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Supplementary information

Physiology and immunology of a pig-to-human decedent kidney xenotransplant

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Supplemental Appendix

Supplement to: Montgomery RA[&], Stern JM[&], Fathi F[&], et al. **Physiology and immunology of pig-to-human decedent kidney xenotransplant**

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Supplemental Data

Zoonosis Testing

PERV-ABC DNA was detected in six recipient PBMC samples, and detection was equivocal in the POD42 sample. We developed a multiplex qPCR for porcine and human mitochondrial DNA (cytochrome B, cytb) to determine whether the PERV signal in recipient samples could be explained by microchimerism (*i.e.* tiny amounts of porcine cell debris carrying porcine RNA or DNA fragments or perhaps small numbers of porcine cells). PCR was performed on selected samples and confirmed the assay to be highly specific; pre-operative PBMCs were positive for human cytb only, while porcine tissues were positive only for porcine cytb (pcytb) (Figure 6C). We found pcytb in 8/11 PBMC samples collected after transplantation as well as evidence for human cell infiltration into the implanted porcine kidney, and trafficking of porcine cells into the recipient spleen, liver, graft-draining (ipsilateral iliac) lymph node and non-draining lymph node (Figure 6C).

The presence of pcytb in recipient PBMCs or tissue samples indicated that porcine cells, or porcine mitochondrial genomic DNA, were co-purified with PERV-ABC DNA in some recipient samples. PERV-ABC DNA was never detected in the absence of pcytb in PBMC samples (Figure 6B). Neither analyte was detected in the remaining samples. Human cytb was detected in all recipient samples, as expected. These results indicate that detection of PERV-ABC DNA in the recipient is associated with the presence of porcine mitochondrial DNA.

PERV-C DNA was detected in all porcine donor samples (PBMCs, lymph node, spleen, and thymus) and the explanted kidney and thymus. The broadly reactive PERV-ABC DNA assay was positive in the same samples with lower Ct values (*i.e.* higher titers). PERV-C RNA was not detected in any tissue sample. To verify assay specificity, we performed sequence alignments for PERV-C and PERV-ABC primers and probes against reference PERV sequences. The specificity of the PERV-C assay was further interrogated by testing serial ten-fold dilutions of synthetic PERV partial env gene DNA for each genotype, starting at 5×10^6 copies per reaction, against the PERV-C qPCR assay. Both approaches confirmed that the PERV-C assay was specific. Sanger sequencing of the PERV-C qPCR amplicon (92 bp) from three porcine tissues (lymph node, spleen, thymus) confirmed PERV-C genomic sequence from the envelope gene. Importantly, this sequence did not match the synthetic PCR positive control (three nucleotide mismatches) indicating that this result was not due to a contamination event that could have occurred during laboratory processing. To further interrogate this result, three separate and specific semi-nested PCR assays were designed to amplify a 300-400 nt region of the PERV-A, PERV-B, and PERV-C env genes located upstream of the PERV-C qPCR assay target region from selected donor tissues. PERV-A and PERV-B PCR products were obtained from the first round of PCR from porcine tissues. PERV-C DNA was not detected in any sample after a single round of PCR; however, the thymus from the donor pig and the explanted kidney were positive after a second round of PCR. Amplicons from the thymus and kidney were sequenced for all PERV subtypes and confirmed specific amplification of the intended target (Table S6).

There was evidence for mixed populations of each PERV-A and PERV-B subtype within the same sample. Sequencing of the PERV-C amplicon yielded non-identical sequences from the thymus and the explanted kidney, indicating mixed populations for this subtype within the host animal. Semi-nested PCRs confirmed the presence of PERV-C proviral DNA but absence of PERV-C RNA in any sample may indicate non-functional integrated sequence.

Porcine Kidney Physiology

Renin concentration measured by ELISA in the donor pig's serum was 207.8 pg/mL. However, pig renin was not measurable in any of the post-transplant decedent sera. A captopril challenge, performed to stimulate renin release from the xenograft on POD 20, resulted in a decrease in aldosterone from 4.8 ng/dL to less than 3 ng/dL and an increase in plasma renin activity from undetectable to 1.0 ng/mL/hr. Despite exogenous active Vitamin D supplementation, vitamin D levels were noted to be low, as typically seen in long-term intensive care unit management.

Supplemental Tables

Table S1. List of antibodies used for flow cytometry to detect different antigens and other materials used in the study

