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ORIGINAL ARTICLE

Glycocalyx dynamics and the inflammatory response of genetically modified porcine endothelial cells

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Abstract

Xenotransplantation is a promising approach to reduce organ shortage, while genetic modification of donor pigs has significantly decreased the immunogenic burden of xenotransplants, organ rejection is still a hurdle. Genetically modified pig organs are used in xenotransplantation research, and the first clinical pig-to-human heart transplantation was performed in 2022. However, the impact of genetic modification has not been investigated on a cellular level yet. Endothelial cells (EC) and their sugar-rich surface known as the glycocalyx are the first barrier encountering the recipient's immune system, making them a target for rejection. We have previously shown that wild type venous but not arterial EC were protected against heparan sulfate (HS) shedding after activation with human serum or human tumor necrosis factor alpha (TNFα). Using a 2D microfluidic system we investigated the glycocalyx dynamics of genetically modified porcine arterial and venous EC (Galα1,3 Gal knock-out, transgenic for human CD46 and thrombomodulin, GTKO/hCD46/hTM) after activation with human serum or human TNFα. Interestingly, we observed that GTKO/hCD46/hTM arterial cells, additionally to venous cells, do not shed HS. Unscathed HS on GTKO/hCD46/hTM EC correlated with reduced complement deposition, suggesting that protection against complement activation contributes to maintaining an intact glycocalyx layer on arterial EC. This protection was lost on GTKO/hCD46/hTM cells after simultaneous perfusion with human serum and human TNFα. HS shedding on arterial cells and increased complement deposition on both arterial and venous cells was observed. These findings suggest that GTKO/hCD46/hTM EC revert to a proinflammatory phenotype in an inflammatory xenotransplantation setting, potentially favoring transplant rejection.

Nicoletta Sorvillo and Robert Rieben are co-senior authors.

KEYWORDS
endothelial cells (EC), genetic modification, glycocalyx, in vitro, microfluidic system, xenogeneic activation, xenotransplantation

1 | INTRODUCTION

A crucial advance in xenotransplantation research was the development of genetically modified donor pigs lacking surface sugar xenoantigens as well as expressing different human genes such as thrombomodulin[1] or CD39 which regulate the plasma cascade systems.[2] These pigs were created to reduce organ rejection and improve transplant acceptance, and their organs have indeed proven to be more successful than non-modified pig organs.[3,4] Nonetheless, rejection of xenografts eventually still occurs.[5] An important player in xenograft rejection is the vasculature, and more precisely endothelial cells (EC). Both cellular and humoral immune responses can be directed against graft EC and trigger different types of rejection.[6] Hyperacute rejection starts minutes after transplantation and is characterized by complement activation mediated by deposition of preformed xenoreactive antibodies. These antibodies recognize carbohydrate antigens expressed on the EC surface of all mammals except higher primates and humans.[7] Different xenoantigens such as the sugars Galα1,3 Gal (αGal), N-Glycolylneuraminic acid (Neu5Gc) and SDa blood group antigen have been identified, and knock-out pigs have been created to overcome hyperacute rejection.[8,9] Furthermore, additional expression of human complement regulatory proteins such as CD46, CD55 or CD59 on porcine EC reduces complement activation and prolongs xenograft survival.[10] However, although hyperacute rejection can be managed, xenotransplantation is still characterized by coagulopathies that eventually lead to organ failure and graft loss.[11,12]

Endothelial surface glycans and proteins that make up the glycocalyx[13] are also known to influence organ rejection. It was shown that damage to the endothelial glycocalyx during liver, lung and kidney transplantation correlated with reduced organ survival and early graft rejection.[14–16] Furthermore, in xenotransplantation, complement activation is linked to glycocalyx shedding which correlates with xenograft rejection.[17,18] While removing carbohydrate xenoantigens from EC is crucial to improve xenograft survival, this also manipulates the endothelial glycan synthesis. In fact, knocking out alpha galactosyltransferase or N-acetylneuraminic acid hydroxylase has been shown to induce expression of novel carbohydrate epitopes[19–21] which could elicit additional immune reactions.

Therefore, carbohydrate remodeling due to genetic manipulation of porcine EC could also lead to changes in the glycocalyx composition and -function, and thereby impact xenograft survival. Here we analyzed the impact of genetic modifications such as alpha galactosyltransferase knockout and transgenic expression of human CD46 and thrombomodulin (GTKO/hCD46/hTM) on endothelial glycocalyx composition and -function in a xenotransplantation setting using a 2D microfluidic system.

