SUPPLEMENTARY APPENDIX

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INVESTIGATORS

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MATERIALS AND METHODS

Autopsies:

All COVID-19 autopsies, as confirmed by antemortem SARS-CoV-2 nasopharyngeal or oropharyngeal polymerase chain reaction (PCR), were performed at Mayo Clinic's Department of Laboratory Medicine and Pathology in Rochester, Minnesota. The study was approved by the Institutional Review Board and the COVID-19 Research Task Force. Kidney samples obtained at autopsy were immediately fixed in glutaraldehyde and then submitted for ultrastructural studies. Urine when available, was collected.

Histology

Sections of kidneys were standardly sampled and after appropriate fixation in formalin were systematically processed. All sections were stained with Hematoxylin/Eosin (HE), Periodic Acid Schiff (PAS), Jones Methenamine Silver, and Massons trichrome stain. Glomerulitis and peritubular capillaritis were scored according to the Banff grading scheme, on a maximum of 100 glomeruli.[1] If g+ptc was ≥ 2, it was considered as microvascular inflammation.

Immunohistochemistry:

SARS-CoV-2**:**

Immunohistochemical assays were performed on the Leica BOND-III platform (Leica, Wetzlar, Germany) using formalin fixed-paraffin embedded specimens sectioned at 3 microns onto positively charged glass slides. Immunohistochemical antigen retrieval was performed using BOND Epitope Retrieval Solution 2 (prediluted, pH 9.0; AR 9640) for 20 minutes at 100°C. Specimens were incubated with mouse monoclonal SARS-CoV-2 nucleocapsid antibody (Catalogue# bs-41408P; Bioss, Woburn, MA) for 15 minutes at room temperature, followed by visualization with the Leica Bond detection kit at room temperature (DS 9800). The specimens were then counterstained with hematoxylin.

In situ hybridization

In situ hybridization was performed with RNAScope (ACD, Newark, CA) using probes directed against SARS-CoV-2 on formalin fixed paraffin embedded tissue sections cut at a thickness of 3 microns. A negative control (bacterial gene *dapB*) was also included to assess background signals as well as positive control probes to the housekeeping gene peptidylprolyl isomerase B (*PPIB*). The ISH sections were counterstained using periodic acid-Schiff.).

Statistical analyses: Because of small number of subjects (cases) per group, we used non-parametric rank-sum test to compare the groups. All statistical analyses were performed using JMP (SAS Institute, Cary, NC; version 14.1). P-values <0.05 were considered to be statistically significant.

TUNEL Assay

Deparaffinize slides in xylene, and hydrate with graded alcohols to water. Pretreat with Proteinase K, followed by EDTA, dH_2O wash, and BSA blocking. Follow buffer incubation with reaction mixture (TdT, dUTP & buffer) incubation. Wash with SSC buffer, then incubate with Anti Dig. Visualize using Fuchsin (Red), counterstain with hematoxylin, then dehydrate with graded alcohols, clear in xylene, and mount in permount.

Transmission electron microscopy

Tissue was fixed in Trump's fixative (1% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer, pH 7.2 (1). Tissue was then rinsed for 30 min in 3 changes of 0.1 M phosphate buffer, pH 7.2, followed by a 50 minute postfix in 1% OsO_4. After rinsing in 3 changes of distilled water for 30 min the tissue was *en bloc* stained with 2% uranyl acetate for 30 min. After *en bloc* staining, the tissue was rinsed in three changes of distilled water, dehydrated in progressive concentrations of ethanol and 100% acetone and embedded in epoxy resin. Thin (90 nm) sections were cut, placed on 200 mesh copper grids and stained with lead citrate. Images were taken on a JEOL 1400 plus electron microscope operating at 80KV.[2]

Mass Cytometry

Tissue Staining

All tissue staining and slide preparation was performed by the Mayo Clinic Pathology Research Core. Formalin fixed paraffin embedded tissue sections derived from COVID 19 autopsy renal blocks were stained with our full antibody panel (**Table S1**). The metal tagged antibodies were acquired directly from the manufacturer Fluidigm. Briefly, slides were baked for 60 minutes in a 60°C oven and then cooled for 5 minutes before loading on to a Bond RX autostainer (Leica) for automated slide preparation prior to staining. Slides were deparaffinized with xylol and rehydrated through a graded alcohol series and were blocked with Superblock solution (ThermoFisher) before a final wash with PBS supplemented with 0.05% Tween and 1% BSA (PBS-TB). Slides were manually stained overnight in a humidity chamber at 4°C with a cocktail of the antibodies diluted at the indicated dilution factors in Table 1. On the following day, slides were washed with PBS-TB and then stained with an iridium nucleic acid intercalator (Fluidigm)

to label cell nuclei. Cells were washed with PBS-TB twice before a final wash with water and drying for 20 minutes at room temperature.

