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Viable pigs after simultaneous inactivation of porcine MHC class I and three xenoreactive antigen genes GGTA1, CMAH and B4GALNT2

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Abstract

Background: Cell surface carbohydrate antigens play a major role in the rejection of porcine xenografts. The most important for human recipients are α-1,3 Gal (Galactose-alpha-1,3-galactose) causing hyperacute rejection, also Neu5Gc (N-glycolylneuraminic acid) and Sd(a) blood group antigens both of which are likely to elicit acute vascular rejection given the known human immune status. Porcine cells with knockouts of the three genes responsible, *GGTA1*, *CMAH* and *B4GALNT2*, revealed minimal xenoreactive antibody binding after incubation with human serum. However, human leucocyte antigen (HLA) antibodies cross-reacted with swine leucocyte antigen class I (SLA-I). We previously demonstrated efficient generation of pigs with multiple xeno-transgenes placed at a single genomic locus. Here we wished to assess whether key xenoreactive antigen genes can be simultaneously inactivated and if combination with the multi-transgenic background further reduces antibody deposition and complement activation.

Methods: Multiplex CRISPR/Cas9 gene editing and somatic cell nuclear transfer were used to generate pigs carrying functional knockouts of *GGTA1*, *CMAH*, *B4GALNT2* and SLA class I. Fibroblasts derived from one- to four-fold knockout animals, and from multi-transgenic cells (human CD46, CD55, CD59, HO1 and A20) with the four-fold knockout were used to examine the effects on human IgG and IgM binding or complement activation in vitro.

Results: Pigs were generated carrying four-fold knockouts of important xenoreactive genes. In vitro assays revealed that combination of all four gene knockouts reduced human IgG and IgM binding to porcine kidney cells more effectively than single or double knockouts. The multi-transgenic background combined with *GGTA1* knockout

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alone reduced C3b/c and C4b/c complement activation to such an extent that further knockouts had no significant additional effect.

Conclusion: We showed that pigs carrying several xenoprotective transgenes and knockouts of xenoreactive antigens can be readily generated and these modifications will have significant effects on xenograft survival.

KEYWORDS

B2M, *B4GALNT2*, *CMAH*, complement regulators, *GGTA1*, MHC-I, multiplex CRISPR/Cas9 gene editing, SLA-I, xenotransplantation

1 | INTRODUCTION

The global shortage of donated human organs and tissues has motivated efforts to genetically engineer pigs as a source of xenografts for human patients. A major advance in overcoming the immunological hurdles involved was identification of galactose-α1,3-galactose (α-1,3Gal) as responsible for eliciting hyperacute rejection of pig tissue by old world primate recipients. Binding of preformed antibodies activates the host complement system leading to complete destruction of the xenograft within minutes.[1] Removing the main enzyme responsible for α-1,3Gal synthesis by inactivating the porcine *GGTA1* gene significantly reduces hyperacute rejection, and this can be further improved by equipping *GGTA1*-knockout pigs with human complement regulator transgenes.[2-6] The focus has now shifted onto delayed immune responses, notably those that target the endothelial lining of the graft vasculature. Acute vascular rejection occurs within a few days or weeks and is characterized by proinflammatory and procoagulatory activation of the vascular endothelium, complement activation, thrombotic microangiopathy and infiltration of innate immune cells into the graft.[7,8] This response is also initiated by preformed antibodies,[9] with evidence indicating a role for non-Gal porcine antigens including N-glycolylneuraminic acid (Neu5Gc), synthesized by cytidine monophospho-N-acetylneuraminic acid hydroxylase (*CMAH*),[10-12] and surface glycans including the Sd(a) blood group antigen produced by β-1,4-N-acetyl-galactosaminyl transferase 2 (*B4GALNT2*).[13-15]

Triple knockout of porcine *GGTA1*, *CMAH* and *B4GALNT2* has been shown to significantly reduce human IgG and IgM antibody binding to porcine peripheral blood monocytes and red blood cells.[15-17] These studies also revealed that human leucocyte antigen (HLA) antibodies cross-react with the porcine major histocompatibility complex class I, also known as swine leucocyte class I (SLA-I) antigens, highlighting the SLA-I complex as a potential target for further genetic modification.[17]

SLA class I molecules consist of a heavy α-chain and a light β-chain (β2-microglobulin; B2M). The α-chain, a transmembrane glycoprotein with three domains (α1, α2 and α3), is encoded by three different genes *SLA-1*, *-2* and *-3*.[18] There are also a number of pseudogenes. The α1 and α2 domains interact to form the peptide-binding domains and are therefore highly polymorphic. The α3 domain contains the cytoplasmic tail and has a constant region (encoded by exon 4)

that is conserved between *SLA-1*, *-2* and *-3*. β2-microglobulin does not contain a transmembrane region, is non-covalently linked with the α-chain and encoded by a highly conserved gene (*B2M*).[19] SLA-I has previously been ablated by targeting either porcine *B2M*,[20] or the conserved region of the α-chain within exon 4.[21]

To minimize antibody-mediated xenograft rejection mechanisms, we produced pigs carrying four-fold knockout of *GGTA1*, *CMAH*, *B4GALNT2* and either the SLA-I heavy α-chain or light β-chain, and assessed how these affected binding of human IgG and IgM. The four-fold knockouts were also combined with a five-fold (human CD46, CD55, CD59, HO1 and A20) transgenic genotype, generated previously and shown to reduce hyperacute and acute vascular rejection,[5] and the effects on C3b/c and C4b/c complement activation tested.

2 | MATERIALS AND METHODS

2.1 | Animal welfare

Animal experiments were approved by the Government of Upper Bavaria (permit number 55.2-1-54-2532-6-13) and performed according to the German Animal Welfare Act and European Union Normative for Care and Use of Experimental Animals.