2 | MATERIAL AND METHODS

2.1 | Endothelial cell isolation

Arterial (Aorta thoracica) and venous (Vena cava) EC from wild type and GTKO/hCD46/hTM male and female juvenile landrace pigs were isolated mechanically (arterial) or enzymatically (venous) from fresh vessel pieces. Shortly, EC from thoracic aorta were harvested by scraping the vessel lumen with a humidified sterile cotton swab. EC from vena cava were isolated by incubation of vessel pieces for 10 min at 37°C with a collagenase II solution (Worthington LS004174, 1.88U/mL final concentration) and subsequent centrifugation of the collagenase II solution to obtain an EC pellet. Isolated cells were maintained in complete cell culture medium (DMEM GlutaMAX, Gibco 21885-025; 10% heat inactivated fetal bovine serum, Sigma F7542 and 1% penicillin/streptomycin, Gibco 15140-122) supplemented with 1% endothelial growth medium 2 supplement mix (PromoCell C-39216). Endothelial phenotype was confirmed by staining EC markers VE-Cadherin or CD31 and von Willebrand Factor while alpha smooth muscle actin staining was used to exclude contamination with fibroblasts or smooth muscle cells (Figure S1). Fibroblast and smooth muscle cell growth was monitored by light microscopy and contaminating cells were removed mechanically from the EC culture. Additionally, transgene expression was evaluated on both arterial and venous isolated GTKO/hCD46/hTM EC by staining for hCD46 (Hycult Biotech HM2103, dilution 1:100) and hTM (Abcam ab6980, dilution 1:100). To confirm knockout of αGal cells were also stained with griffonia simplicifolia isolectin B_4 (GSI B_4, Sigma L-2140, dilution 1:100) (Figure S2). EC from at least 2 different pig donors were used between passages 3–5 to avoid phenotypic drift.

2.2 | Microfluidic experiments

Ibidi μ-Slides (Ibidi μ-Slide VI 0.4, Cat. 80606) were used to study EC in vitro under physiological flow conditions. μ-Slides were coated with 12.5 μg/mL fibronectin (Merck FC010) before cell seeding. All different cell types were seeded at a density of 1 million cells/mL in flow medium (complete cell culture medium containing 4% dextran, Sigma 31390-100G, and 1% bovine serum albumin, Sigma A7030-100G) and left to adhere overnight at 37°C in a humidified incubator with 5% CO_2. For the perfusion, the μ-Slides were connected via silicon tubing to a microfluidic pump (Gilson minipuls 3) and falcon tubes containing flow medium. μ-Slides were perfused for 72 h with 10 dyn/cm^2 shear stress (viscosity of medium 2.1 mPa*s) and medium was changed every 24 h to assure constant nutrient supply. To activate the cells, μ-Slides were

perfused under flow with FBS-depleted flow medium containing 10% pooled normal human serum (xenogeneic activation), 10% heat inactivated pooled normal human serum for 2 h or 100 ng/mL recombinant human TNFα (rhTNFα, R&D 201-TA) for 4 h. To evaluate complement deposition and HS shedding during rhTNFα activation (4 h), channels were perfused with 10% pooled human or porcine serum for the last 2 h of activation. After activation, cells were fixed with 4% formaldehyde solution (Sigma 252549-500ML stock solution 37%) for 15 min at room temperature before immunofluorescence staining was performed.

2.3 | Immunofluorescence staining

Ibidi μ-Slides were blocked for 1 h at room temperature with PBS-3% BSA and subsequently incubated with anti-HS antibody (Amsbio 370-255-1, clone F58-10E4, dilution 1:200), wheat germ agglutinin (WGA) lectin-FITC (Sigma L4895-2MG, dilution 1:100) to stain N-Acetylglucosamine (GlcNAc) and sialic acid (Sia), anti-complement C3b/c antibody (DAKO A0062, dilution 1:500) and anti-E-selectin antibody (Sigma S9555, dilution 1:100) over night at 4°C. Samples were then incubated with the following secondary antibodies (dilution 1:500) for 1.5 h at room temperature under agitation: goat anti-mouse IgM AlexaFluor568 (Invitrogen A21043) for HS, goat anti-rabbit IgG AlexaFluor633 (Invitrogen A21071) for C3b/c and goat anti-mouse AlexaFluor488 (Invitrogen A21121) for E-selectin. Nuclei were stained with DAPI (Sigma, 32670-20MG-F). All antibodies were diluted in PBS-1%BSA-0.05% Tween (Tween 20, AppliChem A4974,0250). Images were acquired with a Zeiss LSM980 confocal microscope and analyzed using Image J (version 2.9.0/1.53q). HS, C3b/c and E-selectin coverage was quantified according to a method adapted from Cheng et al.[22] Briefly, a black and white mask was created from images of HS, C3b/c or E-selectin staining from which the percentage of coverage was measured compared to the channel area. Coverage was normalized to the cell number.

2.4 | Statistical analysis

GraphPad Prism 9 software was used to analyze quantification of HS, C3b/c and E-selectin coverage, $p < .05$ was considered significant. One-way ANOVA followed by multiple comparisons using pairwise Tukey *t*-test was performed for statistical analysis.

3 | RESULTS

3.1 | Galactosyltransferase knockout and transgenic expression of hCD46 and hTM influences heparan sulfate expression on venous but not arterial porcine endothelial cells

To investigate whether genetic modification could influence the glycocalyx composition and sulfation of porcine EC, we evaluated the expression of non-sulfated (GlcNAc/Sia) and single sulfated sugars (HS) on arterial and venous GTKO/hCD46/hTM EC compared to wild type EC. For this, cells were grown in Ibidi μ-Slides for 72 h under static conditions or with laminar shear stress (10 dyn/cm^2).

As shown in Figure 1, GlcNAc/Sia was mainly observed on the cell surface of EC under laminar shear stress (Figure 1A–F) but was absent under static conditions (Figure 1A–C). While wild type arterial EC expressed significantly higher GlcNAc/Sia coverage than wild type venous EC (Figure 1F dark grey bars, 70% and 45%, respectively), GlcNAc/Sia coverage on arterial and venous GTKO/hCD46/hTM EC was comparable, with a mean value of 55% (Figure 1F light grey bars). This suggests that this specific genetic modification does not influence GlcNAc/Sia expression or coverage.