Imaging Mass Cytometry Data Acquisition

Data acquisition was performed on a Helios time-of-flight mass cytometer (CyTOF) connected to a Hyperion Imaging System (Fluidigm). Optical images of slides were acquired prior to laser ablation using the Hyperion software (version 7.0.5189.0). Ablations were performed at a resolution of 1 micron and at a frequency of 200 Hz. Two to four regions of interest were acquired on consecutive days for 3 days. Performance stability was ensured through calibration using a 3 element full coverage tuning slide embedded with the 3 metal elements 89Y, 140Ce and 175 Lu (Fluidigm). All metals are within the mass range of the time-of-flight detector. Images from the renal cortex sections were generated using the MCD Viewer software (version 1.0.560.2; Fluidigm). Additional formatting and layout of images was performed using Adobe Photoshop (version 19.1.3).[3]

Imaging Mass Cytometry Tissue and Cellular Segmentation Image Analysis Methods

Image analysis was performed using a customized algorithm within Definiens Developer version 2.7. Imaging Mass Cytometry channels were normalized using a z-score, and combinations of markers with corresponding thresholds were used to identify kidney tubules (PanK and E-Cadherin) from inter-tubular stroma (collagen, aSMA), from Glomeruli (Vimentin). Following tissue-level segmentation, cell segmentation was performed using the DNA intercalator as a marker of individual cells, with a watershed to separate closely-packed cells. Cells were then classified using a supervised approach, in which thresholds / gates were defined to separate immune cell types from one another. The presence of CD3 and CD4 was used to denote CD4+ T cells, and likewise for CD3/CD8a for CD8a+ T cells. Macrophages were identified with CD68, NK cells with Granzyme B, and HLA-DR+ cells were also identified using the same approach. The number of cells within each tissue region (kidney tubules versus inter-tubular stroma) were summed across the whole region of interest, and the percentage of each cell type (relative to all cells identified within that compartment) was calculated for each of the ROIs within distinct patient samples.

Gene expression analysis

RNA extraction and gene expression analysis were performed as previously described.[4 5 6 7] Briefly, four consecutive 20-µm sections were obtained from each formalin-fixed paraffin-embedded (FFPE) block and RNA was isolated using the RNeasy FFPE Kit (Qiagen, Toronto, ON). RNA concentration and purity were measured using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Gene expression was quantified using a NanoString nCounter FLEX Analysis System (NanoString Technologies, Seattle, WA), as per manufacturer's recommendations. Samples were analyzed with the 770-gene nCounter Human Organ Transplant Panel (https://www.nanostring.com/products/gene-expression-panels/gene-expression-panels-overview/human-organ-

transplant-panel) plus the 10-gene COVID-19 Panel Plus (https://www.nanostring.com/COVID19). Quality control assessment and data normalization were performed using the default settings in nSolver Analysis Software Version 4.0 (NanoString Technologies).

Statistical analysis

Post-normalization differential gene expression analysis was performed using R version 3.6.3 (R Foundation for Statistical Computing, Vienna, Austria). Significant differential expression between COVID-19 cases and control samples was determined using linear regression (lm function in stats package) with a false discovery rate (FDR) threshold of 0.05 (p.adjust function in stats package).

