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Urinary Cell Transcriptome Profiling and Identification of ITM2A, SLAMF6, and IKZF3 as Biomarkers of Acute Rejection in Human Kidney Allografts

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Background. Identification of a shared gene expression pattern between T cell–mediated rejection (TCMR) and antibody-mediated rejection (AMR) in human kidney allografts may help prioritize targets for the treatment of both types of acute rejection. **Methods.** We performed RNA sequencing and bioinformatics of genome-wide transcriptome profiles of urinary cells to identify novel mRNAs shared between TCMR and AMR and of mechanistic relevance. Customized RT-QPCR assays were then used to validate their abundance in urinary cells. Urinary cell transcriptome profiles and mRNA abundance were assessed in 22 urine samples matched to 22 TCMR biopsies, 7 samples matched to 7 AMR biopsies, and 24 samples matched to 24 No Rejection (NR) biopsies and correlated with biopsy diagnosis. **Results.** RNA sequencing data and bioinformatics identified 127 genes in urine to be shared between TCMR and AMR. We selected 3 novel mRNAs—ITM2A, SLAMF6, and IKZF3—for absolute quantification and validation by customized RT-QPCR assays. The abundance of all 3 mRNAs was significantly higher in urine matched to TCMR or AMR than in urine matched to NR biopsies. Receiver-operating-characteristic curve analysis showed that all 3 mRNAs distinguished TCMR or AMR from NR. Their abundance was similar in patients with TCMR and those with AMR. **Conclusions.** State-of-the-art antirejection therapies are mostly effective to treat TCMR but not AMR. Our identification of mRNAs shared between TCMR and AMR and contributing to T cell–B cell interactions may help prioritize therapeutic targets for the simultaneous treatment of TCMR and AMR.

(*Transplantation Direct* 2020;6: e588; doi: 10.1097/TXD.0000000000001035. Published online 22 July, 2020.)

INTRODUCTION

Kidney transplantation has evolved since the first successful kidney transplantation performed by Dr Joseph E. Murray in 1954.[1] Advancements in immunosuppressive therapy and infection prophylaxis have contributed to continued improvements in allograft survival rates, with the number of kidney transplant recipients alive and with functioning kidney allografts projected to exceed 250 000 within the next 2 years.[2]

Although short-term graft survival has improved remarkably, long-term survival has not improved proportionally.[3,4] Acute rejection is a risk factor for graft loss. Between the 2 major types of acute rejection in kidney allografts, acute T cell–mediated rejection (TCMR) is more frequent and more effectively treated compared with active antibody–mediated rejection (AMR). TCMR is characterized by interstitial infiltration and tubulitis, and AMR is characterized by an antibody response mostly directed at donor human leukocyte antigens displayed by endothelial cells, with the kidney allograft

biopsy displaying acute tissue injury, vascular inflammation, and often the deposition of complement factor 4 degradation products.[5]

We developed urinary cell mRNA profiling for the noninvasive assessment of kidney allograft status and identified that urinary cell levels of mRNA encoding cytopathic proteins granzyme B and perforin and mRNAs encoding immunoregulatory proteins are associated with TCMR in human kidney allografts.[6–12] Our single-center studies led to the multicenter Clinical Trials in Organ Transplantation-04 (CTOT-04) in which a urinary cell 3-gene signature of 18S rRNA normalized levels of CD3E mRNA, IP10/CXCL10 mRNA, and 18S rRNA was developed and validated to be diagnostic of TCMR.[13] Our single-center studies, as well as the CTOT-04 study, were designed to investigate a panel of mRNAs selected on the basis of their potential participation in TCMR.

In this study, we sought to identify a shared gene expression pattern between TCMR and AMR to aid diagnosis of both types of acute rejection and to identify shared targets for therapeutic intervention. Our search for shared targets during an episode of TCMR or AMR was based on the following considerations: (i) activation of the antigen-specific B-cell humoral response is mediated through direct contact between T and B cells as well as through T-cell secretion of cytokines[14]; and (ii) T-cell and B-cell interactions are bidirectional, with T cell help required for affinity maturation and immunoglobulin isotype switching in B cells, and B cells functioning as classical antigen-presenting cells (APCs), processing and presenting major histocompatibility (MHC) bound antigens to the T-cell receptor.[15]

We report here that urinary cell abundance of mRNA for ITM2A, SLAMF6, and IKZF3 discriminate patients with TCMR or AMR biopsies from patients with No Rejection (NR) biopsies. Importantly, urinary cell abundance of these mRNAs is diagnostic of both TCMR and AMR.

MATERIALS AND METHODS

Kidney Allograft Recipients

The study cohort was composed of adult recipients of human kidney allografts transplanted at our institution, New York Presbyterian-Weill Cornell Medicine. The patients were treated with a standardized immunosuppression protocol and received their posttransplant care by a single group of transplant physicians. The study was approved by our WCM Institutional Review Board (no 1207012730) with the kidney graft recipients providing written, informed consent to participate. The clinical and research activities reported in this communication are consistent with the principles of the "Declaration of Istanbul on Organ Trafficking and Transplant Tourism."

Total RNA Isolation From Urinary Cells

Our urine collection protocol and isolation of total RNA from urinary cells have been reported.[13] The quantity and quality of the RNA isolated from the urine were measured using the NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific, Waltham, MA). The Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA) was used to measure RNA integrity, reported as RNA integrity number.

RNA-seq of Urinary Cells

The Genomics Core Laboratory at Weill Cornell Medicine performed RNA sample library preparation and RNA

sequencing (RNA-seq). The normalized cDNA libraries were pooled and sequenced on the Illumina HiSeq4000 sequencer with pair-end 100 cycles. Among the 70 urine samples from 60 kidney allograft recipients that were submitted for RNA-seq, 57 urine samples from 53 recipients met quality thresholds and were passed onto downstream data analysis.

Customized RT-QPCR Assays

Total RNA isolated from the urinary cells was reverse transcribed to cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) at a final concentration of 1.0 μg of total RNA in 100 μL volume. Gene-specific oligonucleotide primers and fluorogenic probes were designed using Primer Express software. A 2-step customized RT-QPCR assay was performed via a preamplification step, using gene-specific primer pairs, followed by measurement of the absolute levels of mRNAs, using our previously described standard curve method[10,13] and the Applied Biosystems QuantStudio 6 Flex Real-Time PCR System (ThermoFisher Scientific). The standard curve copy numbers in our PCR assays ranged from 25 copies to 2.5 million copies. Among the 57 biopsy-matched urine samples that passed RNA-seq quality thresholds, 53 urine samples from 49 kidney allograft recipients met the prespecified RNA quality thresholds (18S rRNA $\geq 5.0 \times 10^7$ copies/μg of total RNA and TGFβ1 mRNA $\geq 1.0 \times 10^2$ copies/μg of total RNA) and were included in downstream data analysis.

Banff Classification of Kidney Allograft Biopsies

Urine samples were from a crosssection of patients selected to represent 3 major Banff diagnostic categories: Banff Category 1, normal biopsy or nonspecific changes (designated in this report as NR); Banff Category 2, antibody-mediated changes (AMR); or Banff Category 4, TCMR. All kidney allograft biopsies were performed under ultrasound guidance. The biopsies were read and reported independently by 2 transplant pathologists at our center (S.P.S. and S.V.S.); classification was based on the Banff 2017 update of the Banff 1997 classification of allograft pathology.[5,16] The pathologists were masked to gene expression profiles. Sections of biopsy tissues were stained with hematoxylin and eosin, periodic acid–Schiff, and Masson trichrome. Indirect immunofluorescence for complement factor 4 degradation (C4d) product was performed on cryosections using a monoclonal anti-C4d antibody (Quidel, Santa Clara, CA). Anti-SV40 antibody was used to screen for the presence of the polyoma virus in the allograft.