Next, we evaluated the expression of HS, a sulfated glycocalyx component that mediates fundamental functions such as binding of complement regulatory and anticoagulant proteins to EC.[23] As expected, HS was observed on the cell surface of arterial cells mainly under laminar shear stress (Figure 1J) while in contrast, wild type venous cells also express HS under static conditions[24] (Figure 1H, I). Surprisingly, GTKO/hCD46/hTM venous cells show a different HS expression pattern than wild type venous cells. HS was only observed upon shear stress (Figure 1K). Furthermore, venous GTKO/hCD46/hTM EC expressed higher HS coverage than arterial GTKO/hCD46/hTM EC (Figure 1L light grey bars) whereas no differences were observed for wild type EC (Figure 1L dark grey bars). This indicates that genetic modification of venous EC affects the expression of sulfated glycocalyx components.

3.2 | Arterial and venous GTKO/hCD46/hTM endothelial cells are resistant to heparan sulfate shedding after xenogeneic activation

Endothelial dysfunction and antibody-mediated complement deposition are drivers of glycocalyx shedding, eventually promoting graft failure in xenotransplantation.[17,18] Our lab has previously shown that GTKO/hCD46/HLA-E cells are protected against complement deposition in a xenotransplantation setting.[25] However, whether reduced complement deposition also protects against HS shedding from the endothelial surface is unknown.

To investigate HS dynamics of GTKO/hCD46/hTM EC in an in vitro xenotransplantation setting, both arterial and venous porcine GTKO/hCD46/hTM cells were perfused with 10% human serum and HS shedding was evaluated. In agreement with our previous findings,[24] HS shedding from arterial (Figure 2A, B) but not venous wild type cells (Figure 2C, D) was observed after activation with human serum. Interestingly, analysis of HS dynamics on the cell surface of GTKO/hCD46/hTM cells revealed that both arterial and venous cells are protected from HS shedding, with an average HS coverage of 11% and 23%, respectively (Figure 2B, D). This was comparable to perfusion with heat inactivated serum, where complement activation is impeded by heat, and therefore served as a control. Other glycocalyx components such as the non-sulfated sugars GlcNAc/Sia remained intact on the cell surface after perfusion with human serum (data not shown).