2.2 | Generation of guide RNA constructs

Specific gRNA oligonucleotides for *GGTA1* exon 7 (5′-GTC GTGACCATAACCAGA-3′), *CMAH* exon 10 (5′-AGAAACTCCTGAA CTACA-3′), *B4GALNT2* exon 3 (5′-AGGAAAGCTATAACTTGG-3′) and *B2M* exon 1 (5′-TAGCGATGGCTCCCCTCG-3′) or SLA-I α-chain exon 4 (guide 1 5′-CCAGGACCAGAGCCAGGACA-3′ and guide 2 5′-CCAGAAGTGGGCGGCCCTGG-3′),[21] 18 to 20 bp in length with additional BbsI overhangs were synthesized by MWG Eurofins, Germany. gRNA oligonucleotides were sub-cloned into plasmid pSL1180 that carries a 0.2 kb U6 promoter followed by a BbsI restriction site and gRNA scaffold sequences. Restriction fragments were further sub-cloned into the pX330 plasmid backbone (pX330-U6-Chimeric_BB-CBh-hSpCas9 was a gift from Feng Zhang; Addgene plasmid # 42230; http://n2t.net/addgene:42230; RRID:Addgene_42230). The two final constructs contained three gRNAs specific for *GGTA1*, *CMAH* and *B4GALNT2*; either an

additional gRNA specific for *B2M*, or two additional gRNAs for the SLA-I α-chain; a 0.8 kb chicken β hybrid promoter, followed by a SV40 nuclear localization signal (NLS), a 4.0 kb hSpCas9 gene followed by a second NLS signal, linked via a T2A self-cleaving peptide to a 0.6 kb puromycin resistance cassette with a 0.2 kb bovine growth hormone polyadenylation (BGHpA) signal. A summary of the knockout strategy is provided in Figure S1. An alignment of SLA-1, SLA-2 and SLA-3 of exon 4 with the sgRNA target site is provided in Figure S2. Details of screening primers for the genomic target sites are provided in Table S1.

2.3 | Cell culture

Porcine kidney fibroblasts (PKF) were isolated and cultured as described previously.[5] Transfection of gRNA/Cas9 plasmids was performed using Lipofectamine 2000 (Life Technologies). Twenty-four hours after transfection, transiently transfected cells were selected using 1.5 μg/mL puromycin (InvivoGen) for 2 days.

2.4 | Magnetic bead selection and sequencing

α-1,3Gal-deficient cells were enriched by counter-selection using streptavidin-coated magnetic beads (Dynabeads, Life Technologies) and biotin-conjugated isolectin B4 (Enzo Life Science) in a magnetic field. Gene editing was determined by sequencing across the gRNA target site (MWG Eurofins) and subsequent TIDE (tracking of indels by decomposition) analysis.[22]

2.5 | Somatic cell nuclear transfer

Nuclear transfer was performed as described previously.[5] In short, donor cells were arrested at GO/G1 phase by serum deprivation. Oocytes isolated from prepubertal gilts were matured in vitro, enucleated, single donor cells were inserted in the perivitelline space, and then cell fusion and oocyte activation induced by electric pulse. Reconstructed embryos were transferred into the oviducts of hormonally synchronized recipient gilts by mid-ventral laparotomy.

2.6 | Isolation and culture of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) from pig and human were isolated from blood samples by Ficoll density gradient centrifugation (Biocoll Separating Solution, Biochrom GmbH). Human blood samples were isolated from anonymized Leukotrap filters from the Department of Transfusion Medicine, Hannover Medical School. This procedure was approved by the local ethics committee of Hannover Medical School. Human CD8+ T cells were negatively enriched from PBMC by depletion of CD4+ T cells, B cells, NK cells and monocytes using magnetic separation with goat anti-mouse IgG beads (MACS, Miltenyi Biotec GmbH). The following monoclonal antibodies were used to retain unwanted cells: anti-human HLA-DR (L243, ATCC), CD14 (3C10, ATCC), CD56 (T199, provided by T.

Pietsch, University of Bonn) and anti-human CD4 (OKT4, ATCC). Cell cultures and proliferation assays were performed with RPMI-1640 medium (Lonza) supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mmol/L sodium pyruvate and 0.05 mmol/L β-mercaptoethanol. Cells were cultivated at 5% CO_2 and 37°C for human PBMC or 39°C for porcine PBMC.

2.7 | Flow cytometry

For phenotypic analyses of porcine fibroblasts, cells were stained with anti-human beta-2-microglobulin-PE (B2M-02, Santa Cruz Biotechnology), an isotype-matched control antibody (BD Biosciences), or anti-porcine MHC-I (74-11-10, provided by A. Saalmüller). Unlabelled primary antibody was detected using FITC-labelled goat anti-mouse Ig as a secondary antibody. Porcine PBMC from knockout and wt pigs were stained using anti-porcine MHC-I (PT85A, Kingfisher Biotech) that detected the constant α3-region present in SLA-1, -2 and -3), or an isotype-matched control antibody (Dako). α-1,3Gal expression in PBMC and fibroblasts was detected by staining with FITC-conjugated isolectin B4 (IB4 lectin, isolated from *Bandeira simplicifolia*, Enzo life sciences). FITC-labelled *Dolichos biflorus* lectin (DBA lectin, Vector laboratories) was used to detect β4GalNT2 activity. Neu5GC epitopes were stained using a polyclonal chicken anti-Neu5GC antibody (Poly21469, Biolegend) plus secondary staining with FITC-labelled donkey anti-chicken IgY (Jackson ImmunoResearch). Data were acquired on a FACSCalibur flow cytometer (Becton Dickinson) and analysed with summit 5.1 software (Beckmann Coulter). Dead cells were excluded from the analyses.

2.8 | Western blot analysis

Protein isolation and Western analysis were carried out as described previously.[5] Neu5Gc epitopes were detected using a purified chicken anti-Neu5Gc antibody (clone Poly21469 chicken IgY; diluted 1:10 000 in Neu5Gc free blocking solution) and horseradish peroxidase-labelled goat anti-chicken sc-2428 (diluted 1:5000 in Neu5Gc free blocking solution). GAPDH was detected using mouse monoclonal anti-GAPDH #G8795, (diluted 1:3000) and rabbit anti-mouse IgG H&L (HRP) ab6728 (diluted 1:5000).