Selection of ITM2A, SLAMF6, and IKZF3 as Candidate Genes

Whole transcriptome profiling of urinary cells by RNA-seq and differential gene expression analysis was used to identify genes differentially expressed in urine matched to TCMR biopsies compared with urine matched NR biopsies using $\log_2 FC \geq 2$ and false discovery rate (FDR) <0.05 as the thresholds for differential gene expression. Identical thresholds were used to identify differentially expressed genes in urine matched to AMR biopsies versus urine matched to NR biopsies. An overlapping set of 127 genes shared between urine matched to TCMR biopsies and urine matched to AMR biopsies was then generated. This set of genes was ranked by their abundance using $\log_2 FC$ as the parameter to identify the most highly and differentially expressed genes and those most likely

to be successfully validated via RT-QPCR. For class comparison, TCMR versus NR was chosen instead of AMR versus NR, or a composite score of both types of rejection, due to the higher number of TCMR samples (22 samples for TCMR versus 7 samples for AMR). Of the most highly expressed genes, a search for novel genes excluded previously described markers of TCMR or AMR in kidney allograft rejection, T-cell receptor regions, and histones. Of remaining genes, a literature review was used to identify genes of mechanistic relevance to rejection, prioritizing genes capable of contributing to T cell–B cell interactions.

Statistical Analysis

RNA-seq data were analyzed for differential expression of genes in urine matched to different biopsy diagnostic categories using voom normalized raw counts in the *limma* package (http://bioconductor.org/packages/release/bioc/html/limma.html) in R.[17] *P* values for differences in gene expression were adjusted for FDR using the Benjamini-Hochberg method.

Absolute levels of each mRNA measured using customized RT-QPCR assays were $\log_{10}$-transformed to reduce positive skewness.[13] Before log transformation, all zero values for mRNA copies after 40 cycles of PCR amplification were assigned a value of 6.25 copies of mRNA per microgram of total RNA, and copy numbers between 25 copies to 1 copy were assigned a value of 12.5 copies. *P* values for 3-group comparisons of mRNA abundance were calculated using the Kruskal-Wallis test. Pairwise comparisons among groups were calculated using the Mann-Whitney *U* test; $P < 0.05$ was considered statistically significant. Receiver-operator-characteristic (ROC) curve analysis, sensitivity, and specificity were calculated using Youden's index. GraphPad Prism 8.0.0 (GraphPad Software, San Diego, CA) was used for statistical tests and for generating graphs.

RESULTS

Study Cohort

Table 1 is a summary of characteristics of the study cohort. All 49 kidney allograft recipients received tacrolimus as the calcineurin inhibitor and mycophenolate mofetil, and 5 patients with TCMR, 6 patients with AMR, and 9 patients with NR biopsies received corticosteroid maintenance therapy. All TCMR and AMR biopsies were for-cause (clinically indicated biopsies) and performed to resolve the basis for graft dysfunction. Among 24 NR biopsies, 21 were surveillance biopsies, and the remaining 3 biopsies were clinically indicated biopsies. Figure 1 shows Banff scores of individual biopsies, and the summed Banff scores are shown in Table 1. The purity of the total RNA isolated from the urine specimens and the RNA integrity number are also shown, stratified by biopsy diagnosis, in Table 1.

RNA-seq and Differential Gene Expression Analysis

As recently reported,[18] whole transcriptome profiling of urinary cells by RNA-seq and differential gene expression analysis identified 180 differentially expressed genes in urine matched to TCMR biopsies compared with urine matched to NR biopsies using $\log_2 FC \geq 2$ and FDR <0.05 as the thresholds for differential gene expression. At identical thresholds, 544 differentially expressed genes were identified in urine matched to AMR biopsies compared with urine matched to NR

biopsies. Among the genes over expressed in urine matched TCMR biopsies or AMR biopsies versus urine matched to NR biopsies, 127 genes formed an overlapping set of genes that were shared between the urine matched to TCMR biopsies and urine matched to AMR biopsies.[18] This set of 127 shared genes was then ranked by $\log_2 FC$ >2 for TCMR urine versus NR urine to identify the most highly expressed genes. Of highly expressed genes, ITM2A, SLAMF6, and IKZF3 were selected for their high level of expression, novelty, and mechanistic relevance to rejection. Table 2 shows their ranks among the highly expressed genes in urine matched to acute rejection biopsies, $\log_2 FC$ >2 as compared with urine matched to NR biopsies, and associated nominal *P* values and FDR-adjusted *P* values for each of the genes selected as candidate genes involved in both AMR and TCMR.

Urinary Cell Levels of ITM2A, SLAMF6, and IKZF3

We measured absolute levels of ITM2A, SLAMF6, and IKZF3 urinary cell mRNAs in biopsy matched urine specimens using customized RT-QPCR assays developed and validated in our laboratory.[10,13] The oligonucleotide primers and fluorogenic TaqMan® probes designed for the measurement of mRNA for ITM2A, SLAMF6, and IKZF3 and the primers and probes for the quantification of 18S rRNA (reference gene) are shown in Table 3.

The median (interquartile range [IQR]) $\log_{10}$-transformed level of ITM2A mRNA was 3.87 (2.81–4.68) copies per microgram of total RNA in the 22 urine samples matched to TCMR biopsies and 2.40 (2.11–3.09) copies per microgram of total RNA in the 24 urine samples matched to NR biopsies ($P = 0.0001$; Mann-Whitney test) (Figure 2A; Table 4). The level of ITM2A mRNA was 4.24 (3.16–4.63) in the 7 urine samples matched to AMR biopsies and significantly higher than the copy number in the 24 urine samples matched to NR biopsies ($P = 0.0002$) (Figure 2B; Table 4). In contrast to the significant difference between urine samples matched to TCMR biopsies or AMR biopsies versus urine samples matched to NR biopsies, urinary cell levels of ITM2A mRNA were not significantly different between urine matched to TCMR biopsies and urine matched to AMR biopsies ($P = 0.57$) (Figure 2C; Table 4).

ROC curve analysis of levels of ITM2A mRNA in urine matched to TCMR biopsies versus levels in urine matched to NR biopsy yielded an area under the curve (AUC) of 0.82 (95% confidence interval [CI], 0.69-0.94; $P = 0.0002$). At the Youden cutoff value of 3.73 $\log_{10}$-transformed copies of ITM2A mRNA, the sensitivity was 59% (95% CI, 39-77) and the specificity was 92% (95% CI, 74-99) (Figure 3A). The AUC was 0.93 (95% CI, 0.84-1.00; $P = 0.0007$) comparing urine samples matched to AMR biopsies versus urine samples matched to NR biopsies and the sensitivity was 100% (95% CI, 65-100) and the specificity was 79% (95% CI, 60-91) at the Youden cutoff value of 3.12 $\log_{10}$-transformed copies per microgram of RNA (Figure 3B). ITM2A mRNA levels in urine were similar between urine matched to TCMR biopsies and urine matched to AMR biopsies and the AUC was 0.58 (95% CI, 0.36-0.80; $P = 0.55$) (Figure 3C).

The median (IQR) $\log_{10}$-transformed level of SLAMF6 mRNA was 3.72 (2.88–4.36) copies per microgram of total RNA in the 22 urine samples matched to TCMR biopsies and 3.08 (2.08–3.44) copies per microgram of total RNA in the 24 urine samples matched to NR biopsies ($P = 0.0025$,

TABLE 1.