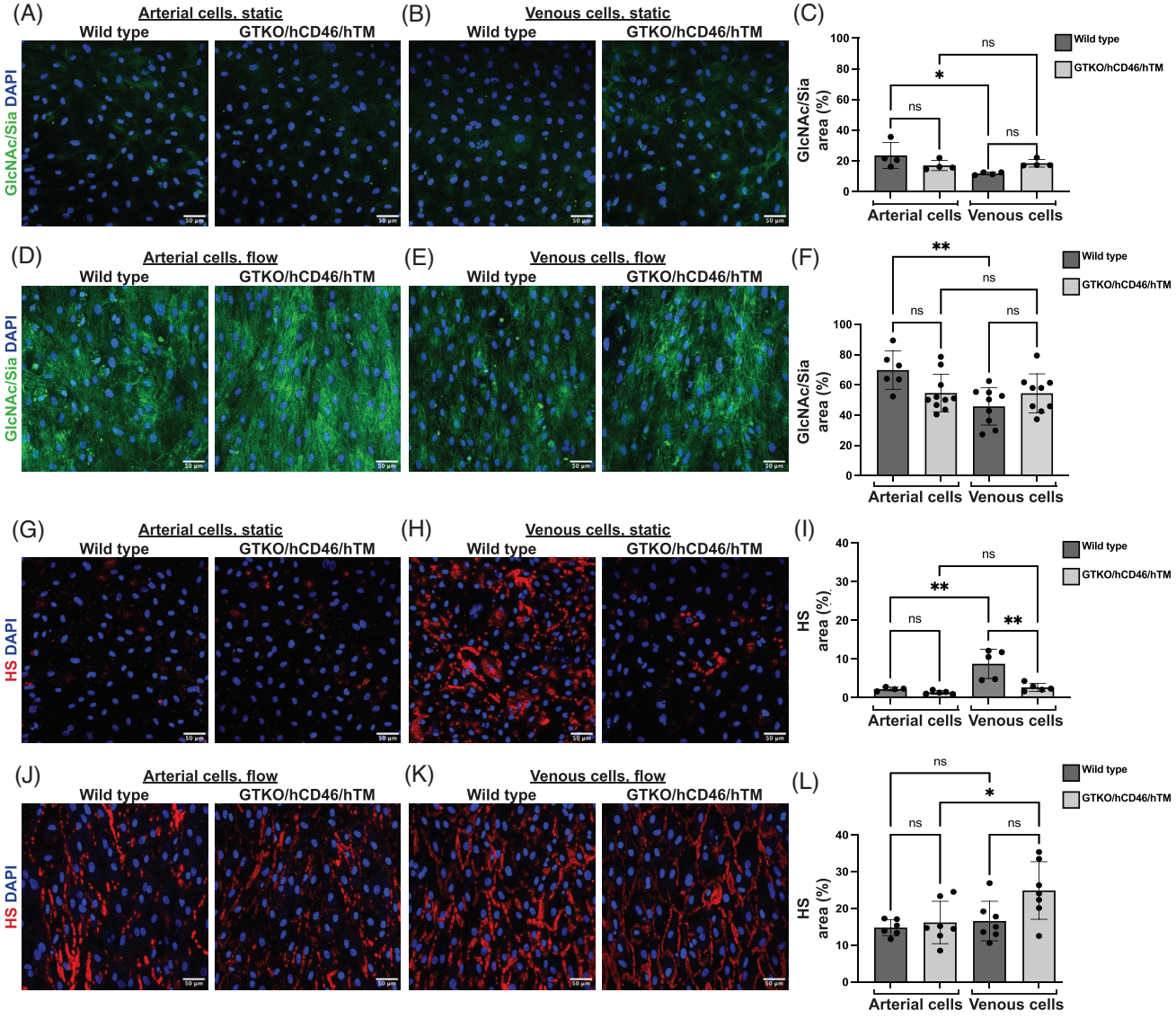

FIGURE 1 Coverage of GlcNAc/Sia and HS on wild type and GTKO/hCD46/hTM arterial and venous porcine EC. Representative confocal images of EC from wild type and GTKO/hCD46/hTM pigs grown in Ibidi μ-Slides. Cells were kept 72 h under static or 10 dyn/cm^2 shear stress conditions (flow). Cells were stained for (A, B, D, E) N-Acetylglucosamine/Sialic acid (GlcNAc/Sia) in green and (G, H, J, K) heparan sulfate (HS) in red. Nuclei are shown in blue (DAPI). All images were acquired with a confocal microscope (Zeiss LSM980). Scale bar: 50 μm. Coverage of GlcNAc/Sia (C, F) and HS (I, L) for each condition (four images/experiment) was calculated as percentage area positive for the respective staining normalized to the cell count. Dark grey bars represent data from wild type EC while light grey bars represent data from GTKO/hCD46/hTM EC. Data are from four or more independent experiments and at least two different porcine EC donors. One-Way ANOVA with multiple comparisons was used for statistical analysis.

To determine whether GTKO/hCD46/hTM EC are also protected against endothelial activation, E-selectin expression was evaluated. E-selectin is an adhesion molecule expressed on activated EC which mediates binding of inflammatory cells to the cell surface.[26] Surprisingly, as shown in Figure 2(E–H), E-selectin expression was increased on the cell surface of GTKO/hCD46/hTM and wild type EC after human serum perfusion, while no E-selectin was observed on EC perfused with heat inactivated serum which was used as control.

To further assess endothelial activation, we analyzed complement C3b/c deposition on GTKO/hCD46/hTM cells. C3b is the main opsonin deposited on the cell surface which propagates activation of the complement cascade.[27] In agreement with our previous findings, both arterial (Figure 3A, B) and venous (Figure 3C, D) GTKO/hCD46/hTM cells showed a 75%–85% reduction in complement deposition compared to wild type cells after perfusion with human serum. Interestingly, while there was no complement deposition on the surface of arterial GTKO/hCD46/hTM cells, a C3b/c coverage of 8% was observed on venous GTKO/hCD46/hTM cells. As expected, no complement deposition was observed after perfusion with heat inactivated human serum (Figure 3B, D). Taken together our data suggest that although HS remains intact on the cell surface of both arterial and venous GTKO/hCD46/hTM EC and complement deposition is reduced, endothelial activation still occurs.