Antibody	**Company**	**Clone**	**Cat # / Identifier**	**Dilution factor**
Mouse monoclonal anti-Human CD68	Dako-Agilent	KP1	Ga60961-2	
Mouse monoclonal anti-Human CD15	Dako-Agilent	Carb-3	Ga06261-2	
Rabbit monoclonal anti-Human CD3	Thermo Scientific	SP7	MA1-90582	
Mouse monoclonal anti-Human CD20	Dako-Agilent	L26	M075501-2	
Rabbit polyclonal anti-Human vWF (von Willebrand Factor)	Dako-Agilent	N/A	A008229-5	
Rabbit monoclonal anti-human C4d	Ventana Medical Systems, Roche	SP91	06732364001	
Mouse monoclonal anti-Human Vδ2 TCR	Miltenyi Biotec	123R3	130-121-339	8
Mouse monoclonal anti-Human γδ TCR	Beckman Coulter	IMMU510	B10247	8
Mouse monoclonal anti-Human CD45	BD Horizon	HI30	562279	64
Mouse monoclonal anti-Human CD8	BD Biosciences	SK1	557834	4
Mouse monoclonal anti-Human CD4	Tonbo	OKT4	80-0048	8
Mouse monoclonal anti-Human CD3	BD Biosciences	SP34-2	552852	128
Mouse monoclonal anti-Human CD45	BD Biosciences	HI30	560777/RRID: AB_1937324	8
Mouse monoclonal anti-Human CD1a	BD Biosciences	HI49	555806/RRID: AB_396140	16
Mouse monoclonal anti-Human γδ TCR	Beckman Coulter	IMMU510	B10247/RRID: AB_3662706	16
Mouse monoclonal anti-Human V γδ TCR	Milteny Biotec	123R3	130-121-399/RRID: AB_2784475	128
Mouse monoclonal anti-Pig CD3e	BD Biosciences	BB23-8E6-8C8 (RUO)	561477/RID: AB_10683312	16
Violet Proliferation Dye 450	BD Biosciences		562158/RRID: AB_2869398	

DAPI	BD Biosciences		564907/RRID: AB_2869624	
Goat anti-human IgG	Jackson Lab	Polyclonal	109-546-088	100
Goat anti-human IgM	Jackson Lab	Polyclonal	709-546-073	100
Mouse monoclonal B7 anti-Human C5b-9 neo-epitope	Professor Paul Morgan Cardiff Institute of Infection & Immunity, UK	N/A	N/A	
Material	**Company**		**Cat #**	
Histopaque	Sigma Aldrich		10771	
PBS	Cytvia		SH30256.02	
ACK lysis buffer	Gibco		A10492-02	
Human serum	Gem Cell		100512	
DMSO	Sigma Aldrich		D2650	
AIM v	Gibco		12055091	
IMDM	Gibco		12440053	
HEPES	Gibco		15630080	
Penicillin/Streptomycin	Gibco		15140122	
Glutamax	Gibco		35050061	
Classical Monocyte Isolation Kit	Milteny Biotec		130-117-337	
Multi Tissue Dissociation Kit 1	Milteny Biotec		130-110-201	
Pan T Cell Isolation Kit	Milteny Biotec		130-096-535	
DNA extraction kit	Qiagen		69504	
Pig GMCSF	bioteche		711-PG	
Pig IL-4	bioteche		654-p4	
Human IL-4	PeproTech		200-04	
Human M-CSF	PeproTech		300-25	
Human IFN-γ	PeproTech		300-02	
LPS	Millipore Sigma		L2387	
CFSE proliferation tracking kit	Invitrogen		C34554	
Round bottom 96 well cell culture plate	Corning		3799	