Characteristics of kidney allograft recipients, organ donors, and biopsy features

Characteristics	Acute T cell–mediated rejection (n = 22 samples, 20 patients)	Active antibody–mediated rejection (n = 7 samples, 7 patients)	No Rejection (n = 24 samples, 22 patients)
At time of transplant			
Recipient information			
Age (y) mean (SD)	47.6 (14.5)	47.0 (15.7)	45.1 (14.4)
Women, n (%)	10 (50.0)	5 (71.4)	10 (45.5)
Racial categories, Black, n (%)	8 (40.0)	1 (14.3)	6 (27.3)
Donor information			
Age (y), mean (SD)	47.3 (12.9)	42.8 (14.5)	42.0 (17.8)
Women, n (%)	11 (55.0)	3 (50.0)	13 (59.1)
Racial categories, Black, n (%)	2 (10.0)	0 (0.0)	3 (13.6)
Deceased donor, n (%)	10 (50.0)	2 (28.6)	7 (31.8)
Induction therapy			
Antithymocyte globulin, n (%)	14 (70.0)	5 (71.4)	19 (90.5)
After transplant and before index biopsy			
Delayed graft function, n (%)	8 (40.0)	2 (28.6)	1 (4.8)
Calcineurin inhibitor, n (%)	20 (100.0)	7 (100.0)	22 (100.0)
Corticosteroids maintenance, n (%)	5 (25.0)	6 (85.7)	9 (40.9)
BK virus nephropathy	0 (0.0)	0 (0.0)	0 (0.0)
Biopsy proven acute rejection, n (%)	1 (5.0)	0 (0.0)	4 (18.2)
At time of index allograft biopsy			
Biopsy clinically indicated: n (%)	22 (100.0)	7 (100.0)	3 (12.5)
Time from transplantation to biopsy (d), median (IQR)	85 (27, 393)	13 (8.0, 1190)	134 (95, 398)
Serum creatinine, mg/dL, median (IQR)	2.5 (1.8, 3.1)	1.8 (1.7, 2.2)	1.4 (1.0, 1.6)
DSA at the time of biopsy, n (%)	4 (19.0)	6 (100.0)	10 (43.4)
Banff lesion scores			
Number of glomeruli, mean (SD)	18.1 (7.1)	23.9 (10.2)	16.0 (9.2)
t score, mean (SD)	2.4 (0.5)	0.3 (0.5)	0.04 (0.2)
i score, mean (SD)	2.7 (0.5)	0.7 (0.5)	0.04 (0.2)
g score, mean (SD)	0.4 (0.6)	2.4 (0.8)	0.0 (0.0)
ptc score, mean (SD)	2.0 (0.9)	2.6 (0.8)	0.1 (0.3)
v score, mean (SD)	0.3 (0.6)	0.0 (0.0)	0.0 (0.0)
C4d intensity, mean (SD)	0.0 (0.0)	2.0 (0.6)	0.0 (0.0)
RNA			
Total RNA purity, A260/A280, median (IQR)	1.97 (1.93, 2.05)	2.03 (1.95, 2.06)	1.94 (1.88, 1.99)
Total RNA integrity, RNA integrity number, median (IQR)	6.95 (5.3, 7.8)	6.9 (6.3, 7.1)	5.4 (3.9, 6.4)

IQR, interquartile range; t score, tubulitis score; i score, interstitial inflammation score; g score, glomerulitis score; ptc score, peritubular capillaritis score; v score, intimal arteritis score; C4d intensity, complement factor 4 degradation product staining intensity.

Mann-Whitney test) (Figure 4A; Table 4). The level of SLAMF6 mRNA was 3.99 (3.42–4.52) copies per microgram of total RNA in the 7 urine samples matched to AMR biopsies and significantly higher than the copy number in the 24 urine samples matched to NR biopsies ($P = 0.0031$) (Figure 4B; Table 4). Urinary cell levels of SLAMF6 mRNA were similar between urine specimens matched to TCMR biopsies and urine specimens matched to AMR biopsies ($P = 0.67$) (Figure 4C; Table 4).

ROC curve analysis of levels of SLAMF6 mRNA in urine samples matched to TCMR biopsies versus levels in urine samples matched to NR biopsy yielded an AUC of 0.76 (95% CI, 0.61-0.90; $P = 0.003$). At the Youden cutoff value of 3.52 $\log_{10}$-transformed copies of SLAMF6 mRNA, the sensitivity was 64% (95% CI, 43-80), and the specificity was 88% (95% CI, 69-96) (Figure 5A). The AUC was 0.86 (95% CI, 0.70-1.00; $P = 0.005$) comparing urine samples matched to AMR biopsies versus urine samples matched to NR biopsies. At the Youden cutoff value of 3.48 $\log_{10}$-transformed copies of SLAMF6 mRNA, the sensitivity was 71% (95% CI, 36-95), and the specificity was 88% (95% CI, 69-96) (Figure 5B).

SLAMF6 mRNA levels were similar between urine matched to TCMR biopsies and urine matched to AMR biopsies and the AUC was 0.56 (95% CI, 0.34-0.77; $P = 0.65$) (Figure 5C).

The median (IQR) $\log_{10}$-transformed level of IKZF3 mRNA was 3.62 (2.89–4.43) copies per microgram of total RNA in the 22 urine samples matched to TCMR biopsies and 2.78 (2.40–3.29) copies per 1 μg of total RNA in the 24 urine samples matched to NR biopsies ($P = 0.0008$; Mann-Whitney test) (Figure 6A; Table 4). The level of IKZF3 mRNA was 3.90 (3.42–4.44) copies per microgram of total RNA in the 7 urine samples matched to AMR biopsies and significantly higher than the copy number in the 24 urine samples matched to NR biopsies ($P = 0.004$) (Figure 6B; Table 4). Urinary cell levels of IKZF3 mRNA were similar between the urine specimens matched to TCMR biopsies and the urine specimens matched to AMR biopsies ($P = 0.64$) (Figure 6C; Table 4).

ROC curve analysis of levels of IKZF3 mRNA in urine samples matched to TCMR biopsies versus levels in urine samples matched to NR biopsy yielded an AUC of 0.78 (95% CI, 0.65-0.91; $P = 0.001$). At the Youden cutoff value of 3.44 $\log_{10}$-transformed copies of IKZF3 mRNA, the sensitivity was
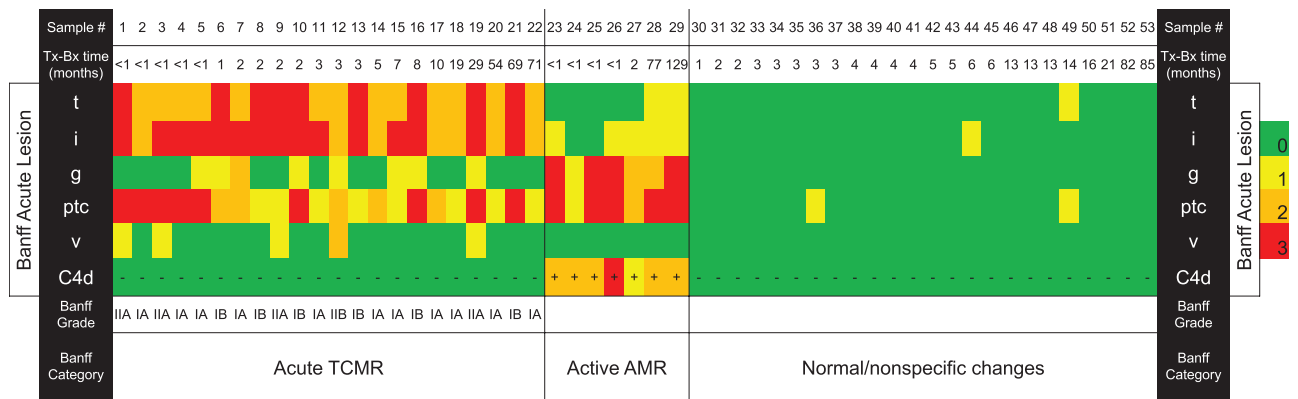
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FIGURE 1. Banff scores of histopathological characteristics of individual kidney allograft biopsies. Kidney allograft biopsy diagnosis is based on the 2017 update of the Banff classification of kidney allograft pathology. Banff diagnostic categories shown include acute TCMR (samples 1–22), AMR lesions (samples 23–29), and normal/nonspecific changes (No Rejection, samples 30–53). Banff acute lesions observed in the 53 kidney allograft biopsies obtained from 47 kidney allograft recipients are color coded based on Banff scores for each of the acute lesions. "Banff grade" denotes overall acute TCMR grade. Colors represent Banff lesion scores 0 through 3 for t (tubulitis), i (interstitial inflammation), g (glomerulitis), ptc (peritubular capillaritis), v (intimal arteritis), and C4d staining. The time from transplant to biopsy (Tx-Bx time) in months is also shown. AMR, active antibody–mediated rejection; TCMR, acute T cell–mediated rejection.

