SUPPLEMENTAL METHODS

A clinically relevant murine model unmasks a 'Two-Hit' mechanism for reactivation and dissemination of cytomegalovirus following kidney transplantation

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EXPANDED MATERIALS AND METHODS

2.1 Mice

BALB/c (H-2^d) and B6 (H-2^b) were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in the animal facility at Center for Comparative Medicine, Northwestern University. Breeders of CD45.1/Ly5.1 on the BALB/c background, were purchased from The Jackson Laboratories and bred in house. Three to 4-week old female BALB/c or B6 mice were used for generating latently infected kidney donors, while male mice aged between 10 and14 weeks were used as transplant recipients. All mice were used according to protocols approved by the Institutional Animal Care and Use Committee of Northwestern University.

2.2 Virus and establishment of latency

The m157 deletion mutant MCMV (Δm157) strain was originally obtained from Professor Ulrich Koszinowski (Ludwig Maximilians-University, Munich, Germany). Virus stocks were derived from salivary glands of BALB/c mice 2 weeks post infection as previously described[13,15] and were titrated by plaque assay on murine embryo fibroblasts using standard techniques. The Δm157 strain was used for generating latently infected donor mice to avoid potential effect of MCMV resistance seen in B6 mice due to interactions between Ly49H and the m157 protein.[15,16] To establish latency, BALB/c or B6 mice were infected with $1x10^7$ plaque forming units of virus through intraperitoneal injection, and were used as transplant donors 4-6 month after infection. Latency was confirmed by serology using EZ-spot (Charles River, Wilmington MA) and DNA analysis.[10,14]

2.3 Kidney Transplantation

Kidneys from latently infected (D+) BALB/c or D+ B6 donor mice were transplanted into nephrectomized naïve (R-) BALB/c or R- B6 recipient mice, respectively, using the previously described technique.[17-19] Recipients were monitored daily and were euthanized at predesignated endpoints. Recipients that died of surgical complications within 4 days after transplantation surgery were considered technical failures and were excluded from the study. Tissues and blood samples were collected at several predesignated endpoints post-transplant for histopathological, viral and molecular analyses. In all cases, the contralateral donor kidney (not transplanted) was used as a control for the graft.

2.4 Immunosuppression

Recipients (with IS) were treated with a clinically relevant IS regimen consisting of 1) FK506 (Tacrolimus, Astellas Pharma US, Inc.), 3mg/kg daily subcutaneously from day 0 to POD7, then every other day until euthanasia, 2) Rabbit anti-mouse lymphocyte serum (ALS, Mybiosource, San Diego, CA), 4 doses of 300ul i.p, starting from 12 hours prior to transplant, then once every other day; and 3) dexamethasone, 1mg/kg, i.p. daily from POD0 to POD7, then once every other day until euthanasia. Other recipients were not treated with IS (no IS). Additional groups of latently infected mice were also with the same IS regimen, but did not undergo transplantation. Control vehicle (PBS) was used for all groups. Mice were euthanized at weekly intervals until 28 days after either transplant or initiation of IS or PBS.

2.5 Quantitative viral DNA analysis and plaque assay

Frozen tissues were immediately added to lysis solution (Gentra Puregene kits; Qiagen, Valencia, CA, USA) and processed as previously described.[10,14] MCMV DNA copy number in the sample was determined from a standard curve generated using the serially diluted plasmid pIE111, from 10^5 to 10 copies, in 400 ng cellular DNA, while GAPDH copy number in the sample was determined from a standard curve generated by fivefold serial dilutions of mouse kidney DNA as previously described. The viral DNA copy number per million cells in each sample was normalized by dividing the average number of MCMV IE-1 copies by the average GAPDH copy number and multiplying by 2×10^6 as previously described.[14]

For Plaque assay, frozen tissues were sonicated on ice in Dulbeccos's Modified Eagle Medium, serially diluted in media, plated in duplicate on sub-confluent murine embryo fibroblasts in 6-well dishes, overlaid with agarose, and incubated at 37 ^{o}C for 5 days. Plaques were visualized by staining with crystal violet and counted under a Zeiss microscope as previously described.

