ORIGINAL ARTICLE

Expression of human thrombomodulin on porcine endothelial cells can reduce platelet aggregation but did not reduce activation of complement or endothelium – an experimental study

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SUMMARY

Clinical xenotransplantation will only be feasible when present limitations can be controlled sufficiently. Activation of endothelium and complement as well as coagulopathy and thrombotic microangiopathy (TMA) is important barriers. Transgenic expression of hTBM on porcine endothelial cells is a reasonable approach to reduce activation of haemostasis. Endothelial cells from wild-type pigs as well from pigs expressing hTBM alone or in combination with hCD46 and knockout of the alpha-1,3,-galactosyltransferase (GTKO) were perfused with platelet-rich plasma in a microfluidic flow chamber. Platelet aggregation and activation, coagulation, complement and endothelial cell activation were assessed. Perfusion of wild-type porcine aortic endothelial cells (PAEC) resulted in distinct platelet aggregation. Expression of hTBM in either mono-transgenic or triple-transgenic (GTKO/hCD46/hTBM) PAEC showed significantly reduced or absent platelet aggregation. Flow cytometric analysis of platelets showed an increased CD62P expression in wild-type PAEC and significantly reduced expression in mono- or triple-transgenic PAEC. Activation of coagulation measured by TAT occured in WT PAEC and was clearly reduced in hTBM and GTKO/hCD46/hTBM PAEC. Activation of complement and endothelial cells was only reduced in GTKO/hCD46/hTBM but not in PAEC expressing hTBM alone. Expression of hTBM was able to prevent activation of coagulation and platelet aggregation in mono- and triple-transgenic PAEC, while activation of complement and endothelial cells was not reduced in mono-transgenic PAEC.

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acute vascular rejection, coagulation, flow chamber, thrombomodulin, thrombotic microangiopathy, xenotransplantation

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Introduction

Pig-to-human xenotransplantation could be a potential future treatment for acute or chronic organ failure. Before a routinely clinical use is achievable, the problem of xenograft rejection needs to be controlled. Immediately after xenotransplantation, hyperacute rejection (HAR) occurs. Preformed xenoreactive natural

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antibodies (XNA) and complement activation are the main drivers of this in the first minutes to hours appearing process [1]. HAR can be overcome by the use of transgenic modified donor animals either with knock out of the alpha-1,3,-galactosyltransferase (GTKO), or expression of human complement regulators or both [2–4].

The acute vascular rejection (AVR) is the next immunological barrier and usually develops after HAR. AVR is characterized by development of coagulopathy following activation of the recipients' coagulation, platelet aggregation, endothelial cell activation and inflammation leading to thrombotic microangiopathy (TMA) and finally organ failure [5].

In a xenotransplantation setting, coagulation is activated by tissue factor and the tissue factor pathway [6]. In addition, inhibition of coagulation is impaired by a molecular incompatibility affecting the regulatory thrombomodulin [6–8]. Ongoing activation and missing regulation of coagulation result in a coagulopathy and TMA [9]. There are two main strategies to deal with the coagulation issue: interfering with the activation [10] or restoring the regulation by transgenic expression of human thrombomodulin on porcine endothelial cells [11].

For successful xenotransplantation, treatment or prevention of coagulopathy is crucial. Several models for assessment of coagulopathy have been developed [10–13]. The variety of these models ranges from simple static cell culture assays [11,12] or shear stress applying assays [13] to dynamic assays like flow chambers [14,15], perfusion circuits [10] or animal models [16]. Cell culture assays with transgenic porcine endothelial cells expressing human thrombomodulin using plasma have been described before [11,12]. Due to the static nature of the assay, it can be used as a proof of principle if the transgene has the potential to interfere with coagulation. But the biological efficacy under flow conditions and the concomitant shear stress cannot be determined using this assay [14,15]. For these purposes, flow chamber assays were developed. In flow chamber assays, an endothelial cell layer is perfused with platelet-rich plasma for minutes. Platelet aggregation over time is recorded using a camera device [14,15]. A limited alternative to the flow chamber model is the concept of applying shear stress by magnetic rotation in a static proincubation setting [13]. In contrast to the flow chamber, these models lack continuous blood flow over the endothelial layer like in vivo with applied shear stress at defined rates. Perfusion circuits using whole blood are closer to the in vivo situation, but have the disadvantage of aberrant thrombocyte activation and loss due to the use of membrane oxygenators and pump devices. The artificial surface of the tubing of the perfusion circuit requires heparin supplementation for prevention of clotting [10]. These factors limit the possibilities of a detailed investigation of coagulation [10].

Animal models are best for evaluation of the overall efficacy of a treatment or genetic modification. The investigation of the effect of a single transgene in these models is difficult with many factors contributing to the result. In addition, these experiments are expensive and the rising concern on animal welfare necessitates a thorough planning and targeted use of the animal model [16].

In the present study, we investigated the effect of transgenic expression of human thrombomodulin in porcine aortic endothelial cells (PAEC) alone or in combination with GTKO/CD46. For this purpose, hTBM transgenic pig lines on the background of either German Landrace wild type (WT) or the Revivicor GTKO/hCD46 transgenic background were used. Consistent endothelial hTBM expression was achieved by using an expression vector with regulatory sequences from the porcine thrombomodulin gene [11]. PAEC from these animals were isolated to examine the platelet aggregation in a dynamic flow chamber assay.

Materials and methods

Generation of hTBM transgenic pigs

The production of the hTBM transgenic founder animals is described elsewhere [11]. In brief, porcine foetal fibroblasts (WT) and kidney cells (GTKO/hCD46) were nucleofected (Lonza, Basel, Switzerland) followed by selection for 7–10 days either with G418 (Gibco®, Carlsbad, CA, USA) for the foetal fibroblasts and blasticidin S (PAA, Pasching, Austria) for the kidney cells. The growing cell clones were mixed during the selection process in a passaging step and used for somatic cell nuclear transfer. The best expressing founder pigs were recloned resulting in the hTBM mono-transgenic animal #9943 and the GTKO/hCD46/hTBM triple-modified animal #1222. The other animals were produced by breeding, #1198 (hTBM) by mating of #9943 with WT and #2865 (GTKO/hCD46) by mating of #9943 and his offspring with GTKO/hCD46 animals.

Collection and expansion of PAEC

The WT PAEC control experiments of this study were taken from our last publication [14]. As previously

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described, WT PAEC were obtained by surgical removal of the aorta. Following explantation, remaining connective tissue layers and lymph nodes were removed, and remnants of aortal side branches were cauterized. Subsequently, the aorta was repeatedly rinsed in phosphate-buffered saline (PBS, Gibco®) supplemented with 1% penicillin and streptomycin (P/S, Gibco®) and subsequently filled up with 10% collagenase/PBS solution (1 mg/ml, Gibco®) for enzymatic loosening of endothelial cells (37 °C and 5% CO_2 saturation, 30 min, three cycles). Upon isolation, PAEC were repeatedly rinsed in PBS and transferred to cell culture flasks (Sarstedt, Nürmbrecht, Germany), holding cell culture media (DMEM (1×) + GlutaMAX, Gibco®), 20% foetal calf serum (FCS, BioChrom®, Merk AG, Darmstadt, Germany), 1% P/S and 50 µg/ml endothelial cell growth supplement (ECGS, Gibco®). Amphotericin B (Gibco®) was added until first passage. Isolation and initial cultivation of PAEC from hTBM mono-transgenic and triple-modified pigs was done as previously described [11] with minor modifications. Briefly, thoracic or abdominal aorta from animals with an age of 2 months to 2 years was washed with PBS containing 100 units/ml penicillin, 100 µg/ml streptomycin (P/S, PAA) and connective tissue was removed. After cutting the aorta longitudinal, it was transferred with the endothelial surface into a tissue culture plate moistened with 0.1% Collagenase II (Gibco®). After 12 min of incubation, the endothelial cells were collected either by rinsing with DMEM (Gibco®) or scratching with a scalpel and transferred into a tube containing DMEM. After washing by centrifugation (5 min, 500 *g*), the cells were seeded on culture plates coated with 0.1% gelatin (Sigma-Aldrich, St. Louis, MO, USA) in medium M199 supplemented with L-glutamine (PAA), PS (PAA) and 20% FCS (Gibco®). After 24 h, the medium was replaced by endothelial specific cell medium containing endothelial medium supplements (PAA), PS, 293 mg/l L-glutamine (PAA) and 5% FCS in case of endothelial cultures PAEC #9943, #1198 and #1222.