2.9 | Immunohistochemistry

Immunohistochemistry was performed as described previously.[23] Tissues were snap-frozen in liquid nitrogen. Five-micrometre cryostat sections were air-dried, acetone fixed and incubated with mouse anti-pig MHC class I mAb 74-11-10[24] (provided by A. Saalmüller), or mouse anti-human beta2 microglobulin mAb B2M-02 (Thermo Fisher Scientific), then horseradish peroxidase-coupled goat anti-mouse antibody (Dianova) and binding visualized with 3-amino-9-ethyl-carbazole (AEC, Sigma). Sections were lightly counterstained with haematoxylin (Merck). Cells were stained using biotinylated anti-porcine MHC-I (diluted 1:100; PT85A, Kingfisher

Biotech), biotinylated mouse anti-human beta2 microglobulin mAb B2M-02 (diluted 1:100; Thermo Fisher Scientific) and Atto-488 streptavidin (diluted 1:1000; ATTO-TEC).

2.10 | Cell proliferation

About 1×10^5 human cells (CD8$^+$ T cells/ PBMC) were co-cultured in triplicate with increasing numbers of irradiated (30 Gy) porcine PBMC from wt or four-fold knockout pigs in a total of 200 µL medium in microtiter plates. Tritiated thymidine (^{3}H-TdR, Perkin Elmer) was added after 5 days. After an additional incubation of 16 hours, incorporated ^{3}H-TdR was measured in a Microbeta scintillator counter (Wallac).

2.11 | Construction of microfluidic channels with round cross section

Microfluidic channels were prepared as described previously.[25] In brief, 10 parts of polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning) were mixed with 1 part of curing agent and cast in a Petri dish (Thermo Fisher Scientific). Sterile- and pyrogen-free support needles (Ø 120 µm, Seirin) were laid in parallel in the liquid PDMS at the bottom of a petri dish. Four mould needles (Ø 550 µm, BD Biosciences) were placed on top of the support needles at a 90° angle. The Petri dish was incubated at 60°C overnight to allow the PDMS to cure. PDMS chips were cut out, and the needles were extracted horizontally. Inlets and outlets were made using 2 mm biopsy punches (Shoney Scientific). Liquid PDMS was used to seal the needle holes between the edge of the PDMS gel and the inlet and outlet and cured at 60°C overnight. The final microfluidic chips contained four 1 cm long microchannels.

2.12 | PDMS coating and cell seeding in microfluidic channels

The luminal surface of the microchannels was coated using human fibronectin (Millipore) and bovine collagen I (Gibco, Thermo Fisher Scientific), as described previously.[25] Briefly, cleaned PDMS chips and standard glass slides were activated with an oxygen plasma cleaner (Harrick Plasma) and bonded together. The luminal surface of the microchannels was treated with a 5% aqueous solution of 3-triethoxysilylpropylamine (APTES, Sigma-Aldrich) and incubated for 20 minutes at room temperature. The channels were then washed with ultrapure water and treated with 0.1% glutaraldehyde (Sigma-Aldrich) for 30 minutes to allow subsequent crosslinking of extracellular matrix proteins. A 50 µg/mL solution of human fibronectin in PBS was added, incubated for 1 hour at 37°C, followed by incubation with 100 µg/mL of bovine collagen I in 0.2 mol/L acetic acid. Unbound collagen I was rinsed from the microchannels with cell culture medium containing 10% FBS. Porcine kidney fibroblasts (PKF) grown to confluence in T75 flasks were disaggregated with 0.05% EDTA-trypsin (Gibco, Thermo Fisher Scientific) and suspended in

FBS-supplemented cell culture medium (DMEM) with 4% dextran from Leuconostoc spp. (Mw ~ 70 000, Sigma-Aldrich) to increase viscosity and promote cell adhesion. PKF cells were loaded into the microfluidic channels at a density of 1×10^6/mL and allowed to reach confluence under static conditions overnight in a 37°C cell culture incubator.

2.13 | Human serum preparation

Human blood was drawn from healthy volunteers into polypropylene tubes containing glass beads (S-Monovette, Sarstedt) and allowed to clot for 30 minutes at room temperature. The clot was removed by centrifugation for 10 minutes, 2000 × g, 4°C and the supernatant collected and stored at −80°C. All human blood samples were obtained with informed consent according to Swiss jurisdiction and the ethics guidelines of the Bern University Hospital.

2.14 | Perfusion of PKFs with normal human serum

A peristaltic pump (Minipuls 3 with 8 channels, Gilson) was connected to the PKF-coated microfluidic channels via sterile silicon tubing with stoppers (Gilson) and extension silicon tubes (Gobatec). The tubing circuit was previously autoclaved and flushed extensively with distilled water, then PBS followed by cell culture medium with 4% dextran. Reservoir tubes of 15 mL (Nalge NUNC) were filled with 10 mL of either serum-free DMEM for controls, or 10% normal human serum in serum-free DMEM and connected to each microchannel. PKF-coated microchannels were perfused at 1 rpm corresponding to a flow rate of 0.09 mL/min in an incubator at 37°C/ 5% CO_2 for 2 hours. Perfusion was in a closed circuit to recirculate the perfusate.

