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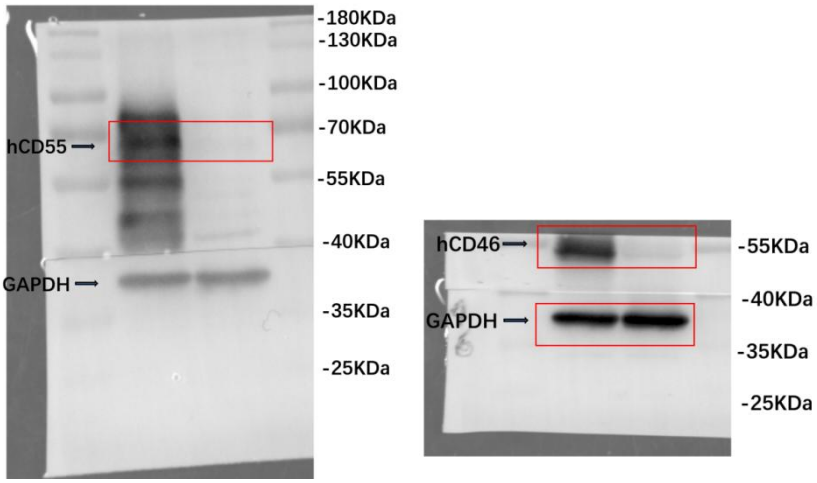
Supplementary information

Gene-modified pig-to-human liver xenotransplantation

In the format provided by the authors and unedited

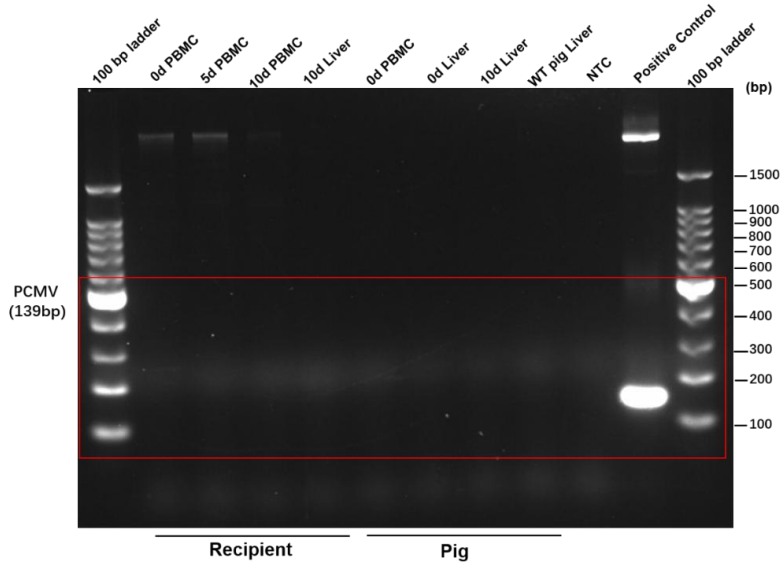
SUPPLEMENTARY FIGURE 1

Fig. 1c

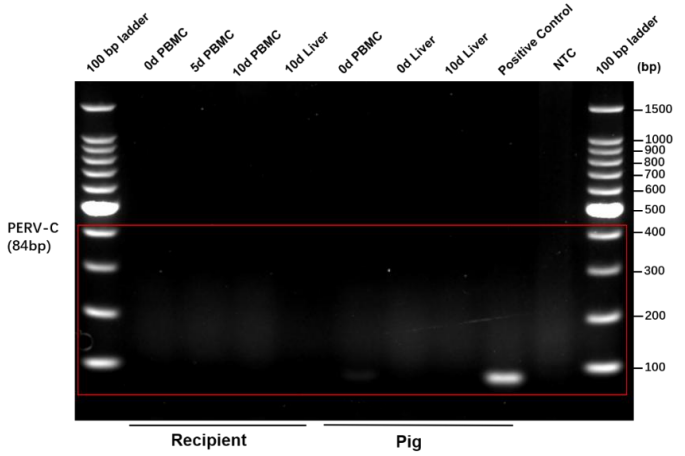


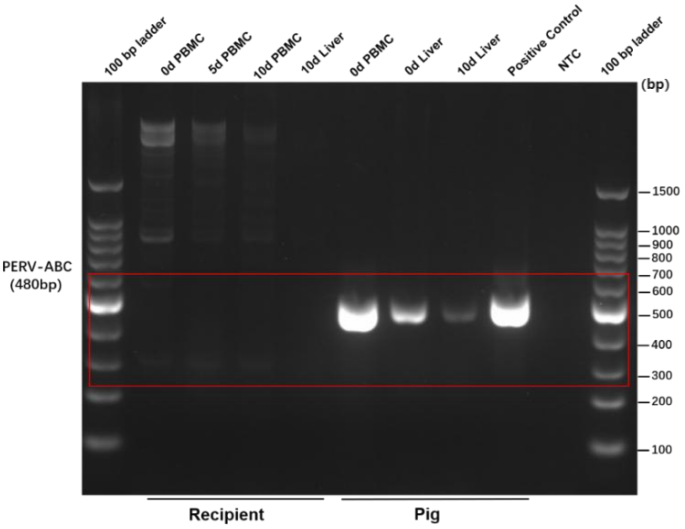
GAPDH was used as the control. For hCD46 and GAPDH, samples obtained from liver tissues of the donor and wild-type pig were run on the same gel, and then transferred to the same PVDF membrane, which were cropped and incubated in different primary antibodies. For hCD55, the same amounts of samples were uploaded on another gel.