2.6 Histone analysis

Histone propionylation and in-solution tryptic digestion: Half of a mouse kidney was dissociated and lysed using gentleMACS Dissociator with M tubes (Miltenyi Biotec, Bergisch Gladbach, Germany) in Nuclear Isolation Buffer [15mM Tris-HCl (pH 7.5), 60mM KCl, 15mM NaCl, 5mM MgCl2, 1mM CaCl2, 250mM Sucrose, 1mM DTT, 1:100 Halt

Protease Inhibitor Cocktail (Thermo Scientific), and 10mM sodium butyrate]. Nuclei were resuspended in 0.2M H2SO4 for 1 hour at ambient temperature and centrifuged at 4000 x g for 5 min. Histones were precipitated from the supernatant by the addition of TCA at a final concentration of 20% TCA (v/v). Precipitated histones were pelleted at 10,000 x g for 5min, washed once with 0.1% HCl in acetone and twice with acetone followed by centrifugation at 14,000 x g for 5min. Histones were air dried then resuspended in 10 µL of 0.1 M (NH)4HCO3 for derivatization and digestion according to Garcia et al.[20] Peptides were resuspended in 100 µL 0.1% TFA in water for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis.

LC-MS/MS analysis and data handling: Multiple reaction monitoring was performed on a triple quadrupole (QqQ) mass spectrometer (ThermoFisher Scientific TSQ Quantiva) coupled with an UltiMate 3000 Dionex nano-LC system. Peptides were loaded with 0.1% TFA in water at 2.5 µl/min for 10 min onto a trapping column (3 cm × 150 µm, Bischoff ProntoSIL C18-AQ, 3 µm, 200 Å resin) and then separated on a New Objective PicoChip analytical column (10 cm × 75 µm, ProntoSIL C18-AQ, 3 µm, 200 Å resin). Separation of peptides was achieved using solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in 95% acetonitrile) with the following gradient: 0 to 35% solvent B at a flow rate of 0.30 µl/min over 45 minutes. The following QqQ settings were used across all analyses: collision gas pressure of 1.5 mTorr; Q1 peak width of 0.7 (FWHM); cycle time of 2 s; skimmer offset of 10 V; electrospray voltage of 2.5 kV. Monitored peptides were selected based on previous reports.[21,22]

Data Analysis: Raw MS files were imported and analyzed in Skyline software with Savitzky-Golay smoothing.[23] Automatic peak assignments from Skyline were manually

confirmed. Peptide peak areas from Skyline were used to determine the relative abundance of each histone modification by calculating the peptide peak area for a peptide of interest and dividing by the sum of the peak areas for all peptides with that sequence [e.g. H3K9ac relative abundance = H3K9acK14un ÷ (H3K9unK14un + H3K9acK14un + H3K9unK14ac + H3K9acK14ac)]. The relative abundances were determined based on the mean of three technical replicates for each sample.

2.7 Bottom-up proteomic analysis

Sample preparation: Kidney tissues were weighted and homogenized using a gentleMACSTM dissociator (MiltenyiBiotec, Cambridge, MA) in 2 mL lysis buffer (1%SDS, 50mM Ammonium Bicarbonate, 50 mM NaCl, Halt protease inhibitor) per 100 mg tissue. Tissue homogenate was tip sonicated for 30 seconds for 3 times, and centrifuged at 4°C for 10 minutes at 10,000 x g. The supernatant was saved and protein concentration was determined by a BCA assay (Thermo Fisher Scientific, Rockville, IL). 50 ug protein was precipitated with 8 volumes of cold acetone and one volume of trichloroacetic acid overnight at -20 °C. After washing the pellet with ice-cold acetone, resulting protein pellet was resuspended in 50 uL 8 M urea in 400 mM ammonium bicarbonate, pH 7.8, reduced with 4 mM dithiothreitol at 50 °C for 30 min., and cysteines were alkylated with 18 mM iodoacetamide in the dark for 30 min. The solution was then diluted to < 2 M urea (final concentration) and trypsin (Promega, Madison, WI) was added at final trypsin/protein ratio of 1:50 prior to overnight incubation at 37 °C with shaking. The resulting peptides were desalted using solid phase extraction on a Pierce C18 Spin column and eluted in 80 mL of 80% acetonitrile in 0.1% formic acid. After lyophilization, peptides were reconstituted with 5% acetonitrile in 0.1% formic acid.

Liquid chromatography tandem mass spectrometry (LC-MS) data acquisition and processing: Peptides were analyzed by LC-MS/MS as abovementioned. Approximately 1 µg of peptide samples was loaded onto the trap column, which was 150 µm x 3 cm in-house packed with 3 um C18 beads. The analytical column was a 75 um x 10.5 cm PicoChip column packed with 3 um C18 beads (New Objective, Inc. Woburn, MA). The flow rate was kept at 300nL/min. Solvent A was 0.1% FA in water and Solvent B was 0.1% FA in ACN. The peptide was separated on a 120-min analytical gradient from 5% ACN/0.1% FA to 40% ACN/0.1% FA. The mass spectrometer was operated in data-dependent mode. The source voltage was 2.10 kV and the capillary temperature was 320 degrees C. MS scans were acquired from 300-2000m/z at 60,000 resolving power and automatic gain control (AGC) set to 3x106. The top 15 most abundant precursor ions in each MS scan were selected for fragmentation. Precursors were selected with an isolation width of 2 Da and fragmented by Higher-energy collisional dissociation (HCD) at 30% normalized collision energy in the HCD cell. Previously selected ions were dynamically excluded from re-selection for 20 seconds. The MS2 AGC was set to 1x105. All samples were run in duplicates.