2.15 | Immunoglobulin binding and complement deposition assessment

Immunofluorescence staining was performed to assess the binding of immunoglobulin (IgG and IgM) and complement activation markers (C3b/c and C4b/c). PKFs in the microfluidic channels were washed with PBS supplemented with calcium and magnesium, fixed with 4% formaldehyde for 15 minutes and blocked with PBS-3% BSA for 45 minutes. Incubation with primary antibodies and 4′,6-diamidino-2-phenylindole (DAPI) was carried out at room temperature for 1 hour. Antibodies used were rabbit anti-human C3b/c-fluorescein isothiocyanate (FITC, F0201, Dako), rabbit anti-human C4b/c-FITC (F0169, Dako), goat anti-human IgM- FITC (F5384, Sigma) and goat anti-human IgG-FITC (F5512, Sigma). Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole, Boehringer, Roche Diagnostics). Images were captured using a Plan-Apochromat 10×/0.3 M27/a = 2.00 mm objective with a confocal laser-scanning microscope (LSM 710, Zeiss) and analysed by ImageJ (National Institutes of Health). Statistical analysis was performed using the ANOVA test (linear model).
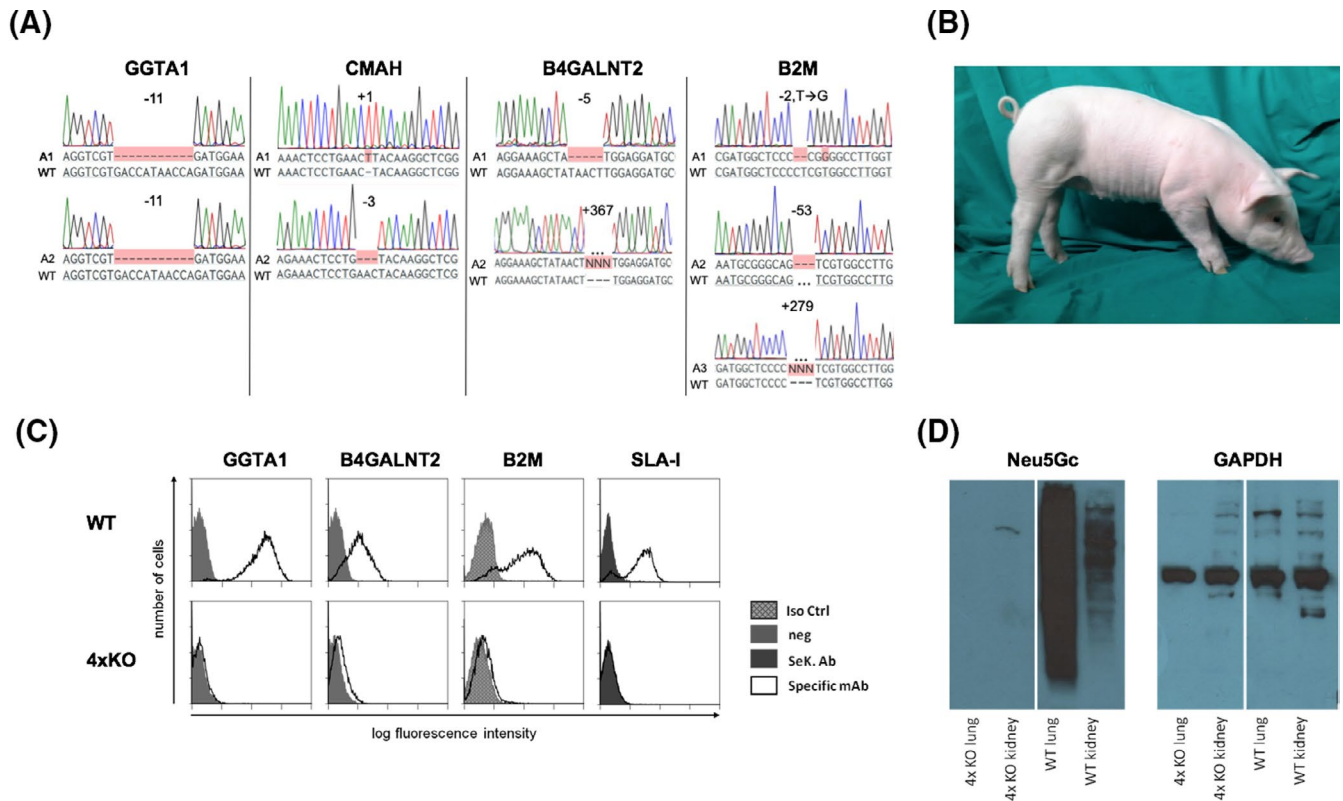
FIGURE 1 A, Genotype analysis of the four-fold knockout piglet 90. PCR and sequencing across the target sites for *GGTA1*, *CMAH*, *B4GALNT2* and *B2M* revealed several indels. A1: allele one, A2: allele two, A3: allele three, WT: wild-type. B, Piglet 90 aged 4 wk. C, Flow cytometry analysis of ECFs revealed the absence of α-1,3Gal (*GGTA1*), Sd(a) (*B4GALNT2*), B2M (antibody: B2M-02) and SLA-I (antibody: 74-11-10). Total cell count was 10 000. Maximum peak at 250-750 cells. X axis from 1 to 10^4. Light grey histograms represent unstained controls, dark grey histograms represent secondary antibody staining only and squared histograms isotype controls. Wild-type (WT) ear clip fibroblasts were used as a positive control. D, Neu5Gc (smear) and GAPDH (35 kDa) were detected by Western blot analysis. Proteins were isolated from lung and kidney of piglet 90, and a wild-type control. GAPDH was used as loading control

3 | RESULTS

We adopted two approaches to removing the key porcine xenoreactive antigens and porcine SLA-I from the cell surface. Both used multiplex genome editing to introduce inactivating mutations into *GGTA1*, *CMAH* and *B4GALNT2*, while surface porcine SLA-I expression was blocked by targeting either the light β-chain (*B2M*) or the constant region of the heavy α-chain.

3.1 | Generation of pigs with gene-edited *GGTA1*, *CMAH*, *B4GALNT2* and *B2M*

Fourteen porcine kidney fibroblast cell clones selected for lack of α-1,3Gal were analysed by DNA sequencing to detect allele-specific mutations of *GGTA1*, *CMAH*, *B4GALNT2* and *B2M*. Twelve clones (86%) revealed at least monoallelic indels in all four genes, while eight (57%) showed bi-allelic mutations in all four genes, most resulting in frameshift or premature stop codons. Selected clones were pooled and used for somatic cell nuclear transfer. A total of 680 reconstructed embryos were transferred to five recipients, and one pregnancy was established resulting in two live-born piglets (89 and 90). Ear clip samples were collected and used for DNA isolation. PCR amplification and subsequent sequencing of the target sites revealed that piglet 89 had compound heterozygous inactivating mutations of *GGTA1*, *B4GALNT2* and *B2M*, and a heterozygous knockout of *CMAH*. This piglet was sacrificed after 4 weeks to isolate cells for further genetic modification. Piglet 90 carried compound heterozygous indel mutations in all four genes. The target site of *GGTA1* exon 7 showed a bi-allelic 11 bp deletion. The target site of *CMAH* exon 10 showed on one allele a single bp insertion leading to a frameshift and an early stop codon. The second allele carried a 3 bp deletion that eliminated a single amino acid. For exon 3 of *B4GALNT2*, two alleles were identified. One carried a 5 bp deletion and the other a 367 bp insertion, both shifting the reading frame. Analysis of *B2M* exon 1 revealed three mutated alleles carrying either a 2 bp deletion combined with a T to G mutation, a 53 bp deletion or 279 bp insertion (Figure 1A). The number of alleles detected for *B2M* accorded with previous reports.[20,26] The complete indel sequences of all mutated alleles are attached in Figure S3.