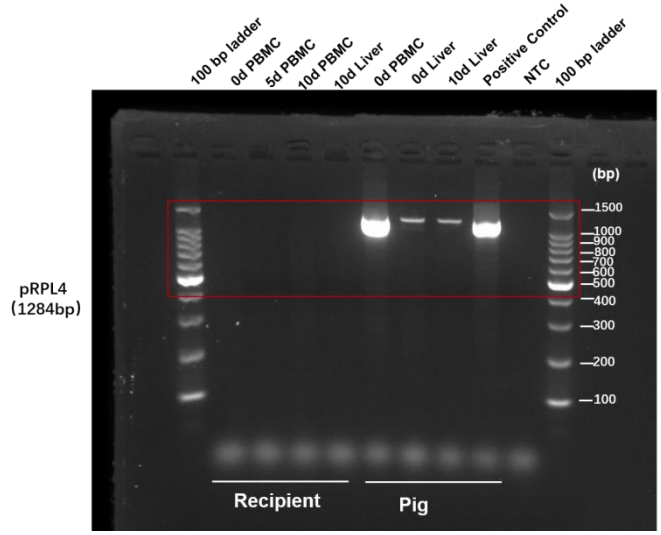
Extended Data Fig. S1a

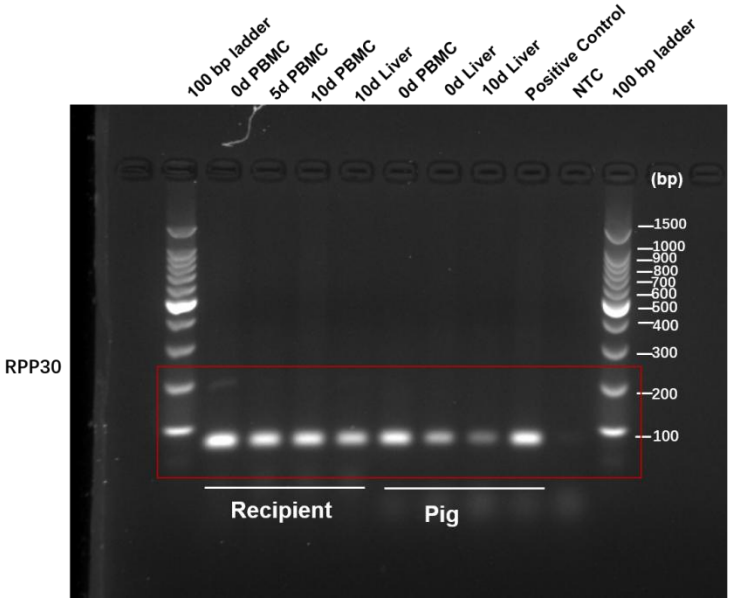


Extended Data Fig. S1b







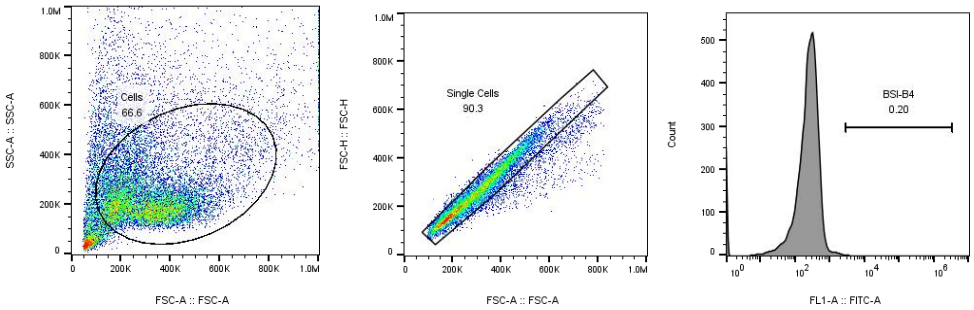


The cDNA were extracted from the same samples, and then went through PCR using the indicated primers. Then the products were running on different gels.

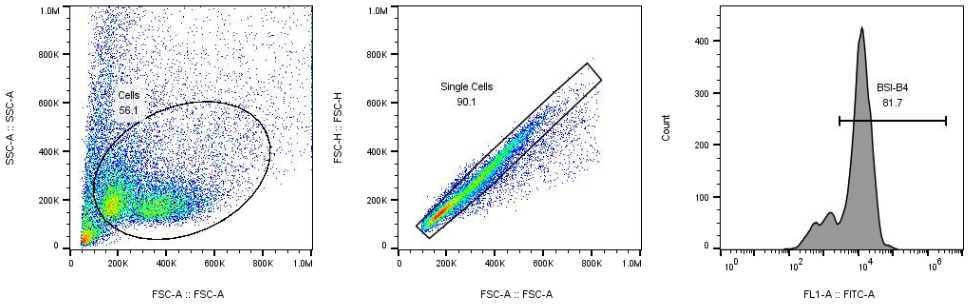
SUPPLEMENTARY FIGURE 2

GGTA1

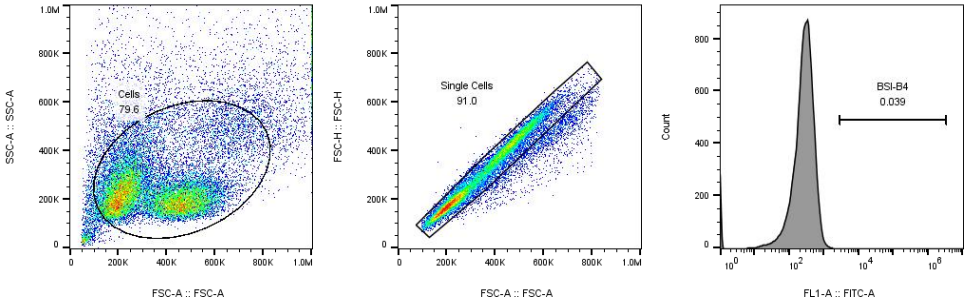
WT pig PBMCs Blank control



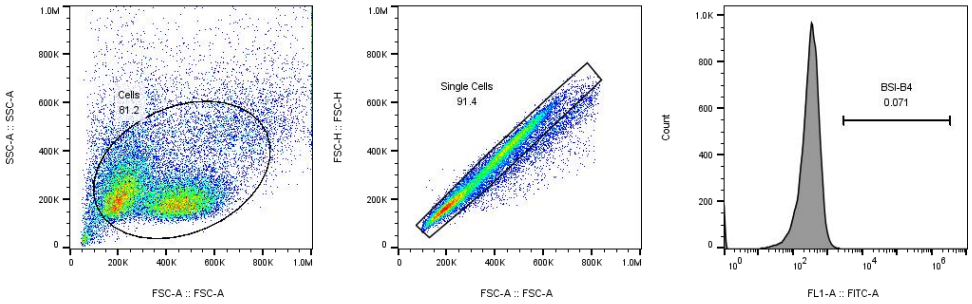
WT pig PBMCs BSI-B4



Donor pig PBMCs Blank control



Donor pig PBMCs BSI-B4



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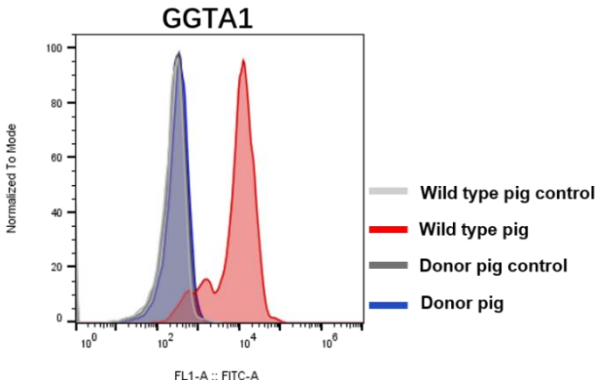
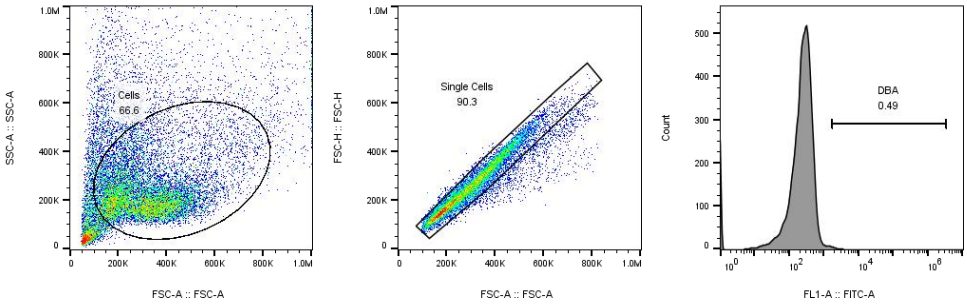
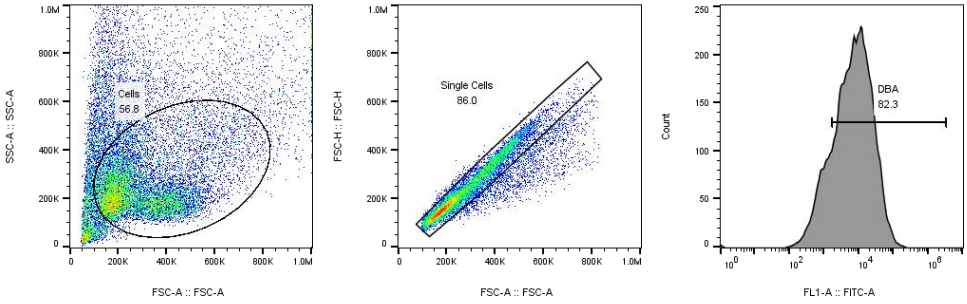