In case of primary culture of PAEC #2865, the cells were seeded with DMEM low glucose GlutaMAX™ (Gibco®) containing 10% Endothelial Cell Growth Medium MV Supplement Mix (PromoCell, Heidelberg, Germany). Isolate purity was checked by (i) cobblestone like morphology and (ii) CD31+ staining using flow cytometry (Mouse Anti-Pig CD31:APC, Kidlington, UK). In order to avoid senescence related artefacts, PAEC were used from passage four to seven.

Porcine aortic endothelial cell were harvested at passage four via enzymatic digestion with accutase solution (Sigma-Aldrich, 30 min, 37 °C, 5% CO_2), repeatedly rinsed in PBS, centrifuged (Eppendorf 5810 R, 10 min, 350 *g*) and counted using trypan blue life/dead staining (Gibco®, 1:30 dilution of 0.4%). Endothelial cells were further used in flow chamber assay with PRP or in six-well culture dish assays using confluent PAEC (24–48 h after seeding procedure) for co-incubation either with human PFP (2 h) or serum (3 h), respectively.

Preparation of human blood

In order to premature platelet activation, blood from healthy human donors was carefully drawn into heparinized (1 U/ml) syringes or citrate (0.106 mol/l). Subsequently, the blood rested for 10 min at room temperature and was then centrifuged at 90 *g* for 15 min or 3000 *g* for 10 min to obtain PRP and PFP, respectively. Platelet concentration was adjusted to 250×10^3/µl via the addition of PFP. Centrifugation of whole blood at 2000 *g* for 10 min after a resting time of 20 min in serum collector tubes was used to obtain serum.

Expression of hTBM in mono- and triple-transgenic PAEC

Expression of hTBM in mono- and triple-transgenic cells was determined by FACS analysis. PAEC were taken up in cell culture medium and subsequently diluted to 5×10^5 cells/ml. Afterwards, cells were washed two times with PBS and centrifuged at 350 *g*. Supernatants were discarded. PAEC were blocked with DPBS (Gibco) for 10 min. PAEC were labelled with a PE-conjugated mouse-anti-human CD141 (Biolegend, San Diego, CA, USA) for 10 min at 4 °C. Followed by centrifugation at 350 *g*. Supernatants were discarded, and cells were washed afterwards with PBS. Finally, hTBM expression of PAEC was measured by flow cytometry (BD FACS Canto, Becton Dickinson, Franklin Lakes, NJ, USA).

Complement and coagulation assays (C3a, C5a, TAT)

Platelet-free plasma supernatants were harvested at different time points (0, 30, 60, 90 and 120 min) for quantification. The supernatants were stored at −80 °C until further analysis. Commercial ELISA kits were used for quantification: Enzygnost® TAT micro (Siemens, Bavaria, Germany), MicroVue C3a Plus (TECOmedical, Bünde, Germany) and MicroVue C5a (TECOmedical).

All measurements were conducted in accordance with the manufacturers' instructions.

Flow-based assays (detection of platelet aggregation and activation)

In order to fluorescently detect perfused platelets, PRP was labelled with Calcein-AM (Sigma-Aldrich, 1:1000 of 4 mM stock) for 30 min at room temperature. PAEC were taken up in cell culture medium and subsequently diluted to 12×10^6 cells/ml. Seeding of PAEC into the area of the viewing field was achieved by a brief torrent of cell culture media flow (1 min, 1 dyn/cm^2). Subsequently, PAEC were subjected to rest for 2 h at 37 °C and 5% CO_2 saturation to firmly attach to the bovine collagen type II (Life Technologies, Carlsbad, CA, USA, 2% of 5 mg/ml) pre-coated polydimethylsiloxane (PDMS) – surface of the flow chamber. The microchannels were blocked with PBS, supplemented with 5% FCS (Biochrom, Berlin, Germany). The tubing of the flow chamber assay has a total length of 2 m with a diameter of 3.2 mm.

Shear rates of 3 dyn/cm^2 or 10 dyn/cm^2 and 9 min of perfusion in total were used.

For quantification of platelet activation, citrated PRP was harvested from the outlet well of the flow chamber after 9 min of perfusion at 10 dyn/cm^2. Subsequently, platelets were diluted in PBS (1:2 ratio) and labelled with anti P-selectin monoclonal antibody (Mouse Anti-Human CD62P:PE, Becton Dickinson) for 30 min at RT. A FACS Canto™ Flow Cytometer (Becton Dickinson) was used to evaluate platelet activation. Non-perfused PRP and PAEC-free perfusions served as controls.

Endothelial cell activation

An RNeasy Mini Kit (Qiagen, Hilden, Germany) was used for total RNA isolation according to manufacturer's guidelines. The isolated RNA was stored at −80 °C until further analysis. 1 µg of RNA was used in a total volume of 20 µl containing RT buffer, 50 µM random hexamers, 20 U RNasin, 50 U MULV reverse transcriptase, 5 mM $MgCl_2$ and 1 nMdNTP (cDNA – Synthesis Kit, Biozym Scientific GmbH, Hessisch Oldendorf, Germany) for cDNA synthesis.

Further preparation was carried out as follows: 10 min at 25 °C, 30 min at 37 °C and 5 min at 85 °C. Negative controls exhibited either no reverse transcriptase or no RNA content. RT-PCR was performed in 96-well optical reaction plates on an Opticon1 Real-Time machine (MJ Research, Quebec, Canada). 25 µl of Power SYBR Green Master Mix (Biozym Scientific GmbH), primers (forward and reverse, 0.25 µM each) and 2 µl cDNA (diluted in sterile water 1:10) were used. The cycle programme consisted of 7 min at 95 °C, 40 total cycles of 15 s at 95 °C, 30 s at 56 °C and 30 s at 72 °C. In order to check reaction specificity, a melting curve analysis was performed. Differential mRNA expression was evaluated via the 2-CT method. Data presentation is expressed as a x-fold change of expression related to baseline (media-CTR). The data were normalized to the housekeeping gene eukaryotic translation elongation factor 1 alpha 1 (pEEF1A1).