3.2 | Multiplex gene editing results in functional inactivation of *GGTA1*, *CMAH*, *B4GALNT2* and *B2M*

While most gene editing events resulted in frameshift or premature stop codons, one *CMAH* allele showed the loss of a single
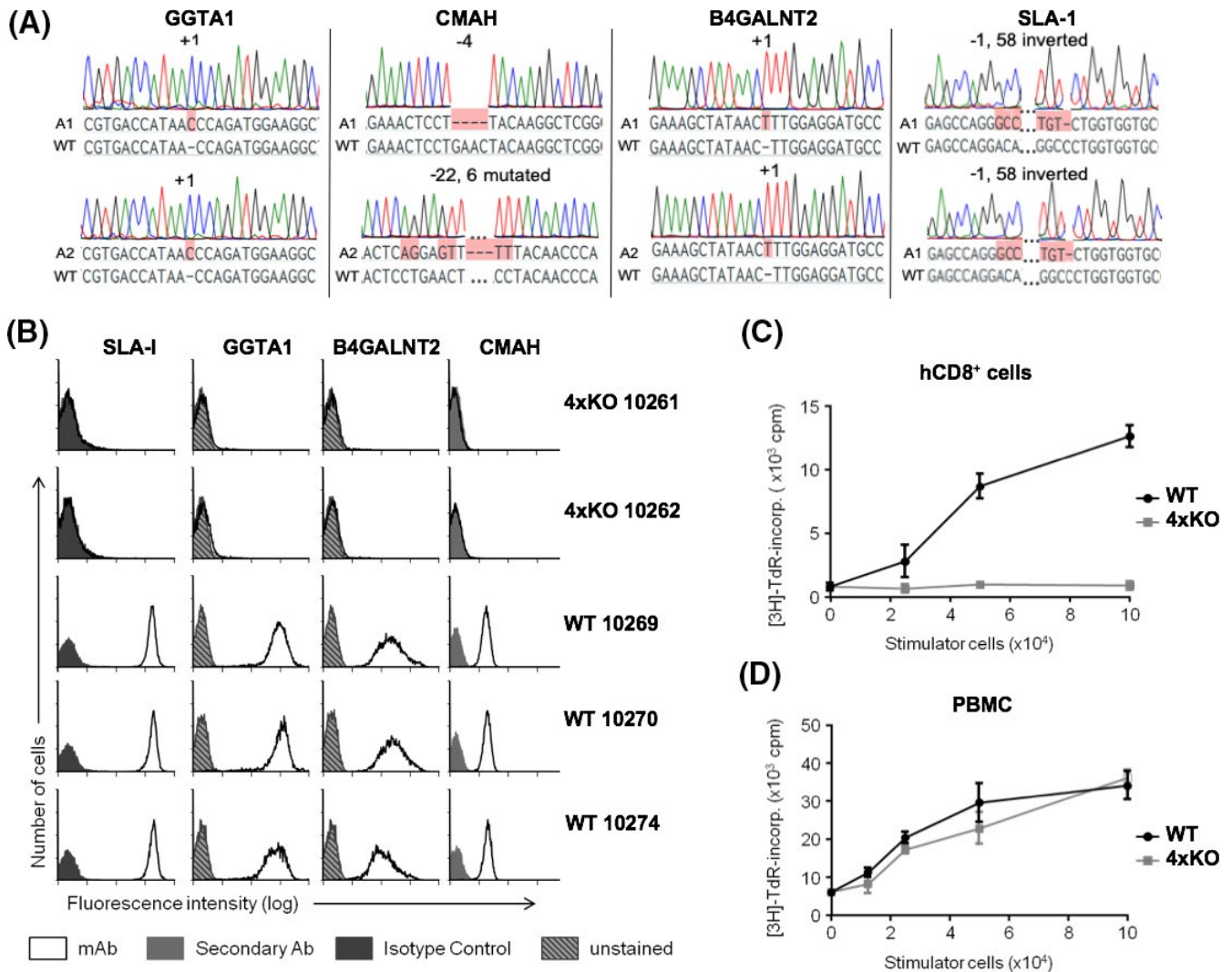
FIGURE 2 A, Genotypic analysis of four-fold knockout (SLA-I heavy) piglets 10261 and 10262. PCR and subsequent sequencing of the target sites for *GGTA1*, *CMAH*, *B4GALNT2* and SLA-I revealed several indels. A1: allele one, A2: allele two, WT: wild-type. Both pigs showed an identical indel pattern. For SLA-I, a PCR fragment could only be amplified for *SLA-1*. B, Flow cytometry analysis of PBMC. Total cell count was 10 000. Maximum peak at 250-750 cells. X axis from 1 to 10^4. Wild-type PBMC from animals 10269, 10270 and 10274 served as controls. Flow cytometry measurements revealed the absence of α-1,3Gal (*GGTA1*), Neu5Gc (*CMAH*), Sd(a) (*B4GALNT2*) and SLA-I (antibody: PT85A). Light grey histograms represent unstained controls, dark grey histograms represent secondary antibody staining only and squared histograms represent isotype controls. 1×10^5 purified human CD8$^+$ T cells (C) or human PBMC (D) were stimulated with increasing numbers of irradiated (30 Gy) porcine PBMC from four-fold knockout pig 10261 or a wild-type pig. Proliferation was measured after 5 d + 16 h by ^{3}H-thymidine incorporation. Data represent mean cpm ± SEM of triplicate cultures obtained with cells from one human blood donor in a single experiment. Similar response patterns were observed using responder cells from a second blood donor and stimulator cells from four-fold knockout pig 10262. Proliferation of human CD8$^+$ T cells decreased after stimulation with four-fold knockout porcine PBMC