β4GalNT4

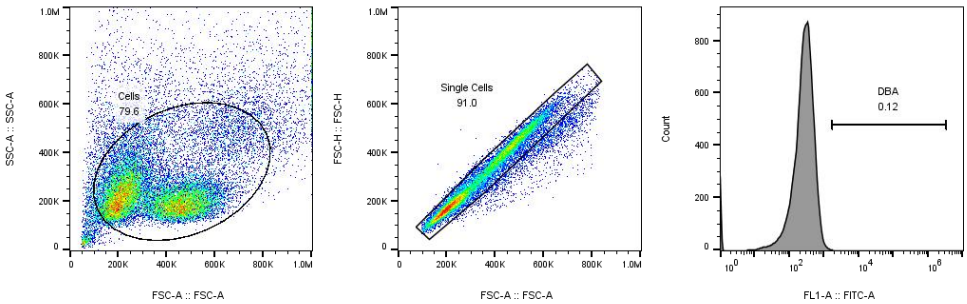
WT pig PBMCs Blank control



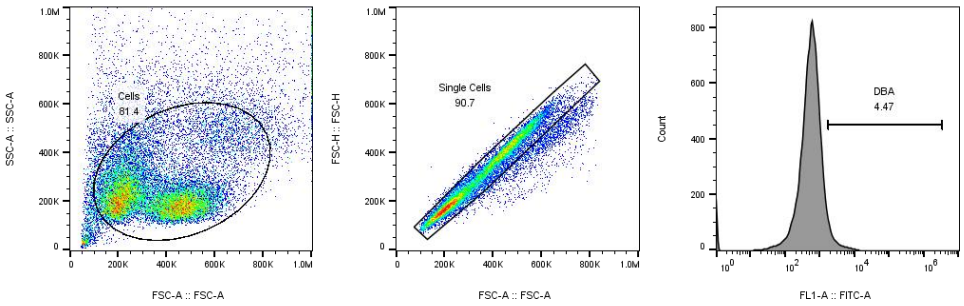
WT pig PBMCs DBA



Donor pig PBMCs Blank control



Donor pig PBMCs DBA



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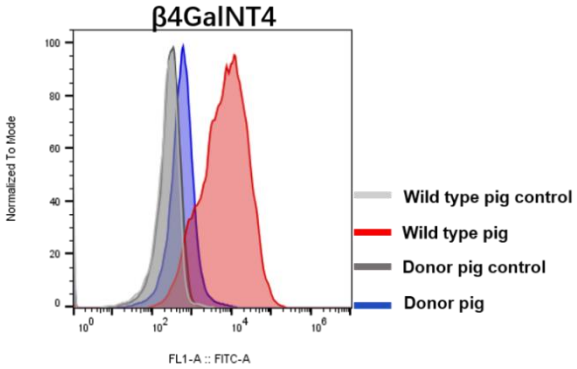
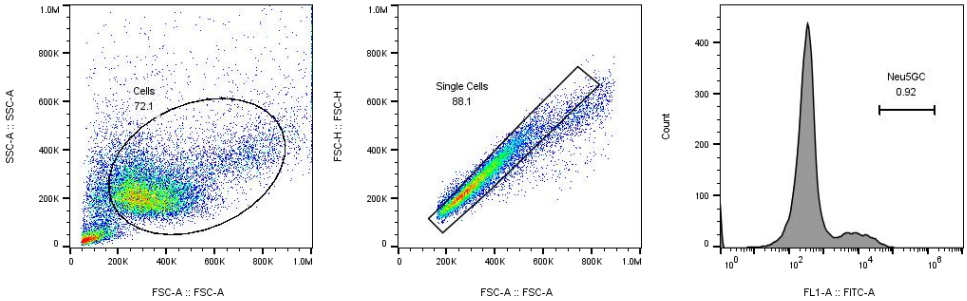
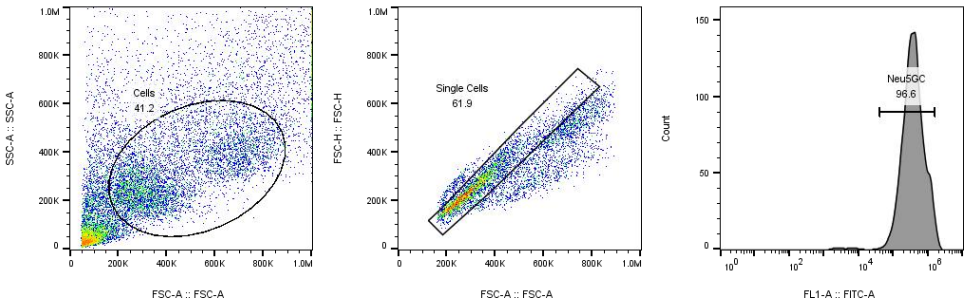