Data Analysis: Protein Tandem MS data was queried for protein identification and label-free quantification against the SwissProt Mus musculus database using MaxQuant v1.6.0.16.[24,25] The following modifications were set as search parameters: peptide mass tolerance at 6 ppm, trypsin digestion cleavage after K or R (except when followed by P), 2 allowed missed cleavage site, carbamidomethylated cysteine (static modification), and oxidized methionine, protein N-term acetylation (variable modification). Search results were validated with peptide and protein FDR both at 0.01. Proteins that were identified

with >1 peptides were subjected to a further statistical analysis using Perseus software v1.6.0.7.[26]

2.8 Plasma protein analysis

Plasma (70 ul) from treated and untreated or untreated transplant recipients was frozen in liquid nitrogen and analyzed for inflammatory proteins at Ampersand Biosciences (Saranac Like, NY) by using Luminex system and the Rodent MAP 4.0 Multiplex assay as previously described.[27]

2.9 Transcriptome analysis

Tissues from treated and untreated recipients were harvested, snap-frozen in liquid nitrogen and stored at -80^{o}C for RNA analysis. RNAs were extracted and purified with Tri-Reagent (Molecular Research Center. Inc.), using DNase treatment as directed by the manufacturer. RNA was quantified and quality was assessed using Affymetrix MG-430 PM mouse microarrays as previously described.[27] Genome-wide RNA expressions were estimated and analyzed using R, an open-source software environment for statistical computing and graphics.

2.10 Cell isolation, antibodies and Flow cytometry

Spleen cells were isolated using standard methods. Kidney graft tissue was cut into small strips of ~5mm thickness and placed in 5ml digestion solution (2 mg/ml collagenase D, 0.2 mg/ml DNase I, both from Roche, dissolved in HBSS with $Ca^{2+}$and Mg^{2+}), then incubated at 37°C for 30 minutes to generate single cell suspensions for the phenotyping analysis.[19,28] All antibodies are were from BD Bioscience unless otherwise indicated.

MHC-tetramer (K^b-m38) was provided by the NIH Tetramer Core Facility (http://tetramer.yerkes.emory.edu/). After incubation with FcR-blocker (Miltenyi Biotec), cells ($1x10^6$) were stained with the tetramer and various fluorescently conjugated antibodies including CD45 (30-F11); CD45.1 (A20), CD45.2 (104), CD11b (M1/70), Ly6G (1A8), F4/80 (T45-2342), CD11c (HL3), MHCII (M5/114.15.2), CD4 (GK1.5), CD8 (53-6.7), CD44 (1M7), CD3 (17A2), B220 (Ly-5), and Ly6C (HK1.4). A fixable viability dye eFluor 506 (Cat# 65-0866-14, eBioscience) was used to gate out dead cells. Data was acquired with a BD LSRII flow cytometer using BD FACSDiva software (BD Bioscience). Flow cytometry data were analyzed with FlowJo v10 software.

2.11 Histology

Kidney samples were bisected transversely and preserved in 10% formalin, and processed and stained by Periodic Acid Schiff (PAS) as previously described[19] and evaluated blindly by experienced renal pathologists (YSK and QC) for the morphologic characteristics of CMV infection.

2.12 Statistical Analysis

All statistical analyses were performed using GraphPad PRISM 7 software unless otherwise indicated. Either the Wilcoxon rank-sum test or T test, as appropriate, were used to for DNA copy number, proteomic and phenotype analyses to compare controls, wo/IS and w/IS groups. A P-value less than 0.05 was considered statistically significant.

For genome-wide RNA expression, Affymetrix Mouse HT_MG-430_PM microarrays were normalized using Robust Multichip Average (RMA)[29] and signal filters of Log2 <3.8 were

used to exclude probe sets with low signal intensities to filter out probesets in the noise range. Pairwise class comparisons were carried out using *limma*, a R-package for microarray data analysis.[30] A False Discovery Rate (FDR) of v5 % was used for all class comparisons. Pathway mapping to biologically significant pathways was done using Ingenuity Pathway Analysis. All pathways were adjusted using the Benjamini-Hochberg correction.