amino acid, which might not perturb its function. Therefore, ear clip fibroblasts (ECF) of piglet 90 were isolated, cultured and used to confirm functional inactivation of all four genes. Loss of cell surface α-1,3Gal (detected by IB4), Sd(a) (detected by DBA), B2M and SLA-I antigens was confirmed by flow cytometry (Figure 1C). Because Neu5Gc can be transmitted by serum components to Neu5Gc-deficient cells during cultivation, ear fibroblasts from piglet 90 were also cultured in xenoserum-free conditions (serum replacement or chicken serum); however, the cells did not proliferate and could not be used for Neu5Gc flow cytometry analysis. Piglet 90 suffered from an infection at age 6 weeks and had to be

sacrificed. Necropsy examination revealed no abnormalities other than an enlarged spleen due to the infection. Tissue samples were collected and Western blot analysis performed to verify the absence of Neu5Gc epitopes (Figure 1D).

3.3 | Generation of pigs with gene edited *GGTA1*, *CMAH*, *B4GALNT2* and constant region of the SLA-I heavy α-chain

For the inactivation of *GGTA1*, *CMAH*, *B4GALNT2* and SLA-I heavy α-chain, a puromycin-selected pool was used to enrich α-1,3

Gal-deficient cells via magnetic bead selection. Sequencing across the gRNA target sites revealed indel efficiencies of 99% for *GGTA1* and up to 81% for the other targeted genes. The cell pool was used for somatic cell nuclear transfer. A total of 436 reconstructed embryos were transferred into three recipients, and one pregnancy was established and two live-born piglets obtained (10261 and 10262). Tail samples were collected and DNA isolated. PCR amplification and subsequent sequencing of the target sites revealed bi-allelic indel mutations in all four genes (Figure 2A). Both pigs showed the same indel pattern and thus probably originated from the same cell clone. The target site of *GGTA1* exon 7 showed a bi-allelic 1 bp insertion. The target site of *CMAH* exon 10 revealed a 4 bp deletion in one allele and a 22 bp deletion, 6 bp mutation in the other. *B4GALNT2* showed a 1 bp insertion in both alleles. Analysis of SLA-I was only possible for *SLA-1* because several PCR primer combinations failed to amplify a PCR fragment from *SLA-2* and *SLA-3* (Figure S4), even though the original wild-type cells did amplify fragments. Thus, the most likely reason is a deletion that either removed *SLA-2* and *SLA-3* sequences or at least the primer-binding site(s). Analysis of *SLA-1* exon 4 revealed a 1 bp deletion and 58 bp inversion. The complete indel sequence of all mutated alleles can be found in Figure S3.

Loss of α-1,3Gal (*GGTA1*), Neu5Gc (*CMAH*), Sd(a) (*B4GALNT2*) and SLA-I epitopes (detection of SLA-1, SLA-2 and SLA-3 by the antibody used) was confirmed by flow cytometry analysis of PBMC from piglets 10261 and 10262 (Figure 2B).

3.4 | CD8^{+} T-cell response

As human T cells can be activated by porcine cells, a direct interaction between porcine MHC molecules and the human T-cell receptor was assumed.[27] Absence of SLA-I from porcine cells should therefore lead to decreased activation, especially of human CD8^{+} T cells. We tested the potential of PBMC from the four-fold knockout pigs 10261 and 10262 to induce proliferation of either isolated purified hCD8^{+} T cells, or total PBMC from human blood donors. Using purified hCD8^{+} T cells as responders, almost no proliferation was observed after coculture with four-fold knockout porcine cells,

compared to a strong proliferative response after stimulation with wild-type porcine cells (Figure 2C). In contrast, only a non-significant difference was observed in the proliferative response of the whole human PBMC population stimulated with wild-type or four-fold knockout cells. The strong proliferating response of hCD4^{+} T cells seemed to obscure any effect on hCD8^{+} T cells in this culture assay (Figure 2D).

3.5 | Immunohistology

Cell and tissue samples were also used to carry out immunohistology for B2M and SLA-I antigens, see Figure 3 and Figure S5. Figure 3 shows kidney fibroblasts of piglet 90 and ear clip fibroblasts of pigs 10261 and 10262. This verified functional knockout of *B2M* in animal 90 and the lack of SLA-I molecules on the cell surface. However, analysis of heart tissue showed a positive signal for what were probably intracellular SLA-I molecules. As the *B2M* knockout affects only the light β-chain and not the heavy α-chain of SLA-I, it seems probable that the α-chain accumulates in the cell cytoplasm, as shown in mice,[28] which would explain the diffuse background signal from the antibody (Figure S5). This accords with the flow cytometry result of animal 90, which detected no SLA-I molecules on the cell surface. Staining of SLA-I molecules on cells of pigs 10261 and 10262 showed no SLA-I molecules on the cell surface, which also accords with the flow cytometry results.

