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Spatial Transcriptomic Signatures of Early Acute T Cell–mediated Rejection in Kidney Transplants

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Background. Kidney transplantation significantly improves the quality of life for those with end-stage renal failure, yet allograft rejection resulting from immune cell interactions remains a persistent challenge. Although T cell–directed immunosuppressive drugs effectively contain graft rejection in most patients, a notable proportion still experiences acute T cell–mediated rejection (TCMR). Despite an emphasis on suppressing T cell–mediated immune responses, successful control over TCMR is not always achieved, suggesting the potential involvement of factors beyond T cells. **Methods.** Biopsy samples from suspicious (borderline) for acute TCMR (borderline TCMR) and non-TCMR patients were obtained 9 d post-surgery, and spatial transcriptomics profiling was conducted using the GeoMx Digital Spatial Profiler platform. Regions of interest in the glomerulus and interstitium were selected on the basis of immunohistochemistry staining anti-CD3 to identify areas with T-lymphocyte infiltration. Differential gene expression analysis was performed using unpaired *t* tests. **Results.** Unbiased clustering of transcriptional profiles across all regions of interest showed distinct transcriptional profiles between glomeruli and interstitium in non-TCMR samples, whereas borderline TCMR samples displayed no distinct transcriptional profiles between these regions. Contrary to the prevailing T cell–centric view, we observed pathways and genes associated with innate immunity-related inflammatory conditions expressed in glomerular regions of borderline TCMR biopsies. Immunofluorescence staining for CD68 confirmed the presence of macrophages in the glomeruli of the post-TCMR sample in a validation cohort, indicating macrophage involvement in the glomerular response after TCMR. **Conclusions.** Activation of the innate immune response in borderline TCMR appears to impact not only the interstitium but also the glomerulus. Glomerulus-specific immune signatures suggest the role of the innate immune system in rejection. This nuanced understanding proposes the necessity for tailored therapeutic interventions targeting both innate and adaptive immune pathways to enhance transplant outcomes.

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Although kidney transplantation significantly improves the quality of life for those with end-stage renal failure, it does not guarantee a complete cure, with persistent risks of delayed graft function, rejection, and graft failure.[1] T cell–mediated rejection (TCMR) is a major concern, as it involves T-lymphocyte activation and migration to the allograft,

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The raw data and processed data in this study are available Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE252137) under accession number "GSM7995010" on reasonable request.

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potentially leading to interstitial inflammation, arteritis, and long-term graft failure.[2] Despite advances in potent immunosuppressive drugs, a substantial portion of patients remain unresponsive to these therapies,[3,4] suggesting that inadequate T-cell management may not be the sole contributor to TCMR pathogenesis.

Previous research, albeit infrequent, has suggested significant contributions from the innate immune response in rejection.[5-7] However, this aspect has not been extensively explored with spatial resolution in various affected areas of kidney graft biopsies.

Herein, we used spatial transcriptomics to examine gene expression patterns in graft rejection, hypothesizing a dynamic transcriptional response to an alloimmune attack. This approach, which allowed us to exclusively profile gene expression within designated regions, unveiled a comprehensive analysis and crucial biological processes that escaped detection through traditional histological analyses.

MATERIALS AND METHODS

Ethics Statement

This retrospective study (institutional review board No. 2205-175-1329) used tissues from protocol biopsies conducted at Seoul National University Hospital, South Korea, with informed consent by the Institutional Review Board. The protocol biopsy samples from borderline TCMR and non-TCMR patients were acquired 9 and 11 d after surgery, respectively, with consent at the aforementioned institution, Seoul National University Hospital. Additionally, we collected kidney biopsy samples from another patient at 2 time points as a validation cohort: first, directly after kidney transplantation, pre-TCMR; and second, 9 d after transplantation by protocol biopsy, post-TCMR. The study was conducted in accordance with the Declaration of Helsinki and the article adheres to the Declaration of Istanbul.

