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Institution/Organization: University of Texas MD Anderson Cancer Center
Initiative Name: Defining the Immune Atlas in Osteosarcoma Lung Metastases
Amount Requested: 98900
Desired Impact: Recent clinical trials of immune checkpoint inhibitors (ICI) have been disappointing in osteosarcoma (OS). While there have been major advances in immunotherapy approaches including adoptive cell therapies (such as TIL and CAR-T) for solid tumors, not enough is known about osteosarcoma and its specific interaction with the lung, the immune system, and the tumor microenvironment to advance these therapies in the clinic. We recently identified three subsets of osteosarcoma tumors that have low, medium, and high levels of immune infiltrating cells from patients with recurrent and metastatic disease. Here, we propose to undertake an in-depth genetic analysis of osteosarcoma lung metastases, immune cells, and the surrounding lung microenvironment to define each cell type comprising the osteosarcoma metastasis and to understand the key features of tumor infiltrating lymphocytes (TILs) that are recognizing OS lung metastasis. Ultimately, the goal is to use these insights to inform the development of new immunotherapy strategies for the treatment of patients with osteosarcoma lung metastasis.
Projected Milestones: Collection of OS lung metastasis (Q1-Q4, ongoing) ssRNAseq, TCRseq, multiplex IF (Q2-Q4) TIL expansion and TIL profiling (Q2-Q4) Data Analysis (Q4) Presentation of research findings (Q4, FACTOR 2021)

Defining the Immune Atlas in Osteosarcoma Lung Metastases

Specific Aims Defining the immune tumor microenvironment (TME) is essential to developing therapeutic strategies to target the immune response and TME in osteosarcoma (OS). Through immunogenomic profiling of relapsed/metastatic OS, we identified suboptimal neoantigen expression and low T cell clonality as contributors to impaired immune response. Both tumor-intrinsic factors and the immune TME appear to contribute to low immunogenicity and primary resistance to immunotherapy in OS. This project seeks to comprehensively define the composition, distribution and state of immune cells within human OS lung metastases and identify mechanisms of immune suppression or suboptimal T cell activation within the TME by:

- **<u>Aim 1.</u> Genomic and immune profiling of resected OS lung metastases through single-cell RNA sequencing (ssRNAseq), T-cell receptor sequencing (TCRseq), and multiplex immunofluorescence (mIF)**
- **<u>Aim 2.</u> Development and characterization of OS tumor infiltrating lymphocytes (TIL) cell lines to identify tumor-reactive TIL vs bystander TIL**

Background There has been a longstanding interest in understanding the role of the tumor microenvironment (TME) in facilitating metastatic disease progression in osteosarcoma (OS) and as a potential therapeutic target. However recent studies with immune checkpoint inhibitors have been disappointing in OS.[1,2] We previously completed a large-scale study to profile the immunogenomic landscape of primary, relapsed, and metastatic OS (manuscript under review, ASCO 2018 poster discussion).[3] In this study, we described three clusters of low, medium, and high immune infiltrates (C1, C2, C3 respectively, Fig. 1) as measured by RNA sequencing (RNAseq) that were validated in the TARGET OS cohort (data not shown) . These levels of immune infiltrate were intermediate as compared to other cancer types (TCGA), but similar to those found in soft tissue sarcoma subtypes where immune checkpoint inhibitors have activity. In our cohort, low immune scores (C1) were associated with a higher number of copy number losses. Multiple immunosuppressive mechanisms were present in patients with high immune scores (C3). Across all profiled OS patients, we observed low levels of T-cell activation which may be due in part to the low levels of neoantigens that were expressed.

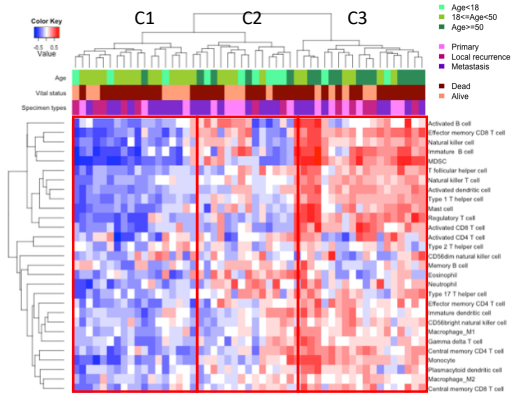

Figure 1. Immune clusters in osteosarcoma (RNAseq)