3.6 | Analysis of off-target events

We screened for five of the most probable off-target sites for each of the guide RNAs, as predicted by the program CRISPOR (crispor.tefor. net). Details of screening primers and off-target sites are provided in Table S2. No off-target events were detected for the guide RNAs targeting *GGTA1*, *CMAH*, *B4GALNT2* and SLA-I (guide RNA 1). For SLA-I guide RNA 2, one off-target event was detected—a 137 bp insertion in an intergenic region between the loci *PRKCDBP* (CAVIN3) and *FAM160A2* on chromosome 9. No genes or miRNAs are annotated in this region of the porcine genome.
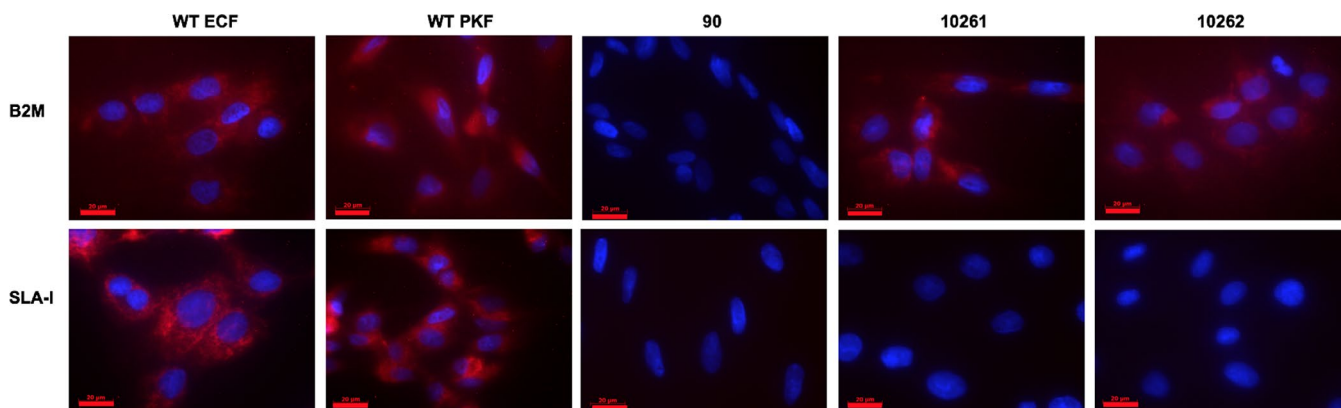

FIGURE 3 Immunofluorescence detection of B2M and SLA-I on porcine kidney fibroblasts. B2M was detected using Biolegend clone 2M2-biotinylated antibody (1:100), and SLA-I using PT85A-biotinylated antibody (1:100). Both were stained with Atto488-streptavidin (1:1000). Shown: porcine kidney fibroblasts (PKF) from animal 90. Ear clip fibroblasts (ECF) of animals 10261 and 10262. Scale bar: 20 μm

3.7 | Effect of removing different xenoreactive epitopes combined with expression of complement regulator genes on antigen deposition

To evaluate the effects of different knockout combinations on human IgG and IgM binding, we perfused porcine kidney fibroblasts previously isolated from our breeding animals that carried *CMAH* knockout, *CMAH/GGTA1* double knockout, *CMAH/GGTA1/B4GALNT2/B2M* four-fold knockout (piglet 90) and wild-type controls, each between passage 5 to 10 and cultured in artificial round section microvessels perfused with normal human serum. *CMAH* and *CMAH/GGTA1* knockout cells showed significantly reduced human IgG and IgM binding compared to wild type, but four-fold knockout cells showed the strongest total reduction (Figure 4A and B). As antibody binding leads to complement activation, we also used the microfluidic system to evaluate the effect of combining four-fold knockout with strong expression of complement regulatory transgenes. For this, multiplex genome editing was carried out in kidney fibroblasts, passage 5 to 10, from multi-transgenic pigs (human CD46, CD55, CD59, HO1 and A20).[5] The knockout profile of these cells was analysed after selection and enrichment by TIDE analysis and indicated almost 100% editing efficiency for each of these four genes. The five-fold transgenic cells showed significant reduction in C3b/c and C4b/c activation even in the absence of *GGTA1* knockout. Inclusion of *GGTA1* knockout to this multi-transgenic background reduced complement activation to a minimum. This reduction was so marked that further effects of the four-fold knockout could not be discerned (Figure 4C and D). However, this does not indicate that additional knockouts are superfluous for complement activation, as other assays or in vivo experiments could very likely reveal additional beneficial effects. The amount of IgG and IgM binding was also assessed in five-fold transgenic cells with various numbers of gene knockouts and revealed reduced antibody binding with increased knockouts (Figure S6).

4 | DISCUSSION

Removal of xenoreactive porcine carbohydrate antigens combined with expression of xenoprotective transgenes holds the promise of protecting xenografts against immune rejection in the short and medium term. The most effective combination of knockouts and transgenes does however remain to be identified and will likely be informed by clinical findings. Previously, we showed that multiple transgenes could be combined and placed at a single genetic locus.[5,23] Here we show that multiple xenoreactive antigens can be inactivated simultaneously and, if required, multi-modified cells can be used to generate viable pigs.

We chose to combine a three-fold knockout of genes responsible for major xenoreactive sugar antigens plus knockout of the light β-chain (*B2M*) or heavy α-chain of SLA-I to block surface SLA-I expression. Additional combination with a panel of five xenoprotective transgenes in cells of an established xenodonor pig line[5] revealed the benefit of combining knockouts and transgenes. We are currently producing 5xtg 4xKO pigs by breeding the 4xKO boars 10261 and 10262 with sows of our established 5xtg *GGTA1*, *CMAH* KO line and living offspring can also be produced by nuclear transfer. The boars 10261 and 10262 are currently 7.5 months old and housed in a SFP facility. Housing should however also be possible in a normal breeding facility. The health problems we observed in pig 90 seem to have been a result of minor stress caused by relocation to another compartment in our facility. In future, such transfers can be minimized or antibiotic treatment provided during the critical timeframe.

While, as previously demonstrated, the three-fold knockout provided beneficial reduction of human immunoglobulin binding,[14,17] but this was further improved by removing surface SLA-I antigens responsible for HLA cross-reactivity.[17] We chose to inactivate the light β-chain (B2M) or heavy α-chain of SLA-I. Although negative effects of *B2M* knockout concerning iron homeostasis have been reported in mice,[29] we obtained viable pigs by both methods. Which form of SLA-I inactivation is the best is open to question. It has been shown in mice that B2M-independent assembly of the heavy α-chain with peptides,[30] stably expressed at the cell surface[31] and interaction with CD8+ T cells, is possible.[30] However, we did not detect cell surface SLA-I heavy α-chain in *B2M* knockout pigs by flow cytometry, rather it seems that the α-chain accumulates in the cytoplasm. On the other hand, inactivation of the α-chain without modification of *B2M* could result in endogenous porcine B2M interacting with human HLAI heavy α-chain after xenotransplantation, and the formation of new xenoreactive hybrid complexes.[32] Future development might thus require deletion of both the light and heavy SLA-I chains to generate pigs lacking an SLA-I immune system to form the basis for completely MHC-I humanized pigs and a new generation of xenodonor animals.