TABLE 2.

RNA-seq and differential gene expression analysis[a]

Ensembl ID	Gene	Ranking	Log$_2$FC for TCMR vs NR	P[b]	FDR-adjusted P[c]	Log$_2$FC for AMR vs NR	P[b]	FDR-adjusted P[c]
ENSG00000078596.9	ITM2A	2	2.691	<0.0001	0.0035	2.761	0.0011	0.0159
ENSG00000162739.12	SLAMF6	21	2.456	<0.0001	0.0033	2.256	0.0046	0.0236
ENSG00000161405.15	IKZF3	14	2.493	<0.0001	0.0033	2.094	0.0035	0.0212

[a]Whole transcriptome profiling of urinary cells by RNA-seq and differential gene expression (DGE) analysis identified a set of 127 overlapping genes differentially expressed in urine matched to TCMR or AMR biopsies compared with urine matched to No Rejection biopsies. Differential expression was based on meeting both log$_2$FC ≥2 and FDR P value <0.05 thresholds.[18] This set of shared genes in urine was then ranked by log$_2$FC in urine matched to TCMR biopsies vs urine matched to No Rejection biopsies to identify the most highly expressed genes. Of the highly expressed genes, the 3 genes shown in the Table were selected for their novelty, mechanistic relevance to both types of rejection, and the high ranking among the top 25 genes differentially expressed between urine matched to TCMR biopsies vs urine matched to No Rejection biopsies.
[b]RNA-seq data were analyzed for differential expression of genes in urine matched to TCMR biopsies vs urine matched to No Rejection biopsies and for differential expression of genes in urine matched to AMR biopsies vs urine matched to No Rejection biopsies using voom normalized raw counts in the *limma* package (http://bioconductor.org/packages/release/bioc/html/limma.html) in R.[17]
[c]P values were adjusted for FDR using the Benjamini-Hochberg method.
TCMR, acute T cell–mediated rejection; AMR, active antibody–mediated rejection; NR, No Rejection; FC, fold change; FDR, false discovery rate.

TABLE 3.

Oligonucleotide primers and TaqMan probes used for mRNA quantification[a]

Target mRNA	GenBank accession number	Sequence	Location
ITM2A	NM_004867.4	Sense: 5′ CGCCTTCGTCGCAGAGA 3′	1013-1029
		Antisense: 5′ AAGTGTCTAATCTTCCAGCATTTATCAA 3′	1086-1059
		Probe: 5′ FAM TTGCTGGGTTTCAAC MGB 3′	1034-1048
SLAMF6	NM_052931.4	Sense: 5′ TCATCATACTGCTGTTACTTGTTTTGA 3′	789-815
		Antisense: 5′ CCTGTGTTCGCTGAGTAGACAAAG 3′	860-837
		Probe: 5′ FAM TAGGGAATCTCTTCTTTTC MGB 3′	817-835
IKZF3	NM_12481.4	Sense: 5′ GGCAGCGACATGGAAGATATACA 3′	54-76
		Antisense: 5′ CACAGACTGCTCCTGAGTGCTT 3′	116-95
		Probe: 5′ FAM ACAAATGCGGAACTGA MGB 3′	78-93
18S rRNA	K03432	Sense: 5′ GCCCGAAGCGTTTACTTTGA 3′	929-948
		Antisense: 5′ TCCATTATTCCTAGCTGCGGTATC 3′	1009-986
		Probe: 5′ FAM AAAGCAGGCCCGAGCCGCC TAMRA 3′	965-983

[a]The sequences and locations of the oligonucleotide primers and probes designed and validated for the measurement of mRNA levels in urinary cells are shown. Fluorogenic TaqMan probes were labeled with 6-carboxyfluorescein (FAM) at the 5′ end and with 6-carboxytetramethylrodamine (TAMRA) or minor groove binder (MGB) at the 3′ end. FAM functioned as the reporter dye and TAMRA or MGB as the quencher.

64% (95% CI, 43-80), and the specificity was 83% (95% CI, 64-93) (Figure 7A). The AUC was 0.85 (95% CI, 0.69-1.00; $P = 0.005$) comparing urine samples matched to AMR biopsies, versus urine samples matched to NR biopsies. At the Youden cutoff value of 3.38 log$_{10}$-transformed copies of IKZF3 mRNA, the sensitivity was 86% (95% CI, 49-99) and the specificity was 79% (95% CI, 60-91) (Figure 7B). IKZF3 mRNA levels were similar between urine matched to TCMR biopsies and urine matched to AMR biopsies, and the AUC was 0.56 (95% CI, 0.34-0.79; $P = 0.61$) (Figure 7C).
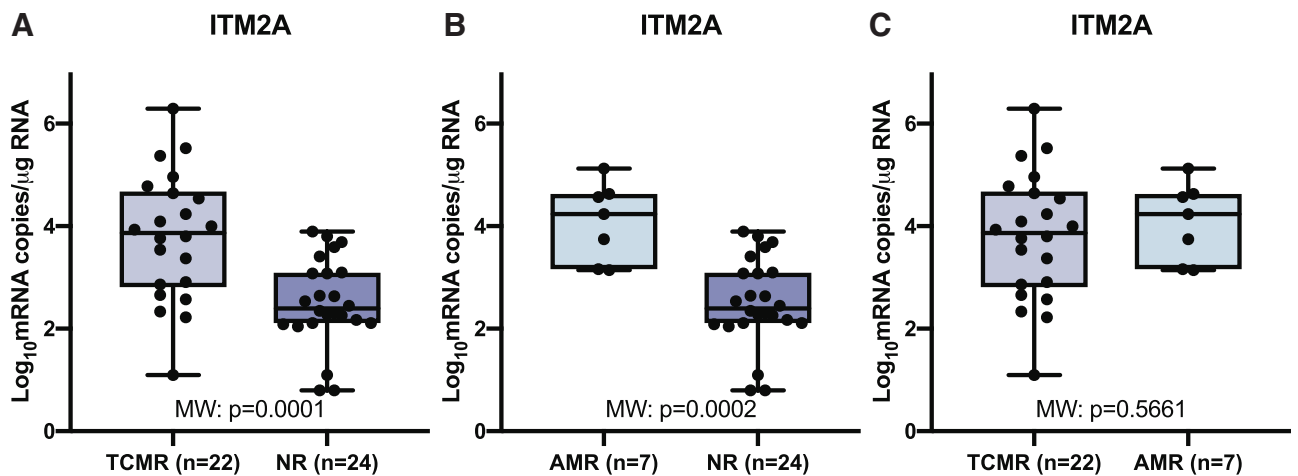
A   ITM2A   **B**   ITM2A   **C**   ITM2A

FIGURE 2. Box plots of urinary cell levels of ITM2A mRNA. (A) through (C) depict $\log_{10}$-transformed urinary cell mRNA copies per microgram RNA on the Y axis for urine samples matched to acute TCMR biopsies (n = 22) vs NR biopsies (n = 24), AMR biopsies (n = 7) vs NR biopsies (n = 24), and acute TCMR biopsies (n = 22) vs AMR biopsies (n = 7), respectively. All panels show minimum, maximum, median, and interquartile range. *P* values for pairwise comparisons are calculated using the Mann-Whitney *U* test. AMR, active antibody–mediated rejection; ITM2A, integral membrane protein 2A; NR, No Rejection; TCMR, acute T cell–mediated rejection.

