**ABSTRACT**

Osteosarcoma (OS) is the most common bone tumor in pediatric and adolescent/young adult patients. The targeted gene expression profiling and immunohistochemistry to characterize the microenvironment of metastatic and non-metastatic OS specimens from pediatric patients exhibiting poor histologic response to chemotherapy. Our results indicate that metastatic specimens exhibit lymphocyte exclusion as T cells are confined to the periphery of the pulmonary lesions. Furthermore, our data provides evidence of vascular dysfunction in metastatic OS indicated by increased expression of VEGFA, an increased ANGPT2:ANGPT1 gene expression ratio, and decreased expression of SELE, the gene encoding the adhesion molecule E-selectin. Moreover, correlation analyses show an inverse relationship between lymphocyte abundance and markers of vascular dysfunction exclusively in the metastatic specimens. Together, our data shows that the non-metastatic OS specimens demonstrate increased expression of various immunotherapeutic targets in comparison metastatic specimens and identifies vascular dysfunction and lymphocyte exclusion as important processes for therapeutic intervention in metastatic disease.

**Introduction:**

Osteosarcoma (OS) is the most common malignant bone tumor observed in pediatric and adolescent/young adult patients. The 5-year overall survival rate for patients with localized disease is approximately 70% whereas for patients with metastatic disease, the survival rate plummets to 30%. These incredibly complex hyperdiploid tumors are hallmarked by substantial genomic instability and highly prevalent chromothripsis. However, despite our current understanding of the genomic characteristics of OS, the molecular and cellular mechanisms utilized by these tumors to modulate their microenvironment and favor disease progression is unknown.

Our collective understanding of cancer genomics, immunology, and the tumor microenvironment is rapidly increasing and the integration of these data is shaping the scientific and clinical landscapes of various malignancies. The current literature states that OS tumors express PD-L1, PD-L2, PD-1, and exhibit varying degrees of lymphocyte abundance with increased CD8 T cells correlating with increased survival. Bioinformatic analysis of DNA and RNA sequencing data shows that OS tumors express neoantigens, albeit at a low level. Why OS patients with advanced and metastatic disease have not benefited from immune checkpoint blockade is an important, yet unresolved, question. Increasing our comprehension of the OS microenvironment could provide valuable insight into this clinical dilemma and potentially elucidate novel scientific and therapeutic advances towards improved management of this disease.

To address this knowledge gap, we used a targeted gene expression panel to profile the microenvironment of both metastatic and non-metastatic specimens obtained from pediatric patients demonstrating a poor histological response to chemotherapy. This sensitive yet robust commercially available panel has been used to profile the microenvironment of various tumor types by utilizing several metagene signatures corresponding to different infiltrating immune-effector and stromal cell types, as well as various functional processes. Our data demonstrates that T cells are confined to the periphery of the pulmonary OS lesions and suggests that the metastatic OS vasculature is unresponsive and destabilized, thereby reducing lymphocyte extravasation and subsequent tumor infiltration. The data presented here provides rationale for additional functional, mechanistic, and clinical investigations that employ vascular normalization therapy as a strategy to facilitate lymphocyte extravasation and subsequent tumor infiltration in metastatic OS.

**Results:**

**Gene expression profiling of the tumor microenvironment and immuno-oncology landscape in pediatric OS**

Targeted gene expression profiling was performed on archived FFPE specimens from unmatched pediatric OS...
Table 1. Specimen Details.