Study Subjects and Sample Collection

Kidney function tests, assessing creatinine levels and glomerular filtration rates, were performed before transplantation and 7 d after the transplantation. One patient exhibited borderline TCMR, whereas the other showed no signs of acute TCMR. Biopsy samples were histopathologically diagnosed using the Banff classification system. The first patient, assessed 9 d posttransplant, showed signs consistent with rejection pathology and was diagnosed as suspicious (borderline) for acute TCMR (borderline TCMR). The findings included mild inflammation (i1), mild tubulitis (t1), absent endarteritis (v0), absent glomerulitis (g0), and minimal peritubular capillaritis (ptc0). Additional findings included 52 glomeruli with 9.6% global sclerosis, focal slight hypercellularity, and eosinophil infiltration in the interstitium. The second patient, evaluated 11 d posttransplant, showed no evidence of acute rejection. Findings included minimal inflammation (i0), absent tubulitis (t0), mild glomerulitis (g1), and minimal peritubular capillaritis (ptc0). Additional information for each patient can be found in Table 1.

Slide Preparation for In Situ Spatial Transcriptome Analysis

The GeoMx Digital Spatial Profiler (DSP), developed by Nanostring in Washington, served as the spatial transcriptomics profiling platform. Slide preparation was conducted following the manufacturer's guidelines (NanoString GeoMx DSP Manual Slide Preparation, MAN-10150-01). Kidney biopsy formalin-fixed paraffin-embedded blocks were sectioned to 5-mm thickness and mounted on a charged slide (Leica BOND Plus slides, Germany). The slide was baked at 60 for 30 min, followed by deparaffinization with CitriSolv and rehydration gradually through 100%, 95% ethanol, and 1X PBS. Epitopes were retrieved and exposed by immerging in 1X Tris-EDTA, pH 9 for 15 min, and 1/mL proteinase K for 15 min. Post-fix preservation was performed with 10%

TABLE 1.

Baseline characteristics of subjects

	Kidney allograft biopsy	
Characteristics	**Borderline TCMR**	**Non-TCMR**
Age, y	63	59
Sex	F	M
Cause of ESKD	Kidney biopsy-proven IgAN	Clinically diagnosed DM-ESRD
Creatinine levels, mg/dL		
Pretransplantation	6.27	10.15
Posttransplantation 7 d	0.76	61.10
Glomerular filtration rate, mL/min		
Pretransplantation	5.0	6.5
Posttransplantation 7 d	72.6	83.8
ABO	Incompatible	Compatible
DSA	Negative	Negative
Graft function	Immediate graft function	Immediate graft function
Pathological diagnosis	Suspicious (borderline) for acute T cell–mediated rejection (i1, t1, v0, g0, ptc0/ ci0, ct0, cv0, cg0/ ti0, i-IFTA0, t-IFTA0/ mm0, ah0, aah0)	1. No evidence of acute rejection 2. Interstitial fibrosis and tubular atrophy, grade I (i0, t0, v0, g1, ptc0/ ci1, ct1, cv1, cg0/ ti0, i-IFTA0, t-IFTA0/ mm0, ah0, aah0)
Immunosuppression regimen	Tacrolimus, MMF, prednisolone	Tacrolimus, MMF, prednisolone

Data are presented as number (%) or median (range) values.
DM-ESRD, diabetes mellitus-end-stage renal disease; DSA, donor specific antibody; ESKD, end-stage kidney disease; IFTA, interstitial fibrosis and tubular atrophy; IgAN, IgA nephropathy; MMF, mycophenolate mofetil; TCMR, T cell–mediated rejection.

neutral buffered formalin and neutral buffered formalin stop buffer, which was made by mixing Tris and glycine to preserve the morphology of kidney tissues. Finally, the slide was incubated with RNA hybridization probes that consist of target complementary sequence and DNA oligo barcode for Next Generation Sequencing readout using Whole Transcriptome Atlas overnight at 37 °C. On the next day, the slide was stained with SYTO 13 (NanoString; 121300303, 1:10), anti-CD45 (Cell signaling technology; 14579, 1:100), anti-PanCK (Novus; NBP2-33200AF594, 1:100), and anti-CD31 (Abcam; ab215912, 1:100) fluorescence antibodies for 1 h at room temperature.