TABLE 4.

Log10-transformed absolute levels of mRNA in urinary cells[a]

Gene	TCMR (n = 22 samples, 20 patients)	AMR (n = 7 samples, 7 patients)	NR (n = 24 samples, 22 patients)	*P* TCMR/AMR/NR[b]	*P* TCMR/NR[c]	AMR/NR[c]	TCMR/AMR[c]
ITM2A	3.867	4.238	2.397	<0.0001	0.0001	0.0002	0.5661
	(2.812, 4.677)	(3.161, 4.626)	(2.110, 3.091)				
SLAMF6	3.716	3.988	3.076	0.0019	0.0025	0.0031	0.6718
	(2.881, 4.365)	(3.424, 4.516)	(2.080, 3.445)				
IKZF3	3.622	3.889	2.776	0.0009	0.0008	0.0038	0.6357
	(2.895, 4.432)	(3.420, 4.445)	(2.399, 3.289)				
18S rRNA	9.761	9.869	9.430	0.0570	0.0538	0.0610	0.4383
	(9.362, 10.067)	(9.653, 10.239)	(8.865, 9.897)				

[a]Median (IQR) $\log_{10}$-transformed copy numbers of urinary cell mRNA and 18S rRNA per microgram RNA are shown.
[b]*P* values for 3 sample comparisons were calculated using the Kruskal-Wallis test.
[c]*P* values for pairwise comparisons were calculated using the Mann-Whitney U test.
AMR, active antibody–mediated rejection; NR, No Rejection; TCMR, acute T cell–mediated rejection.

Gene Expression in Early TCMR Versus Late TCMR

We classified acute rejection occurring within 12 months of transplantation as early acute rejection and those occurring after 1 year as late acute rejection and examined whether urinary cell levels of mRNA for ITM2A, SLAMF6, and IKZF3 discriminate early acute rejection from late acute rejection. This analysis showed that the levels do not discriminate early from late rejection. The median (IQR) $\log_{10}$-transformed level of ITM2A mRNA was 3.93 (2.78–4.71) copies per microgram of total RNA in 17 urine samples matched to early TCMR biopsies and 3.37 (2.52–4.69) copies per microgram of total RNA in the 5 urine samples matched to late TCMR biopsies (*P* = 0.54; Mann-Whitney test). The median (IQR) $\log_{10}$-transformed level of SLAMF6 mRNA was 3.78 (2.81–4.27) copies per microgram of total RNA in 17 urine samples matched to early TCMR biopsies and 3.18 (2.73–4.71) copies per microgram of total RNA in the 5 urine samples matched to late TCMR biopsies (*P* = 0.94). The median (IQR) $\log_{10}$-transformed level of IKZF3 mRNA was 3.69 (2.89–4.26) copies per microgram of total RNA in 17 urine samples matched to early TCMR biopsies and 3.15 (2.79–4.83) copies per microgram of total RNA in the 5 urine samples matched to late TCMR biopsies (*P* = 0.88).

Urinary Cell mRNA Expression and Response to Antirejection Therapy

Our antirejection therapy consists of 250 mg IV methylprednisolone twice a day for 3 days followed by rapid taper for those with biopsies classified as TCMR. Those with recalcitrant TCMR are treated with antithymocyte globulin, 1.5 mg/kg for a 5-day course. The patients with AMR, in addition to treatment with 250 mg IV methylprednisolone twice a day for 3 days, are also treated with the proteasome inhibitor bortezomib. Among the 20 patients with 22 TCMR biopsies, 15 TCMR episodes responded to antirejection therapy as reflected by a return of serum creatinine to 15% of baseline within 4 weeks of antirejection therapy, and the remaining 7 did not respond and were classified as nonresponders.