The following primer sequences were used:

pEEF1A1:
5′CAA AAA YGA CCC ACC AAT GG 3′
3′GGC CTG GAT GGT TCA GGA TA 5′
pICAM-1:
5′CAG AGG CTA CGG TCC ACC T 3′
3′TGT CAC CAC CTT CTC GTC TG 5′
pE-Selectin:
5′GAT GGA TGC TCA ATG GCT CT 3′
3′AGA CTC CAC CAG CAG CAA GT 5′
pVCAM-1:
5′AGA AGC TGA GGG ATG GGA AT 3′
3′GTG TCG CCT GTC TCT GCT TT 5′

Statistics and data quantification

ImageJ (http://imagej.nih.gov/ij/) was utilized to quantify platelet aggregation, via conversion into binary data files, followed by percentile evaluation of the flow chamber viewing field. Statistics were performed using either unpaired *t*-tests (flow-based assays) or one-way or two-way ANOVA tests in conjunction with Tukey's test procedure (six-well culture dish assays). Test parameters were set to assume a Gaussian distribution (GRAPHPAD PRISM 6).

Results

hTBM expression reduces platelet aggregation under flow

Perfusion of WT PAEC resulted in a shear rate dependent formation of platelet aggregates with complex morphology. In more detail, composition of aggregates visually ranged from small non-connected round to irregular patches, either along the centre or in the periphery of the viewing field, up to larger interconnected accumulations in between. The nature and magnitude of platelet aggregates correlated directly with the

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shear rate. If a higher shear rate was used (10 dyn/cm^2), aggregation occurred more rapidly and was further characterized by an increased amount of interconnected sections rather than small clusters after 9 min (Fig. 1). Platelet aggregation in hTBM mono-transgenic and GTKO/hCD46/hTBM PAEC, irrespective of the shear rate, was either significantly reduced, with only thin strand-like residues at the periphery (mono-transgenic donor #9943 and triple-modified donor #2865), or almost completely absent throughout the entire experiment (mono-transgenic donor #1198 and triple-modified #1222, Fig. 2). We noted that in experiments with these aforementioned transgenic lines, the small aggregates that developed tended to disintegrate and were less firmly attached to the PAEC monolayer. These observations were made for both shear rates.

Platelet aggregate coverage per viewing field after perfusion of WT PAEC was next analysed for three different human donors and four different transgenic PAEC cultures in comparison to WT PAEC (Tables 1 and 2). The amount of platelet aggregation was similar for repeated experiments with blood from given donors at 3 dyn/cm^2, with platelet aggregate coverage ranging between 20% and 27% (Table 1). In contrast, the variation between experiments using blood from different donors was higher at 10 dyn/cm^2, exhibiting a minimum of 10% and a maximum of 33% (Table 2). However, the total mean of platelet aggregate coverage per viewing field was similar across different shear rates (24.5% at 3 dyn/cm^2 (Table 1) vs. 19.6% at 10 dyn/cm^2 (Table 2).

Perfusions of PEAC from different hTBM transgenic donors revealed that #1198 showed very little platelet aggregation of <1% at 3 dyn/cm^2 (Table 1) or 10 dyn/cm^2 (Table 2) in all three blood donors. PAEC from #9943 showed residual platelet aggregation in response to donor #3 [5.2% at 3 dyn/cm^2 (Table 1) vs. 4.6% at 10 dyn/cm^2 (Table 2)], but not to donor #1 and #2. Cells from triple-modified pig #1222 showed no detectable platelet aggregation at both shear rates in any donor. PAEC from #2865 showed residual platelet aggregation at 3 dyn/cm^2 (donor #1, Table 1) and at 10 dyn/cm^2 (donor #3, Table 2). Taken together, platelet aggregation was reduced in hTBM mono- and GTKO/hCD46/hTBM triple-modified PAEC compared to WT PAEC, but some combination of PAEC and blood donors exhibited residual platelet aggregation.

Platelet activation in response to perfusion is reduced in hTBM PAEC

Platelets perfused over WT PAEC and recovered from the outlet well of the flow chamber showed an increase in surface expression of CD62P (29.5 ± 3%) as compared to freshly prepared non-perfused PRP (5.5 ± 1.6%) and cell free flow chamber perfusions (4.8 ± 1.4%) (Fig. 3a). The contact to either hTBM mono-transgenic (#1198 and #9943) or GTKO/hCD46/hTBM (#1222 and #2865) PAEC upon perfusion did not activate circulating platelets above the level of native PRP preparations and cell free perfusions, respectively (#1198: 4.0 ± 2.9% and #9943: 4.8 ± 2.2%; #1222: 6.5 ± 1.7% and #2865: 5.7 ± 1.2%, Fig. 3a).

hTBM expression reduces the activation of a coagulation

Activation markers of coagulation were assessed after incubation of PAEC with human PFP for 120 min (Fig. 3b). WT PAEC induced a significant increase in

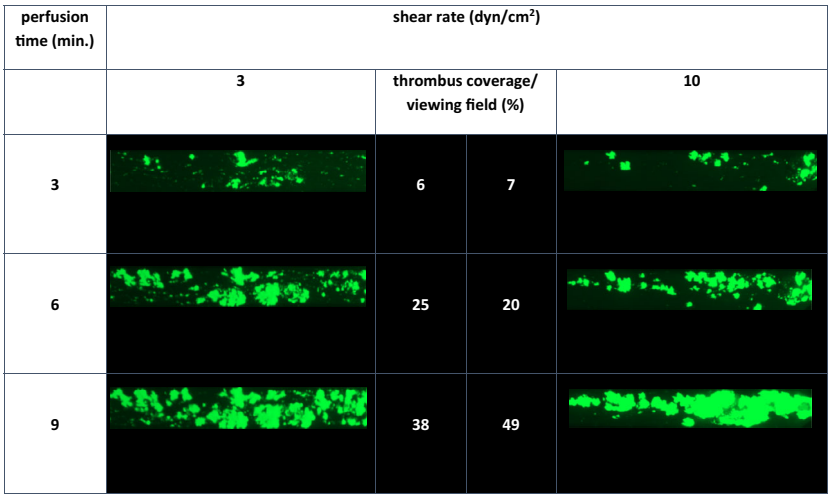

Figure 1 Representative images (4× magnification, fluorescent microscopy) depicting platelet aggregation of Calcein-AM labelled human platelets upon perfusion of WT PAEC at 3 dyn/cm^2 (left-hand section) and 10 dyn/cm^2 (right hand section) for 3, 6 and 9 min, including thrombi quantification (middle section).

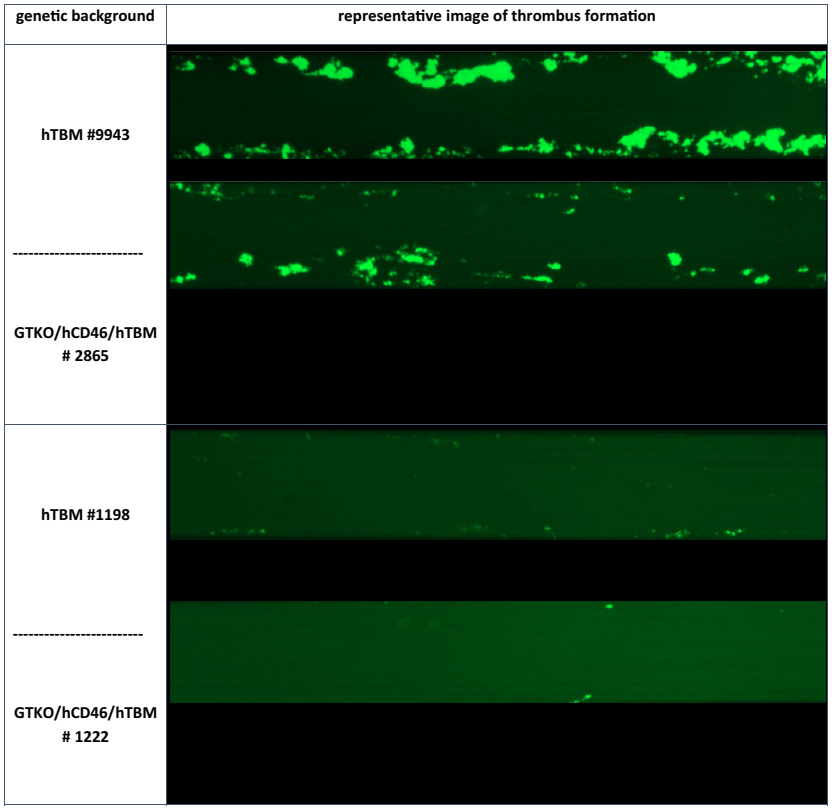

Figure 2 Representative images (4× magnification, fluorescent microscopy) illustrating reduced (hTBM donor #9943 and GTKO/hCD46/hTBM donor #2865) or absent (hTBM donor #1198 and GTKO/hCD46/hTBM donor #1222) platelet aggregation upon perfusion of transgenic PAEC at 3 dyn/cm^2 and 9 min runtime.