For CD68 immunofluorescence (IF) staining, the slide preparation process proceeded similarly to that for in situ spatial transcriptome studies, including deparaffinization and antigen retrieval using 1X Tris-EDTA. Following this, the slides were blocked with 5% BSA for 30 min. Anti-CD68 AF532-conjugated antibody (NBP2-34587AF532, [C68/684], 1:100) for macrophage detection and anti-αSMA AF647-conjugated antibody (ab202296, [EPR5368], 1:5000) for glomeruli detection were then incubated on the slides in a humid chamber for 1 h. Subsequently, the slides were washed twice with distilled water for 5 min each. Finally, images were obtained by scanning with the GeoMx DSP instrument.

Spatial Profiling and Library Construction

The slides were loaded into the GeoMx DSP instrument to select regions of interest (ROIs). Two glomeruli were randomly selected and 4 regions of interstitium with intense CD3 or dense infiltration of immune cells were strategically selected for each patient. Transcripts from an average area of 86 751.1/ROI were collected into a DSP collection plate. Primer pairs, i5/i7 dual-indexing sequences, were used to index the oligonucleotides during polymerase chain reaction. Polymerase chain reaction products from each ROI were pooled into a single tube, followed by cleaning up with AMPure XP (A63881, BECKMAN COULTER, CA) and washing with 80% ethanol.

Spatial Transcriptome Data Processing and Analysis

GeoMx DSP Analysis Suite (GEOMX-0094, version 2.4.2.2) was used to analyze the data. Target genes with 5% of the segment above the threshold were retained in the data sets and gene expressions with lower than the limit of quantitation were initially excluded. The limit of quantitation was determined as the negative probe geomean multiplied by the geometric SDs of the negative probes, resulting in a total 16 164 genes for further analysis. The read counts of these filtered genes were subjected to normalization using the Q3 normalization method.

Differentially expressed genes (DEGs) within each region were subsequently identified. To account for multiple testing, we initially analyzed over 20 000 transcripts and retained 16 164 genes after applying a threshold based on the limit of quantitation. We then applied the Benjamini-Hochberg correction method to control the false discovery rate. This nonparametric approach allowed us to identify DEGs with a P value of <0.05, considering genes with log2 fold change ≥1 as upregulated DEGs.

Statistical Analysis

Statistical comparison of each gene between borderline TCMR and non-TCMR was performed using unpaired t tests. Analyses were conducted using GraphPad Prism software (version 10.1.0; GraphPad Software, La Jolla, CA). Significance was defined with a P value threshold of <0.05. In our results, statistically significant differences were denoted using asterisks, where * denotes $P < 0.05$, ** signifies $P < 0.005$, and *** indicates $P < 0.0005$. The measurement of gene expression levels was performed in arbitrary units.

RESULTS

Flowchart and Baseline Characteristics of the Biopsies

We conducted spatial transcriptome analysis to characterize the transcriptional response in specific kidney regions after kidney transplants (Figure 1A). Both patients received living donor kidney transplants under an identical immunosuppression regimen with a 6-d interval between their respective operations. Microscopic findings from the biopsy of the patient with borderline TCMR revealed interstitial alterations, including tubulitis and inflammation within the nonscarred cortex (Table 1). The non-TCMR biopsy was acquired from a matched individual who did not show evidence of acute rejection based on the Banff classification, as described in the Methods section.