Table S2. Summary of xenograft histopathologic evaluation findings

Time point	Banff Score	General	Acute tubular injury	% IFTA	Glomerular Findings	Tubulo-interstitium	PTC	IgA	IgM	C3	C1q	Electron Microscopy
Post-reperfusion	g0, t0, i0, v0, ptc0, c4d0. cg0, mm0, ct0, ci0, ti0, i-ifta0, cv0, ah0	Mesangial hyper-cellularity	mild	5	0/6 GS		C4d negative	Negative	Negative	Negative	Negative	Patent capillary lumens, no subendothelial/subepithelial deposits. Mild mesangial cellularity. Glomerular endothelial swelling. Preserved foot processes (FP), intact fenestration. Peritubular capillaries (PTC) patent.
POD 10	g0, t0, i0, v0, ptc0, c4d3. cg0, mm2, ct0, ci0, ti0, i-ifta0, cv0, ah0.	Mesangial hyper-cellularity	mild	5	Weak IgA, C3 0/6 GS	Non-specific vacuoles (negative for lymphatic markers)	C4d positivity in >50% of PTC	Mesangial and parietal positivity	Weak parietal staining	Weak parietal, mesangial positivity	Negative	Patent capillary lumens, no subendothelial/subepithelial deposits. Mild mesangial hypercellularity. No electron dense (ED) deposits in glomerular basement membrane (GBM). Preserved FP.
POD 14	g0, t0, i0, v0, ptc0, c4d3. cg0, mm2, ct0, ci0, ti0, i-ifta0, cv0, ah0.	Mesangial hyper-cellularity	mild	5	GS 0/3		C4d positivity in >10-50% of PTC	N/A	N/A	N/A	N/A	Patent capillary lumens, no subendothelial/subepithelial deposits. Mild mesangial cellularity. No ED deposits in GBM. Preserved FP.
POD 21	g0, t0, i0, v0, ptc0, c4d3. cg0, mm2, ct0, ci0, ti0, i-ifta0, cv0, ah0	Mesangial hyper-cellularity	mild	5	weak IgA, C3, C1q (IF) GS 0/4	Interstitial nonspecific vacuoles	C4d3	Mesangial and parietal positivity	Mesangial and parietal positivity	Mesangial positivity	Mesangial and parietal positivity	No glomeruli present; unable to fully evaluate.
POD 28	g0, t0, i0, v0, ptc0, c4d3. cg0, mm2, ct0, ci0, ti0, i-ifta0, cv0, ah0	Mesangial hyper-cellularity	mild	5	GS 0/3	Interstitial nonspecific vacuoles. Arterioles with myocyte vacuolization consistent with tacrolimus toxicity	C4d3	N/A	N/A	N/A	N/A	Patent capillary lumens. No subendothelial/subepithelial ED deposits. Mild glomerular endothelial cell swelling. Rare intracapillary leukocytes. Mesangial lucencies suggestive of mesangiolysis. PTC patent. Minimally effaced podocyte FP.
POD 33	g1, t0, i0, v0, ptc0-1, c4d3. cg0, mm2, ct0, ci0, ti0, i-ifta0, cv0, ah0	Mild mesangio-lysis	0	5	IgA, C3, C1q GS 0/7	Isometric cytoplasmic vacuolization. Arterioles with myocyte vacuolization	C4d3	Mesangial and parietal positivity	Mesangial, parietal, and arterial positivity	Mesangial positivity	Mesangial, parietal, and arterial positivity	Capillary lumen obliterated by markedly swollen endothelial cells. Glomerular intracapillary leukocytes. Visceral epithelial cell FP preserved. Fused fenestration. Significant mesangial lucencies mesangiolysis. Scattered ED deposits. Polymorphonuclear (PMN) cells in tubular lumens and PTC. Findings diagnostic of acute antibody-mediated rejection (AMR).
POD 45	g2, t0, i0, v0, ptc0, c4d3. cg1, mm2, ct0, ci0, ti0, i-ifta0, cv0, ah0	Moderate to severe mesangio-lysis	0	5	IgA, C3, C1q GS 0/14	Isometric cytoplasmic vacuolization. Arterioles with myocyte vacuolization	C4d3	Mesangial and parietal positivity	Mesangial, parietal, and arterial positivity	Mesangial and parietal positivity	Mesangial, and parietal positivity	No glomeruli seen. PMNs in tubular lumens.
POD 49	g2, t0, i0, v0-1, ptc0, c4d2. cg1, mm1, ct0, ci0, ti0, i-ifta0, cv0, ah0	Mild to moderate mesangio-lysis	0	5	IgA, IgM, C3, C1q GS 0/13	Interstitial hemorrhage, activated endothelial cells, possible endotheliality is	C4d2	Mesangial and parietal positivity	Mesangial, parietal, and arterial positivity	Mesangial positivity	Mesangial, parietal, and arterial positivity	Capillary lumens patent. No subendothelial/subepithelial ED deposits. Mild endothelial cell swelling. Mesangiolysis, rare small ED deposits. Tubular epithelial cells with cytoplasmic vacuolization. PTC patent. Minimally effaced podocyte FP.

POD 56	g0, t0, i0, v0, ptc0, c4d2. cg1, mm1, ct0, ci0, ti0, i-ifta0, cv0, ah0	Mild mesangio-lysis	0	10	IgA, IgM, C1q GS 0/14	Interstitial nonspecific vacuoles	C4d2	Mesangial and parietal positivity	Mesangial, parietal, and arterial positivity	Negative	Mesangial and parietal positivity	Patent capillary lumens. No subendothelial/subepithelial ED deposits. Mild endothelial cell swelling. Intact fenestration. Tubular epithelial cells with cytoplasmic vacuolization. PTC patent. Resolution of AMR. Preserved FP.
POD 61	g0-1, t0, i0, v0, ptc0, c4d2. cg1, mm1, ct0, ci0, ti0, i-ifta0, cv0, ah0	Absent to mild mesangio-lysis	0	5	IgA, IgM, C1q GS 0/23		C4d2	Mesangial and parietal positivity	Mesangial, parietal, and arterial positivity	Negative	Mesangial and parietal positivity	Capillary lumens patent. Mild endothelial cell swelling. Intraluminal and mesangial ED deposits. Tubular epithelial cells with cytoplasmic vacuolization. PTC patent. No AMR. Preserved FP.

Table S3. (A) Pig-Human Homology of Nanostring Transcript Probes and (B) Differentially Expressed Genes and Their Annotated Pathways.

Nanostring probe sequences for the six transcripts upregulated at the post-reperfusion time point were aligned against the Sus scrofa genome and transcriptome (Sscrofa11.1) using BLASTn. Probes for S100A8, FOS, and S100A12 showed no high-identity match to the porcine genome, indicating human specificity. S100A9, IFITM2, and CTSS displayed moderate homology (83–86 %), near the lower limit of reliable cross-species hybridization. These data support that the detected transcripts at reperfusion are predominantly of human origin, reflecting infiltration of human innate immune cells.

A.