TAT formation (mean of 23 917 ± 9156 µg/l after 120 min) compared to freshly prepared PFP which was not subjected to PAEC co-incubation (mean of 10.3 ± 4 µg/l, Fig. 3b). Both hTBM mono-transgenic PAEC cultures reduced TAT formation roughly twenty-fold (#1198: 1401 ± 545 and #9943: 809 ± 311 µg/l), whereas the triple-modified PAEC cultures decreased TAT formation almost completely (#1222: 48 ± 15 µg/l and #2865: 71 ± 23 µg/l, Fig. 3b).

The activation of complement is reduced in GTKO/hCD46/hTBM PAEC

WT PAEC exhibited a time dependent increase of C3a with a mean of 1194 ± 363 ng/ml after 120 min incubation (Fig. 4a). The hTBM mono-transgenic PAEC showed comparable C3a generation after 120 min (#1198: 1161 ± 377 and #9943: 1160 ± 385 ng/ml) that was not reduced compared to the WT PAEC (Fig. 4a). In contrast, triple-modified PAEC cultures showed a 30% reduced C3a generation (#1222: 762 ± 192 and #2865: 804 ± 220 ng/ml, Fig. 4a).

C5a concentration increased during WT PAEC incubation with a mean of 30.4 ± 8 ng/ml after 120 min (Fig. 4b). Human TBM mono-transgenic PAEC cultures did not reduce C5a generation compared to WT PAEC (#1198: 33 ± 10 and #9943: 35 ± 10 ng/ml, Fig. 4b). The triple-modified PAEC cultures revealed concentrations of approximately 20 ng/ml, thus reflecting about two-third of the hTBM and WT values (#1222: 22 ± 5 and #2865: 18 + 4 ng/ml, Fig. 4b).

Endothelial cell activation

qPCR was used to assess the expression of E-selectin, VCAM-1 and ICAM-1 on PAEC, after incubation of 3 h with human serum (Fig. 5a–c). Porcine E-selectin (mean 21 ± 7) and VCAM-1 (mean 14 ± 2) mRNA expression was increased several fold in WT PAEC (Fig. 5a,c), whereas ICAM-1 (mean 2.8 ± 0.9) mRNA was only slightly increased (Fig. 5b). Human TBM mono-transgenic PAEC cultures exhibited no reductive effect, whereas the triple-modified PAEC cultures showed a reduced expression of E-selectin and VCAM-1 (Fig. 5a,c). In contrast, the expression of ICAM-1 was not reduced (Fig. 5b).

Expression of hTBM in mono- and triple-transgenic PAEC

To exclude that the observed variations in platelet aggregation may be due to varying expression of hTBM,

Table 1. Quantification of platelet aggregation after perfusion of WT, hTBM mono-transgenic (#1198 and #9943) and GTKO/hCD46/hTBM triple-modified (#1222 and #2865) PAEC for 9 min and 3 dyn/cm^2

	PAEC source						
		hTBM PAEC			GTKO/hCD46/hTBM PAEC		
Blood donor	WT PAEC	#1198	#9943	Total	#1222	#2865	Total
#1	26.1 ± 9.2 (*n* = 9)	0.0 ± 0.0 (*n* = 4)	0.1 ± 0.1 (*n* = 3)	0.1 ± 0.1 (*n* = 7)****	0.0 ± 0.0 (*n* = 8)	3.1 ± 4.0 (*n* = 7)	1.6 ± 2.1 (*n* = 15)****
#2	27.1 ± 13.0 (*n* = 9)	0.0 ± 0.0 (*n* = 4)	0.1 ± 0.1 (*n* = 4)	0.0 ± 0.1 (*n* = 8)****	0.0 ± 0.0 (*n* = 8)	0.1 ± 0.0 (*n* = 4)	0.1 ± 0.1 (*n* = 12)****
#3	20.5 ± 12.9 (*n* = 11)	0.2 ± 0.1 (*n* = 8)	5.2 ± 2.4 (*n* = 8)	2.7 ± 3.5 (*n* = 16)****	0.1 ± 0.0 (*n* = 8)	0.1 ± 0.0 (*n* = 4)	0.1 ± 0.0 (*n* = 12)****
Total	24.5 ± 3.5 (*n* = 29)	0.1 ± 0.1 (*n* = 16)	2.0 ± 2.9 (*n* = 15)	0.9 ± 1.5 (*n* = 31)****	0.1 ± 0.1 (*n* = 24)	1.1 ± 1.7 (*n* = 15)	0.6 ± 0.9 (*n* = 39)****

Data points depict the mean of platelet aggregation ± SD, *n* equals the number of perfused wells in the individual experiment. All three individual blood donors were the same throughout the experiments. WT data are based on ten (3 dyn/cm^2) and three (10 dyn/cm^2) individual perfusions with three identical blood donors in total, and data of transgenic PAEC are based on one individual perfusion per blood donor and shear rate.

****$P \leq 0.0001$.

Table 2. Quantification of platelet aggregation after perfusion of WT, hTBM mono-transgenic (#1198 and #9943) and GTKO/hCD46/hTBM triple-modified (#1222 and #2865) PAEC for 9 min and 10 dyn/cm^2

	PAEC source						
		hTBM PAEC			GTKO/hCD46/hTBM PAEC		
Blood donor	WT PAEC	#1198	#9943	Total	#1222	#2865	Total
#1	32.7 ± 9.4 (*n* = 4)	0.4 ± 0.2 (*n* = 4)	1.9 ± 2.3 (*n* = 3)	1.2 ± 1.1 (*n* = 7)****	0.0 ± 0.0 (*n* = 8)	0.4 ± 0.5 (*n* = 6)	0.2 ± 0.2 (*n* = 14)****
#2	9.9 ± 12.3 (*n* = 8)	0.5 ± 0.1 (*n* = 4)	0.5 ± 0.3 (*n* = 3)	0.5 ± 0.0 (*n* = 7)***	0.1 ± 0.0 (*n* = 8)	0.2 ± 0.2 (*n* = 4)	0.1 ± 0.0 (*n* = 12)****
#3	16.3 ± 4.5 (*n* = 7)	1.4 ± 0.5 (*n* = 8)	4.6 ± 2.0 (*n* = 8)	3.0 ± 2.3 (*n* = 16)****	0.0 ± 0.0 (*n* = 8)s	4 ± 3.3 (*n* = 6)	2.0 ± 2.8 (*n* = 14)****
Total	19.6 ± 11.8 (*n* = 19)	0.7 ± 0.6 (*n* = 16)	2.3 ± 2.1 (*n* = 14)	1.6 ± 1.3 (*n* = 30)****	0.0 ± 0.0 (*n* = 24)	1.5 ± 2.2 (*n* = 16)	0.8 ± 1.1 (*n* = 40)****

Data points depict the mean of platelet aggregation ± SD, *n* equals the number of perfused wells in the individual experiment. All three individual blood donors were the same throughout the experiments. WT data are based on ten (3 dyn/cm^2) and three (10 dyn/cm^2) individual perfusions with three identical blood donors in total, and data of transgenic PAEC are based on one individual perfusion per blood donor and shear rate.

