD1^{HIGH}$ $CD200^{HIGH}$ CXCR6- T_{FH} cells will be sorted separately from CD4+ CD127- CD25- $PD1^{HIGH}$ $CD200^{HIGH}$ CXCR6+ effector cells and rested overnight in 5ng/ml IL-7 followed by expansion with anti CD3 (OKT3), IL-2 (6,000U/ml) and irradiated allogeneic feeder cells. CD200 will be used in addition to PD-1 to identify cells to facilitate analysis of samples with clinical PD-1 blocking antibodies present. Cytokines will be replaced every 7 days and growth will be monitored for 3 weeks. Because tumor specific CD4+ T cell populations uniformly express PD-1, it is possible that PD-1 binding to ligands on costimulatory feeder cells could mediate suppression of proliferation of these cells ex vivo. In order to test whether blocking this potential interaction might be beneficial, we will perform parallel stimulations without the presence of an PD-1 blocking antibody (Atezolizumab biosimilar, BioXcell).

The representation of T cell clonotypes from either validated tumor antigen specific CD4+ T cell clones (n=5 patients) and T cell clones inferred to be enriched for antigen specificity based on presence in at least two cells within the CXCL13+ CD4+ cell clusters seen by single cell RNA sequencing (preliminary data, n=10 patients) will be quantitated by TCRVb deep sequencing in initial TIL and expanded cultures using the human TCRB kit (Adaptive Biotechnologies). Preservation of the confirmed and inferred tumor antigen specific T cell clonal repertoire will then be compared between standard TIL culture and our method to see whether enrichment of these cells prior to expansion increases their purity in expanded cultures.

Biostatistics/analysis plan

TCRVb sequencing data will be analyzed by the Immunoseq analyzer software (Adaptive Biotechnologies). Fractions of T cell clones with confirmed specificity for tumor antigens or inferred specificity based on presence in CXCL13+ clusters on single cell sequencing of primary tumor samples (preliminary data) will be compared between the initial tumor sample, sorted T cells, and expanded T cells from either the tumor fragment culture (control) or after culture of sorted T cells. Comparison of the total number of clones and cumulative frequency of clones will be performed using a ratio paired t test.

Expected results/pitfalls

We expect that isolation of tumor reactive subpopulations away from competing bystander cells will allow for expansion of products containing a higher representation of these cells. We expect that the TCF7- population will have impaired proliferative potential relative to the TCF7+ population (**preliminary data**). If the TCR repertoire of sorted cells does not match the populations identified by single cell sequencing, we will explore additional surface markers such as Tim-3 and CXCR5 for separation of these populations. If we see insufficient growth or skewing or repertoires with our initial culture conditions, we can use isolated cells to develop optimized culture conditions, by varying method of stimulation (anti CD3 versus phytohemagglutinin), presence of additional costimulatory feeder cells (LCL lines[31]) and additional cytokine support (IL-15, IL-21[32]). It is possible that multiple rounds of culture will be necessary to obtain sufficient cells for clinical translation, and also possible that the Tcf1+ subset will expand to a greater degree than the Tcf1- subset.

Aim#2: Develop strategies for minimizing differentiation and exhaustion in expanded CD4+ T cells

Maintained proliferative potential of cultured tumor reactive T cell populations prior to adoptive transfer is a critical component of the efficacy of these cells, and terminally differentiated cells show less therapeutic efficacy in animal models and patients treated with TIL.[21,33] We will leverage several methods that maintain a stem like phenotype in CD8+ T cells to determine if these methods also apply to CD4+ T cells, focusing on methods that would be straightforward to apply to clinical production of a cellular product.