Proteomics

Quantitative proteomics of glomeruli microdissected from FFPE kidney sections

Formalin-fixed paraffin embedded (FFPE) sections of kidney from five COVID-19 positive autopsy cases, five bronchopneumonia cases and five controls were placed onto polyethylene naphthalate **(**PEN) membrane glass slides (ThermoFisher Scientific). The tissue sections were heated at 65°C for 60 minutes, deparaffinized in xylene (2 x 10 minutes) followed by absolute alcohol (10 minutes), 95% alcohol (10 minutes), 70% alcohol (10 minutes), water (15 minutes) and dried at room temperature. Tubulointerstitiawere microdissected using the ZEISS PALM MicroBeam laser microdissection system. The microdissected tubulointerstitumwere heated with 100 µl of lysis buffer (50% trifluoroethanol (TFE) in 300 mM Tris-HCl) at 95°C for 90 minutes. The tissue lysates were cleared by centrifugation at 10,000×g for 15 minutes. Samples were reduced with 5 mM dithiothreitol for 30 minutes at 37ºC and alkylated with 25 mM iodoacetamide for 30 minutes in dark. The samples were dried in a vacuum concentrator and trypsin digestion was performed in 10% TFE buffer at 1:20 enzyme to protein ratio and incubated at 37ºC overnight. Peptide digests were acidified to a final concentration of 1% TFA and cleaned using SDB-RPS stage tips. Peptide digests from each sample were resuspended in 100 mM triethyl ammonium bicarbonate buffer and labelled with 16-plex TMTPro reagents as per manufacturer's instructions. The reaction was quenched with 5% hydroxylamine for 15min. TMT-labelled peptides were then pooled, cleaned using C_{18} stage tip and dried using speed vac concentrator. Dried peptides were then fractionated by basic pH reverse-phase liquid chromatography (RPLC) using UltiMate 3000 HPLC system (Thermo Fisher Scientific, Waltham, MA). In basic pH-RPLC, TMT labeled samples were resuspended in solvent A (5 mM TEABC, pH 8.5) and fractionated on a C18 XBridge column (5

um, 250 X 4.6 mm, Waters) using increasing gradient of solvent B (5 mM TEABC, pH 8.5, 90% acetonitrile) on UltiMate 3000 HPLC system (Thermo Fisher Scientific, Waltham, MA). The absorbance of eluted peptides was measured at 280 nm. The total run time was 120 min and 96 fractions were collected, which were subsequently concatenated into 24 fractions. These fractions were then vacuum dried.

LC-MS/MS analysis

Each fraction was suspended in 0.1% formic acid (Solvent A) and analyzed on Orbitrap Eclipse mass spectrometer (Thermo Scientific, San Jose, CA). Dionex Ultimate RSLC 3000 system (Thermo Scientific, San Jose, CA) was used for the online separation of peptides using a 2-column setup. Peptides were first loaded onto a trap column (Thermo Scientific, Acclaim Pepmap 100, 75 µm x 2 cm, 5 µm C_{18} 100A˚) followed by separation on analytical column (Thermo Scientific, Acclaim PepMap RSLC, 75 µm x 50 cm, 2 µm C_{18}) using a gradient of 8-28% solvent B (80% Acetonitrile, 0.1% formic acid) for 45 min and 28 – 40% solvent B for 7min. MS/MS analysis was performed in a data dependent top speed mode with 2 sec cycle time. Precursor ions within mass range 350-1600 m/z were resolved in Orbitrap mass analyzer with a resolution of 120,000 (at m/z 200). Precursor ions were sequentially isolated in quadrupole with an isolation width of 0.8 Da and subjected to high energy collision dissociation with 35% normalized collision energy (NCE). Product ions were collected using an AGC setting of 100,000 or an injection time of 100 ms and analyzed in the Orbitrap with 30,000 resolution (at m/z 200). Enhanced resolution mode was enabled for TMTpro masses. Additional settings used for the MS/MS analysis were charge state filter of 2-6, minimum precursor threshold of 25,000, monoisotopic precursor selection and dynamic exclusion of 40 sec.

Raw data files were processed using Proteome Discoverer 2.5 version. Protein identification was performed by Sequest search engine using Uniprot human protein sequence database. Database searching was done using tryptic cleavage specificity, two missed cleavages, precursor ion tolerance of 10 ppm and fragment ion tolerance of 0.02 Da. Carbamidomethylation at cysteine, TMTpro reporter mass at lysine and peptide N-terminus were used as static modifications. Oxidation at M and acetylation at protein N-terminus were used as dynamic modifications. Quantitation was performed by reporter ion node which calculates the reporter ion intensities using 20 ppm integration tolerance around the TMT reporter mass. Protein identifications were filtered for a false discovery rate of 1% at protein, peptide and PSM levels.