We identified 2 glomeruli and 4 interstitial ROIs from each borderline TCMR and non-TCMR biopsy (Figure 1B). To closely approximate the molecular signatures characteristic of definitive TCMR, ROIs were carefully selected from interstitial areas demonstrating significant T-cell infiltration in the borderline TCMR sample.

Unbiased Clustering of Transcriptional Profiles Across all ROIs Reveals Differential Gene Expression Patterns in Borderline and Non-TCMR Samples

To investigate transcriptional changes between ROIs, we selected the top 500 DEGs across all 12 ROIs and conducted a hierarchical analysis based on unsupervised clustering. In the non-TCMR sample, the resulting heatmap with dendrogram revealed distinct transcriptional profiles between the glomerulus and interstitium, corroborating the clear histological differentiation between these regions. Conversely, in the borderline TCMR sample, no clear distinction between glomerular and interstitial regions could be identified (Figure 2A; **Table S1, SDC,** http://links.lww.com/TXD/A696). This suggested the existence of a common factor leading to comparable alterations in both glomerular and interstitial areas, resulting in a convergence in their respective gene expression profiles.

To identify the potential common factor, we first compared gene expression differences between all 6 ROIs, including both the interstitium and glomeruli in the borderline TCMR biopsy and 6 analogous ROIs in the non-TCMR biopsy (**Table S2, SDC,** http://links.lww.com/TXD/A696). Notably, we observed heightened expression of genes associated with innate immunity-related inflammatory conditions, including *C4B, CLU*, and *WFDC2*[8-10] (Figure 2B). These genes also appeared when we conducted the same analysis only with interstitial regions (Figure 2C; **Table S3, SDC,** http://links.lww.com/TXD/A696), suggesting that these genes were more elevated in the interstitium than the glomerulus. To obtain candidate genes expected to be expressed especially in glomeruli, we compiled all DEGs