Neu5Gc

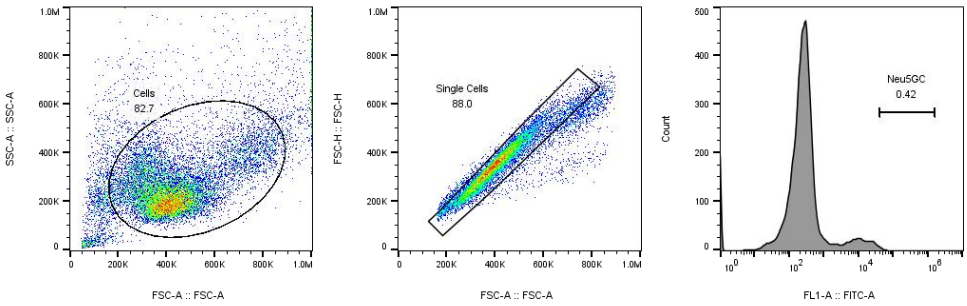
WT pig PBMCs Isotype control



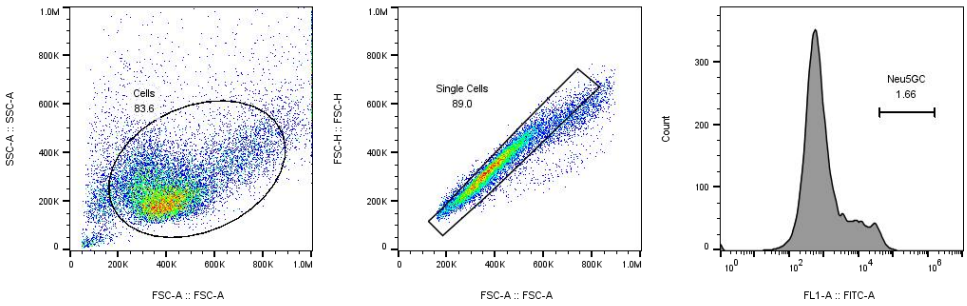
WT pig PBMCs Neu5GC antibody



Donor pig PBMCs Isotype control



Donor pig PBMCs Neu5GC antibody



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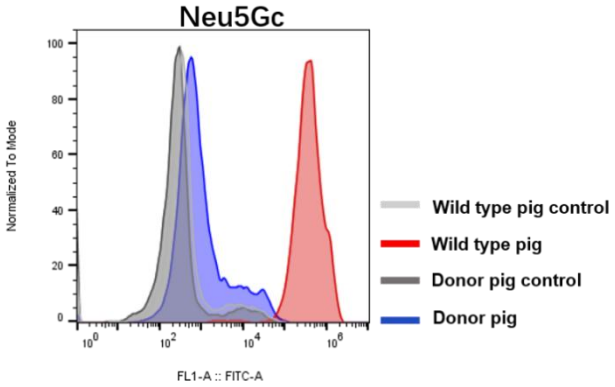
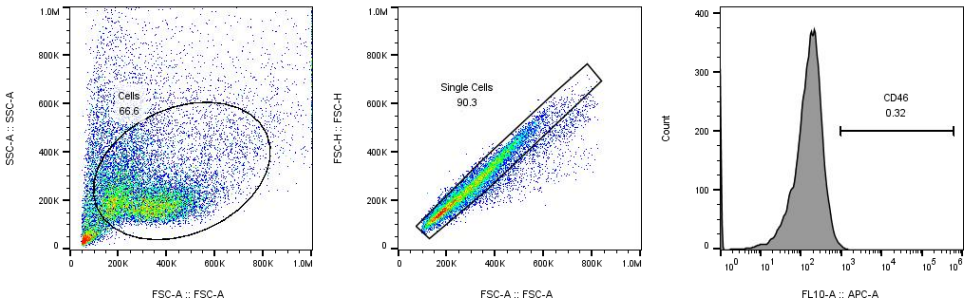
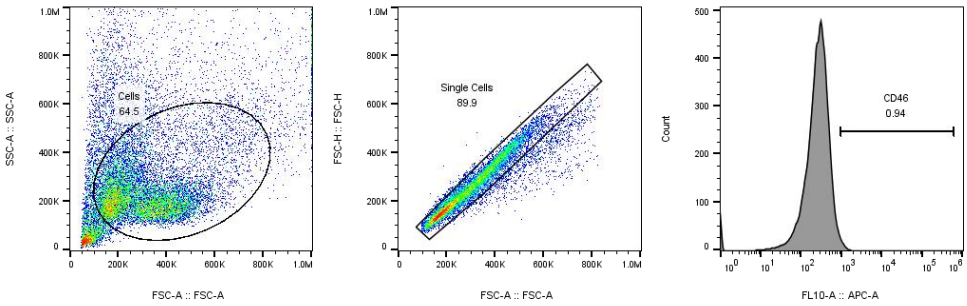


CD46

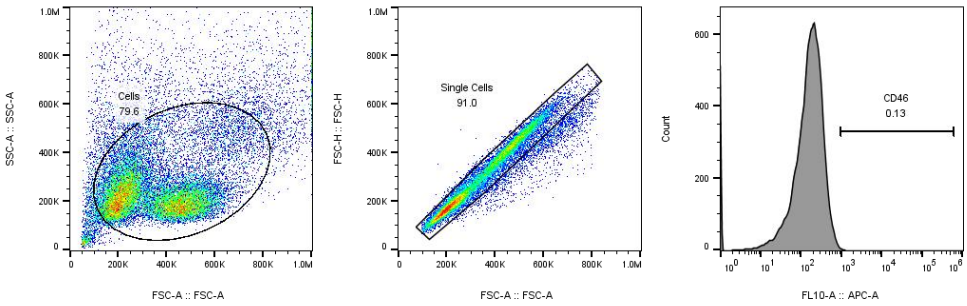
WT pig PBMCs Blank control



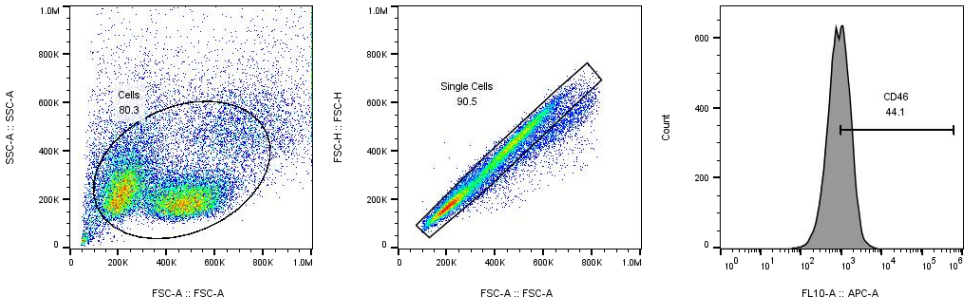
WT pig PBMCs CD46 antibody



Donor pig PBMCs Blank control



Donor pig PBMCs CD46 antibody



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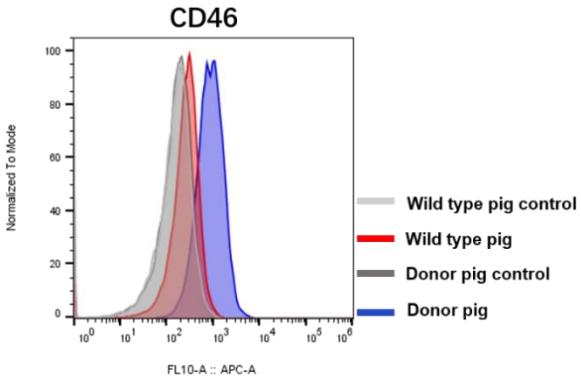
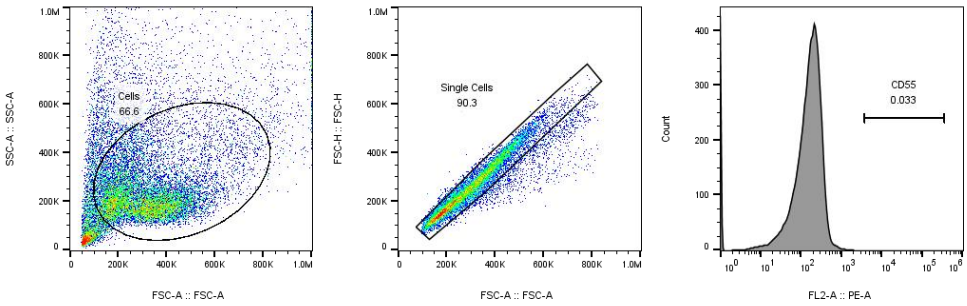
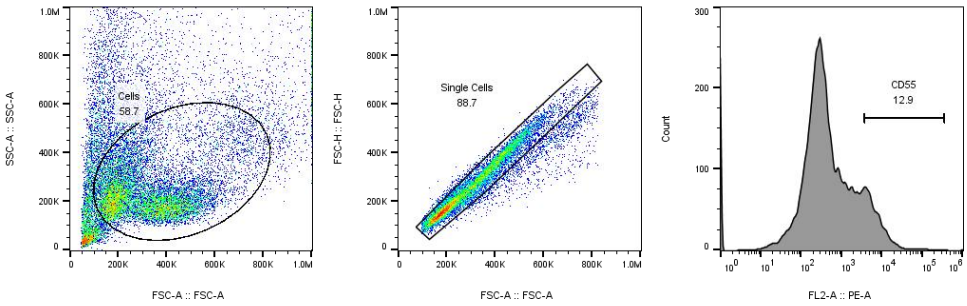