| Sample ID | Age | Sex | Site       | pTNM      | Disease Status |
|-----------|-----|-----|------------|-----------|----------------|
| OS-0456   | 17  | F   | Lung       | N/A       | Metastatic     |
| OS-0457   | 14  | F   | Lung       | N/A       | Metastatic     |
| OS-0458   | 21  | F   | Lung       | N/A       | Metastatic     |
| OS-0459   | 9   | F   | Femur      | yPT2, NX, MX | Non-Metastatic |
| OS-0461   | 9   | F   | Humerus    | yPT2, NX, MX | Non-Metastatic |
| OS-0463   | 18  | M   | Lung       | N/A       | Metastatic     |
| OS-0464   | 16  | M   | Lung       | N/A       | Metastatic     |
| OS-0465   | 13  | M   | Lung       | N/A       | Metastatic     |
| OS-0466   | 19  | M   | Lung       | N/A       | Metastatic     |
| OS-0467   | 19  | M   | Lung       | N/A       | Metastatic     |
| OS-0469   | 18  | M   | Femur      | yPT1, NX, MX | Non-Metastatic |
| OS-0470   | 18  | F   | Femur      | yPT1, NX, MX | Non-Metastatic |
| OS-0471   | 7   | M   | Femur      | yPT2, N0, M1a | Non-Metastatic |
| OS-0472   | 12  | F   | Radius     | yPT1, MX   | Non-Metastatic |
| OS-0473   | 11  | M   | Tibia      | yPT2, NX, MX | Non-Metastatic |
| OS-0474   | 10  | F   | Radius     | yPT2, MX   | Non-Metastatic |
| OS-0476   | 21  | M   | Lower Extremity, Diaphysis Tibia | t2, N0, MX | Non-Metastatic |
| OS-0479   | 9   | F   | Tibia      | yPT2, NX, MX | Non-Metastatic |
| OS-0480   | 16  | M   | Humerus    | yPT3, NX, MX | Non-Metastatic |

patients with pulmonary metastases (n = 8) and non-metastatic disease (n = 11). Age, sex, site, and pathological staging data are listed in Table 1. Unsupervised hierarchical clustering of the normalized gene expression data faithfully partitioned the metastatic and non-metastatic specimens into distinct non-overlapping clusters (Figure 1a). The data were further analyzed to examine the expression levels of genes encoding various stimulatory and inhibitory immune checkpoints (Figure 1b). Notably, with few exceptions, we observed a decrease in the expression of immune checkpoint genes in the metastatic OS specimens. Cell-type specific metagene signatures were used to identify and quantify different immune cell subsets in the OS microenvironment. With the exception of mast cells, our analysis reveals that metastatic OS lesions contain fewer immune cells than non-metastatic tumors (Figure 2).

The gene expression data were also used to generate metagene signatures that describe functional processes within the microenvironment. Unsupervised hierarchical clustering of the functional metagene signatures demonstrates that metastatic and non-metastatic OS specimens cluster independently of one another (Figure 3a). Our analysis also shows that the Tumor Sensitivity to Attack, Inhibitory Tumor Mechanisms, Inhibitory Metabolism, Inhibitory Immune Signaling, and Immune Cell Population Abundance signatures are significantly enriched in the non-metastatic OS specimens (Figure 3b). While not quite statistically significant (p = .0672), we observed an increased enrichment of the Tumor Immunogenicity signature in the metastatic specimens. The Tumor Inflammation Signature (TIS) is a weighted metagene signature that measures the degree to which the pre-existing anti-tumor adaptive immune response is suppressed where a higher TIS equates to an increasingly suppressed anti-tumor immune response. We observed a similar enrichment of the TIS in both the metastatic and non-metastatic specimens (Figure 4).

Examing the distribution, localization, and activation status of T cells within OS tissues

Lymphocyte exclusion is a means by which tumors evade the immune system and negate the therapeutic effects of checkpoint blockade. Given the lack of therapeutic efficacy of immune checkpoint blockade in patients with metastatic OS, and the decreased abundance of various immune cells in metastatic OS (Figure 2), we sought to determine if metastatic OS tumors exhibit lymphocyte exclusion. To visualize the abundance and localization of T cells within the specimens, immunohistochemistry (IHC) was performed using an antibody against CD3ε. This pan-T cell marker was chosen in order to indiscriminately visualize all subpopulations of the T cell lineage present within the tissues. With few exceptions, we consistently detected T cells in the periphery of the metastatic lesions adjacent to the lung parenchyma (Figure 5a, D) and very few T cells penetrating the tumor core (Figure 5b, e). Similar results were observed in experimental models showing that activated NK cells were able to traffic to, but not infiltrate, pulmonary metastatic OS lesions. The distribution of the T cells within the non-metastatic tumors was more diffuse and infiltrative in comparison to the confined peritumoral localization of T cells in the metastatic specimens (Figure 5c, f). Quantification of the IHC data revealed increased CD3ε immunoreactivity in the non-metastatic OS specimens (Figure 5g). This data was consistent with the T cell abundance data generated by gene expression profiling (Figure 2).