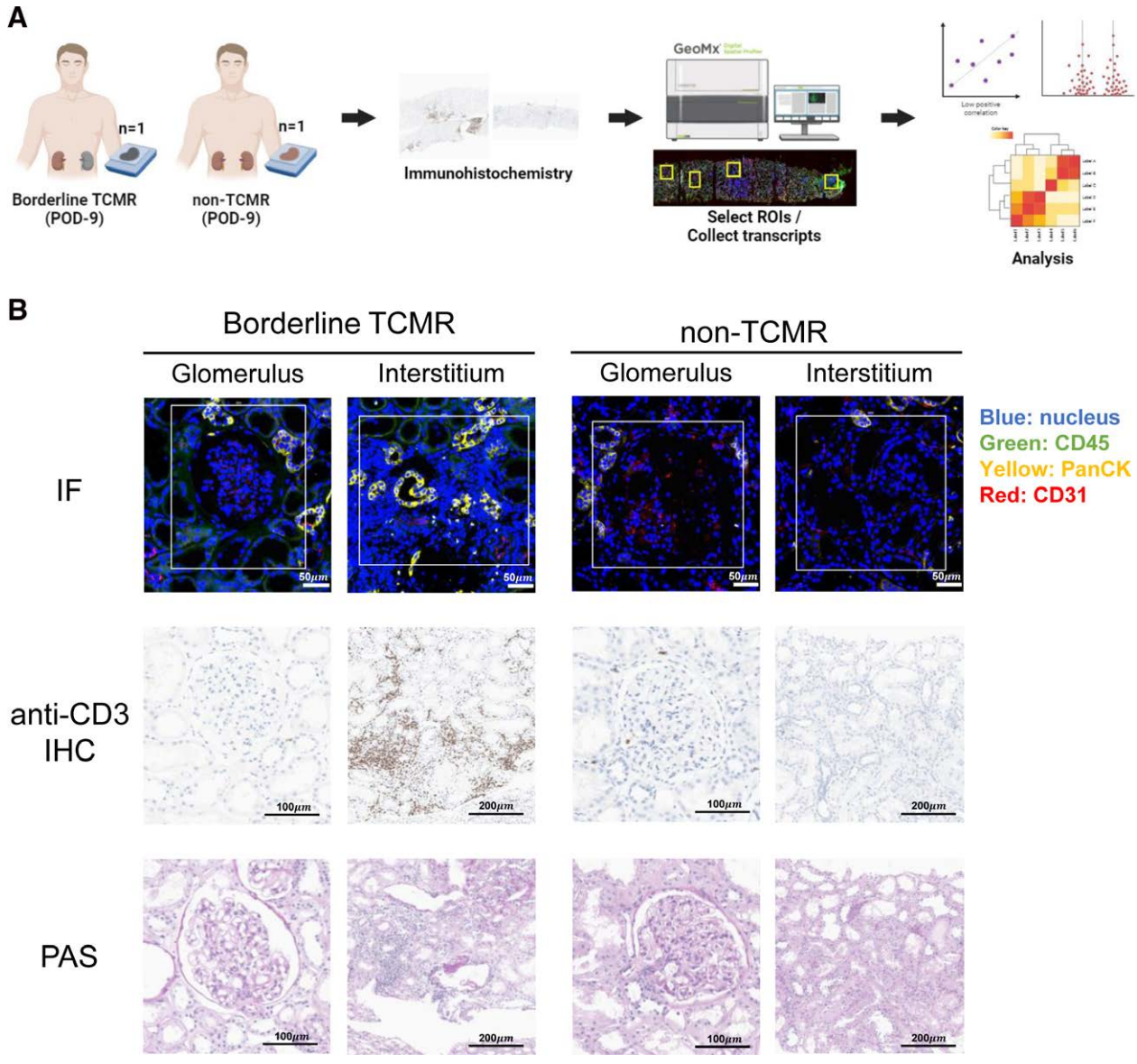

FIGURE 1. Geometric ROI selection using the GeoMx platform. A, Overview of the GeoMx workflow. Tissue samples for spatial transcriptome analysis were acquired 9 d after surgery (POD-9). B, IF, anti-CD3 IHC, and PAS stain images depicting glomerulus and interstitium of TCMR (left) and non-TCMR (right) samples with selected ROIs. Two glomeruli and 4 regions of interstitium were strategically selected for each patient. Morphology markers include SYTO13 (blue), CD45 (green), PanCK (yellow), and CD31 (red). Scale bars in all images of IF, glomerulus of IHC and PAS, and interstitium of IHC and PAS indicate 50, 100, and 200 μm, respectively. IF, immunofluorescence; IHC, immunohistochemistry; PAS, periodic acid-schiff; POD, postoperative day; TCMR, T cell–mediated rejection; ROI, region of interest.

across all 12 regions and then excluded those exclusive to interstitial comparisons. This yielded 52 genes (Figure 2D; **Table S4, SDC,** http://links.lww.com/TXD/A696). When performing gene set enrichment analysis on these 52 genes to gain hints into biological changes occurring in the borderline TCMR sample, we identified that these genes were involved in the following 4 biological changes: innate immune response, positive regulation of cytokine production, allograft rejection, and interferon-gamma signaling (Figure 2E; **Figure S2, SDC,** http://links.lww.com/TXD/A696). Additionally, normalized expression levels of each of these genes were compared using scatter plots (**Figure S1, SDC,** http://links.lww.com/TXD/A696). Remarkably, 4 genes (*MT1-G*, *MT2A*, *TXNIP*, and *PSME1*) showed significant upregulation across both glomerular and interstitial regions, indicating that these genes may contribute to the distinct transcriptional changes that blurred the distinction between these 2 regions in the borderline TCMR sample (Figure 2F).