We examined whether urinary cell levels of mRNA for ITM2A, SLAMF6, and IKZF3 are prognostic of acute rejection reversal. Our analysis showed that urinary cell levels of ITM2A mRNA were not significantly different between the 15 urine specimens matched to reversible TCMR biopsies versus 7 urine specimens matched to nonreversible TCMR (*P* = 0.95; Mann-Whitney test); and urinary cell levels of SLAMF6 mRNA (*P* = 0.73) and of
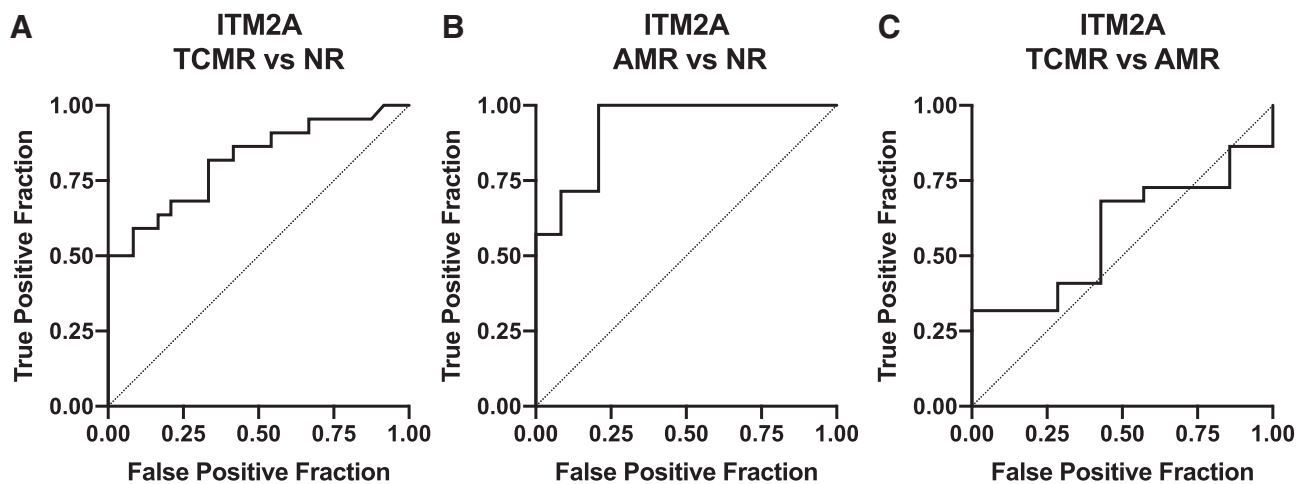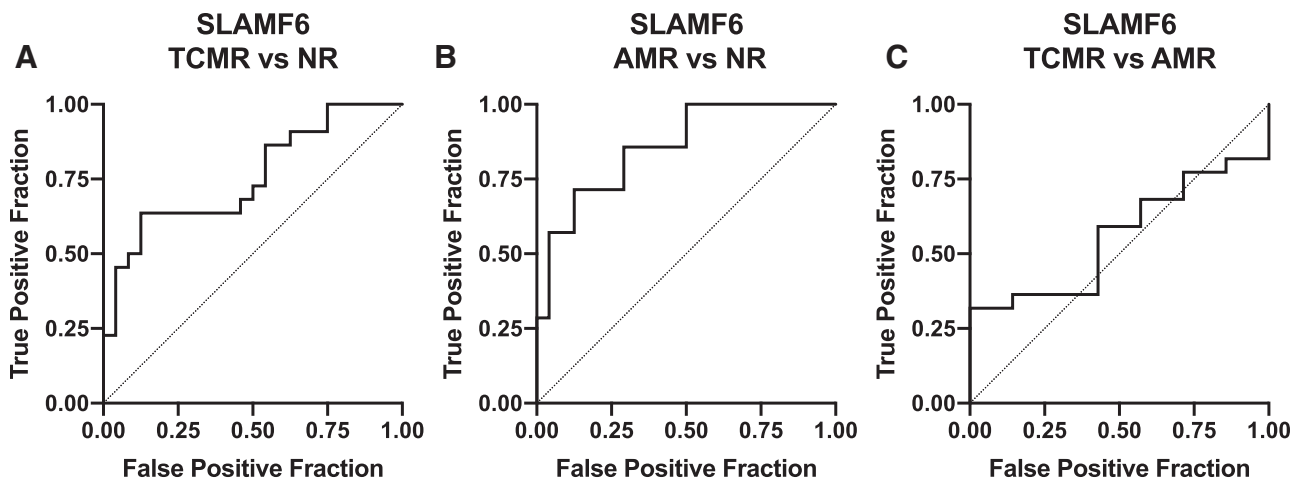
FIGURE 3. Receiver-operating-characteristic curves of urinary cell levels of ITM2A mRNA. (A) through (C) depict the fraction of true positive results (sensitivity) and the fraction of false positive results (1-specificity) for log-transformed urinary cell levels of ITM2A mRNA in urine samples matched to acute TCMR biopsies (n=22) vs NR biopsies (n=24), AMR biopsies (n=7) vs NR biopsies (n=24), and acute TCMR biopsies (n=22) vs AMR biopsies (n=7), respectively. In comparing TCMR vs NR biopsy matched urine samples, the area under the curve was 0.82 (95% CI, 0.69-0.94), with 59% sensitivity (95% CI, 39-77) and 92% specificity (95% CI, 74-99) (A). In comparing AMR vs NR biopsy matched urine samples, the area under the curve was 0.93 (95% CI, 0.84-1.00), with 100% sensitivity (95% CI, 65-100) and 79% specificity (95% CI, 60-91) (B). In comparing TCMR vs AMR biopsy matched urine samples, the area under the curve was 0.58 (95% CI, 0.36-0.80), with 32% sensitivity (95% CI, 16-53) and 100% specificity (95% CI, 65-100) (C). AMR, active antibody–mediated rejection; ITM2A, integral membrane protein 2A; NR, No Rejection; TCMR, acute T cell–mediated rejection.
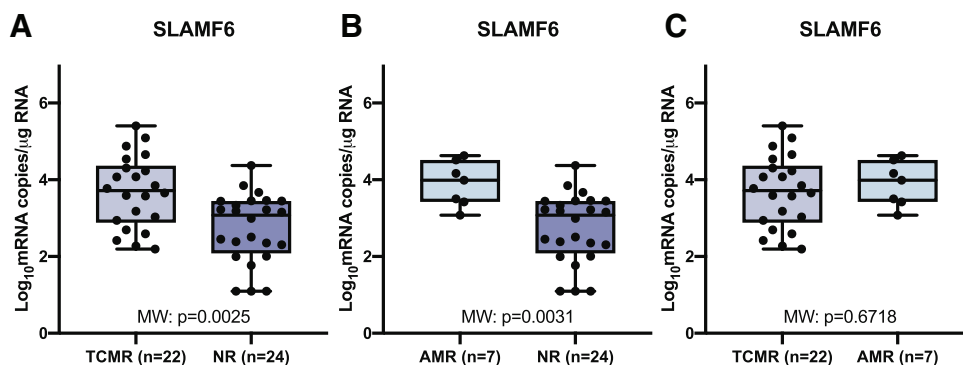
FIGURE 4. Box plots of urinary cell levels of SLAMF6 mRNA. (A) through (C) depict log-transformed urinary cell mRNA copies per microgram RNA on the Y axis for urine samples matched to acute TCMR biopsies (n=22) vs NR biopsies (n=24), AMR biopsies (n=7) vs NR biopsies (n=24), and acute TCMR biopsies (n=22) vs AMR biopsies (n=7), respectively. All panels show minimum, maximum, median, and interquartile range. *P* values for pairwise comparisons are calculated using the Mann-Whitney *U* test.

IKZF3 mRNA ($P = 0.78$) were also not significantly different between the 15 urine specimens matched to reversible TCMR biopsies versus 7 urine specimens matched to nonreversible TCMR.

DISCUSSION

In the CTOT-04 study, we measured urinary cell levels of mRNA for CD3E, perforin, granzyme B, proteinase inhibitor-9, CD103, interferon inducible protein-10 (IP-10/CXCL10), CXCR3, TGFB1, and 18S rRNA and reported their association with TCMR.[13] Among these mRNAs, only CD3E, CXCL10, and granzyme B were among the 127 genes identified by RNA-seq to be shared between urine matched to TCMR and urine matched to AMR. Based on $\log_2$FC ranking, CD3E ranked fifth, CXCL10 ranked fourth, and granzyme B ranked 23rd in urine matched to TCMR biopsies versus urine matched to NR biopsies. ITM2A ranked second, IKZF3 ranked 14th and SLAMF6 ranked 21st. Of the other top 25 highly expressed shared genes, CCL5,

