OBJECTIVE
To perform immune-cell enumeration and programmed death-ligand 1 (PD-L1) expression in clear cell renal cell carcinoma (cc-RCC) with tumor thrombus (TT) to guide therapeutic decisions.

METHODS
After obtaining IRB approval and surgical consent, 6 patients underwent radical nephrectomy with venous tumor thrombectomy. We utilized RNA Sequencing to obtain RNAseq expression profiles. Computational calculation and enumeration of immune cells were performed using CIBERSORT, xCell, and ingenuity pathway analysis software. Statistical assessment was conducted using a t test, chi-square, ANOVA and Spearman rank correlations using SPSS v21.

RESULTS
We observed a higher proportion of M1 macrophages in the primary tumor and tumor thrombus, while we noted no difference in M2 macrophages despite M2 representing a high number in thrombus samples. (ANOVA, $P = .032$, and $P = .89$, respectively). Validation with xCell and ingenuity pathway analysis showed a high involvement of macrophages. We observed a higher number of M1 macrophages (CIBERSORT mean 0.11 vs 0.03, $P < .01$) and (nonactivated) resting Natural Killer (NK) cells (0.077 vs 0.017, $P = .02$) associated PD-L1 high expression of the primary tumor. PDL1 expression was variable without differences in tumor stage, level, or immune cell detection. We observed an inverse correlation of mean platelet volume with PD-L1 expression within the primary tumor (Spearman, $-0.89$, $P = .02$) and the TT (Spearman, $-0.77$, $P = .07$).

CONCLUSION
Renal tumor thrombus has higher levels of M1 macrophages that could be utilized as additional targets for future drug development. The PD-L1 expression on clear cell RCC biopsy may not represent its corresponding TT. Future studies are needed to confirm mean platelet volume as a potential blood-based biomarker for PD-L1 expression in RCC.

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**METHODS**

**Population**

We prospectively identified 6 patients and consented before nephrectomy and thrombectomy for fresh tumor collection. None of the patients received neoadjuvant chemotherapy. We collected tissue prospectively by flash frozen processing for preservation. Additionally, we sent the specimen for standard pathologic processing and recording. We obtained demographic and standard laboratory values before surgery and recorded cancer recurrence and clinical outcomes.

**RNA-Seq**

We performed sequencing with an Illumina HiSeq 3000 system using 100bp paired-end protocol following the manufacturer's protocol to attain mRNAs of all samples. The obtained short sequence reads were aligned to UCSC human genome build hg19 using TopHat. The bam files from alignment were processed using HTSeq-count to compute the counts per gene in all samples.

**Bioinformatics**

**CIBERSORT.** Using RNA expression data, we utilized the CIBERSORT program (https://cibersort.stanford.edu/) that provides an enumeration of immune cells in tumor samples based on RNA expression profile. CIBERSORT is a method to enumerate immune subsets using RNA-Seq without limitations of immunohistochemistry or flow cytometry. CIBERSORT uses an alternative approach to acquire aggregate high dimensional data from tumor cellular mixtures and computationally infer the cellular components from prior knowledge of expression profiles from purified leukocyte subsets.

**xCell Analysis.** We performed xCell analysis to validate the immune cell heterogeneity findings from the CIBERSORT program of our collected ccRCC samples. We submitted the gene expression level in Reads per Kilobase Million unit obtained from RNA-seq to the xCell website (xcell.ucsf.edu) and each tissue’s enrichment score to evaluate P values. P value <.05 is considered significant enrichment.

**Ingenuity Pathway Analysis.** Ingenuity pathway analysis (IPA) was performed on 26 differentially expressed genes identified by comparison between ccRCC tumor samples vs tumor thrombus using edgeR algorithms. We identified networks of potentially interacting proteins and placed into node-edge diagrams comprised of focused genes (upregulated: red; downregulated: green) detected by RNA-seq and other interacting molecules (gray or white). We also plotted the expression of various gene networks and canonical pathways and their corresponding enrichment P values to overlap ratios.

**Statistical Analysis**

The programmed death-ligand 1 (PD-L1) expression profile cutoff was a 2-fold change over adjacent normal kidney tissue. We analyzed the primary tumor and the tumor thrombus against adjacent normal. We utilized the student’s t test for continuous variables and the chi-squared test for categorical variables. We performed correlations with the Spearman test due to the small numbers of samples.

**RESULTS**

**Demographics**

We identify variability in PD-L1 expression in 6 patients with renal tumor thrombus. We display the patient demographics in Table 1. The PD-L1 expression did not correlate any demographic information (all P > .05). Figure 1 displays imaging from 6 patients who underwent nephrectomy and tumor thrombectomy. We displayed the PD-L1 expression profiles and immune cell enumeration below each clinical image of the tumor thrombus.