Gene	Pig ID	Human transcript (Nanostring accession)	Nanostring Probe Sequence	Identity (/100 nt)	Homology
S100A8	NM_001160271	NM_002964.4	CCTGCATGTCTCTTGTCAGCTGTCTTTCAGAAGACCTGGTGGGGCAAGTCCGTGGGCATCATGTTGACCGAGCTGGAGAAAGCCTTGAACTCTATCATCG	No match	NA
S100A9	NM_001177906	NM_002965.3	AAGGAGAATAAGAATGAAAAGGTCATAGAACACATCATGGAGGACCTGGACACAAATGCAGACAAGCAGCTGAGCTTCGAGGAGTTCATCATGCTGATGG	83/100	83 %
FOS	NM_001123113	NM_005252.2	ACTCAAGTCCTTACCTCTTCCGGAGATGTAGCAAAACGCATGGAGTGTGTATTGTTCCCAGTGACACTTCAGAGAGCTGGTAGTTAGTAGCATGTTGAGC	No match	NA
IFITM2	NM_001246214.1	NM_006435.2	CCAAGTGCCTGAACATCTGGGCCCTGATTTTGGGCATCTTCATGACCATTCTGCTCATCATCATCCCAGTGTTGGTCGTCCAGGCCCAGCGATAGATCAG	42/49	86 %
S100A12	NM_001160272	NM_005621.1	CAAGATGAACAGGTCGACTTTCAAGAATTCATATCCCTGGTAGCCATTGCGCTGAAGGCTGCCCATTACCACACCCACAAAGAGTAGGTAGCTCTCTGAA	No match	NA
CTSS	XM_021089893.1	NM_004079.3	ATGACAACGGCTTTCCAGTACATCATTGATAACAAGGGCATCGACTCAGACGCTTCCTATCCCTACAAAGCCATGGATCAGAAATGTCAATATGACTCAA	85/100	85 %

B.

Comparison	Gene	Associated Pathways
Post-reperfusion	S100A9	Damage-associated molecular pattern transcripts (DAMP), Quantitative Constitutive Macrophage-Associated Transcripts (QCMAT), Neutrophil degranulation, Toll-like Receptor Signaling, Transcripts associated with T cell-mediated rejection (TCMR), Innate Immune System
Post-reperfusion	S100A8	Damage-associated molecular pattern transcripts (DAMP), Neutrophil degranulation, Toll-like Receptor Signaling, Transcripts associated with T cell-mediated rejection (TCMR), Innate Immune System
Post-reperfusion	FOS	Cell Cycle Regulation, Injury-repair response associated transcripts (IRRAT), Regulation, T-cell Receptor Signaling, TNF Family Signaling, MAPK, Toll-like Receptor Signaling, Oxidative Stress, B-cell Receptor Signaling / B-cell associated transcripts (BATs), Innate Immune System
Post-reperfusion	S100A12	Damage-associated molecular pattern transcripts (DAMP), IL-1 signaling, Neutrophil degranulation, Toll-like Receptor Signaling, Transcripts associated with T cell-mediated rejection (TCMR), Cytokine Signaling, Innate Immune System
Post-reperfusion	ERRFI1	MAPK
Post-reperfusion	IFITM2	Type I Interferon Signaling
POD33	CD74	MHC Class II Antigen Presentation, Antibody-mediated rejection transcripts (ABMR-RATs), IFNg Signaling and Rejection Induced Transcripts (GRIT3), Transcripts associated with T cell-mediated rejection (TCMR), Adaptive Immune system