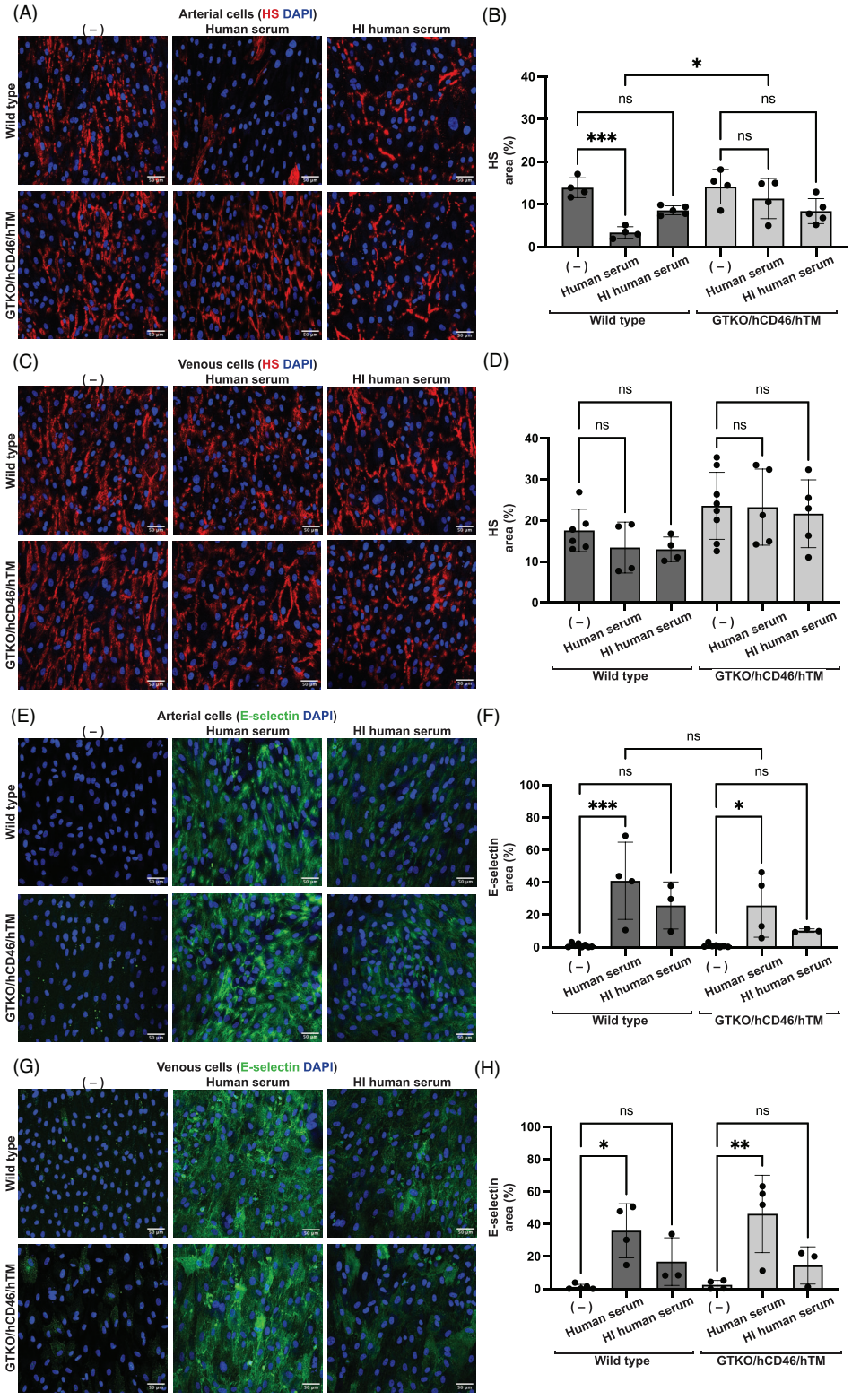

FIGURE 2 Cell surface expression of HS and E-selectin on wild type and GTKO/hCD46/hTM porcine arterial and venous EC after xenogeneic activation. Representative confocal images of Ibidi μ-slide channels containing (A, E) arterial and (C, G) venous EC from wild type and GTKO/hCD46/hTM pigs. Cells were continuously perfused for 72 h under 10 dyn/cm^2 shear stress conditions and subsequently either left untreated (–) or activated with 10% human serum or 10% heat-inactivated human serum (HI human serum) during 2 h. (A, C) Heparan sulfate (HS) is shown in red, (E, G) E-selectin in green and nuclei are shown in blue (DAPI). Scale bar: 50 μm. All images were acquired with a confocal microscope (Zeiss LSM980). (B, D, F, H) coverage of HS and E-selectin was calculated for each image (3–4 images/condition/experiment) as percentage of area positive for the respective staining normalized to the cell count. Data are from three or more independent experiments and at least two different porcine EC donors. One-way ANOVA with multiple comparisons was used for statistical analysis.