CD55

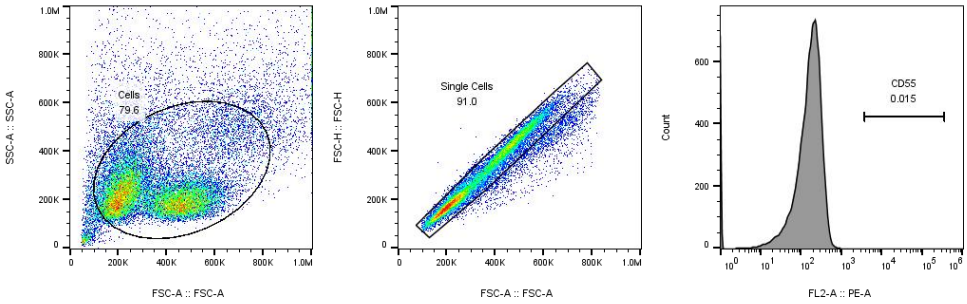
WT pig PBMCs Blank control



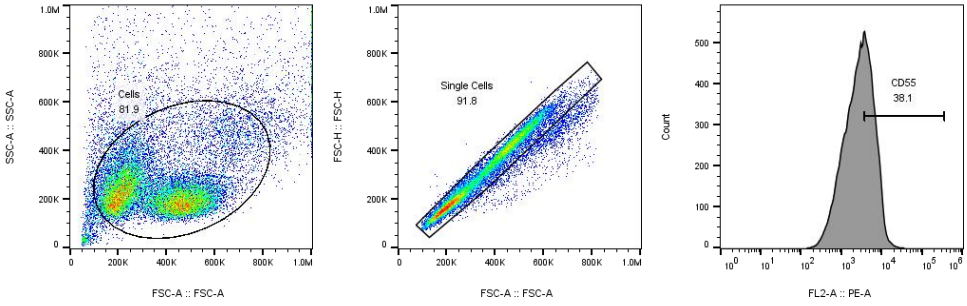
WT pig PBMCs CD55 antibody



Donor pig PBMCs Blank control



Donor pig PBMCs CD55 antibody



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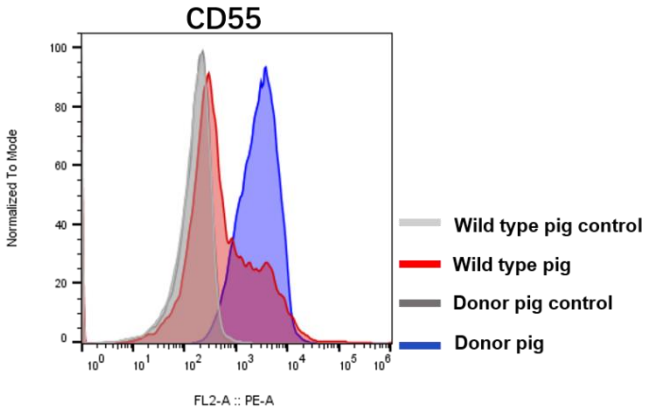


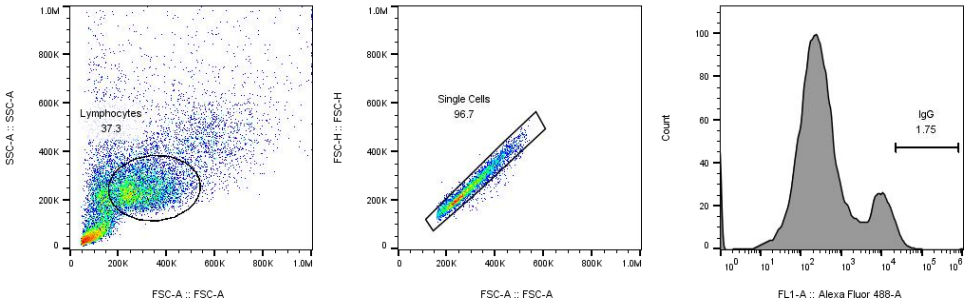
Supplementary figure 2. The gating strategy of Fig. 1c.

These images are the source data of Fig. 1**c**. The four groups of histograms of the above genes were superimposed respectively to obtain Fig. 1**c**.

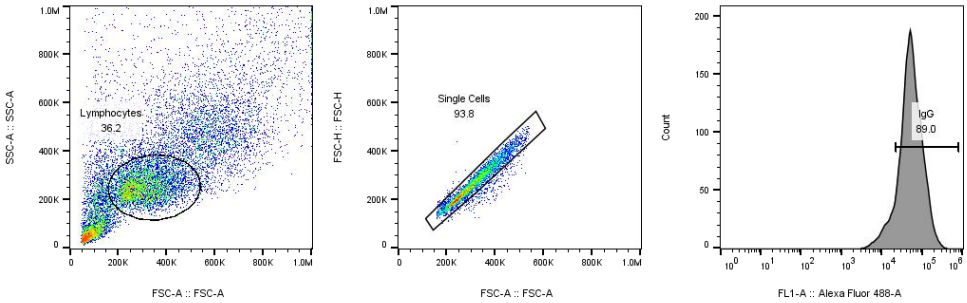
SUPPLEMENTARY FIGURE 3

IgG

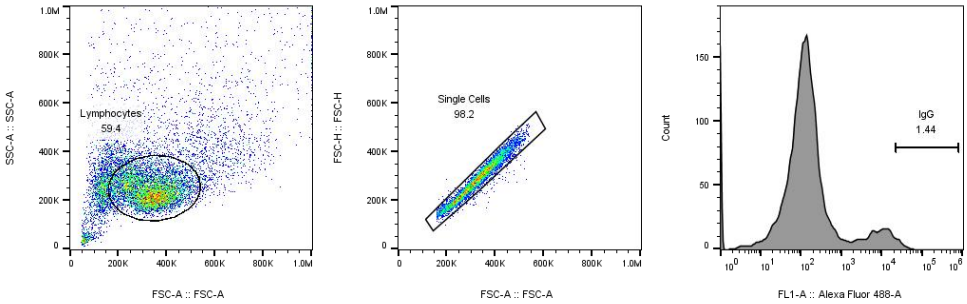
WT pig PBMCs IgG antibody control (Negative Control)