NKG7, CTLA4, PD-1, GZMA, and CD96 have been previously associated with acute rejection in kidney allografts. Of these, NKG7 and CXCL10 have been validated as robust markers of both AMR and TCMR.[19] Of remaining genes, after excluding histones and T-cell receptor regions, we selected the 3 novel and mechanistically relevant mRNAs—ITM2A, SLAMF6, and IKZF3—as candidate genes from the 127 genes identified by RNA-seq to be among the most highly and differentially expressed mRNAs shared between TCMR and AMR for cross validation using customized RT-QPCR assays. Their selection was also based on mechanistic relevance and prioritization placed on T cell–B cell interactions to identify genes of increased relevance to both types of rejection. In brief, ITM2A is involved in thymocyte selection, its expression is increased following activation, and it has been implicated in the helper T cell–mediated immune response; SLAMF6 is expressed in T cells, B cells, and natural killer (NK) cells; and IKZF3 plays a critical role in B-cell proliferation and differentiation. Both SLAMF6 and IKZF3 have been reported to be involved in T- and
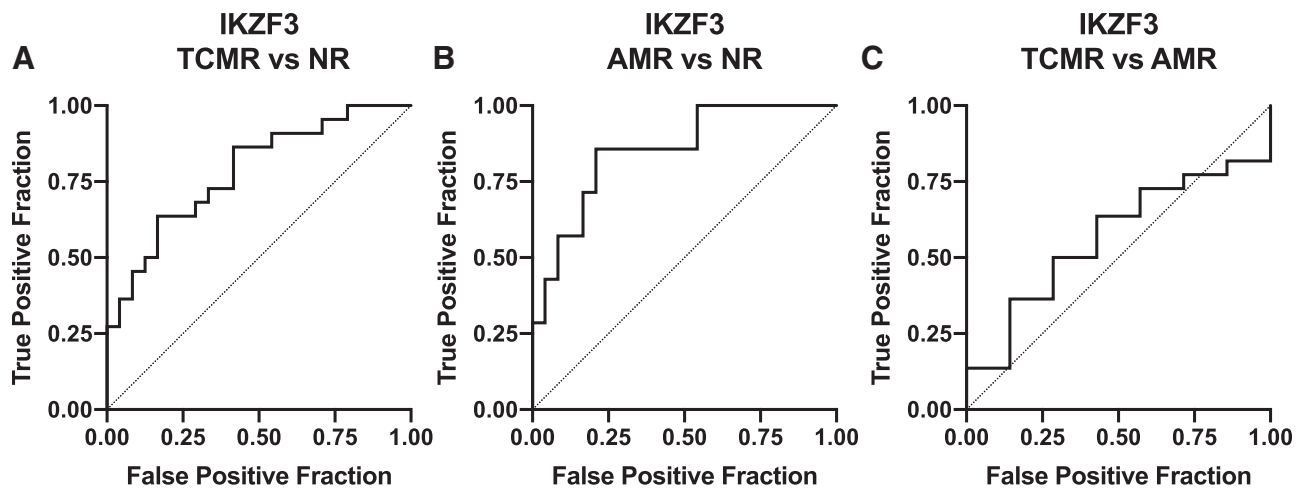
FIGURE 5. Receiver-operating-characteristic curves of urinary cell levels of SLAMF6 mRNA. (A) through (C) depict the fraction of true positive results (sensitivity) and the fraction of false positive results (1-specificity) for log-transformed urinary cell levels of SLAMF6 mRNA in urine samples matched to acute TCMR biopsies (n = 22) vs NR biopsies (n = 24), AMR biopsies (n = 7) vs NR biopsies (n = 24), and acute TCMR biopsies (n = 22) vs AMR biopsies (n = 7), respectively. In comparing TCMR vs NR biopsy matched urine samples, the area under the curve was 0.76 (95% CI, 0.61-0.90), with 64% sensitivity (95% CI, 43-80) and 88% specificity (95% CI, 69-96) (A). In comparing AMR vs NR biopsy matched urine samples, the area under the curve was 0.86 (95% CI, 0.70-1.00), with 71% sensitivity (95% CI, 36-95) and 88% specificity (95% CI, 69-96) (B). In comparing TCMR vs AMR biopsy matched urine samples, the area under the curve was 0.56 (95% CI, 0.34-0.77), with 32% sensitivity (95% CI, 16-53) and 100% specificity (95% CI, 65-100) (C). AMR, active antibody–mediated rejection; NR, No Rejection; SLAMF6, Signaling Lymphocyte Activation Molecule Family Member 6; TCMR, acute T cell–mediated rejection.
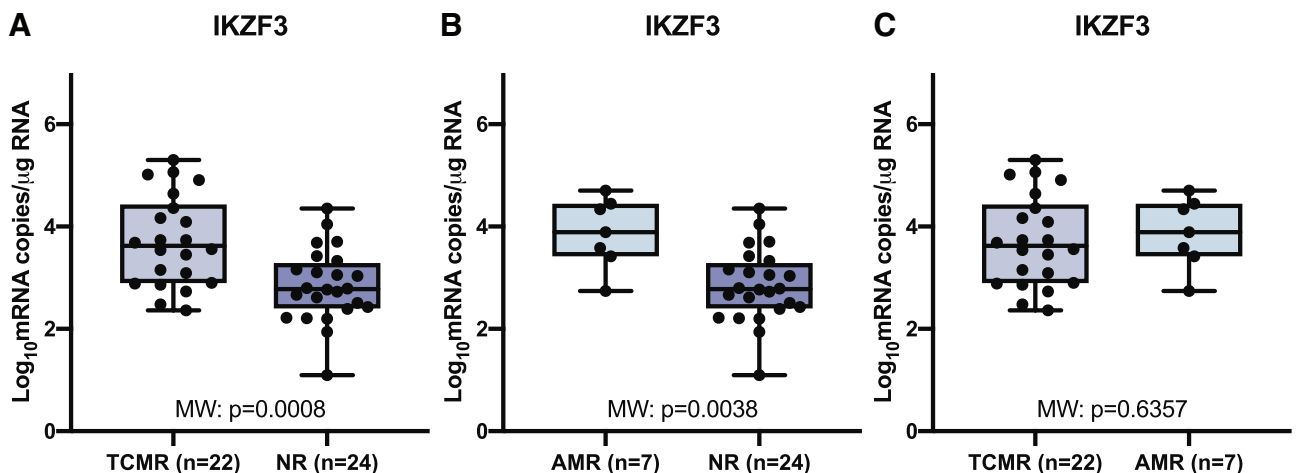
FIGURE 6. Box plots of urinary cell levels of IKZF3 mRNA. (A) through (C) depict log-transformed urinary cell mRNA copies per microgram RNA on the Y axis for urine samples matched to acute TCMR biopsies (n = 22) vs NR biopsies (NR, n = 24), AMR biopsies (n = 7) vs NR biopsies (n = 24), and acute TCMR biopsies (n = 22) vs AMR biopsies (n = 7), respectively. All panels show minimum, maximum, median, and interquartile range. P values for pairwise comparisons are calculated using the Mann-Whitney U test. AMR, active antibody–mediated rejection; IKZF3, Ikaros Family Zinc Finger Protein 3; NR, No Rejection; TCMR, acute T cell–mediated rejection.

B-cell interactions. Although ITM2A has been associated with acute rejection in the liver and lung,[19–21] none of the 3 mRNAs reported here have been investigated in the context of kidney allograft rejection.

To our knowledge, this is the first report to show a significant association between the levels of ITM2A, SLAMF6, or IKZF3 mRNA in urinary cells and acute rejection in human kidney allografts. Our strategy—RNA-seq and bioinformatic analysis to identify a shared gene expression pattern in urine matched to TCMR biopsies and urine matched to AMR biopsies followed by quantification and validation of their abundance using customized RT-QPCR assays—has identified genes diagnostic of both TCMR and AMR. Our identification and verification of shared genes may help prioritize targets for the treatment of both types of acute rejection.

Significance of ITM2A

The ITM2A protein is part of a family of integral membrane proteins of the BRICHOS domain superfamily,[22] X-linked,[23] and was discovered initially as a marker of chondroosteogenic differentiation.[24] Although research continues to support its role in the early stages of chondrogenic differentiation,[25–27] ITM2A is widely expressed, most prominently in the ovary, thyroid, fat, and lymph node.[28] ITM2A is implicated in a number of physiologic processes, ranging from adipogenesis and cell cycle arrest to calcium channel gene expression,[29–31] and in several diseases including Graves' disease and ankylosing spondylitis.[32–34]

ITM2A is expressed in T cells during thymic selection as well as in peripheral CD4+ and CD8+ T cells, and it is upregulated in activated-peripheral T cells.[35] ITM2A is a downstream target of GATA-3, a transcription factor critical for the

FIGURE 7. Receiver-operating-characteristic curves of urinary cell levels of IKZF3 mRNA. (A) through (C) depict the fraction of true positive results (sensitivity) and the fraction of false positive results (1-specificity) for log-transformed urinary cell levels of IKZF3 mRNA in urine samples matched to acute TCMR biopsies (n = 22) vs NR biopsies (n = 24), AMR biopsies (n = 7) vs NR biopsies (n = 24), and acute TCMR biopsies (n = 22) vs AMR biopsies (n = 7), respectively. In comparing TCMR vs NR biopsy matched urine samples, the area under the curve was 0.78 (95% CI, 0.65-0.91), with 64% sensitivity (95% CI, 43-80) and 83% specificity (95% CI, 64-93) (A). In comparing AMR vs NR biopsy matched urine samples, the area under the curve was 0.85 (95% CI, 0.69-1.00), with 86% sensitivity (95% CI, 49-99) and 79% specificity (95% CI, 60-91) (B). In comparing TCMR vs AMR biopsy matched urine samples, the area under the curve was 0.56 (95% CI, 0.34-0.79), with 36% sensitivity (95% CI, 20-57) and 86% specificity (95% CI, 49 99) (C). AMR, active antibody–mediated rejection; IKZF3, Ikaros Family Zinc Finger Protein 3; NR, No Rejection; TCMR, acute T cell–mediated rejection.

development of helper T cells, and ITM2A deficient mice have an attenuated helper T cell–dependent immune response in vivo, producing fewer plasma cells and antigen-specific IgG. ITM2A deficient mice exhibit normal development, including normal expression of T, B, and myeloid cells, presumably due to functional redundancy within the ITM2 family.[36] Because helper T cells are involved in activation of cytotoxic T cells as well as B cells, our finding that ITM2A is upregulated in both TCMR and AMR is consistent with the existing literature implicating ITM2A in the helper T cell–mediated immune response.