POD33	HLA-DPA1	MHC Class II Antigen Presentation, Co-Stimulation by CD28, T-cell Receptor Signaling, Antibody-mediated rejection transcripts (ABMR-RATs), IFNg Signalling and Rejection Induced Transcripts (GRIT3), Adaptive Immune system
POD33	CTSS	Neutrophil degranulation, Injury and repair induced transcripts - Late (IRITD5), Acute kidney injury (AKI), Injury-repair response associated transcripts (IRRAT), MHC Class II Antigen Presentation, Interstitial Fibrosis and Tubular Atrophy (IFTA), MHC Class I Antigen Presentation, Toll-like Receptor Signaling, Extracellular Matrix Organization, IFNg Signaling and Rejection Induced Transcripts (GRIT3), Adaptive Immune system, Innate Immune System
POD33	HLA-A	Type I Interferon Signaling, Cytotoxicity, Antibody-mediated rejection transcripts (ABMR-RATs), IFNg Signaling and Rejection Induced Transcripts (GRIT3), Adaptive Immune system
POD33	S100A9	Damage-associated molecular pattern transcripts (DAMP), Quantitative Constitutive Macrophage-Associated Transcripts (QCMAT), Neutrophil degranulation, Toll-like Receptor Signaling, Transcripts associated with T cell-mediated rejection (TCMR), Innate Immune System
POD33	HLA-DRA	MHC Class II Antigen Presentation, T-cell Receptor Signaling, Antibody-mediated rejection transcripts (ABMR-RATs), IFNg Signaling and Rejection Induced Transcripts (GRIT3), Adaptive Immune system
POD33	HLA-DRB3	Co-Stimulation by CD28, MHC Class II Antigen Presentation, T-cell Receptor Signaling, Adaptive Immune system
POD33	HLA-B	MHC Class I Antigen Presentation, Type I Interferon Signaling, Cytotoxicity, Antibody-mediated rejection transcripts (ABMR-RATs), IFNg Signaling and Rejection Induced Transcripts (GRIT3), Adaptive Immune system, Innate Immune System
POD33	WARS	Antibody-mediated rejection transcripts (ABMR-RATs), IFNg Signaling and Rejection Induced Transcripts (GRIT3)
POD33	HLA-DRB1	MHC Class II Antigen Presentation, T-cell Receptor Signaling, Antibody-mediated rejection transcripts (ABMR-RATs), IFNg Signaling and Rejection Induced Transcripts (GRIT3), Adaptive Immune system
POD33	S100A8	Damage-associated molecular pattern transcripts (DAMP), Neutrophil degranulation, Toll-like Receptor Signaling, Transcripts associated with T cell-mediated rejection (TCMR), Innate Immune System
POD33	HLA-DPB1	MHC Class II Antigen Presentation, Co-Stimulation by CD28, T-cell Receptor Signaling, Antibody-mediated rejection transcripts (ABMR-RATs), IFNg Signaling and Rejection Induced Transcripts (GRIT3), Adaptive Immune system
POD33	CALHM6	Transcripts associated with T cell-mediated rejection (TCMR)
POD33	CD81	Complement Receptors, B-cell Receptor Signaling / B-cell associated transcripts (BATs), Innate Immune System, Adaptive Immune system
POD33	FCGR3A/B	Acute kidney injury (AKI), Neutrophil degranulation, Natural killer cells (NK cells), Antibody-mediated rejection transcripts (ABMR-RATs), Transcripts associated with T cell-mediated rejection (TCMR)
POD33	ARHGDIB	Injury and repair induced transcripts - Late (IRITD5)
POD33	C1QB	Complement Components, Innate Immune System, IFNg Signaling and Rejection Induced Transcripts (GRIT3), Transcripts associated with T cell-mediated rejection (TCMR)
POD33	HLA-E	MHC Class I Antigen Presentation, Type I Interferon Signaling, Cytotoxicity, Antibody-mediated rejection transcripts (ABMR-RATs), IFNg Signaling and Rejection Induced Transcripts (GRIT3), Adaptive Immune system, Innate Immune System
POD33	KLHL13	MHC Class I Antigen Presentation, Adaptive Immune system
POD33	HLA-DQB1	MHC Class II Antigen Presentation, Co-Stimulation by CD28, T-cell Receptor Signaling, IFNg Signaling and Rejection Induced Transcripts (GRIT3), Adaptive Immune system
POD33	PTPRC	Injury-repair response associated transcripts (IRRAT), Lymphocyte Trafficking, Lymphocyte Trafficking, Lymphocyte Trafficking, Neutrophil degranulation, T-cell Receptor Signaling, T-cell Receptor Signaling, T-cell Receptor Signaling, T-cell Receptor Signaling, Adaptive Immune system, Innate Immune System
POD33	FOS	Cell Cycle Regulation, Injury-repair response associated transcripts (IRRAT), Regulation, T-cell Receptor Signaling, TNF Family Signaling, MAPK, Toll-like Receptor Signaling, Oxidative Stress, B-cell Receptor Signaling / B-cell associated transcripts (BATs), Innate Immune System
POD61	CD74	MHC Class II Antigen Presentation, Antibody-mediated rejection transcripts (ABMR-RATs), IFNg Signaling and Rejection Induced Transcripts (GRIT3), Transcripts associated with T cell-mediated rejection (TCMR), Adaptive Immune system

POD61	S100A9	Damage-associated molecular pattern transcripts (DAMP), Quantitative Constitutive Macrophage-Associated Transcripts (QCMAT), Neutrophil degranulation, Toll-like Receptor Signaling, Transcripts associated with T cell-mediated rejection (TCMR), Innate Immune System
POD61	S100A8	Damage-associated molecular pattern transcripts (DAMP), Neutrophil degranulation, Toll-like Receptor Signaling, Transcripts associated with T cell-mediated rejection (TCMR), Innate Immune System
POD61	HLA-DPA1	MHC Class II Antigen Presentation, Co-Stimulation by CD28, T-cell Receptor Signaling, Antibody-mediated rejection transcripts (ABMR-RATs), IFNg Signaling and Rejection Induced Transcripts (GRIT3), Adaptive Immune system
POD61	CTSS	Neutrophil degranulation, Injury and repair induced transcripts - Late (IRITD5), Acute kidney injury (AKI), Injury-repair response associated transcripts (IRRAT), MHC Class II Antigen Presentation, Interstitial Fibrosis and Tubular Atrophy (IFTA), MHC Class I Antigen Presentation, Toll-like Receptor Signaling, Extracellular Matrix Organization, IFNg Signaling and Rejection Induced Transcripts (GRIT3), Adaptive Immune system, Innate Immune System
POD61	HLA-DPB1	MHC Class II Antigen Presentation, Co-Stimulation by CD28, T-cell Receptor Signaling, Antibody-mediated rejection transcripts (ABMR-RATs), IFNg Signaling and Rejection Induced Transcripts (GRIT3), Adaptive Immune system
POD61	HLA-DRA	MHC Class II Antigen Presentation, T-cell Receptor Signaling, Antibody-mediated rejection transcripts (ABMR-RATs), IFNg Signaling and Rejection Induced Transcripts (GRIT3), Adaptive Immune system
POD61	HLA-DRB3	Co-Stimulation by CD28, MHC Class II Antigen Presentation, T-cell Receptor Signaling, Adaptive Immune system
POD61	HLA-DRB1	MHC Class II Antigen Presentation, T-cell Receptor Signaling, Antibody-mediated rejection transcripts (ABMR-RATs), IFNg Signaling and Rejection Induced Transcripts (GRIT3), Adaptive Immune system
POD61	HLA-A	Type I Interferon Signaling, Cytotoxicity, Antibody-mediated rejection transcripts (ABMR-RATs), IFNg Signaling and Rejection Induced Transcripts (GRIT3), Adaptive Immune system
POD61	CD81	Complement Receptors, B-cell Receptor Signaling / B-cell associated transcripts (BATs), Innate Immune System, Adaptive Immune system
POD61	IGHG3	Immunoglobulin transcripts (IGT), Plasma cells