***$P \leq 0.001$.

****$P \leq 0.0001$.

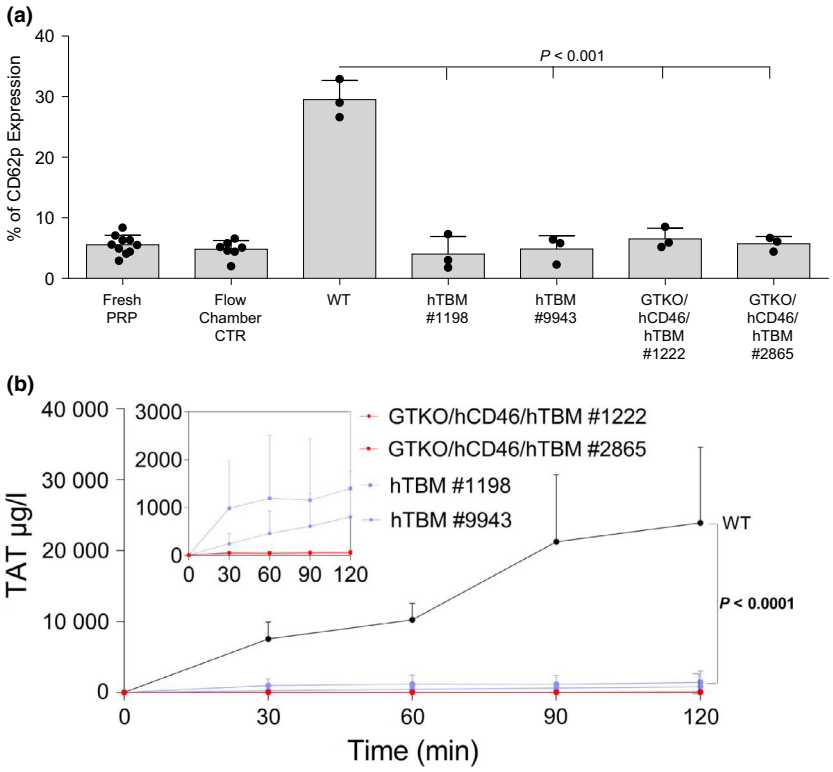

Figure 3 (a) PAEC induced activation of human platelets, evaluated by flow cytometry (P-selectin, $CD62P^+$ staining, 10 dyn/cm^2 and 9 min), compared to fresh PRP and empty perfused flow chamber. Data points depict mean and standard deviation of three individual experiments for WT and transgenic PAEC cultures from four pooled flow chamber wells each, originating from three different blood donors ($P < 0.001$). (b) TAT concentration during incubation of human platelet-free plasma with either WT, hTBM mono-transgenic (#1198 and #9943) or GTKO/hCD46/hTBM triple-modified PAEC for 120 min. Data points depict mean with standard deviation from two (WT) or three (transgenic) individual experiments with different blood donors in triplicate analyses ($P < 0.0001$).

the endothelial expression was assessed in the used mono- and triple-transgenic cells and revealed expression in all used cells (Fig. 6).

Discussion

Despite tremendous progress in the field [16–19], xenotransplantation won't become clinical routine until the remaining issues have been thoroughly addressed. Currently, hyperacute rejection can be successfully prevented by transgenic expression of human complement regulators and knocking out the gene of the 1,3 galactosyltransferase. However, a persisting issue is the acute vascular rejection associated with endothelial cell activation, inflammation, coagulopathy and TMA [20,21]. Whereas endothelial cell activation and inflammation can be prevented by transgenic expression of hHO1 or blocking TNF-alpha by antibodies, coagulopathy and TMA remain major problems [20–22].

To tackle the coagulation issue, hTBM has been identified as a promising target based on the known molecular incompatibility regarding human thrombin and porcine thrombomodulin resulting in an absence of activated protein c, leading to a dysregulated coagulation with excessive uncontrolled thrombin generation [7,20,23,24]. Transgenic expression of hTBM should restore the generation of activated protein C and improve regulation of coagulation and therefore prevent excessive thrombin formation.

The effect of hTBM has been studied extensively in static cell culture assays [7,12,24–27]. Studies in dynamic assays are less common and rarely focus on hTBM specifically [14,15,23].

In animal studies, hTBM is often used as one of multiple transgenes [28–30]. Therefore, it is difficult to rule out the effect of hTBM alone in this setting. There are only few studies focusing on the effect of hTBM alone [7,12,24,26,27,31]. Two major effects of hTBM in this context have been described: first, regulation of the procoagulant activity [12] by prevention of thrombin generation and second, anti-inflammatory and complement regulatory properties [26,27].

To explore the regulation of procoagulant activity, complement activation and inflammation by hTBM in our study, mono- and triple-transgenic porcine endothelial cells expressing hTBM alone or in combination with GALTKO and hCD46 were perfused in vitro or incubated with human plasma and analysed regarding activation of coagulation, endothelial cells, complement as well as platelet activation and aggregation formation. Perfusion of WT PAEC resulted in platelet aggregation with complex morphology and visible

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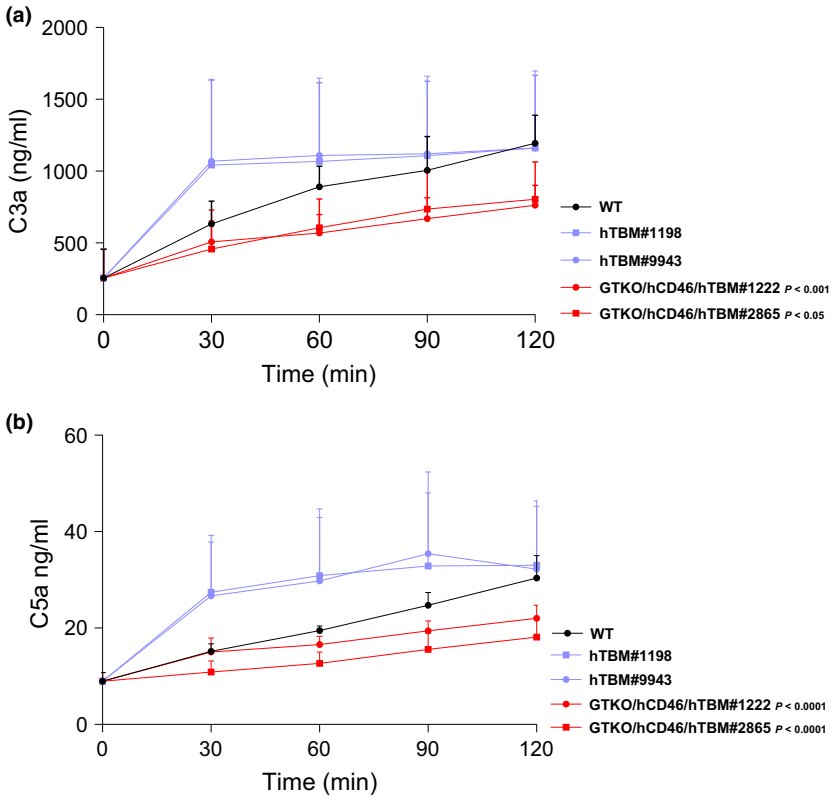