4-1BB, encoded by TNFRSF9, is a transcriptional marker of lymphocyte activation. Pearson correlation analyses were performed to determine the linear relationship between lymphocyte abundance and TNFRSF9 expression to examine lymphocyte activation status. Strong correlations were observed between TNFRSF9 expression and the abundance of each of the lymphocyte subsets in the non-metastatic specimens, indicative of activation (Table 2). Statistically significant correlations were not observed in the metastatic specimens. Together, these data demonstrate that metastatic OS specimens contain fewer T cells than non-metastatic specimens, that T cells are largely excluded from penetrating the metastatic OS lesions, and that the lymphocytes present in metastatic OS specimens are hyporesponsive.

Assessment of the tumor vasculature in metastatic and non-metastatic OS

The tumor vasculature is a key mediator of lymphocyte exclusion whereby the vessels themselves can impede leukocyte tethering and subsequent extravasation into tumor tissues. Due to the T cell exclusion observed in metastatic OS (Figure 5), we focused our attention towards further investigating the relationship between vascular function and lymphocyte abundance. E-selectin, encoded by the SEL gene, is an endothelial cell adhesion molecule that facilitates leukocyte tethering and rolling on the vascular wall, the first step in...
leukocyte extravasation.\textsuperscript{32–34} Whereas ICAM1 and PECAM1 can be expressed in both endothelial cells and tumor cells, SELE is expressed exclusively in endothelial cells and is transcriptionally upregulated in response to inflammation, thus serving as a marker of an activated and functional endothelium.\textsuperscript{34–40} Our results demonstrate that the expression of \textit{SELE} is significantly lower in the metastatic OS specimens (Figure 6a). To determine if the differences in \textit{SELE} expression reflect a decrease in tumor vascularity, we also examined endothelial cell abundance. Our data illustrates that the endothelial content does not significantly differ between metastatic and non-metastatic specimens (Figure 6b).

\textbf{Figure 1.} The tumor microenvironment and immuno-oncology landscape of pediatric OS. (a) Heatmap and hierarchical clustering of the entire 770-gene panel. Columns represent individual genes while rows indicate individual samples. Scale bar indicates z-score. (b) Box plots showing the expression of inhibitory and stimulatory immune checkpoints in metastatic (gray bars) and non-metastatic (black bars) OS. Y-axis is displayed as log2-transformed gene expression values. Statistical significance (p < .05) determined by unpaired t-test with Welch’s correction.
Pearson correlation analysis shows a significant linear relationship between endothelial cell abundance and the expression of SELE in non-metastatic OS specimens (Figure 6c). SELE expression did not significantly correlate with endothelial cell abundance in the metastatic OS specimens (Figure 6d), indicative of a hyporesponsive/inactive tumor vasculature.

Next, we investigated the expression of angiogenic mediators. ANGPT1 and ANGPT2 opposingly regulate vessel stabilization and angiogenesis. Expression of ANGPT1 facilitates pericyte coverage, vessel maturity, and vascular stabilization, whereas expression of ANGPT2 induces pericyte detachment and vascular instability. Our analysis shows that ANGPT1 expression is elevated in the non-metastatic OS specimens (Figure 7a). We did not observe statistically significant differences in ANGPT2 expression between the two groups (Figure 7b). We calculated the ANGPT2:ANGPT1 gene expression ratio to serve as a surrogate for vessel stabilization where an increased ratio favors vascular destabilization while a decreased ratio favors pericyte coverage, vessel stability, and maturation. This analysis demonstrates that the metastatic specimens exhibit an increased ANGPT2:ANGPT1 ratio (Figure 7c), indicative of decreased...
pericyte coverage and increased vascular instability. Pathophysiological levels of VEGFA can induce endothelial cell dysfunction. Expression of VEGFA was assessed at both the protein and mRNA levels, revealing that metastatic OS specimens express significantly more VEGFA than the non-metastatic OS specimens (Figure 8a-b). Quantification of the IHC data revealed increased VEGFA immunoreactivity in the metastatic OS specimens (Figure 8c), consistent with the VEGFA expression data generated by gene expression profiling (Figure 8d). Taken together, these data suggest that endothelial cell function and integrity of the tumor vasculature differs between metastatic and non-metastatic OS.