A) Evaluate the effect of SRC kinase inhibition before and during growth phases of T cell expansion. T cell dysfunction in tumors is mediated in part by chronic antigen exposure which leads to epigenetic changes that limit proliferation and effector functions.[34] Proximal T cell receptor signaling is rapidly and reversibly inhibited by the SRC kinase inhibitor dasatanib, and transient treatment of cells in a model of T cell exhaustion improved T cell functionality and therapeutic efficacy.[22]

Tumor specific CD4+ T cells within tumors have persistent exposure to TCR stimulation which contributes to their exhaustion and get strong and sustained TCR signals during ex vivo expansion. It is possible that residual TCR signaling following tumor dissociation or sustained TCR signaling during ex vivo culture contribute to exhaustion and be reversed by interrupting TCR signaling with Dasatinib. We will compare standard culture expansion to conditions where dasatinib (1μM, Sigma) is included during a 24-hour rest period prior to expansion, and to a condition where dasatinib is included starting at day 5 of ex vivo culture to limit duration of TCR signaling. Maintenance of memory like characteristics will be measured at day 21 by flow cytometry for the markers IL7R, CD62L, and TCF1 as well as the coinhibitory receptors and TIM-3 and CD39. In order to assess functionality as well as potential phenotypic skewing of CD4+ T cells, cytokine profiles will be assessed following T cell activation using the Luminex platform. CD4+ PD-1+ CXCR6- T_{FH} subsets will be examined separately from CD4+ PD-1+ CXCR6+ effector subsets for effects on expansion and phenotype.

B) Evaluate the effect of AKT inhibition during expansion of sorted tumor specific CD4+ TIL subsets. Cellular metabolism can alter T cell differentiation, and interventions that skew T cell metabolism toward partial starvation states including pharmacological inhibition of AKT[23] and growth in high potassium[24] have been shown to preserve T cell stemness in CD8+ T cells cultured from tumors without compromising cell yield. We will ask if similar interventions preserve stem like phenotypes in tumor specific CD4+ T cell subsets cultured from melanoma tumors. T cells will be sorted and cultured as above in **2A** with the addition of either AKT inhibitor VIII (1μM, Calbiochem) or supplemental potassium (40mM). Memory and coinhibitory receptors will be assessed following 3 weeks of culture and following an additional round of culture if necessary to obtain sufficient cellular numbers. If culture conditions are identified in aim **2A** or **2B** that increase stem like features of the expanded tumor antigen specific subsets of CD4+ T cell from melanoma tumors, then post-expansion cells will be interrogated by ATTAC-seq in these conditions to identify whether chromatin remodeling changes associated with exhaustion have been limited or reversed as has been seen in CD8+ T cells.

Biostatistics and analysis plan. Cell numbers and markers of memory function (percentage of cells expressing CD62L, TCF7, CCR7, CD127) and exhaustion markers (Tim-3, CD39) and proliferation after restimulation by CFSE dye dilution will compared after 21 days of culture between culture methods for n=10 donors by t test or non-parametric test, as appropriate for each T_{FH} and effector subsets. Analysis of cytokine production will be analyzed by one way ANOVA and trends will be hypothesis generating for future experiments with a larger number of donors.

Anticipated results and potential pitfalls. We expect that one or more of the interventions shown to be effective in delaying differentiation of CD8+ T cells in expansion from tumors will also be effective in CD4+ T cells. If they are not, it would suggest that molecular mechanisms of exhaustion could fundamentally differ between these populations and suggest additional avenues of research into the nature of these differences. Additional strategies that could be tried to facilitate increased stem-like features of expanded CD4+ T cells include additional cytokine support with IL-15[35] or IL-21,[36,37] and transient mTOR inhibition.[38] It is also possible that AKT inhibition or metabolic changes will skew the differentiation of CD4+ T cells away from the T_{FH} or TH1 phenotype seen in cell in situ in tumors,[39] which we will evaluate using cytokine production in expanded cells.

Impact

Adoptive T cell transfer of ex-vivo expanded tumor infiltrating lymphocyte cell products have shown the ability to lead to clinical responses across a large number of tumor types, and in some cases to overcome PD-1 inhibitor resistance. The CD4+ T cell component of these products is a key gap in our current knowledge, given the critical role they could play in efficacy either alone or in combination with CD8+ T cells. This proposal includes two strategies that could significantly enhance the potency of CD4+ T cell products through increasing the diversity and purity of tumor antigen specific T cell populations and decreasing the dysfunction of expanded T cell products after culture. If this proposal and subsequent clinical trials show promise, this strategy could be a transformative approach to adoptive therapy that could apply to a large number of cancer types.

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