Figure 4 Effect of WT, hTBM mono-transgenic and GTKO/hCD46/hTBM triple-modified PAEC on C3a (a) and C5a (b) formation, during incubation of human platelet-free plasma for 120 min. Each data point depicts mean with standard deviation from three human blood donors analysed in triplicates. Donors #1198 and #9943 were not statistically significant, #2865 $P < 0.05$, #1222 $P < 0.001$.

adhesion and aggregation of human platelets under flow on the porcine endothelial cells (Figs 1 and 2). In contrast, platelet aggregation was either reduced or completely prevented in PAEC expressing hTBM alone as well as in triple-transgenic endothelial cells (Tables 1 and 2). This observation implies that platelet aggregation strongly depends on the activation of coagulation with consecutive thrombin generation, as thrombin is directly inhibited by thrombomodulin. The perfusion of the mono-transgenic PAEC revealed that activation of coagulation measured by TAT and expression of P-selectin derived from platelet activation could be reduced by transgenic expression of hTBM, whereas endothelial cell and complement activation were not affected (Figs 3–5). Only in combination with GTKO and hCD46, activation of coagulation was inhibited as both complement and endothelial cell activation were reduced (Figs 3–5). The interference of hTBM with thrombin generation is in line with the data from other studies [12,27]. In regard to the aspects of anti-inflammatory and complement regulatory properties and in contrast to other studies [26,27], we found no protective effect of the mono-transgenic PAEC expressing hTBM. The complement regulatory effect of hTBM observed in other studies was described as based on thrombin inhibition, since thrombin can activate the complement system via cleavage of C3 and C5 to C3a and C5a [27]. In our study, in hTBM mono-transgenic cells C3a and C5a levels were highly elevated, indicating that the regulatory effect was not sufficient and may be overruled by other factors contributing to complement activation like endothelial cell activation and antibody binding. C3a and C5a are general markers of complement activation, as the classical, alternative and lectin pathway converge on this level: C3 is activated by C4bC2b (classical and lectin pathway) or factors B and D (alternative pathway). C5 is activated by C3b (all pathways). Although binding of XNA is likely driven by the classical pathway [32], by measurement of C3a and C5a all sources of complement activation are covered. Therefore, we can exclude that hTBM expression favourably affects complement activation in our model.

The anti-inflammatory effect of hTBM originates from HMGB-1 inhibition of PAEC, making them less sensitive to TNF [26]. While the principal potential of hTBM to reduce activation of endothelial cells was shown before, we could not confirm this effect in our study. The anti-inflammatory effect of hTBM may be overruled by other factors resulting in endothelial cell activation such as endothelial cell damage, binding of antibodies and complement activation. By expression of hTBM alone, thrombin as activator of endothelium

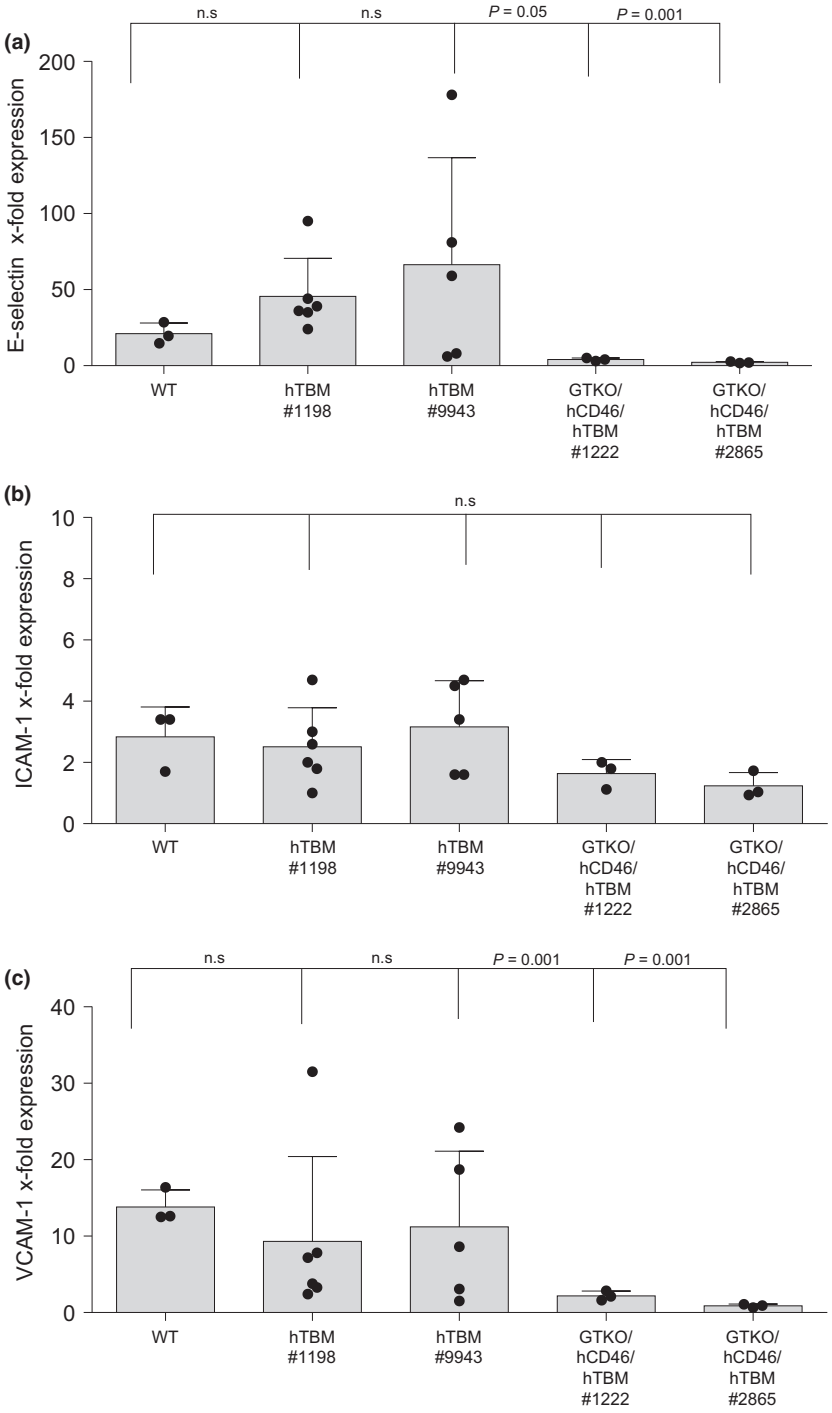

Figure 5 Endothelial cell activation after 3h of incubation with human serum evaluated by expression of porcine E-selectin, ICAM-1 and VCAM-1. Data points depict mean with standard deviation of three individual experiments in triplicate analysis from three different blood donors. (E-selectin #1198 and #9943 n.s., #1222 $P < 0.05$, #2865 $P < 0.001$; ICAM-1 #1198, #9943, #1222, #2865 n.s; VCAM-1 #1198 n.s., #9943 n.s., #1222 $P < 0.001$, #2865 $P < 0.001$).

and complement can be inhibited [20,27] and presumably the susceptibility of PAEC to TNF reduced [26]. The other factors contributing to endothelial cell and complement activation are not affected, such as antibody binding or endothelial damage. In this light, our study strongly suggests that hTBM should by combined with GTKO and a human complement regulator.