Table S4. Top-ranked differentially expressed genes comparing xenograft at 33 days post-reperfusion to genetically modified pig kidneys before implantation

Xenograft at 33 days post-reperfusion versus xenografts before implantation			
Gene	**log2-Fold Change**	**p-value**	**adjusted p-value**
CD74	7.62594741	3.38E-24	2.27E-21
HLA-DPA1	5.923173845	1.43E-13	9.61E-11
CTSS	6.300745844	7.91E-13	5.32E-10
HLA-A	4.658952995	1.34E-09	8.99E-07
S100A9	5.12760222	1.41E-09	9.48E-07
HLA-DRA	4.457660602	4.86E-09	3.27E-06
HLA-DRB3	4.416576985	1.09E-08	7.33E-06
HLA-B	4.333985487	1.56E-08	1.05E-05
WARS	4.797704045	1.27E-07	8.50E-05
HLA-DRB1	4.38086721	1.49E-07	1.00E-04
S100A8	4.693665692	3.21E-07	2.16E-04
HLA-DPB1	4.632644616	4.54E-07	3.05E-04
CALHM6	4.154929197	1.16E-06	7.82E-04
CD81	4.299248171	1.25E-06	8.37E-04
FCGR3A/B	4.028265342	1.72E-06	1.16E-03
ARHGDIB	3.974740419	2.28E-06	1.53E-03
C1QB	3.786437044	6.27E-06	4.21E-03
HLA-E	3.71066322	7.48E-06	5.03E-03
KLHL13	-3.087369131	2.99E-05	2.01E-02
HLA-DQB1	3.50157386	3.71E-05	2.49E-02
PTPRC	3.32527387	5.05E-05	3.40E-02
FOS	-3.234619831	7.14E-05	4.80E-02
IFNGR1	3.847785636	7.18E-05	4.82E-02
ANXA1	3.671243972	8.61E-05	5.78E-02
ERRFI1	-2.593665451	9.65E-05	6.49E-02
GZMB	3.966637617	1.08E-04	7.24E-02
SLAMF7	4.003434092	1.35E-04	9.09E-02
RORC	-2.812411305	1.78E-04	1.20E-01
RTN4	-2.151860783	4.90E-04	3.29E-01
CXL10	3.1386096155	9.64E-04	6.48E-01

Table S5. Top-ranked differentially expressed genes comparing xenograft at 33 days post-reperfusion to wild-type non-transplanted pig kidneys

Xenograft at 33 days post-reperfusion versus wild-type non-transplanted pig-kidney			
Gene	**log2-Fold Change**	**p-value**	**adjusted p-value**
B2M	6.12012248	1.64E-105	1.10E-102
CD74	7.58957967	1.43E-83	9.59E-81
CTNNB1	-3.21988507	2.24E-57	1.50E-54
GAPDH	-3.09379328	3.28E-52	2.21E-49
RPS6	-2.75221678	9.25E-51	6.22E-48
RTN4	-2.92131487	2.16E-50	1.45E-47
THBS1	-3.00897959	4.10E-46	2.75E-43
HMGB1	-3.29853545	4.74E-44	3.19E-41
ERRFI1	-4.14427667	1.23E-42	8.30E-40
MYL9	-2.71481987	1.92E-39	1.29E-36
HLA-A	5.58485268	8.00E-39	5.37E-36
ARG2	-3.36805968	8.04E-39	5.40E-36
COL4A1	-2.26006565	7.10E-36	4.77E-33
MAPK14	-3.09146565	7.91E-36	5.32E-33
EEF1A1	-2.27155296	3.16E-35	2.13E-32
PLK2	-3.65842329	1.51E-33	1.02E-30
STAT3	-2.56701611	2.14E-32	1.44E-29
VEGFA	-2.48940116	2.44E-32	1.64E-29
TGFBR2	-2.71252578	7.07E-32	4.75E-29
VMP1	-2.97004313	1.15E-31	7.71E-29
RPS6KB1	-2.78705344	2.78E-31	1.87E-28
IGHG3	-4.24797378	3.39E-31	2.28E-28
HLA-DRA	2.92300872	2.87E-30	1.93E-27
MIF	-2.40025195	9.39E-30	6.31E-27
KLHL13	-4.37230408	1.80E-29	1.21E-26
CHCHD10	-2.57060294	1.99E-29	1.34E-26
CTSS	5.00906658	1.39E-28	9.36E-26
NOTCH2	-3.20548418	2.36E-28	1.58E-25
PSEN1	-2.85864996	1.43E-27	9.62E-25
ABCA1	-3.01467061	2.68E-27	1.80E-24

Table S6. Bulk TCRB CDR3 Data Summary.

(A) Number of CD4 and CD8 unique clones and templates present in the different unstimulated samples. (B) Number of unique CD4 and CD8 clones remaining after removing the clones whose frequencies in the CFSE^{low} subpopulations of the different MLRs performed were not at least twice that in the pre-transplant unstimulated population, the criterion for XDRTCC definition. (C) Number of unique CD4 and CD8 XDRTCCs identified after pooling from the different MLRs and removing duplicate clones.