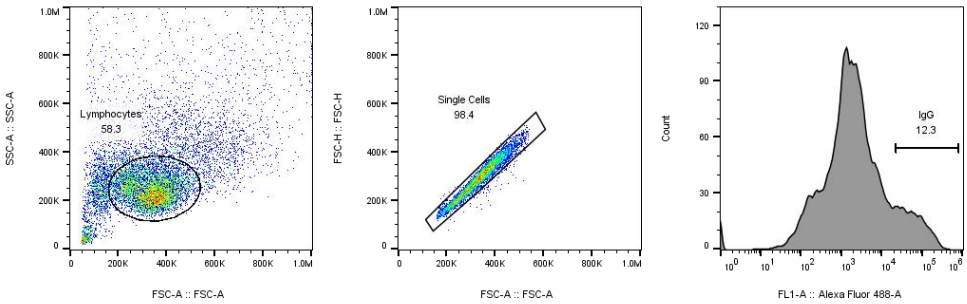
WT pig PBMCs and Recipient sera IgG antibody



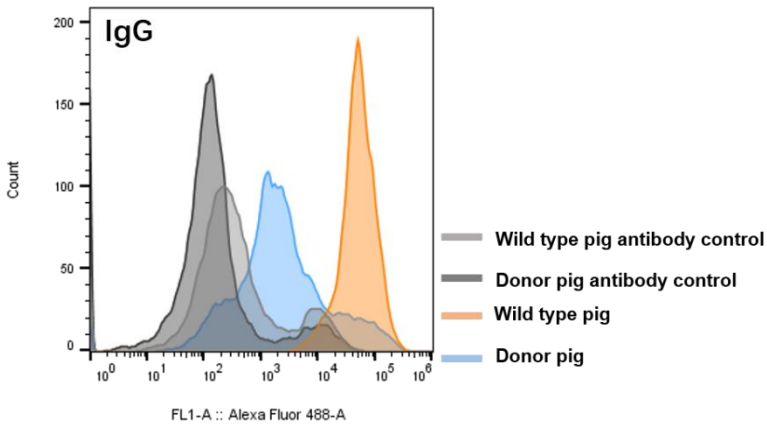
Donor pig PBMCs IgG antibody control (Negative Control)



Donor pig PBMCs and Recipient sera IgG antibody

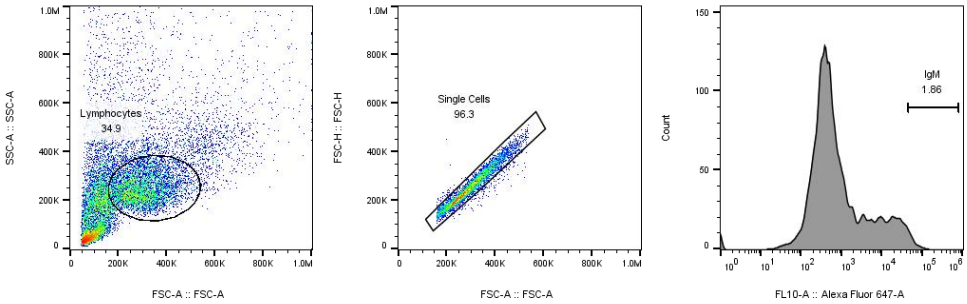


The above four groups of histograms are superimposed to obtain the following figure

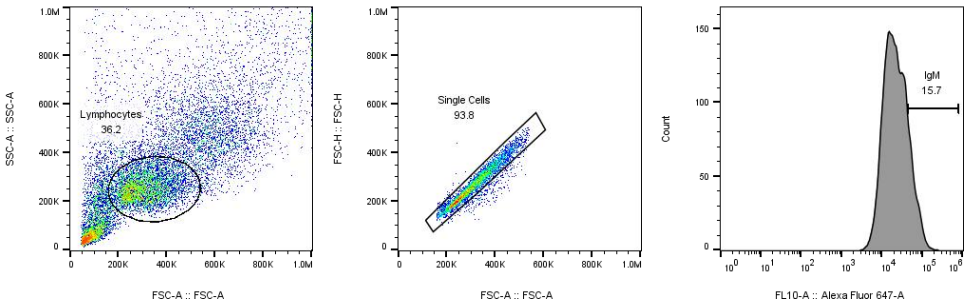


IgM

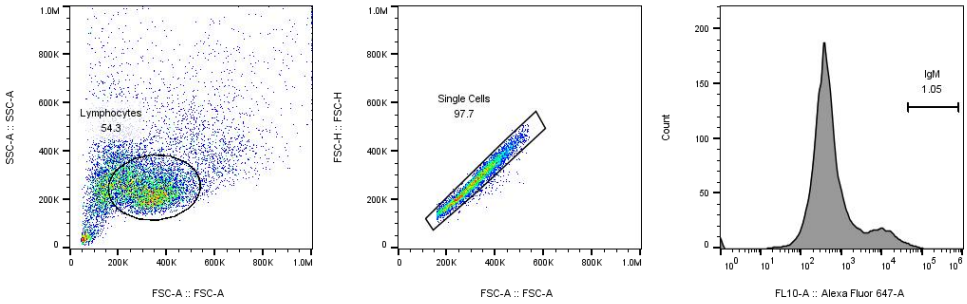
WT pig PBMCs IgM antibody control (Negative Control)



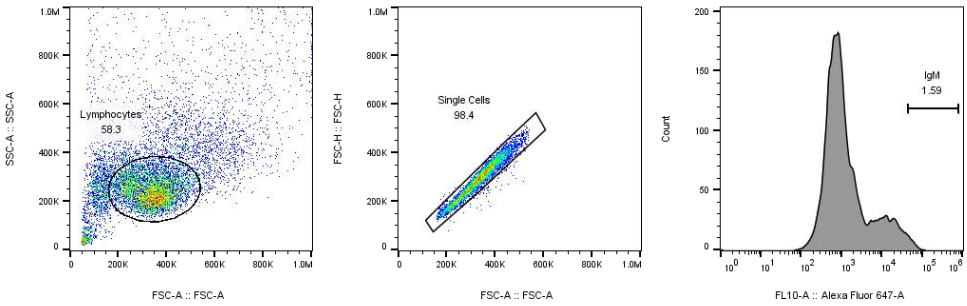
WT pig PBMCs and Recipient sera IgM antibody



Donor pig PBMCs IgM antibody control (Negative Control)



Donor pig PBMCs and Recipient sera IgM antibody



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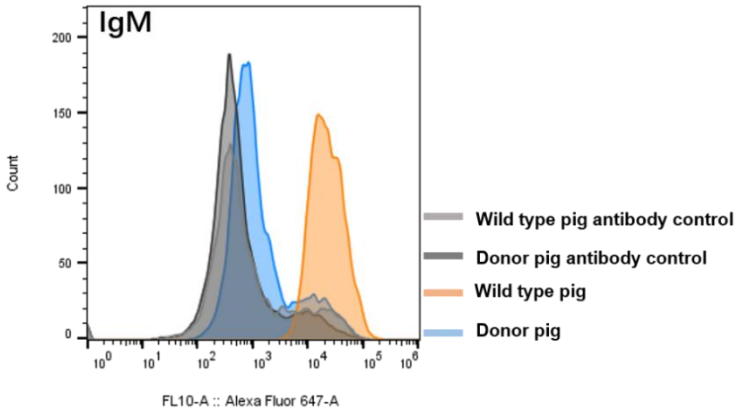

Supplementary figure 3. The gating strategy of Fig. 1e.

These images are the source data of Fig. 1**e**. The four groups of histograms of the above genes were superimposed respectively to obtain Fig. 1e.