Another target for regulation of coagulation could be CD39. While hTBM regulates coagulation by generation of activated protein C which inhibits coagulation factors II and V and therefore interferes with amplification of plasmatic coagulation, CD39 interferes with platelet activation. CD39 is an endothelial enzyme that generates AMP by conversion from ATP and ADP. While ADP is a platelet activator, AMP has protective and antithrombotic

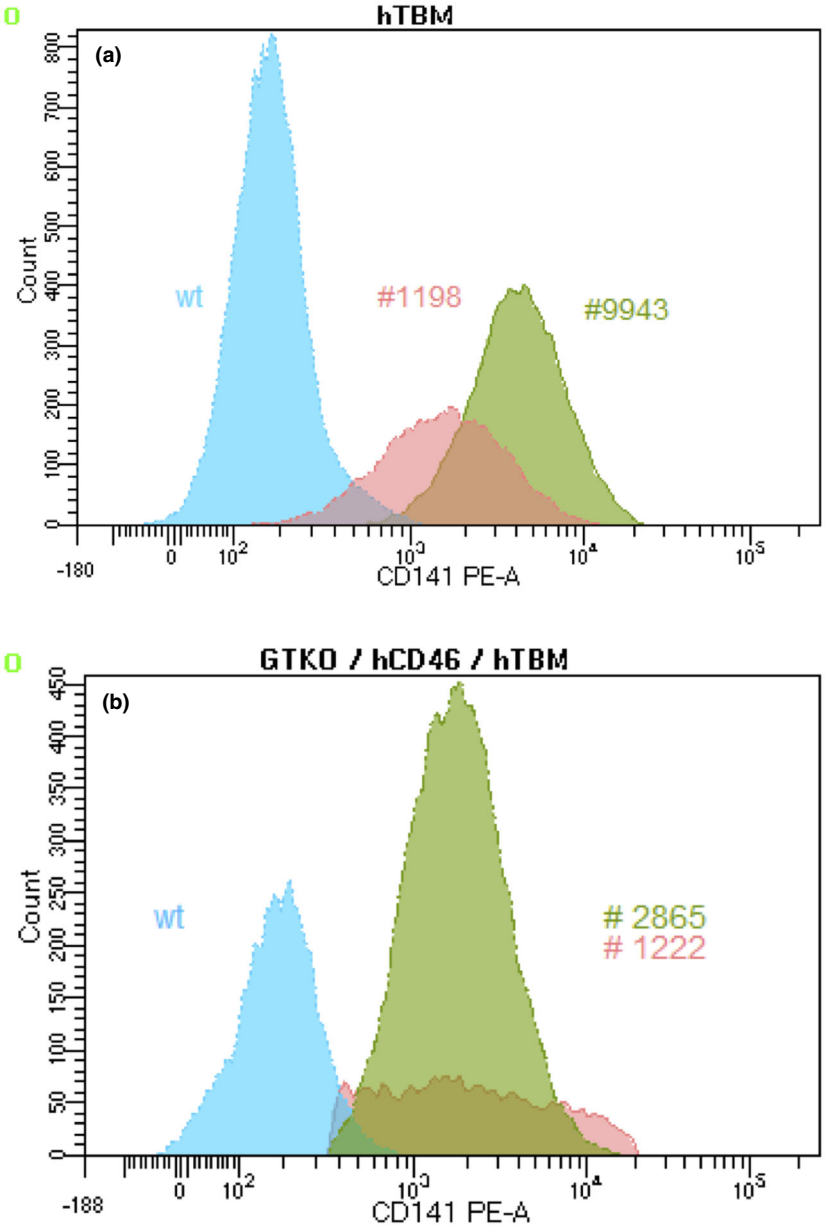

Figure 6 Endothelial hTBM expression in mono-transgenic (a) and triple-transgenic (b) cells by FACS analysis (red and green peak). Wild-type PAEC served as control (blue peak).

effects including vasodilation and interference with platelet aggregation. Both molecules may contribute to antithrombotic mechanisms during transplantation, but via different pathways (coagulation versus platelet aggregation). Our data suggest that additional inhibition of platelet aggregation may be useful to diminish even residual platelet aggregation. Therefore, we think a combination of hTBM and C39 as regulators of plasmatic coagulation and platelet aggregation should be considered in future transgenic animals.

We observed in some combinations of blood donors and mono- or triple-transgenic cells platelet aggregation, whereas in other combination was no platelet aggregation detectable. Analysis of hTBM expression showed no weaker expression in the respective cells. The underlying cause of this variation remains unclear and should be further investigated in future studies. Furthermore, before experimental or clinical xenotransplantation donor ECs should be tested with the assays described in our study in combination with recipient blood to find out difficult recipient/donor combinations and to assess the risk of coagulopathy after transplantation.

Limitation of the study is that we were unable to recover endothelial cells from our flow chamber system with sufficient quality and reproducibility for the downstream assay used here. Therefore, we used the static 6-well assay as a preliminary approach to study EC activation markers as shown in Fig. 5. Another limitation is

the use of plasma. Whole blood adds a lot more complexity to the assay that may affect conclusions. However, our goal was to study the aspects of platelet and complement activation. This is better studied in systems that separate these biological systems from the many other aspects that whole blood would certainly add. Importantly, our experimental procedure only removes red cells and part of the leucocytes from the sample while platelets and antibodies, that may activate ECs, remain within. Therefore, we believe that the conclusions drawn with regard to complement and platelet activation are valid.

In recent animal studies, long survival times have been reported for transgenic kidneys with GTKO and human complement regulator in absence of thrombomodulin. The results showed that TMA was still developing, indicating that this might be an unsolved issue in the long term [17,33]. In these models, GTKO and a human complement regulator were combined with an immunosuppressive regime including a costimulation blockade.

While survival after xenogeneic kidney transplantation obtained by Adam et al. was impressive, animal studies with cardiac xenotransplants also emphasize the importance of hTBM. Both studies were performed on a GTKO background expressing a human complement regulator and based on immunosuppression with costimulation blockade. While immunosuppression without hTBM appeared quite effective, TMA was still present in these animals. In contrast, additional expression of hTBM lead to better survival and could prevent TMA, underlining the importance for clinical xenotransplantation [28,30,34]. The longest survival after cardiac xenotransplantation could be achieved only with triple-transgenic animals expressing hTBM in combination with GTKO and hCD46 [16].

In summary, data from this study indicate that expression of human thrombomodulin on PAEC conveys protection towards activation of coagulation but did not prevent activation of complement or endothelium. Some combination of donor and recipient revealed residual platelet aggregation. Our study underlines the importance of multi-transgenic pigs, based on GTKO in combination with a human complement regulator, human thrombomodulin and an additional transgene with platelet regulatory properties like CD39 in combination with a recipient screening using donor ECs.

Authorship

WR: wrote the paper, analysed and interpreted the data and performed the surgical procedures. DR: executed the experiments and drafted the manuscript. SW and AT: involved in research design and scientific supervision. SB: performed as technical assistance. MW: involved in scientific guidance. AW, AB, EW, NK and DA: contributed transgenic pig cells.

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Conflicts of interest

The authors have declared no conflicts of interest.