**Numeration of Immune Cell Profile**

We identified that there are differences to include a higher number of M1 macrophages (CIBERSORT mean 0.11 vs 0.03, P < .01) and (nonactivated) resting NK cells (0.077 vs 0.017, P = .02) in PD-L1 high expression of the primary tumor, respectively. However, when applying this high PD-L1 expression within the tumor thrombus compared to adjacent normal 2 subjects were unable to identify any CIBERSORT immune cell variables that were significant. We then examined normal adjacent, primary tumor, and tumor thrombus; we only detected M1 macrophages that are distinctly different (higher) in the primary tumor and tumor thrombus. We identified no difference in M2 macrophages (ANOVA, P = .32, and P = .89, respectively, Supplemental Figure 1a and b).

**Confirmation of M1/M2 Polarization**

We confirmed M1 and M2 macrophages with individual marker genes to validate the CIBERSORT data. We selected CSF1 (Colony-stimulating factor 1, also called macrophage colony-stimulating factor 1) and CD86 as M1 markers (Supplemental Figure 2). We used the paired t test for statistical comparison and noted significance for both markers. For M2, we selected MRC1 (or CD206) and CD163 as markers. CD206 is significant between normal renal tissue and local tumor. However, we noted an association between CD163 and cancer in the kidney rather than the tumor thrombus (second row).

**Table 1.** Demographics

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<th>ID*</th>
<th>Age</th>
<th>Ethnicity</th>
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<th>BMI</th>
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<th>Stage</th>
<th>Thrombus Level</th>
<th>Fuhrman Grade</th>
<th>DM</th>
<th>HTN</th>
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</table>

* ID is the last digit from patient ID (eg TB31325).
Validation of Enumeration and IPA Analysis

We performed 2 levels of bioinformatic validation of enumeration. First, to confirm CIBERSORT analysis, we conducted a study using the xCell platform to establish the presence of various cell types (Figure 2). In this figure, we noted the dominant representation of macrophages, while T cells are less represented in the tumors. There is some representation from T helper cells. We identified networks of potentially interacting proteins and placed into node-edge diagrams comprised of focused genes (Figure 3a). We also examined enriched canonical pathways represented in a bar graph (Figure 3b, top 10 pathways). Three pathways emerged as most important: agranulocyte adhesion and diapedesis differential regulation of cytokine production, differential regulation of cytokine prediction in intestinal epithelial cells by IL17-A and IL17F, and glutathione-mediated detoxification.

Figure 1. Comparison of clinical tumor images, PD-L1 expression, and CIBERSORT inflammatory cell expression data.

PD-L1 Expression

Examining tumor compared to adjacent normal using a 2-fold change or higher cut off from the normal adjacent PD-L1 expression, we noted 3 tumors (IDs #0, 5, 7) were of high PD-L1 expression, and 3 others (IDs #1, 8, 9) were of low PD-L1 expression. Of note, there was variability in 2 subjects that showed the primary tumor and thrombus could have different PD-L1 expression. Subject #5 had 2.9-fold higher expression in the primary tumor but low expression in the tumor thrombus. Subject #8 had low expression in the primary tumor but increased in the tumor thrombus (2.7-fold expression). Taking these into account, the tumor thrombus PD-L1 expression groups (high vs low) in the tumor thrombus did not show any differences in immune cell detection.

Mean Platelet Volume

The mean platelet volume did have an inverse correlation with PD-L1 expression within the primary tumor (Spearman, −0.89, $P = 0.02$, Supplemental Figure 2a) and near significance with PD-L1 expression within the tumor thrombus (Spearman, −0.77, $P = 0.07$, Supplemental Figure 2b).

DISCUSSION

Our findings indicate that the inflammatory processes inside ccRCC with tumor thrombus (TT) are complex varying between patients and within the primary tumor of the same patient. A consistent theme regarding cell enumeration is the presence of inflammatory macrophages in higher proportions in the tumor thrombus. Unfortunately, PD-L1 expression was not consistent and renal mass biopsy may not reflect PD-L1 expression within the TT. However, mean platelet volume (MPV) may be a possible biomarker regarding PD-L1 expression within TT. Further studies are required to confirm if MPV would predict PD-L1 inhibitor response. Recently, a low MPV was associated with adverse renal cancer outcomes and may indicate a high-risk group for which PD-L1 inhibition may provide greater benefit.19

Our primary aim was to investigate the types of cells predominating in tumor thrombus compared to the renal tumor and adjacent normal tissue, as well as, to investigate the PD-L1 expression of ccRCC. The significance of PD-L1 expression in different immune cell subpopulations in the tumor microenvironment remains poorly understood in ccRCC. Neoadjuvant studies have shown partial...
responses but only rarely does a mammalian target of rapamycin or tyrosine kinase inhibitor inhibition cause dramatic reductions in tumor thrombus size to improve surgical resection and morbidity (approximately 12%).

RCC is known for its genomic complexity and high mutational burden. Therefore, the presence of complex genomic heterogeneity and a wide range of mutational load in RCC may predict better responses to recently approved checkpoint inhibitors in patients with RCC with tumor thrombus. The physician may utilize checkpoint inhibitors as newer medications to attempt to reduce renal thrombus size. Unfortunately, our data speak to the complexity of the immune system and the heterogeneity of renal cancer. In Figure 1, we illustrate the various tumor thrombus and their respective PD-L1 expression profiles. It is unknown if immunohistochemistry of the tumor would provide helpful information regarding potential responders. However, it is likely that only a small piece of the primary tumor would be biopsied and not the tumor thrombus. Therefore, a renal mass biopsy may not provide accurate information as a PD-L1 biomarker for therapeutic response.