<u>Specific Aim 1:</u> Genomic and Immune Profiling of OS Lung Metastasis. Although the prior RNAseq and TCRseq data in OS gave us insights into the levels of immune infiltrate and some immune cell types, the information is incomplete: bulk RNAseq allows for comparison of levels of various immune cell types between patients but does not provide sufficient resolution to quantify the abundance and relative proportions of each subset of immune cells within a single patient. For instance, the CD8+/FoxP3 ratios are used to determine the interactions between cytotoxic T-cells and immunosuppressive Tregs. Another example is the relationship between macrophages and lymphocyte markers to determine whether tumor-associated macrophages are eliciting an immunosuppressive effect on T cells. ssRNAseq will provide the ability to examine these relationships. We will also complete multiplex immunofluorescence (IF) of multiple immune markers to assess protein expression and cellular localization of these markers as well as the spatial relationship of immune cells relative

to tumor. **Specific Aim 2. Develop and Characterize Tumor Infiltrating lymphocyte (TIL) Lines.** In the MD Anderson TIL laboratory, we previously showed the feasibility of isolating and expanding TILs from multiple sarcoma subtypes including a limited number of OS specimens. This work led to two current clinical trials of autologous TIL therapy in multiple solid tumors including osteosarcoma (clinicaltrials.gov, NCT03449108 and NCT03610490). In the current study, we will isolate and expand TIL lines from OS pulmonary metastases to comprehensively characterize the TIL population pre- and post-expansion, use sequencing data for neoantigen identification, and evaluate tumor-reactive vs bystander TIL. This will include identification of suppression mechanism(s) or suboptimal T-cell activation within the TME.

Research Design Sample collection. Patients undergoing standard of care resection of OS pulmonary metastasis will be consented to an existing IRB-approved APOLLO protocol which allows for serial biospecimen collection. Over a 10-year period, 85 patients underwent wedge resection of osteosarcoma pulmonary metastases within our center. We anticipate collecting 6-8 resection specimens along with matched normal blood samples within a 1-year period. Bioinformatics analysis will be done in the Department of Genomic Medicine. Whole exome sequencing (WES) will be used for mutation, copy number, and neoantigen prediction. Cells with similar single-cell RNA sequencing profiles will be clustered together with tSNE plots and the top variable genes used for deconvolution into specific immune and stromal cell types. We will analyze the TCRseq for clonality, and diversity. T cell isolation and expansion is performed in the MDA TIL Lab for multiple cancer types including OS. Briefly, TIL are expanded from fragments of tumor tissue with the help of anti-CD3 ab, agonistic anti-4-1BB ab, and Interleukin-2 (IL2) using G-Rex devices as previously described (Fig. 2).[4] Tumor cells and TIL will be co-cultured to assess tumor-reactivity. TCR sequencing will be performed in the tissue and the expanded TIL to assess clonality. The elucidation of neoantigen-specific TCRs will be done by ssRNAseq.

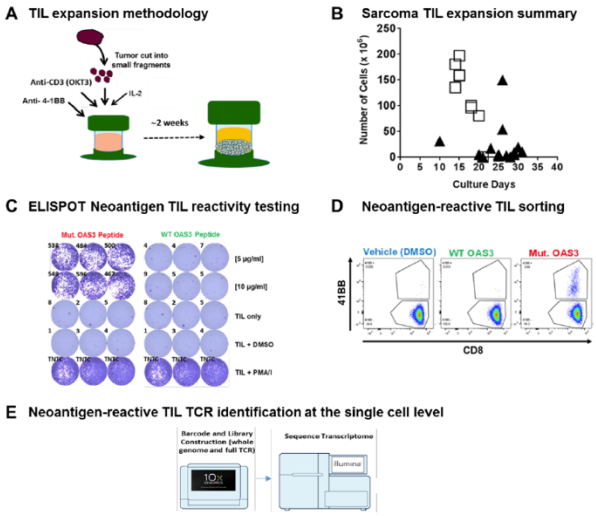

Figure 2. TIL expansion and reactivity testing.

Expected Outcomes The ability to perform all assays simultaneously in a single lung metastasis specimen is dependent on the size of the tumor tissue. The take rate for TIL expansion is over 80%. Due to the expansion platform, we expect the expanded TIL to be enriched in CD8+ T cells and therefore we are confident that we will be able to uncover cytotoxic T cells reactive to their autologous tumor and to neoantigens. If initial resection specimens are inadequate or unsuccessful for genomic profiling or TIL expansion, we will collect additional surgical specimens. **Research Findings** Results will be rapidly disseminated through presentations at national/international meetings and scientific publication. **Other Relevant Information** This project will take place as part of a larger ongoing institutional initiative profiling rare tumors. As such, institutional resources will be leveraged to identify and accrue patients, assure quality sample collection, and complete the proposed profiling studies through an established genomics and immune profiling platform under a current IRB-approved protocol.