Presence of CD68+ Macrophages in Glomeruli of TCMR

Upon comparison of the expression levels of the 52 selected genes exclusively in the glomeruli of non-TCMR and borderline TCMR patients, 23 genes showed significant differences (**Figure S3, SDC,** http://links.lww.com/TXD/A696). After excluding ribosomal protein-related genes from those 23 genes, we found that approximately half (7/15) of the remaining genes (*MT1-G*,[11] *ZFP36L1*,[12] *MT2A*,[13,14] *UXT*,[15] *ALDH2*,[16] *RACK1*,[17] *CCDC92*[18]) played roles in with macrophage activity or innate immunity (Figure 2G). Consequently, to confirm the presence of macrophages in the glomeruli, we stained with anti-CD68 using IF in a validation cohort of pre- and post-TCMR samples

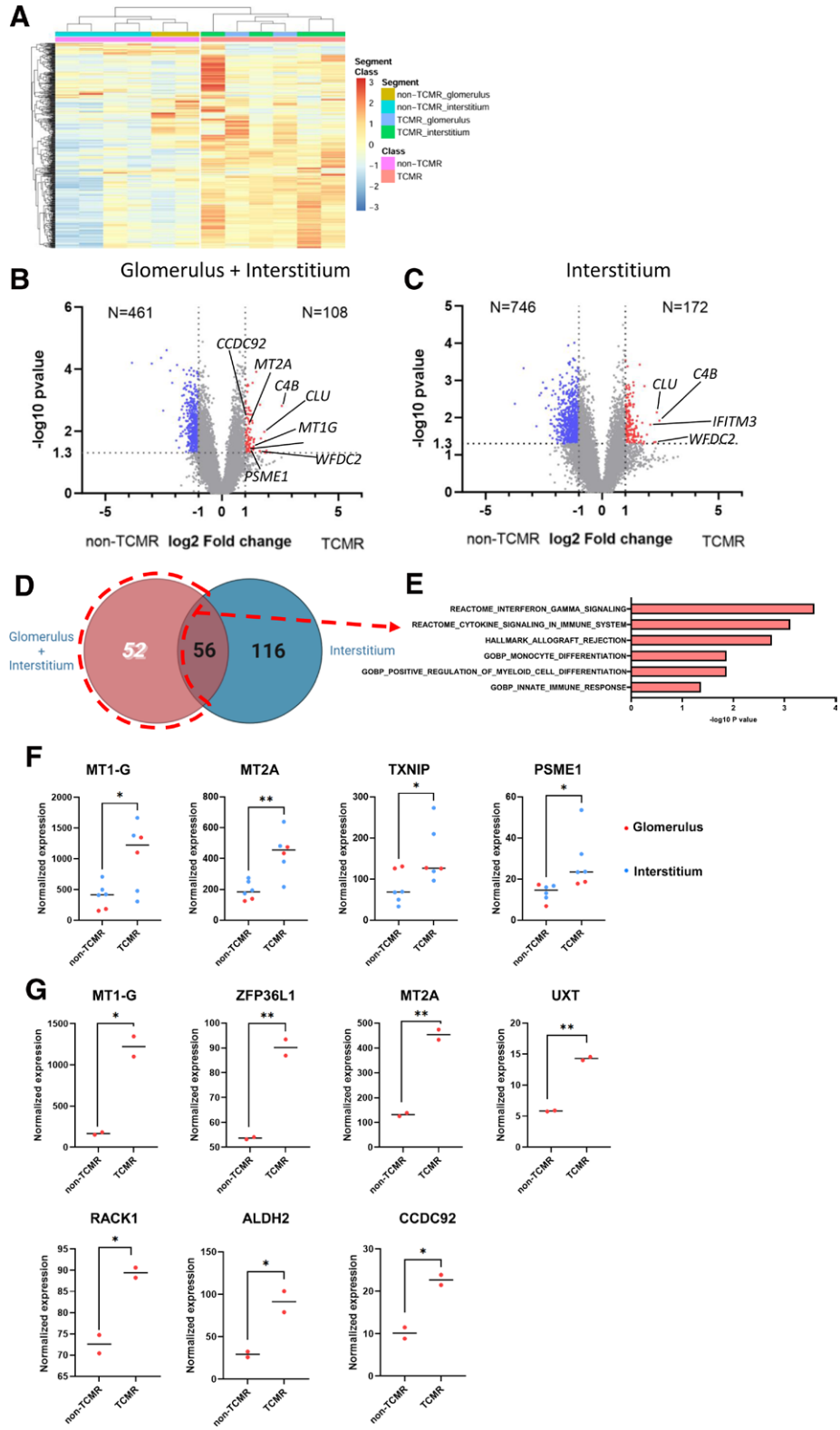

FIGURE 2. Innate immune signatures in TCMR. A, 500 most variable genes were selected to cluster the ROIs unbiasedly and displayed here as a heatmap. In TCMR, the difference between the glomerulus and interstitium was not dichotomized. B, Volcano plot of DEGs within 6 ROIs, including both glomerulus and interstitium in both TCMR and non-TCMR samples. This analysis identified 108 upregulated and 461 downregulated DEGs with log2 fold change ≥1 and *P < 0.05. C, Volcano plot of DEGs in interstitial regions in TCMR and non-TCMR samples, excluding the glomerulus. Analysis revealed 172 upregulated and 746 downregulated DEGs with log2 fold change ≥1 and *P < 0.05. D, Venn diagram displaying 52 candidate genes potentially linked to glomerulus-specific transcriptional changes in TCMR patients. E, Biological changes based on the 52 candidate genes were confirmed by GSEA. F, Scatter plots depicting the expression levels of 4 glomerulus-specific DEGs (*MT1-G, MT2A, TXNIP,* and *PSME1*) in ROIs from both a control patient and a patient with TCMR. G, Scatter plots showing significantly upregulated expression levels of genes related to the innate immunity signature in the glomeruli of borderline TCMR patients. DEG, differential expressed gene; GESA, gene set enrichment analysis; ROI, region of interest; TCMR, T cell–mediated rejection.