REFERENCES

1. Ierino FL, Sandrin MS. Spectrum of the early xenograft response: from hyperacute rejection to delayed xenograft injury. *Crit Rev immunol* 2007; **27**: 153.
2. Dai Y, Vaught TD, Boone J, *et al.* Targeted disruption of the [alpha] 1,3-galactosyltransferase gene in cloned pigs. *Nat Biotech* 2002; **20**: 251.
3. Byrne GW, McCurry KR, Martin MJ, McClellan SM, Platt JL, Logan JS. Transgenic pigs expressing human CD59 and decay-accelerating factor produce an intrinsic barrier to complement-mediated damage. *Transplantation* 1997; **63**: 149.
4. McGregor CG, Ricci D, Miyagi N, *et al.* Human CD 55 expression blocks hyperacute rejection and restricts complement activation in Gal knockout cardiac grafts. *Transplantation* 2012; **93**: 686.
5. Gollackner B, Goh SK, Qawi I, *et al.* Acute vascular rejection of xenografts: roles of natural and elicited xenoreactive antibodies in activation of vascular endothelial cells and induction of procoagulant activity. *Transplantation* 2004; **77**: 1735.
6. Cowan PJ, Roussel JC, d'Apice AJ. The vascular and coagulation issues in xenotransplantation. *Curr Opin Organ Transplant* 2009; **14**: 161.
7. Roussel JC, Moran CJ, Salvaris EJ, Nandurkar HH, D'Apice AJF, Cowan PJ. Pig thrombomodulin binds human thrombin but is a poor cofactor for activation of human protein C and TAFI. *Am J Transplant* 2008; **8**: 1101.
8. Kopp CW, Grey ST, Siegel JB, *et al.* Expression of human thrombomodulin cofactor activity in porcine endothelial cells. *Transplantation* 1998; **66**: 244.
9. Shimizu A, Hisashi Y, Kuwaki K, *et al.* Thrombotic microangiopathy associated with humoral rejection of cardiac xenografts from alpha1,3-galactosyltransferase gene-knockout pigs in baboons. *Am J Pathol* 2008; **172**: 1471.

10. Ramackers W, Friedrich L, Klose J, *et al*. Recombinant human antithrombin prevents xenogenic activation of hemostasis in a model of pig-to-human kidney transplantation. *Xenotransplantation* 2014; **21**: 367.
11. Wuensch A, Baehr A, Bogoni AK, *et al*. Regulatory sequences of the porcine THBD gene facilitate endothelial-specific expression of bioactive human thrombomodulin in single- and multitransgenic pigs. *Transplantation* 2014; **97**: 138.
12. Miwa Y, Yamamoto K, Onishi A, *et al*. Potential value of human thrombomodulin and DAF expression for coagulation control in pig-to-human xenotransplantation. *Xenotransplantation* 2010; **17**: 26.
13. Iwase H, Ekser B, Hara H, *et al*. Regulation of human platelet aggregation by genetically modified pifg endothelial cells and thrombin inhibition. *Xenotransplantation* 2014; **21**: 72.
14. Rataj D, Werwitzke S, Haarmeijer B, *et al*. Inhibition of complement component C5 prevents clotting in an ex vivo model of xenogeneic activation of coagulation. *Xenotransplantation* 2016; **23**: 117.
15. Galbusera M, Buelli S, Gastoldi S, *et al*. Activation of porcine endothelium in response to xenogeneic serum causes thrombosis independently of platelet activation. *Xenotrasnplantation* 2005; **12**: 110.
16. Mohiuddin MM. Chimeric 2C10R4 anti-CD40 antibody therapy is critical for long-term survival of GTKO.hCD46.hTBM pig-to-primate cardiac xenograft. *Nat Commun* 2016; **7**: 11138.
17. Adams AB, Kim SC, Martens GR, *et al*. Xenoantigen deletion and chemical immunosuppression can prolong renal xenograft survival. *Ann Surg* 2018; **268**: 564.
18. Längin M, Mayr T, Reichart B, *et al*. Consistent success in life supporting porcine cardiac xenotransplantation. *Nature* 2018; **564**: 430.
19. Wang L, Cooper DKC, Burdorf L, *et al*. Overcoming coagulation dysregulation in pig solid organ transplantation in nonhuman primates: recent progress. *Transplantation* 2018; **102**: 1050.
20. Cowan PJ, Robson SC. Progress towards overcoming coagulopathy and hemostatic dysfunction associated with xenotransplantation. *Int J Surg* 2015; **23**(Pt B): 296.
21. Ramackers W, Klose J, Tiede A, *et al*. Effect of TNF-alpha blockade on coagulopathy and endothelial cell activation in xenoperfused porcine kidneys. *Xenotransplantation* 2015; **22**: 284.
22. Petersen B, Ramackers W, Lucas-Hahn A, *et al*. Transgenic expression of human heme oxygenase-1 in pigs confers resistance against xenograft rejection during ex vivo perfusion of porcine kidneys. *Xenotransplantation* 2011; **18**: 355.
23. Ramackers W, Friedrich L, Tiede A, *et al*. Effects of pharmacological intervention on coagulopathy and organ function in xenoperfused kidneys. *Xenotransplantation* 2008; **15**: 46.
24. Schulte am EschJ, RogiersX, RobsonSC. Molecular incompatibilities in hemostasis between swine and men–impact on xenografting. *Ann Transplant* 2001; **6**: 12.
25. Sfrisio R, Bongoni AK, Banz Y, *et al*. Assessment of the anticoagulant and anti-inflammatory properties of endothelial cells using 3D cell culture and non-anticoagulated whole blood. *J Vis Exp* 2017; **127**: e56227.
26. Bongoni AK, Klymiuk N, Wolf E. Transgenic expression of human thrombomodulin inhibits HMGB1-induced porcine aortic endothelial cell activation. *Transplantation* 2016; **100**: 1871.
27. Kim H, Hawthorne WJ, Kang HJ, *et al*. Human thrombomodulin regulates complement activation as well as the coagulation cascade in xeno-immune response. *Xenotransplantation* 2015; **22**: 260.
28. Singh AK, Chan JL, DiChiaccio L, *et al*. Cardiac xenografts show reduced survival in the absence of transgenic human thrombomodulin expression in donor pigs. *Xenotransplantation* 2019; **26**: e12465.
29. Chan JL, Singh AK, Corcoran PC. Encouraging experience using multi-transgenic xenografts in a pig-to-baboon cardiac xenotransplantation model. *Xenotransplantation* 2017; **24**: e12330.
30. Mohiuddin MM, Singh AK, Corcoran PC. Genetically engineered pigs and target-specific immunomodulation provide significant graft survival and hope for clinical cardiac xenotransplantation. *J Thorac Cardiovasc Surg* 2014; **148**: 1106.
31. Yazaki S, Iwamoto M, Onishi A. Production of cloned pigs expressing human thrombomodulin in endothelial cells. *Xenotransplantation* 2012; **19**: 82.
32. Platt JL, Fischel RJ, Matas AJ, *et al*. Immunopathology of hyperacute xenograft rejection in a swine-to-primate model. *Transplantation* 1991; **52**: 214.
33. Kim SC, Mathes DV, Breeden CP. Long-term survival of pig-to-rhesus macaque renal xenografts is dependent on CD4 T cell depletion. *Am J Transplant.* 2019; **19**: 2174.
34. Iwase H, Ekser B, Satyananda V. Pig-to-baboon heterotopic heart transplantation–exploratory preliminary experience with pigs transgenic for human thrombomodulin and comparison of three costimulation blockade-based regimens. *Xenotransplantation* 2015; **22**: 211.