A Unstimulated Samples

Pre-Tx	POD28		POD33		POD49	
CD3	CD4	CD8	CD4	CD8	CD4	CD8
18,254 (19,783)	22,716 (29,518)	25,972 (41,140)	6460 (8079)	5549 (12,872)	20,279 (24,460)	26,953 (60,142)

Unique Clones (Templates)

B XDRTCCs Across MLRs

Pre-Tx Direct LN		Pre-Tx Direct PBMC		Pre-Tx Indirect PBMC		POD28 Direct PBMC		POD49 PBMC vs PBMC	
CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
81	14	61	18	261	37	333	67	376	258

↓ Pool & Remove Duplicates

C Total XDRTCCs

CD4	CD8
1058	386

Table S7. The number of isolated leukocytes from each sample.

The indicated number of leucocytes was isolated from kidney biopsies/lymphocele sample and submitted for single-cell RNA sequencing

Time Point	Sample	Isolated leukocytes*	Number of leukocytes used for scRNAseq
POD14	Kidney biopsy	30,000	30,000
POD14	Lymphocele	20,000	20,000
POD28	Kidney biopsy	12,000	12,000
POD33	Kidney biopsy	6002	6002
POD61	Kidney biopsy	100,000	30,000

***** Leukocytes were isolated from kidney biopsy tissue through enzymatic digestion followed by CD45 FACS sorting, and from lymphocele fluid using Ficoll gradient centrifugation

$^{+}$ CD45+ cells were defined as RNA expression of PTPRC > 1

Table S8. Real-time PCR surveillance for porcine-origin viruses in donor and recipient samples using RNA template

ND, Not detected. Duplicate Ct values in parentheses where Detected or Equivocal results were obtained. Weak positive results (Ct > 35) that were not reproduced in duplicate samples were considered Not Detected. Human or porcine RPP30 was detected for all samples, indicating successful nucleic acid purification.

	Sample ID	PERV-C	PERV-ABC	PLHV-1	PLHV-2	PLHV-3	PCMV	PCV-1	PCV-2	PCV-3
Recipient PBMC	POD -1	ND	ND	ND	ND	ND	ND	ND	ND	ND
	POD 0 (0 hr)	ND	Equivocal (37, 40)	ND	ND	ND	ND	ND	ND	ND
	POD 0 (1hr)	ND	ND	ND	ND	ND	ND	ND	ND	ND
	POD 0.5	ND	ND	ND	ND	ND	ND	ND	ND	ND
	POD 1	ND	ND	ND	ND	ND	ND	ND	ND	ND
	POD 2	ND	ND	ND	ND	ND	ND	ND	ND	ND
	POD 3	ND	ND	ND	ND	ND	ND	ND	ND	ND
	POD 7	ND	ND	ND	ND	ND	ND	ND	ND	ND
	POD 8	ND	ND	ND	ND	ND	ND	ND	ND	ND
	POD 10	ND	ND	ND	ND	ND	ND	ND	ND	ND
	POD 14	ND	ND	ND	ND	ND	ND	ND	ND	ND
	POD 21	ND	ND	ND	ND	ND	ND	ND	ND	ND
	POD 28	ND	ND	ND	ND	ND	ND	ND	ND	ND
	POD 35	ND	ND	ND	ND	ND	ND	ND	ND	ND
	POD 42	ND	ND	ND	ND	ND	ND	ND	ND	ND
	POD 49	ND	ND	ND	ND	ND	ND	ND	ND	ND
	POD 56	ND	ND	ND	ND	ND	ND	ND	ND	ND
	POD 61	ND	ND	ND	ND	ND	ND	ND	ND	ND
Recipient other	POD 13 lymphocele	ND	ND	ND	ND	ND	ND	ND	ND	ND
Porcine samples	PBMC	ND	Detected (17, 17)	ND	ND	ND	ND	ND	ND	ND
	Lymph node	ND	Detected (24, 25)	ND	ND	ND	ND	ND	ND	ND
	Spleen	ND	Detected (22, 23)	ND	ND	ND	ND	ND	ND	ND
	Thymus	ND	Detected (24, 24)	ND	ND	ND	ND	ND	ND	ND
	Kidney (explant)	ND	Detected (24, 24)	ND	ND	ND	ND	ND	ND	ND
	Thymus (explant)	ND	Detected (25, 25)	ND	ND	ND	ND	ND	ND	ND
Terminal decedent samples	CSF	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Graft-draining lymph node	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Non-draining lymph node	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Mesenteric lymph node	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Throat lymph node	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Spleen	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Liver	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Thymus	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Pancreas	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Heart	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table S9. Real-time PCR surveillance for porcine-origin viruses in donor and recipient samples using DNA

ND, Not detected; INS, insufficient material available for repeat testing; *, Sample POD 0 (0hr) was unavailable for repeat testing and a POD 0 (1hr) sample was used to substitute; Ct values in parentheses where Detected or Equivocal results were obtained. Weak positive results (Ct > 35) that were not reproduced in duplicate samples were considered Not Detected. Human or porcine RPP30 was detected for all human and porcine samples, respectively, indicating successful nucleic acid purification.