**Correlations between vascular function/integrity and immune cell abundance**

Correlation analyses were performed to determine if the \( \text{ANGPT}2: \text{ANGPT}1 \) ratio and/or VEGFA expression correlate with immune cell abundance. Our results show that both the \( \text{ANGPT}2: \text{ANGPT}1 \) ratio and VEGFA expression inversely correlate with the abundance of various lymphocyte populations in the metastatic specimens (Table 3). These correlations were

| Comparison                      | Metastatic OS | Non-Metastatic OS |
|---------------------------------|---------------|-------------------|
|                                 | \( r \) (Pearson) | \( p \) value   | \( r \) (Pearson) | \( p \) value   |
| \( \text{TNFRSF}9 \) vs. CD8 T cells | 0.4715        | 0.2382            | 0.9706            | <0.0001         |
| \( \text{TNFRSF}9 \) vs. Exhausted CD8 T cells | 0.5298        | 0.584             | 0.8796            | 0.0004          |
| \( \text{TNFRSF}9 \) vs. T Cells | 0.4223        | 0.2973            | 0.9635            | <0.0001         |
| \( \text{TNFRSF}9 \) vs. Regulatory T Cells | 0.5506        | 0.1573            | 0.9158            | <0.0001         |
| \( \text{TNFRSF}9 \) vs. Th1 cells | 0.4369        | 0.279             | 0.943             | <0.0001         |
| \( \text{TNFRSF}9 \) vs. NK cells | 0.5637        | 0.1456            | 0.8734            | 0.0004          |

Figure 4. Tumor Inflammation Signature (TIS). Box plot showing the TIS in metastatic (gray) and non-metastatic (black) OS. Y-axis is displayed as log2-transformed Tumor Inflammation Score. Statistical significance (\( p < .05 \)) determined by unpaired t-test with Welch’s correction.

Figure 5. Immunohistochemical analysis of T cell infiltration in metastatic and non-metastatic OS. Hematoxylin and eosin staining of (a-b) metastatic and (c) non-metastatic OS. CD3ε immunohistochemistry demonstrating the localization and distribution of T cells in (d-e) metastatic versus (f) non-metastatic OS. For metastatic OS, images from (a, d) the tumor periphery and (b, e) the tumor core were captured to demonstrate viable tumor and highlight regional differences in T cell localization within the same tissue. A representative image from a single metastatic and non-metastatic OS specimen are shown. Scale bar = 1000μm. (g) Box plot of the quantified CD3ε immunoreactivity in metastatic (gray) and non-metastatic (black) OS specimens. Quantified immunoreactivity data expressed as arbitrary units (A.U.). Statistical significance (\( p < .05 \)) determined by unpaired t-test with Welch’s correction.
exclusive to lymphoid lineage cells as statistical significance was not achieved when comparing myeloid lineage cells. At the protein level, we observed a significant inverse correlation between VEGFA and CD3ε immunoreactivity in the metastatic OS specimens (Figure 9a) but not in the non-metastatic specimens (Figure 9b), thus validating the gene expression data. We also examined the correlation between SELE expression and lymphocyte abundance in the metastatic and non-metastatic specimens. We observed a significant positive correlation between the expression of SELE and the different lymphocyte subsets in the non-metastatic OS specimens (Table 4), as would be expected for a functional and activated endothelium. Significant correlations were not observed in the metastatic OS specimens (Table 4), indicative of an unresponsive endothelium. Together, these data provide additional evidence to suggest that the dysfunctional tumor vasculature in metastatic OS is associated with lymphocyte exclusion.