The mechanism of ITM2A's effect on the helper T cell–dependent immune response is possibly explained by its effect on autophagic flux. Autophagy is a survival mechanism by which cellular integrity is maintained through the regeneration of metabolic precursors and the clearing of subcellular debris.[37] In transplantation, intact autophagic machinery is required for induction of allograft tolerance.[38] ITM2A has been found to interfere with autophagic flux, as evidenced by autophagosome accumulation and autolysosome inhibition in ITM2A expressing cells; ITM2A expression is also upregulated through autophagic signaling pathway protein kinase A-cAMP responsive element binding protein through a cAMP response element site on its promoter.[39]

Helper T-cell development and activation are likewise autophagy dependent. For example, in MHC class II positive cells, including B cells, autophagosomes fuse with MHC class II loading compartments leading to enhanced MHC class II presentation of cytosolic antigens to helper T cells.[40] In thymic epithelial cells, high-constitutive expression of autophagy contributes to T-cell selection and the generation of self-tolerant helper T cells through the delivery of intracellular proteins to MHC class II molecules. Interference with autophagic pathways results in altered selection of MHC-II-restricted T cells, leading to colitis and multiorgan failure.[41] This could also explain the connection between ITM2A and autoimmune diseases.

Significance of SLAMF6

SLAMF6 is a member of the SLAM family of type 1 transmembrane surface receptors.[42] Control and magnitude of SLAMF receptor signaling is regulated through the binding of SLAM associated protein (SAP) and Ewing's sarcoma-associated transcript 2 adaptors to SLAMF cytoplasmic tails.[42,43] SLAMF6 mRNA is expressed most prominently in the lymph node and spleen,[28] and it is found in multiple types of immune cells, including T cells, B cells,[42] and NK cells.[44] It is involved in NK cell activation,[44] and as described in Yigit's comprehensive review, in T cell–B cell signaling.[45]

SLAMF6 as well as other SLAMF proteins have been shown to function as SAP-dependent adhesion receptors promoting direct T cell-B–cell conjugation.[46] In vivo blocking of SLAMF6 interaction with its ligands results in inhibited B-cell isotype switching,[47] and mice with targeted disruption of the SLAMF6 gene also exhibit decreased interleukin 4 production in CD4+ T cells and an inhibited adaptive immune response.[48] Furthermore, treatment of mice with anti-SLAMF6 monoclonal antibody has been shown to cause severe inhibition of development of follicular helper T cells and germinal center B cells, similar to results observed in mice deficient in SAP adaptor protein.[49] In addition to facilitating interactions between helper T cells and B cells, blocking of SLAMF6 on APCs has been shown to decrease cytokine production in CD8+ lymphocytes.[50] Our finding that SLAMF6 mRNA is upregulated in both TCMR and AMR supports our hypothesis that T cell-B–cell interactions are contributory to both TCMR and AMR. SLAMF6 has already been recognized as a potential therapeutic target in the management of chronic lymphocytic leukemia.[51]

Significance of IKZF3

IKZF3 is part of a family of transcription factors, each containing a common C terminal domain to which different combinations of N-terminal zinc fingers are attached.[52] IKZF3, expressed mostly in the lymph and spleen,[28] is found in a

number of immune cell types including B cells, NK cells, CD4+, and CD8+ T cells.[53] It is expressed most strongly in B cells and studies of IKZF3 knockout mice indicate a critical role for IKZF3 in B-cell differentiation, maturation, and proliferation, and T-cell dependent B-cell responses.[54] IKZF3 is upregulated in pre-B cells,[52] and it has been found to play a role in executing the transition from large pre-B cell to small pre-B cell during normal B-cell development.[55] IKZF3 has also been found to play an important role in T-cell regulation. It is expressed in interleukin-17 producing helper T cells and promotes differentiation through silencing of interleukin 2 production.[56] It has also been shown to influence regulation of the B-cell lymphoma-6 transcriptional repressor protein and through this, differentiation of T follicular helper cells.[57] Our finding that IKZF3 is upregulated not only in AMR but also in TCMR biopsy matched urinary cell specimens suggests that B cells may play a more active role in TCMR than previously recognized, perhaps functioning as classical APCs. As a target for treatment, IKZF3 has also been shown to be involved in the mechanism of immunomodulatory drug–induced cell death in multiple myeloma cells.[58]

There are several limitations to our study. The study sample is relatively small, and our novel observations that urinary cell levels of mRNA for ITM2A, SLAMF6, and IKZF3 are higher in urine matched to TCMR or AMR than in urine matched to NR biopsies require validation using a larger, external cohort of kidney allograft recipients. In this regard, it is worth noting that the heightened expression of these mRNAs in urine was validated using 2 orthogonal technologies—RNA-seq and customized PCR assays. We did not measure urinary cell levels of ITM2A, SLAMF6, and IKZF3 following antirejection therapy. The impact of antirejection therapy on the expression of these genes is therefore not known and represents a limitation of this study.

A shortcoming that should also be acknowledged is that we have not resolved the cellular basis for the heighted abundance of these mRNAs during an episode of TCMR or AMR; that is, whether the graft infiltrating cells, kidney parenchymal cells, or both contribute to the observed high abundance. Our initial profiling of human papillomavirus 16 transformed human kidney proximal tubular (HK2) cells and human peripheral blood mononuclear cells (PBMC) suggest that mRNA for IKZF3 is expressed in high abundance (>100 000 copies of mRNA per microgram of total RNA) in both HK2 cells and PBMC, whereas all 3 mRNAs are expressed in high abundance in normal human PBMC. We plan to complement our ensemble RNA-seq studies with single-cell RNA-seq, and this may help resolve this unresolved issue.

Moreover, RNA-seq of urine samples and of allograft biopsies showed that SLAMF6 and IKZF3 are enriched in TCMR biopsies and in TCMR urine as well as in AMR biopsies and in AMR urine. ITM2A, however, was uniquely enriched in TCMR and AMR urine but not in AMR or TCMR biopsy.[18] The reason for the enrichment in urinary cells and not in kidneys may reside in their differential expression in lymphoid cells compared with kidney parenchymal cells.

Although we showed that mRNA expression levels of these 3 novel mRNAs were not predictors of response to antirejection therapy, this analysis was limited by small sample size. Results of mRNA expression levels for ITM2A, SLAMF6, and IKZF3 for early versus late TCMR, which showed no significant difference, should likewise be interpreted with caution as there were only 17 urine samples matched to early TCMR and

5 urine samples matched to late TCMR using 1 year as the cutoff time to classify an acute rejection as Early or Late rejection. Data analysis was restricted to TCMR biopsies (N = 22) since the number of AMR biopsies was only 7.

To our knowledge, this is the first study associating the abundance of mRNA for ITM2A, SLAMF6, and IKZF3 with human kidney allograft rejection. Moreover, the abundance of these mRNAs in urinary cells are similar between urine matched to TCMR biopsies and urine matched to AMR biopsies. State-of-the-art antirejection therapies are relatively effective for the treatment of TCMR but not for AMR. The mRNAs shared between TCMR and AMR may help prioritize therapeutic targets for the development of more effective antirejection therapies.

ACKNOWLEDGMENTS

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