	Sample ID	PERV-C	PERV-ABC	PLHV-1	PLHV-2	PLHV-3	PCMV	PCV-1	PCV-2	PCV-3
Recipient PBMC	POD -1	ND	ND	ND	ND	ND	ND	ND	ND	ND
	POD 0 (0hr)	INS	INS	INS	INS	INS	INS	INS	INS	INS
	POD 0* (1hr)	ND	Detected (28, 29)	ND	ND	ND	ND	ND	ND	ND
	POD 0.5	ND	Detected (32, 31)	ND	ND	ND	ND	ND	ND	ND
	POD 1	INS	INS	INS	ND	ND	ND	ND	ND	ND
	POD 2	ND	ND	ND	ND	ND	ND	ND	ND	ND
	POD 3	ND	ND	ND	ND	ND	ND	ND	ND	ND
	POD 7	ND	INS	ND	ND	ND	ND	ND	ND	ND
	POD 8	ND	ND	ND	ND	ND	ND	ND	ND	ND
	POD 10	ND	INS	INS	ND	ND	ND	ND	ND	ND
	POD 14	ND	ND	ND	ND	ND	ND	ND	ND	ND
	POD 21	ND	Detected (30, 31)	ND	ND	ND	ND	ND	ND	ND
	POD 28	ND	Detected (33, 32)	ND	ND	ND	ND	ND	ND	ND
	POD 35	ND	ND	ND	ND	ND	ND	ND	ND	ND
	POD 42	ND	Equivocal (ND, 33)	ND	ND	ND	ND	ND	ND	ND
	POD 49	ND	Detected (33, 34)	ND	ND	ND	ND	ND	ND	ND
	POD 56	ND	ND	ND	ND	ND	ND	ND	ND	ND
	POD 61	ND	Detected (32, 31)	ND	ND	ND	ND	ND	ND	ND
Recipient other	POD 13 lymphocele	ND	Detected (30, 30)	ND	ND	ND	ND	ND	ND	ND
Porcine samples	PBMC	Detected (35, 35)	Detected (17, 17)	ND	ND	ND	ND	ND	ND	ND
	Lymph node	Detected (31, 30)	Detected (12, 11)	ND	ND	ND	ND	ND	ND	ND
	Spleen	Detected (33, 34)	Detected (16, 16)	ND	ND	ND	ND	ND	ND	ND
	Thymus	Detected (35, 35)	Detected (18, 17)	ND	ND	ND	ND	ND	ND	ND
	Kidney (explant)	Detected (30, 30)	Detected (14, 14)	ND	ND	ND	ND	ND	ND	ND
	Thymus (explant)	Detected (35, 36)	Detected (19, 21)	ND	ND	ND	ND	ND	ND	ND
Terminal decedent samples	CSF	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Graft-draining lymph node	ND	Detected (32, 32)	ND	ND	ND	ND	ND	ND	ND
	Non-draining lymph node	ND	Detected (35, 34)	ND	ND	ND	ND	ND	ND	ND
	Mesenteric lymph node	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Throat lymph node	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Spleen	ND	Detected (31, 31)	ND	ND	ND	ND	ND	ND	ND
	Liver	ND	Detected (36, 34)	ND	ND	ND	ND	ND	ND	ND
	Thymus	ND	Detected (33, 34)	ND	ND	ND	ND	ND	ND	ND
	Pancreas	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Heart	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table S10. PCR probe sequences and results

(A) Primer and probe sequences for confirmation of PERV using semi-nested PCR

Primer name	Sequence (5' to 3')
PERVA-env-F	CCTCAAGTTAATGGTAAACGCC
PERVA-env-F2	TAAACGCCTTGTGGACAGCC
PERVA-env-R	CGCTGTTGCCAATCTTTCC
PERVB-env-F	AGGCCAGTAGTAAACGCC
PERVB-env-F2	GCCTTATAGACAGCTCGAACC
PERVB-env-R	TAAGTCTGATGGGGAGCAGC
PERVC-env-F	CTCTCAGACTAATGGTATGCGC
PERVC-env-F2	ATAGGAGACAGCCTGAACTCC
PERVC-env-R	GCTTCCAGTTCTAATCCAGGTC

(B) Sequencing results of partial PERV envelope gene PCR products

PCR assay	Sample	Closest match	Accession	Amplicon (bp)	Cover	Identity	e-score
PERV-A	Thymus (pig)	PERV A	HQ540592.1	396	100%	99.75%	0
	Kidney (explant)	PERV A	HQ540592.1	396	100%	99.75%	0
PERV-B	Thymus (pig)	PERV B	HQ540593.1	390	100%	99.74%	0
	Kidney (explant)	PERV B	HQ540593.1	390	100%	99.74%	0
PERV-C	Thymus (pig)	PERV C	DQ996276.1	338	100%	100.00%	2.00E-174
	Kidney (explant)	PERV C	AF402663.1	338	100%	98.22%	2.00E-164

(C) Primer and probe sequences for the multiplexed real-time PCR for microchimerism

Oligo name	Sequence (5' to 3')
P-cytb-F	GGCTTTTCCGTCGACAAAGC
P-cytb-R	ATAGGAGATGTACGGCTGCG
P-cytb-P (HEX)	TGAATGGCAGGATAAAGTGGAAGGCGA
H-cytb-F	AGCCCTAGCAACACTCCAC
H-cytb-R	GTGTAGTAAGGGTGGAAGGTG
H-cytb-P (FAM)	CCCTAGGAATCACCTCCCATTCC