Discussion:
Prior to enrollment on any experimental therapeutic trial, pediatric OS patients would have already received standard of care chemotherapy consisting of high-dose methotrexate, doxorubicin, and cisplatin. For that reason, we intentionally...
utilized post-treatment specimens from pediatric patients that have demonstrated poor histological responses to chemotherapy for our analysis as this is the context within which these patients would encounter therapeutic immune checkpoint blockade. Our study provides an overview of the gene expression profiles of the metastatic and non-metastatic OS microenvironments with a particular focus on the tumor vasculature and lymphocyte abundance. Our investigation primarily focused on lymphocytes and not myeloid cells for the following reasons: (1) osteoclasts, which are present in OS in varying abundance, are specialized bone macrophages and thus express various myeloid lineage markers, thereby compromising the interpretation of the data; (2) the remarkable plasticity and phenotypic/functional diversity exhibited by myeloid-derived cells in human tumors is an active area of investigation for which this specific panel was not designed to capture in sufficient detail; (3) current FDA approved immuno-therapeutics are designed to enhance the cytotoxic activity of T cells. Nevertheless, we cannot disregard the biologic, prognostic, and potential therapeutic significance of myeloid lineage cells in OS as these tumors often invade the bone marrow, the site of myelopoiesis, and are heavily infiltrated by macrophages.

To fully elucidate how the various myeloid-lineage cell populations shape the metastatic and non-metastatic OS microenvironments and contribute to disease progression, additional functional and mechanistic studies are needed.

Profiling the microenvironment of metastatic and non-metastatic pediatric OS has revealed that the non-metastatic OS lesions offer more immunotherapeutic targets than the metastatic specimens as evidenced by the increased abundance of various activated immune cell subsets and increased expression

Figure 8. VEGFA expression in metastatic and non-metastatic OS. IHC analysis of VEGFA expression levels in (a) metastatic and (b) non-metastatic OS tumor sections. Three different representative specimens are shown for each group. Scale bar = 1000µm. (c-d) Quantification of (c) IHC data and (d) gene expression data. VEGFA IHC data expressed as arbitrary units (A.U.) and VEGFA gene expression data is displayed as log2 transformed gene expression values. Statistical significance (p < .05) determined by unpaired t-test with Welch’s correction.

Table 3. Pearson Correlation analysis of immune cell abundance versus ANGPT2/ANGPT1 ratio and VEGFA expression.