SUPPLEMENTARY TABLES

Table S1. Patient medication management

Category	Drug	Dosage	Day
Proton pump inhibitor	Esomeprazole	40mg bid	POD: -1，1 - 10
		40mg tid	POD: 0
Hepatoprotective drug	Magnesium Isoglycyrrhizinate	200mg qd	POD:0, 6 - 10
		200mg bid	POD:1 - 5
	Ademetionine butanedisulfonate	1g bid	POD: 0 - 6,9-10
		0.5g bid	POD: 7 - 8
	Polyene Phosphatidylcholine	465mg bid	POD: 6 - 10
Enteral nutrition	Glucose and Sodium Chloride Injection	300ml qd	POD: 3 - 6
	Probiotics	500mg tid	POD: 3 - 10
	Enteral Nutritional Powder	27.9g tid	POD: 5 - 6
	Enteral Nutritional Powder	9.3g qd	POD: 7
Parenteral nutrition	10% Glucose	1000ml qd	POD: -1, 7 - 9
	50% Glucose	200ml qd	POD: -1, 7 - 9
	Compound Amino Acid	500ml qd	POD: -1, 7 - 9
	Fat-soluble Vitamin(II)/Water-soluble Vitamin	10ml qd	POD: -1, 7 - 9
	Alanyl-Glutamine	20g qd	POD: -1, 7 - 9
	Fructose	250ml bid	POD: 1 - 6
		500ml qd	POD: 7 - 10
	Insulin	Adjust insulin dosage according to the blood glucose level	
Protein	Albutein	40g qd	POD: -1 - 2
		60g qd	POD: 3 - 8
		80g qd	POD: 9
		30g qd	POD: 10
	Human Immunoglobulin	10g qd	POD: 0 - 6
micronutrients	Vitamin C	3g qd	POD: 0 - 2
		6g qd	POD: 3
	Vitamin B6	200mg qd	POD: 0 - 2
		400mg qd	POD: 3
	Calcium Gluconate	1g pre-transfusion; Adjust dosage according to the blood calcium level	
Antiviral drug	Ganciclovir	0.25g bid	POD: 0 - 10
Antifungal drug	Caspofungin Acetate	50mg qd	POD: 3 - 10

Antimicrobial drug	Meropenem	0.5g tid	POD: -1 - 10
	Linezolid	0.6g bid	POD: 8 - 10
Anti-inflammatory drug	Ulinastatin	0.5 MIU bid	POD: -1 - 10
Anticoagulation drug	Heparin Sodium Injection	12500 IU qd	POD: 0 - 10
Vasoactive drug	Noradrenaline	Adjust dosage according to the blood pressure	

Table S2. Biochemical indexes of the recipient

Sample	Item category	Representative indexes
Whole blood	Lymphocytes	T cell, B cell, $CD3^+CD4^+$T cell, $CD3^+CD8^+$T cell
Whole blood	Plasma concentration	FK506
Whole blood	Cytokines	IL-6, TNF-α, IFN-α, IFN-γ
Serum	Liver function	ALT, AST, TB, DBil, IDBil, ALP, γ-GGT
Serum	Immunoglobin	IgG, IgM
Serum	Inflammatory mediator	CRP, PCT
Serum	Myocardial enzyme	CK, CK-MB
Plasma	Coagulation function	PLT, PT, APTT, D-dimer, PTA, PTA, INR
Plasma	Coagulation factors	FII, FV, FVII, FVIII, FIX, FX, FXI
Plasma	Plasma concentration	MMF

Table S3. Reagents and antibodies

Reagent	Dilution Ratio	Product Number	Clone Name	Brand
Isolectin BSI-B4	FC(1:500)	L2895	Bandeirea simplicifolia agglutinin, BS-I	SIGMA
Dolichos Biflorus Agglutinin	FC(1:500)	FL-1031	Fluorescein	Vector
Neu5Gc Polyclonal antibody)	FC(1:200)	146901	Poly21469	Biolegend
Goat Anti-Chicken IgY H&L (DyLight® 488)	FC(1:200)	ab96947		Abcam
CD55 monoclonal antibody	5ul/10^6 cells	SC-59092PE	BRIC 216	Santa-cruz
CD46 monoclonal antibody	10ul/10^6 cells	MAB4439	MEM-258	Abnova
CD55 Polyclonal antibody	WB(1:5000), IHC-P(1:200)	26580-1-AP		Proteintech
CD46 Polyclonal antibody	WB(1:2000), IHC-P(1:500)	12494-1-AP		Proteintech
TBM monoclonal antibody	IHC-P(1:500)	67831-1-Ig	5A4C5	Proteintech
GAPDH Monoclonal antibody	WB(1:20000)	60004-1-Ig	1E6D9	Proteintech
Goat anti-Human IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	FC(1:200)	A11013		invitrogen
Goat anti-Human IgM (Heavy chain) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	FC(1:200)	A21249		invitrogen
Pig Albumin (Alb) ELISA Kit		CSB-E16207p		CUSABIO
LEGENDplex™ Human		740809		Biolegend

Inflammation Panel 1 (13-plex) with Filter Plate				
C3d monoclonal antibody	IHC-P(1:150)	NBP3-07741	C3D/2891	NOVUS
C4d monoclonal antibody	IHC-P(1:200)	NBP2-34234	C4D204	NOVUS
C5b-9 Polyclonal antibody	IHC-P(1:1000)	ab55811		Abcam
IgM monoclonal antibody	IHC-P(1:100)	ab200541	IM260	Abcam
IgG monoclonal antibody	IHC-P(1:1000)	ab109489	EPR4421	Abcam
Ki-67 Polyclonal antibody	IHC-P(1:500)	27309-1-AP		Proteintech
smooth muscle actin specific Recombinant antibody	IHC-P(1:400)	80008-1-RR	5H7	Proteintech
CD31 monoclonal antibody	IF(1:50)	MCA1746GA	LCI-4	BIO-RAD

Table S4. Primer sequence

Virus/primer name	**Oligonucleotide sequence**	**Accession numbers**
porcine cytomegalovirus		HQ113116.1
PCMV-F	CGCTGTGAGGACCCTATGTTGG	
PCMV-R	TGGTGATGGACGCCGCTAGA	
porcine endogenous retrovirus ABC		PERV-A: HQ540592.1 PERV-B: HQ540595.1 PERV-C: HM159246.1
PERV-ABC-F	GCAGCCCCTCCAGTATTGGCC	
PERV-ABC-R	GGGGTGAACCGCCTGAAGGC	
Porcine endogenous retrovirus C		AM229312.2
PERV-C-F	GGCGCCACCTCCCGATTCGGG	

PERV-C-R	GAGTAAGGAACCACGCGATGG	
RPL4 ribosomal protein L4 [Sus scrofa (pig)]		100038029
pRPL4-F	ATGGCGTGTGCTCGTCC	
pRPL4-R	TTATGCAACAGCCTTCTTTTCTTCTGT	
Pig RPP30 (housekeeping)		XM_001924771
Pig RPP30- F	GATTTGGACCTGCGAGCGG	
Pig RPP30-R	GTAAGCGGCGTTCTCCACGA	
Human RPP30 (housekeeping)		NM_001104546
Human RPP30-F	AGATTTGGACCTGCGAGCG	
Human RPP30-R	GAGCGGCTGTCTCCACAAGT	

SUPPLEMENTARY METHODS

Postoperative management of recipient

After surgery, the recipient, whose vital signs were closely monitored, was administered with vasoactive drugs, anticoagulation drugs, anti-inflammatory drugs, antibiotics, hepatoprotective drugs, proton pump inhibitors, parenteral nutrition, enteral nutrition and other supportive therapies (Table S1 of the Supplementary Information). Meanwhile, the abdominal cavity and bile drainage were observed and recorded. Biopsy of the xenograft (0, 2, and 10 d) and the recipient's native liver (10 d) were performed for hematoxylin and eosin (H&E) staining. Immunohistochemical staining (C3d, C4d, C5b-9, IgM and IgG) of a pig liver biopsy was also performed at specific time points (0 d, 2 h and 10 d) to test for signs of hyperacute rejection. Concurrently, immune-related biochemical indicators in the recipient's whole blood or serum were measured daily beginning on 1 day before surgery (-1 d) until the trial endpoint (10 d) (Table S2 of the Supplementary Information).