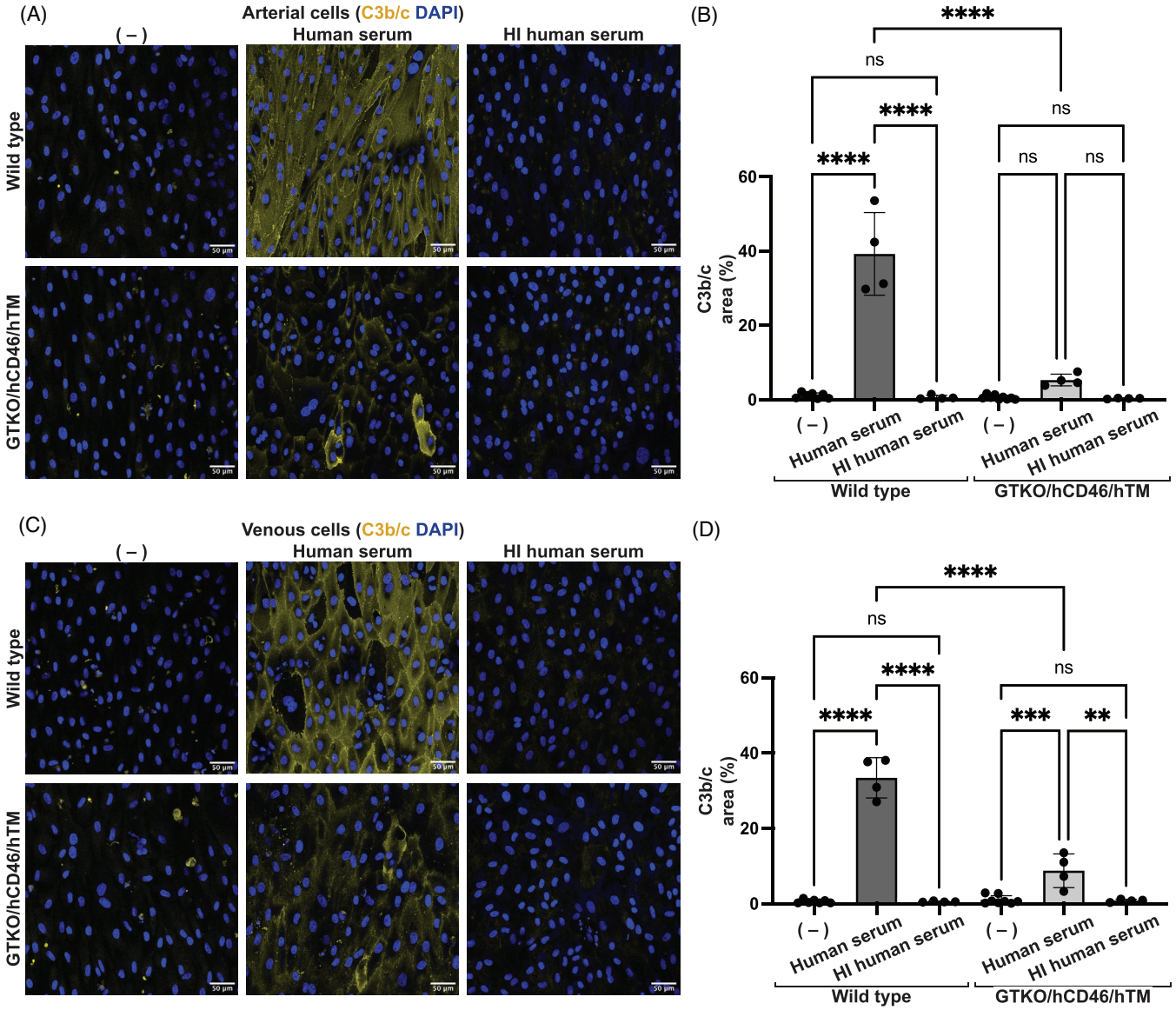

FIGURE 3 Deposition of complement C3b/c on the surface of wild type and GTKO/hCD46/hTM porcine arterial and venous EC after xenogeneic activation. Representative confocal images of Ibidi μ-Slide channels containing (A) arterial and (B) venous EC from wild type and GTKO/hCD46/hTM pigs. Cells were continuously perfused for 72 h under 10 dyn/cm^2 shear stress conditions and subsequently either left untreated (–) or activated with 10% human serum or 10% heat-inactivated human serum (HI human serum) during 2 h. C3b/c is shown in yellow, and nuclei are shown in blue (DAPI). Scale bar: 50 μm. All images were acquired with a confocal microscope (Zeiss LSM980). (B, D) Coverage of C3b/c was calculated for each image (four images/condition/experiment) as percentage of area positive for C3b/c normalized to the cell count. Data are from four or more independent experiments and at least two different porcine EC donors. One-way ANOVA with multiple comparisons was used for statistical analysis.

3.3 | Inflammation in a xenogeneic setting induces an inflammatory phenotype on arterial and venous GTKO/hCD46/hTM endothelial cells

Inflammation plays an important role in xenotransplantation, it promotes coagulation and activates the immune response which is detrimental to xenograft survival.[28] Proinflammatory cytokines such as TNFα were shown to increase in plasma after xenotransplantation and contribute to xenograft rejection.[29]

To investigate the effect of TNFα on HS expression and endothelial activation, both arterial and venous GTKO/hCD46/hTM EC were activated with rhTNFα and then perfused with human serum to mimic an inflammatory xenotransplantation environment. Interestingly, shedding of HS (80%) from the cell surface of arterial but not venous GTKO/hCD46/hTM EC was observed after rhTNFα activation and perfusion with human serum but not after rhTNFα activation alone (Figure 4A, B). Again, venous GTKO/hCD46/hTM EC showed no HS shedding (Figure 4C, B). As a control, ECs were treated with