| Comparison                               | Metastatic OS |          |          | Non-Metastatic OS |          |
|------------------------------------------|---------------|----------|----------|-------------------|----------|
| ANGPT2/ANGPT1 Ratio vs. CD8 T cells      | -0.7379       | 0.0366   | 0.2234   | 0.5091            |
| ANGPT2/ANGPT1 Ratio vs. Exhausted CD8 T cells | -0.8304       | 0.0107   | 0.1769   | 0.6028            |
| ANGPT2/ANGPT1 Ratio vs. T Cells          | -0.8989       | 0.0024   | 0.3047   | 0.3622            |
| ANGPT2/ANGPT1 Ratio vs. Regulatory T Cells | -0.5763       | 0.1349   | 0.2883   | 0.39              |
| ANGPT2/ANGPT1 Ratio vs. Th1 T Cells      | -0.8006       | 0.017    | 0.3498   | 0.2917            |
| ANGPT2/ANGPT1 Ratio vs. NK cells         | -0.9005       | 0.0023   | -0.193   | 0.5697            |
| ANGPT2/ANGPT1 Ratio vs. Macrophages      | 0.7036        | 0.0515   | 0.0396   | 0.908             |
| ANGPT2/ANGPT1 Ratio vs. Dendritic Cells  | -0.5626       | 0.1466   | 0.06606  | 0.847             |
| ANGPT2/ANGPT1 Ratio vs. Mast Cells       | -0.3074       | 0.4589   | 0.1056   | 0.7573            |
| ANGPT2/ANGPT1 Ratio vs. Neutrophils      | -0.372        | 0.3641   | -0.03874 | 0.91              |
| VEGFA vs. CD8 T cells                    | -0.7887       | 0.02     | 0.04826  | 0.8879            |
| VEGFA vs. Exhausted CD8 T cells          | -0.7293       | 0.0401   | -0.002287| 0.9947            |
| VEGFA vs. T Cells                        | -0.7893       | 0.0198   | 0.03061  | 0.9288            |
| VEGFA vs. Regulatory T Cells             | -0.6229       | 0.99     | 0.3288   | 0.3236            |
| VEGFA vs. Th1 T Cells                    | -0.5949       | 0.1198   | 0.07096  | 0.8358            |
| VEGFA vs. NK cells                       | -0.7856       | 0.0208   | -0.05889 | 0.8635            |
| VEGFA vs. Macrophages                    | 0.3265        | 0.4299   | -0.2885  | 0.3896            |
| VEGFA vs. Dendritic Cells                | -0.4553       | 0.257    | -0.2664  | 0.4284            |
| VEGFA vs. Mast Cells                     | -0.1163       | 0.7839   | 0.09643  | 0.7779            |
| VEGFA vs. Neutrophils                    | -0.1237       | 0.7703   | -0.02629 | 0.9389            |
of several inhibitory and activating immune checkpoints. For instance, non-metastatic OS specimens contain more regulatory T cells than metastatic specimens. Regulatory T cells are a heterogeneous group of lymphocytes that mediate immunosuppression using various mechanisms, including PD-1 and CTLA-4 mediated suppression, and are considered therapeutic targets.\textsuperscript{53,54} The increased abundance of NK cells is also an important finding as adoptive NK cell therapies are currently being explored and refined.\textsuperscript{55,56} The potential of this therapeutic context will need to be experimentally validated as the presence of myeloid derived suppressor cells and M2 macrophages will present additional immunosuppressive hurdles.

With the significant differences in lymphocyte abundance, activation status, and immune checkpoint expression between the metastatic and non-metastatic specimens, we expected to also observe significant variation in the TIS between the two sets of specimens. That there were no differences between the metastatic and non-metastatic OS specimens was a surprising result as this indicates that the pre-existing lymphocytes in both the metastatic and non-metastatic microenvironments are equally suppressed.\textsuperscript{23,24,27} From this we conclude that the TIS, which was derived from adult carcinoma patients, may not accurately reflect the biology of pediatric osteosarcoma as there are likely additional factors that differentially mediate immunosuppression that are not accounted for in this signature. These factors may include the presence/activity of myeloid-derived suppressor cells, tumor associated macrophages, and/or tumor associated fibroblasts as each of these cell types can influence the activity of cytotoxic and regulatory lymphocytes and polarize the microenvironment. As additional predictive and/or prognostic immuno-oncology gene signatures become available, understanding how these signatures, or derivatives thereof, can be utilized in OS will benefit future clinical trials and correlative biological studies.

In addition to its role in tumor vascularization, excessive angiogenesis is a means of immune escape as increased angiogenesis is associated with resistance to anti-PD1 therapy.\textsuperscript{57} In the presence of inflammatory cytokines, excessive pathologic VEGFA signaling renders endothelial cells unresponsive to inflammatory signals, resulting in decreased expression and/or cluster density of adhesion molecules and impaired leukocyte extravasation, a phenomenon termed “endothelial cell anergy.”\textsuperscript{46,58,59} E-selectin is decreased in anergic endothelial cells and its downregulation reduces lymphocyte extravasation into tumor tissues resulting in lymphocyte exclusion.\textsuperscript{60–63} Additionally, under these pathologic angiogenic conditions, an increased ANG2:ANG1 ratio favors pericyte detachment from the abluminal surface of the endothelial cells and subsequent destabilization of the tumor vasculature.\textsuperscript{61–63} Destabilized tumor vessels have been shown to decrease immune cell extravasation and tumor infiltration \textit{in vivo}.\textsuperscript{64}

Collectively, our data and the available literature supports the hypothesis that lymphocytes are actively excluded from successfully penetrating the metastatic OS lesions as a consequence of pathologic angiogenesis and a dysfunctional tumor vasculature.