of a different patient. Although CD68 was not detected in the pre-TCMR samples, it was observed in the glomeruli of post-TCMR samples, indicating that CD68+ macrophages are present in the glomeruli after TCMR (**Figure S4, SDC,** http://links.lww.com/TXD/A696).

DISCUSSION

In this study, we used spatial transcriptomics to explore gene expression profiles within the glomeruli and interstitium of both borderline TCMR and non-TCMR patients. Prior studies of TCMR immunopathogenesis primarily focused on the characteristics of tubulointerstitial regions,[19] due to 2 reasons: first, T cells predominantly infiltrate the tubulointerstitium, and second, indicators of glomerular injury, such as proteinuria, are relatively infrequent in TCMR patients.[20]

However, in our analysis, clustering of DEGs across all ROIs in the borderline TCMR biopsy uncovered homogeneity in transcriptional profiles within glomerular and tubulointerstitial regions. This implied potential transcriptional abnormalities in the glomeruli, even in the absence of visible infiltration of T lymphocytes.

Initially, across all ROIs, *C4B* displayed the highest log2 fold change among the identified genes, indicating a correlation between borderline TCMR onset and activation of the classical complement pathway. Activated complements may amplify effector T-cell function and TCMR through a positive feedback loop and play a role in TCMR by attracting inflammatory cells, including macrophages, neutrophils, and natural killer cells.[21] The heightened expression of *C4B*, along with supporting findings from prior research,[22-24] suggests the potential effect of controlling complements in cell-mediated transplant rejection.