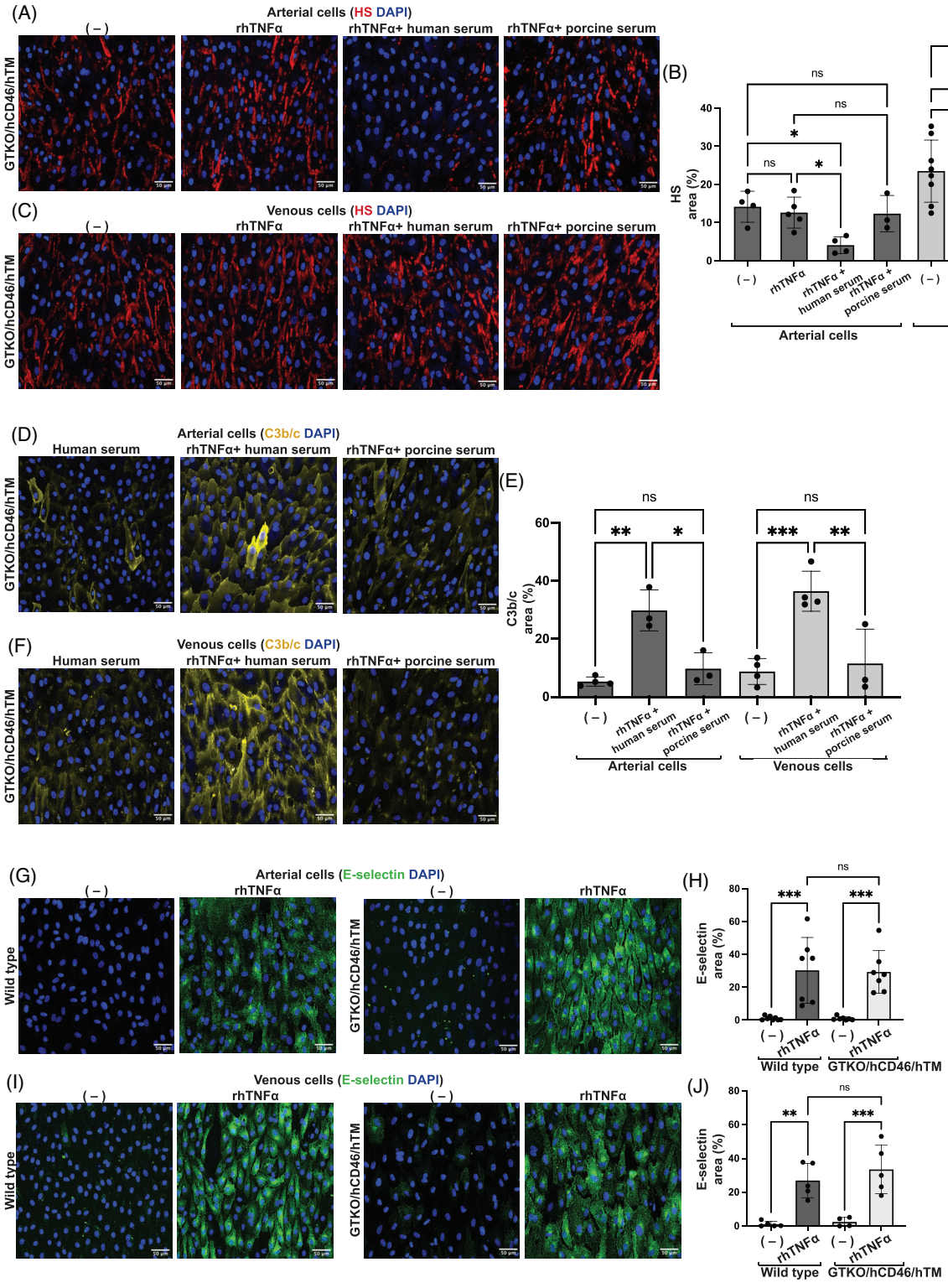

FIGURE 4 Expression of HS, complement C3b/c deposition and E-selectin coverage on the cell surface of GTKO/hCD46/hTM porcine arterial and venous EC in an inflammatory xenogeneic environment. Representative confocal images of Ibidi μ-slide channels containing (A, D, G) arterial (C, F, I) venous GTKO/hCD46/hTM EC. Cells were continuously perfused for 72 h under 10 dyn/cm^2 shear stress conditions and subsequently either perfused with only human serum or simultaneously perfused with rhTNFα and human or porcine serum. To evaluate E-selectin expression, channels were perfused with rhTNFα alone during 4 h (A, C) Heparan sulfate (HS) is shown in red, (D, F) complement C3b/c in yellow, (G, I) E-selectin in green and nuclei are shown in blue (DAPI). Scale bar: 50 μm. All images were acquired with a confocal microscope (Zeiss LSM980). (B, E, H, J,) Coverage of HS, C3b/c and E-selectin was calculated for each image (3–4 images/condition/experiment) as percentage of area positive for the respective staining normalized to the cell count. Data are from three or more independent experiments and at least two different porcine EC donors. One-way ANOVA with multiple comparisons was used for statistical analysis.

rhTNFα and then perfused with porcine serum to mimic an allogenic environment. As expected, HS was not shed from the cell surface (Figure 4A–C). The coverage of the non-sulfated components GlcNAc/Sia on GTKO/hCD46/hTM EC was not affected by these inflammatory conditions (data not shown).

Next, we evaluated the effect of rhTNFα on complement activation. As observed in Figure 4(D–F), C3b/c deposition increased by 83% and 75% on both arterial and venous GTKO/hCD46/hTM EC, respectively, when compared to cells perfused with human serum alone. While complement deposition on arterial cells correlated with HS shedding, no correlation was observed for venous cells where complement deposition occurred even with an intact HS layer. This finding is in agreement with our previous studies on wild type EC where venous cells showed an inflammatory phenotype without HS shedding.[24] Complement deposition was not increased in an allogenic environment after activation with rhTNFα and porcine serum (Figure 4F).

To establish whether rhTNFα activates GTKO/hCD46/hTM EC and potentially contributes to an inflammatory cell phenotype, we investigated the expression of E-selectin on arterial and venous GTKO/hCD46/hTM EC after activation with rhTNFα. Interestingly, GTKO/hCD46/hTM EC were not protected against E-selectin expression. An increase of 50% compared to non-stimulated cells was observed for both cell types (Figure 4G–J).

Taken together our findings suggest that both arterial and venous GTKO/hCD46/hTM EC revert to an inflammatory phenotype when proinflammatory cytokines, such as TNFα, are present during xenotransplantation, thus favoring graft rejection.

4 | DISCUSSION

Organs from genetically modified pigs are currently used for preclinical cardiac xenotransplantation experiments,[3] and inflammatory reactions leading to graft rejection have been studied on a systemic- and organ level. However, knowledge on the immune response of different EC and their glycocalyx contained within such xenografts is still lacking. Carbohydrate remodeling following genetic modification, particularly after *GGTA1*-KO/*CMAH*-KO, has been shown,[19–21] but it is not clear to which extent it changes the inherent cell surface composition or function of the endothelial glycocalyx. Such changes could potentially impact the ability of EC to regulate vascular homeostasis. Here we show that wild type and GTKO/hCD46/hTM EC have distinct HS dynamics. While the expression of HS on GTKO/hCD46/hTM venous cells is flow dependent, wild type venous cells express HS already under static conditions.[24] Furthermore, venous GTKO/hCD46/hTM EC have a higher HS coverage than arterial GTKO/hCD46/hTM EC, thus differing from wild type arterial and venous EC which instead have a similar HS coverage. This suggests that genetic modification of porcine EC correlates with modifications in glycan expression on the EC surface. Next, we investigated whether this could impact endothelial function in a xenotransplantation setting.