Vascular normalization describes the ability of low dose anti-angiogenic agents to facilitate a transient window within which the tumor vasculature regains functionality.\textsuperscript{65} The findings of our study are timely as vascular normalization is quickly becoming an attractive therapeutic strategy.\textsuperscript{31,66,67} Dual targeting of both ANG2 and VEGFA signaling has demonstrated efficacy in pre-clinical animal models of breast cancer, colon cancer, and glioblastoma.\textsuperscript{68–70} ANG2 itself is an attractive therapeutic target as studies have shown that inhibition of ANG2 signaling decreases tumor growth and metastasis.\textsuperscript{71} Additionally, normalizing the tumor vasculature via inhibition of VEGF and/or ANG2 promotes lymphocyte infiltration into tumors, reprograms the suppressive tumor microenvironment, and can sensitize tumors to immune checkpoint blockade.\textsuperscript{69–72} Recent results from the REGOBONE study (NCT02389244) and the SARCO024 study
(NCT02048371) report that single-agent regorafenib (pan-VEGFR small molecule inhibitor) prolonged progression-free survival in patients with recurrent, progressive, and/or metastatic OS who have failed standard of care chemotherapy.\textsuperscript{73,74} Both of these studies highlight the therapeutic relevance of VEGFA signaling in advanced OS.

Investigating the therapeutic potential of combining anti-angiogenic therapy with immune checkpoint blockade is an active area of research. The combination of axitinib, a pan-VEGFR inhibitor, and pembrolizumab is currently being studied in patients with advanced soft tissue sarcomas (NCT02301039). Combination sunitinib plus nivolumab is currently being investigated in patients with advanced soft tissue and bone sarcomas in Europe (NCT03277924). The combination of bevacizumab and PD-L1 blockade improved immune cell trafficking in patients diagnosed with metastatic renal cell carcinoma while also increasing the therapeutic response rate (NCT01633970).\textsuperscript{75} Furthermore, metastatic melanoma patients treated with a combination of bevacizumab and ipilimumab experienced encouraging clinical results and increased lymphocyte infiltration (NCT00790010).\textsuperscript{76} Given the dysfunctional vasculature in metastatic OS specimens, vascular normalization combined with immune checkpoint blockade may be of clinical utility as it pertains to improving lymphocyte infiltration into tumors to enhance the efficacy of checkpoint inhibition.

Collectively our data suggests that, in the context of metastatic OS, vascular instability and endothelial cell anergy promotes lymphocyte exclusion and that mounting an effective immune response against metastatic OS requires interventions that will both increase lymphocyte infiltration as well as attenuate immunosuppression. While the small sample size is indeed a limitation of our study, to our knowledge, this is the first report to describe such findings in addition to providing an initial characterization of the microenvironment of metastatic and non-metastatic OS specimens. Our data invites further mechanistic investigations to functionally validate the role of the tumor vasculature in metastatic OS. Nevertheless, the data presented here identify therapeutically actionable targets in a dismal disease.

**Materials and methods:**

**Patient samples**

Archived formalin-fixed paraffin-embedded (FFPE) specimens from post-treatment pediatric OS patients were obtained from the Department of Pathology at Children’s Hospital Los Angeles under IRB protocol HS-16-588 and in compliance with the Declaration of Helsinki. Non-metastatic primary tumor specimens and metastatic lung lesions were obtained from individual unmatched patients. The specimens in this study were obtained from resected tumors status post chemotherapy. The average viability of each specimen was 65% (necrosis average: 35%; necrosis range: 10–60%) indicating poor histological response to chemotherapy. Supplemental Table 1 lists the percent viability of each non-metastatic OS specimen. Slides from each specimen block containing the least amount of necrotic tissue possible were selected for downstream use. All specimens were subjected to acid decalcification to ensure homogeneity of the nucleic acid quality across both metastatic and non-metastatic specimens. Serial 5μm-thick sections were cut from each block and used for downstream analysis.