Table S1. Imaging Mass Cytometry Antibody Panel

No.	Target	Tag	Clone	Dilution Factor
1	**Alpha-SMA**	141Pr	1A4	200
2	**CD19**	142Nd	6OMP31	400
3	**Vimentin**	143Nd	D21H3	100
4	**CD14**	144Nd	EPR3653	200
5	**CD16**	146Nd	EPR16784	100
6	**Pan-Keratin**	148Nd	C11	200
7	**CD11b**	149Sm	EPR1344	100
8	**CD45**	152Sm	2B11	100
9	**CD11c**	154Sm	Polyclonal	50
10	**FoxP3**	155Gd	236A/E7	1:50
11	**CD4**	156Gd	EPR6855	400
12	**E-Cadherin**	158Gd	24E10	50
13	**CD68**	159Tb	KP1	100
14	**Vista**	160Gd	D1L2G	50
15	**CD20**	161Dy	H1	800
16	**CD8a**	162Dy	C8/144B	100
17	**CD45RA**	166Er	HI100	100
18	**Granzyme B**	167Er	EPR20129-217	50
19	**CD127 (IL-7Ra)**	168Er	EPR2955(2)	100
20	**Collagen type I**	169Tm	Polyclonal	600
21	**CD3**	170Er	Poly	100
22	**CD45RO**	173Yb	UCHL1	50
23	**HLA-DR**	174Yb	YE2/36 HLK	50

Table S2: Proteins in the proteomic pathway analysis

		z-score		
Canonical Pathways	**Molecules**	**COVID_19/S-AKI**	**S-AKI/NS-AKI**	**COVID-19/NS-AKI**
Sirtuin Signaling Pathway	ATG3,NAMPT,NDUFA8,NDUFB11,NDUFS8,PCK2,PPID,TIMM17B,TOMM6	1.633	0.816	1.633
Ceramide Signaling	CYCS,MRAS,PPP2CB,RRAS	2	0	2
Necroptosis Signaling Pathway	FKBP1A,PPID,SLC25A13,TIMM17B,TOMM6	0.447	0.447	2.236
Synaptic Long Term Depression	CACNA2D1,MRAS,PPP2CB,RRAS	1	1	1
Oxidative Phosphorylation	CYCS,NDUFA8,NDUFB11,NDUFS8	-1	0	-1
mTOR Signaling	EIF3L,FKBP1A,MRAS,PPP2CB,RRAS	0	1	1
EIF2 Signaling	EIF3L,HNRNPA1,MRAS,RPL29,RRAS,WARS1	1	0	0
NRF2-mediated Oxidative Stress Response	AKR1A1,DNAJA3,MRAS,RRAS,TXN	0	0	1

REFERENCES

1. Roufosse C, Simmonds N, Clahsen-van Groningen M, et al. A 2018 Reference Guide to the Banff Classification of Renal Allograft Pathology. Transplantation 2018;102:1795-814.
2. McDowell EM, Trump BF. Histologic fixatives suitable for diagnostic light and electron microscopy. Arch Pathol Lab Med 1976;100:405-14.
3. Chang Q, Ornatsky OI, Siddiqui I, Loboda A, Baranov VI, Hedley DW. Imaging Mass Cytometry. Cytometry A 2017;91:160-9.
4. Adam B, Afzali B, Dominy KM, et al. Multiplexed color-coded probe-based gene expression assessment for clinical molecular diagnostics in formalin-fixed paraffin-embedded human renal allograft tissue. Clin Transplant 2016;30:295-305.
5. Adam BA, Smith RN, Rosales IA, et al. Chronic Antibody-Mediated Rejection in Nonhuman Primate Renal Allografts: Validation of Human Histological and Molecular Phenotypes. Am J Transplant 2017;17:2841-50.
6. Dromparis P, Aboelnazar NS, Wagner S, et al. Ex vivo perfusion induces a time- and perfusate-dependent molecular repair response in explanted porcine lungs. Am J Transplant 2019;19:1024-36.
7. Adam BA, Kikic Z, Wagner S, et al. Intragraft gene expression in native kidney BK virus nephropathy versus T cell-mediated rejection: Prospects for molecular diagnosis and risk prediction. Am J Transplant 2020.