Most in vitro research using genetically modified EC has been performed with arterial cells,[30,31] therefore, to address inter-vascular heterogeneity, we analyzed HS shedding from both arterial and venous GTKO/hCD46/hTM EC in a xenotransplantation environment. We have demonstrated previously that HS was shed from arterial but not venous wild type porcine EC after perfusion with human serum or rhTNFα.[24] Here, we show that HS remains intact on both arterial and venous GTKO/hCD46/hTM cells after xenogeneic activation with human serum, and correlates with reduced complement C3b/c deposition. While reduced complement deposition on single transgenic arterial EC has been shown,[30] little is known for venous cells. Surprisingly, C3b/c was still significantly increased after human serum perfusion of venous GTKO/hCD46/hTM cells compared to non-stimulated cells. This suggests that genetically modified venous cells are more prone to complement deposition than arterial cells, where no increase in complement deposition upon human serum perfusion was detected. The mechanism behind the resistance of venous EC to HS shedding is not known. Shedding of HS and other glycocalyx components is mainly mediated by heparanase, an enzyme which processes carbohydrates, and endopeptidases belonging to the family of matrix metalloproteases (MMPs). Such enzymes are upregulated during inflammation and physiologically regulated by inhibitory proteins.[32,33] We therefore hypothesize that venous cells show either less activation of sheddases or better protection through regulatory factors; however, we did not observe any changes in the gene expression of MMP2 and its inhibitor (data not shown). Further research is needed to elucidate possible protective mechanisms in venous EC.

As inflammation plays an important role in xenotransplantation,[28] we also investigated the synergistic effect of human serum and rhTNFα on HS shedding and complement activation on GTKO/hCD46/hTM EC. HS shedding was observed only on arterial but not venous GTKO/hCD46/hTM EC. However, complement deposition was observed on both cell types. This indicates that GTKO/hCD46/hTM EC in an inflammatory xenogeneic environment have the same inflammatory phenotype as wild type EC after xenogeneic stimulation.[24] Increased complement deposition can occur due to changes in the expression of complement regulatory proteins, such as hCD46. Other groups working with EC in vitro have shown either minimal or no changes in the expression of various complement regulatory molecules upon EC activation with different cytokines.[34,35] However, no data exists on simultaneous activation with multiple inflammatory stimuli. Synergistic stimulation could impair CD46 expression and favor complement activation and needs to be further investigated in GTKO/hCD46/hTM EC after activation with inflammatory stimuli in a xenogeneic setting.

Interestingly, we also observed a comparable increase in E-selectin expression for wild type and GTKO/hCD46/hTM EC in all experimental conditions, suggesting that galactosyltransferase knock-out and transgenic expression of human CD46 and thrombomodulin does not prevent E-selectin expression. However, additional genetic modifications, such as removing three xenoantigens and two swine-leukocyte antigen I chains, were shown to lead to a reduction of endothelial E-selectin expression.[36]

Taken together, our findings suggest that GTKO/hCD46/hTM EC revert to a proinflammatory phenotype similar to that of wild type EC in an inflammatory xenotransplantation setting. This could contribute to transplant rejection, and strategies to reduce

inflammation are essential to prolong xenograft survival. Indeed, EC from genetically modified pigs expressing a human anti-apoptotic and anti-inflammatory gene (hA20) were shown to be protected against TNFα-mediated apoptosis.[37] Further studies could therefore focus on investigating the HS dynamics of such EC as they might be better protected than GTKO/hCD46/hTM EC during xenotransplantation.

While we have focused on HS, representing one type of sulfated sugar, heterogeneity should be further investigated by analyzing the glycocalyx with the use of sulfation-specific phage-derived antibodies, which recognize different sulfated sugars.[38] It would be important to investigate differences in sulfation not only after xenogeneic activation, but also under non-inflammatory conditions to understand inherent diversity between wild type- and genetically modified EC.

AUTHOR CONTRIBUTIONS

Anastasia Milusev was responsible for conceptualization, investigation, methodology, visualization figure preparation and writing of the original draft. Jianfang Ren helped with investigation and visualization. Nicoletta Sorvillo helped with conceptualization, writing of the original draft, review, and editing. Robert Rieben developed the original idea and was responsible of this investigation, funding acquisition as well as help with review and editing. Alain Despont and Jane Shaw provided technical support. Elisabeth Kemter, Nikolai Klymiuk, and Eckhard Wolf produced the genetically modified pigs. Matthias Längin, Jan-Michael Abicht, Maren Mokelke, Julia Radan, and Elisabeth Neumann were responsible for organ procurement from pig-to-baboon xenotransplantation experiments, for which Bruno Reichart carried the overall responsibility, and helped with pig vessel isolation. David Ayares produced the original construct of hCD46 on a GTKO background.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

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