**RNA extraction**

Serial 5μm sections (n = 10–12 per specimen) were used for RNA extraction by scraping the entire tissue sections (tumor and surrounding stroma). RNA was extracted from the scraped FFPE sections using the Agencourt FormaPure RNA kit (Beckman Coulter Life Sciences) according to the manufacturer’s protocol with the following modification, the recommended elution volume was decreased to 10μL. The qualitative and quantitative analysis of the RNA was performed using the Agilent TapeStation 4200 (Agilent Technologies) to determine the percentage and concentration of RNA fragments greater than 300 nucleotides in length (DV300). Based on the DV300 metric, 100ng of RNA was used as the input for gene expression analysis.

**Nanostring gene expression profiling**

RNA was profiled using the PanCancer IO 360 Panel Gene Expression Panel (NanoString Technologies) and analyzed on the nCounter MAX Analysis System (NanoString Technologies) according to the manufacturers recommendations. The NanoString IO360 panel has 770 genes that characterize the interplay between the tumor, microenvironment, and immune response in cancer. For this analysis, raw data was log2 transformed and then samples were assessed for quality by evaluating the arithmetic mean of the housekeeping genes for every sample to generate a quality threshold of 5. If samples fell below this threshold they were removed from analysis. The passing samples were then normalized using the 10 Tumor Inflammation Signature (TIS) reference genes and panel standard. The Panel Standard is a NanoString developed assay standard, which includes synthetic DNA targets for all 770 probes to allow for the correction of batch effects on a per-probe basis. In order to normalize the data, the arithmetic mean of the reference genes for each sample were calculated to form a normalization factor. The normalization factor was subtracted from all other genes to produce reference gene-normalized data. Similarly, the arithmetic mean of the panel standard for each gene was calculated and subtracted from that specific gene across all samples. Normalized data was used to calculate all the biological signatures, including TIS. All but three of the biological signatures are computed as a linear combination of weighted normalized logarithmic gene expression for a specific set of genes. The three “loss” signatures: MMR loss, APM loss, and JAK-STAT loss utilize the whole dataset to uncover outlier samples for specific gene sets. All data are presented as log2 transformed gene expression values.
**Immunohistochemistry**

OS FFPE sections were deparaffinized in xylenes, rehydrated through graded ethanol solutions, and subjected to heat-mediated antigen retrieval. The slides were quenched of endogenous peroxidases, blocked in 2% normal horse serum, and incubated with a rabbit monoclonal antibody against CD3e (#85061; Cell Signaling Technologies) or a rabbit monoclonal antibody against VEGFA (#ab52917; Abcam) overnight at 4°C. Biotinylated anti-rabbit IgG was used as the secondary antibody (BAF008; R&D Systems). Antibody signals were amplified using the VectaStain Elite ABC kit and 3,3’-diaminobenzidine (DAB) was used for enzymatic color development (Vector Laboratories). IHC quantification was performed using whole slide images acquired on an Ultra-Fast Scanner (Phillips). Optimized color vectors were deconvoluted using Image J and DAB immunoreactivity was quantified. Snapshots (Figures 5 and 8) were captured on an Evos FL digital imaging system (Thermo Scientific) with a 10X objective.

**Statistical analysis**

Box plots and scatter plots were generated using Prism8 (GraphPad). The ANGPT2:ANGPT1 ratio was derived as follows: normalized log2(ANGPT2) – normalized log2(ANGPT1). Scatter plots were analyzed using Pearson correlation tests to specifically identify the linear relationships between the variables of interest. Box plots were analyzed using unpaired two-tailed t-tests with Welch’s correction. P ≤ 0.05 was deemed statistically significant. Heatmaps and dendrograms were generated using statistical analysis software (https://software.broadinstitute.org/morpheus/).

**Conflict of Interest Disclosure**

YF, TH, and SW are employees of NanoString Technologies, Inc. The authors have no further conflicts of interest.

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**ORCID**

Troy A. McEachron http://orcid.org/0000-0003-0695-5783

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