Immunosuppression regimen

The core principle of immunosuppression, to prevent recipient organ rejection and inflammation, is derived from our previous clinical protocol. This primarily included treatment with intravenous antihuman thymoglobulin (ATG) (75 mg) to deplete T cells 1 day before surgery (-1 d) and on the day of surgery (0 d). Rituximab (100–200 mg) was administered intravenously 3–5 d after surgery to inhibit B cells. Eculizumab

(900 mg) was administered intravenously to inhibit complement on the day of surgery (0 d). Etanercept (25 mg/0.47 ml) was injected subcutaneously on the day of surgery (0 d) and 3 and 7 d after surgery to inhibit TNF and control inflammation. Pulsed intravenous methylprednisolone sodium succinate (Solu-Medrol) (650 mg three times on the day of surgery) was administered to maintain immunosuppression, in addition to Mycophenolate mofetil (MMF, 1 g after surgery and twice daily from 1 d onwards), Tacrolimus (FK506, 5 mg twice daily to 0.6 mg daily), and a daily tapering of Solu-Medrol (750–190 mg daily). Recipient plasma and whole blood were assessed daily to monitor the plasma concentrations of MMF and FK506, respectively. Meanwhile, the recipient received a heparin sodium infusion and was adjusted to the range of systemic anticoagulation therapy, in addition to other anti-pathogen drugs and hepatoprotective and nutritional support therapy. The specific dosing records are found in Extended Data Fig. 6.

Infectious disease prevention and surveillance

Porcine endogenous retrovirus (PERV) is the major pathogen transferred from xenografts involving pigs. The donor pig in this study was bred and raised in facilities free of designated pathogen (DPF) and regularly monitored for porcine cytomegalovirus (PCMV), porcine circovirus (PCV) and other viruses to minimize the risk of transmission. In this study, virus expression was monitored by qRT-PCR and gel electrophoresis. Briefly, RNA was isolated from the PBMCs of the recipient and the donor pig. Reverse transcription and qRT-PCR were simultaneously

performed to collect FAM fluorescence signals. After 40 cycles of reverse transcription (50℃/10 min), predenaturation (95℃/10 min) and the PCR reaction (95℃/15 sec + 60℃/1 min), virus expression was analyzed according to the *Ct* value. Additionally, the products were gone through gel electrophoresis. All the sequences of the primers are provided in Table S4 of the Supplementary Information.

Liver function assessment and histological analyses

To evaluate the pig liver function and hemodynamics, the levels of porcine albumin, liver injury-related indicators (ALT, AST, etc.), coagulation-related indicators (PLT, PT, APTT, etc.), and other biochemical indexes were measured in the recipient's peripheral blood beginning at 1 day before surgery (-1 d) (Table S2 in the Supplementary Information). The blood flow velocity of the hepatic vein, the PV, and the hepatic artery were also monitored by ultrasound after surgery. Liver biopsies from the pig and recipient were H&E stained, and hemorrhage, necrosis, and cell infiltration in the sections were semi-quantitatively analyzed at the time of surgery (0 d), 2 h post-surgery (2 h) and at the trial endpoint (10 d). The differentiation and function of hepatocytes, hepatic stellate cells (HSCs), and liver sinusoidal endothelial cells (LSECs) were assessed in porcine liver-tissue samples obtained during surgery (0 h) and at the trial endpoint (10 d) using IHC testing (Ki67 and αSMA), IF staining (CD31), and scanning electron microscopy (SEM) (Supplementary Methods).

Histology

Liver samples were preserved in 4% paraformaldehyde (PFA), embedded in paraffin, sectioned, and routinely stained with H&E. Samples preserved in PFA were embedded with an optimum cutting temperature compound and cut into 8-μm sections. For IHC, sections were incubated at 4°C overnight with primary antibodies, washed, and incubated for 2 hours at room temperature with horseradish peroxidase-conjugated secondary antibodies. The sections were then developed using a 3,3'-diaminobenzidine substrate kit (Zhongshan Jinqiao Biotech, Beijing, China). Immunofluorescence (IF) was conducted using fluorescent antibodies and counter-stained with DAPI (Thermofisher). Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were conducted as previously described. [22] All the antibodies were listed in Table S3 of the Supplementary Information.

Flow cytometry IgG and IgM binding assay

Porcine peripheral blood mononuclear cells (PBMCs) were isolated using porcine lymphocyte separation medium (Dakewe Biotech, Shenzhen, China, Cat. No.7411011), and PBMCs were diluted with heat-inactivated recipient serum (final concentration of 25%. In brief, 1×10^5 cells were resuspended in 100 μl FACS buffer, mixed with 100 μl recipient serum diluted 1:1) and incubated at 4°C for 1 h. The cells were washed twice with FACS buffer (calcium-free magnesium PBS containing 1% BSA). After incubating PBMCs at 4°C for 20 min using 10% heat-inactivated goat serum as the blocking solution, PBMCs and Goat anti-Human IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488, Invitrogen, Cat. No.

A11013) or Goat anti-Human IgM (Heavy chain) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 647, Invitrogen, Cat. No. A21249) antibody was incubated at 4℃ in the dark for 30 min. After washing twice, the cells were resuspended in FACS buffer and assessed using a MA900 flow cytometer (Sony, MA900). The results were analyzed using FlowJo 10.8.1 software. Results are expressed as the specific median fluorescence intensity (MFI).

Inflammation-related multiple cytokine assays

Assays were performed according to the instructions of LEGENDplex™ Human Inflammation Panel 1 (13-plex) with Filter Plate (BioLegend, Cat.No.740809) and the recipient plasma (Assay Buffer) was diluted twice before testing.

SUPPLEMENTAL REFERENCES

22. Wang L, Wang CM, Hou LH, Dou GR, Wang YC, Hu XB, et al. Disruption of the transcription factor recombination signal-binding protein-Jkappa (RBP-J) leads to veno-occlusive disease and interfered liver regeneration in mice. Hepatology 2009, 49(1): 268-277.

SUPPLEMENTARY VIDEO

Supplementary video. Gene-modified pig to brain-dead recipient heterotopic auxiliary liver transplantation

The video illustrates the main process of the donor organ source, the position of the graft, the surgical operation, and the postoperative management in this porcine-brain death receptor heterotopic auxiliary liver transplantation through cartoon.