We previously showed that multiple transgenes can be combined and placed at a single genetic locus; here we show that multiple xenoreactive antigens can be inactivated simultaneously and viable pigs generated. This allows fast track generation of xenodonor pigs once the most effective combination of gene addition and inactivation has been determined. Such an approach also lends itself to the generation of different pig lines each with a set of genetic modifications tailored to the transplantation of particular organs or tissues.

FIGURE 4 Incubation of wild-type and various knockout porcine cells with human serum using microfluidic channels. A, Binding of human IgG antibodies. Mean ± SD. Numbers of replicates of each group as shown on X axis: N = 5, 5, 5, 5, 7, 5, 5. B, Binding of human IgM antibodies. Mean ± SD. N = 5, 5, 10, 10, 10, 10, 5, 5. C, C3b/c complement activation. Mean ± SD. N = 5, 5, 5, 3, 5, 3, 5, 5. D, C4b/c complement activation. Mean ± SD. N = 5, 5, 5, 4, 5, 3, 5, 5. Micrographic images are on the right side. NHS: normal human serum; 5xtg: cells isolated from pigs expressing CD46, CD55, CD59, HO1 and A20; 4xKO: cells isolated from pigs with inactivation of *GGTA1*, *CMAH*, *B4GALNT2* and *B2M*. The 4xKO was performed on 5xtg cells
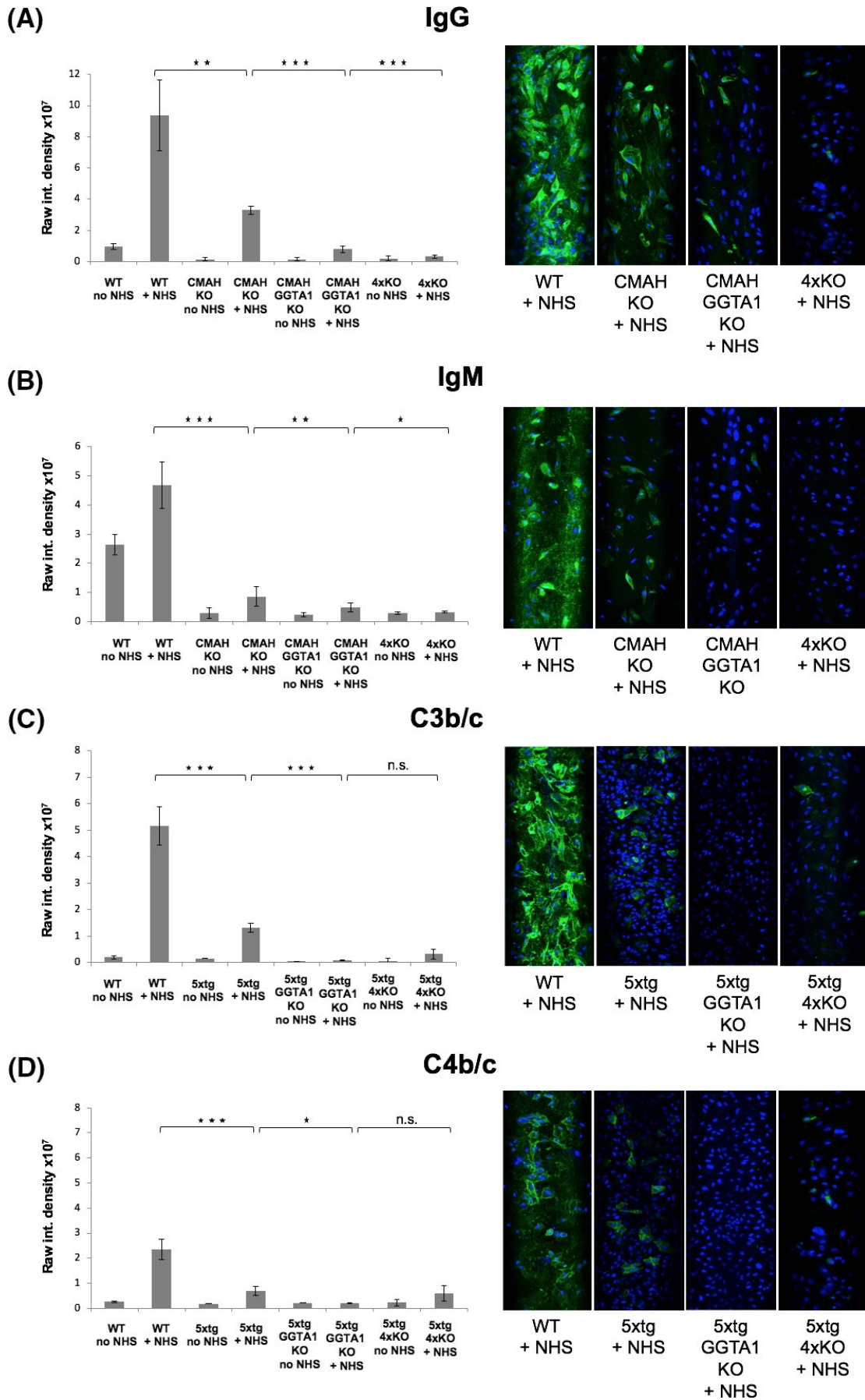
(A) IgG

(B) IgM

(C) C3b/c

(D) C4b/c

CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

AS, KFis, BR and AK designed the experiments. KFis, BR, RH, RS, JZ, ASchae, BK, LW, RR and RS generated and analysed genetically modified animals and cells. MK, KFli, VZ, BK and EW carried out nuclear transfer and embryo transfer. AK, AS, BR and KFis cowrote the manuscript. All authors discussed the results and commented on the manuscript.

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SUPPORTING INFORMATION

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