We utilize the CIBERSORT software program to provide an overview of various immune cells that populate the primary tumor and tumor thrombus compared to normal adjacent tissue. The most abundant immune cells in thrombus were M2 Macrophages and resting (nonactivated) CD4 T cells. Usually, M1 macrophages are considered inflammatory (antitumor); whereas, M2 macrophages are believed to be more anti-inflammatory and tumor promoting. However, current literature has questioned the simplification of complex macrophage processes. After testing all 22 immune cells in the CIBERSORT profile, we only identified differences in M1 Macrophage across normal adjacent, primary tumor, and tumor thrombus (ANOVA, P = .032). Using an xCell bioinformatics platform for cell type enumeration profiling, we confirmed that macrophages are likely to have a significant role in renal tumor thrombus based on their overall abundance. Profiling studies in RCC have recently shown increased complexity in the assumption of M1/M2 macrophage profiling. Macrophages will express genes as a mixture of both M1 and M2 genes rather than exhibit traditional phenotypes. One could repolarize macrophages in combination with other therapies with mammalian target of rapamycin inhibition, which has been shown to modulate the immune system.

We used the IPA analysis to explicitly identify particular pathways used in the tumor thrombus compared to the tumor resident in the kidney proper. While we have confirmed there is a large presence of macrophages by cell enumeration; we utilized the IPA program to determine pathways based on our RNAseq expression profiles. We identified that IL1b as a potential primary driver in interconnecting protein pathway networks. IL1b is a proinflammatory cytokine utilized by M1 macrophages to induce an inflammatory reaction and recruit other cells; however, when consistently activated can promote tumorigenesis.

Additionally, diapedesis was the identified as the most statistically significant pathway utilized by the tumor. The finding indicates that the tumor cells are not likely bathing in the bloodstream but continue to require migration of immune cells across barriers.

Figure 2. xCell analysis. The figure demonstrates each patient number at the top to include the letters N (adjacent normal), T (tumor within the kidney), and TT (tumor thrombus). The right side of the figure displays 64 cell types, and the boxes represent the differential expression representing the presence of that cell. Each box with the darker staining has a stronger association with the number of cells detected and associated with a stronger P value located in the figure legend at the bottom right of the figure.
IL17 cytokines may allow an autoimmune type reaction to the thrombus enabling thrombus survival and propagation. Lastly, glutathione-mediated detoxification may render a mechanism for the tumor thrombus to handle reactive oxygen species (ROS) and mitigate oxidative damage from internal and external sources to preserve maintenance of tumor function. ROS are free radicals that efficiently react with DNA, RNA, and proteins and in high levels can cause cell death.25 Interestingly, platelets involved in wound repair release ROS to recruit additional platelets and leukocytes and could be an external source of ROS.26 We hypothesize that if platelets are attracted to the tumor, the platelet volume may be a potential biomarker of aggressiveness.

A recently published article by Seles et al. associated a low blood platelet volume with adverse renal cell cancer outcomes.19 A cut off of 9.5 had the worst prognosis. We identified 2 patients with this value and both had the highest PD-L1 expression in the tumor and the tumor thrombus. In our correlative studies, we note an inverse relationship of MPV and PD-L1 expression within tumors. MPV is a surrogate marker of platelet activation. Several mechanistic studies have shown that increased platelets facilitate cancer progression and metastasis by promoting angiogenesis.27,28 Given the inverse correlation (Supplemental Figure 2) demonstrated in our research, MPV could be a potential biomarker for PD-L1 expression in the context of a clinical trial.

Limitations of the current study include the low sample size to produce conclusive evidence of PD-L1 expression and inflammatory markers. This study was performed to influence further investigation regarding the types of inflammatory cells compromising the tumor thrombus and could be targeted before surgery. Another limitation is that we did not confirm our bioinformatic findings on inflammatory cells with flow cytometry or immunohistochemistry. Due to the cost of immunohistochemical and pathologic evaluation, we utilized this platform to identify the most promising pathways and cellular interactions to evaluate. Based on this work we have applied for funding to engage in investigations regarding the role of the macrophage and its function in tumor thrombus development and propagation. Due to the small sample size, we would not have been able to provide definitive conclusions even if we performed additional confirmatory investigations.

**CONCLUSION**

Thrombus has higher levels of M1 macrophages that could be additional targets in renal cancer thrombus. The PD-L1 expression is variable between primary clear cell renal tumors and its corresponding tumor thrombus; therefore, a renal mass biopsy may not provide actionable data regarding the PD-L1 profile of the tumor thrombus. Confirmatory studies should explore macrophage-specific functions in tumor thrombus development and propagation along with MPV as a potential blood-based biomarker for PD-L1 expression in renal cancer.
ACKNOWLEDGMENT

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.urology.2018.09.018.

References