Next, to investigate further, we excluded overlapping DEGs specific to interstitial ROIs from upregulated DEGs in all 12 ROIs. This process allowed us to infer that the remaining 52 genes are likely to be expressed especially in the glomeruli. Among these genes, we detected *MT1-G, MT2A, TXNIP*, and *PSME1*, which are related to inflammatory signatures and the production of reactive oxygen species (ROS). This result illustrates the potential induction of damage-associated molecular patterns (DAMPs) and subsequent activation of innate immune responses in TCMR pathogenesis, as well as the aggravation of adaptive immune responses. *MT1-G* and *MT2A* are metallothionein-encoding genes, mainly induced in response to cellular oxidative stress[25]; therefore, the heightened expression of these genes may indicate oxidative damage. *TXNIP*, which stands for thioredoxin-interacting protein, is known to act as a DAMP, impeding the antioxidative capacity of thioredoxin.[26] Consequently, this inhibition leads to the buildup of ROS and induces cellular stress.[27] *PSME1* encodes a subunit of PA28, a multimeric complex that acts as a proteasome activator.[28] This protein plays a major role in antigen presentation through major histocompatibility complex class I,[29,30] and activates inflammatory signatures related to both innate and adaptive immunity. Products of cellular damage indicated by these genes may lead to a full-fledged activation of the innate immune system. Additionally, we identified 7 significantly upregulated genes related to innate immune signatures, especially those associated with macrophages in the glomeruli of TCMR patients.[11-18] Finally, through anti-CD68 IF, we observed that macrophages were recruited to the glomeruli of TCMR patients. Hence, it may be implied that macrophages are activated and play a role in the pathogenesis of TCMR onset. We interpret this as macrophages sensing DAMPs, leading to their increased migration to the glomeruli and production of ROS. Finally, we searched for overlapping innate immune activation signatures with the Banff Human Organ Transplant (panel. Specifically, among the 52 genes we highlighted, 10, including *MT2A, PSME1*, and *SOD2*, were genes listed in the Banff Human Organ Transplant panel. This reinforces the relevance of our findings within the established framework of transplant immunology and highlights key players in the innate immune response during rejection.

Although these changes may not be histologically apparent within 9 d posttransplant surgery, our region-specific spatial transcriptomic analysis provides evidence that the innate immune response in borderline TCMR impacts the interstitium and the glomerulus.

Our study has some limitations. First, the borderline TCMR patient was ABO-incompatible (ABO-i) and the cause of end-stage kidney disease in this case was IgA nephropathy. To reduce the anti-B IgG titer, allowing transplantation, borderline TCMR patients underwent a total of 4 sessions of plasmapheresis. Despite ABO-i, no antibody-mediated rejection was diagnosed, making it unlikely that ABO-i alone caused transcriptional changes in interstitial and glomerular areas. In the context of end-stage kidney disease, the biopsy taken a week posttransplant, was likely not influenced by IgA deposits, and there was no IgA nephropathy recurrence in the 1-y follow-up biopsy. Future analyses should use immunohistochemistry to check for IgA deposits in a larger sample size.

Second, our analysis is based on a single case, thereby limiting the immediate generalization of our findings to other biopsies. The included case is diagnosed as borderline rather than definitive TCMR based on histological findings, although clinically, they are viewed not as discrete conditions but as part of a continuous spectrum of rejection phenotypes. To address this issue, we focused on T cell–infiltrated regions in spatial transcriptomics analysis to minimize differences between borderline rejection and confirmed TCMR. Nonetheless, to achieve a more comprehensive understanding of innate immunity activation within glomeruli during rejection, further studies with larger sample size and a longitudinal approach incorporating zero-time biopsy data are warranted. Furthermore, a comprehensive comparative study including both borderline and definitive TCMR samples would validate our findings and provide deeper insights into the molecular distinctions and parallels between these histological entities.

This study is the first study, to our knowledge, to demonstrate abnormalities in glomerular transcripts in regions of a patient experiencing early TCMR. This supports the hypothesis that immune-related changes, not detectable in histological testing, occur in various areas of the graft biopsy, particularly in the glomerulus. By expanding our understanding of rejection mechanisms, this research provides insights for exploring innate immune responses that may impact overall transplant outcomes.

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