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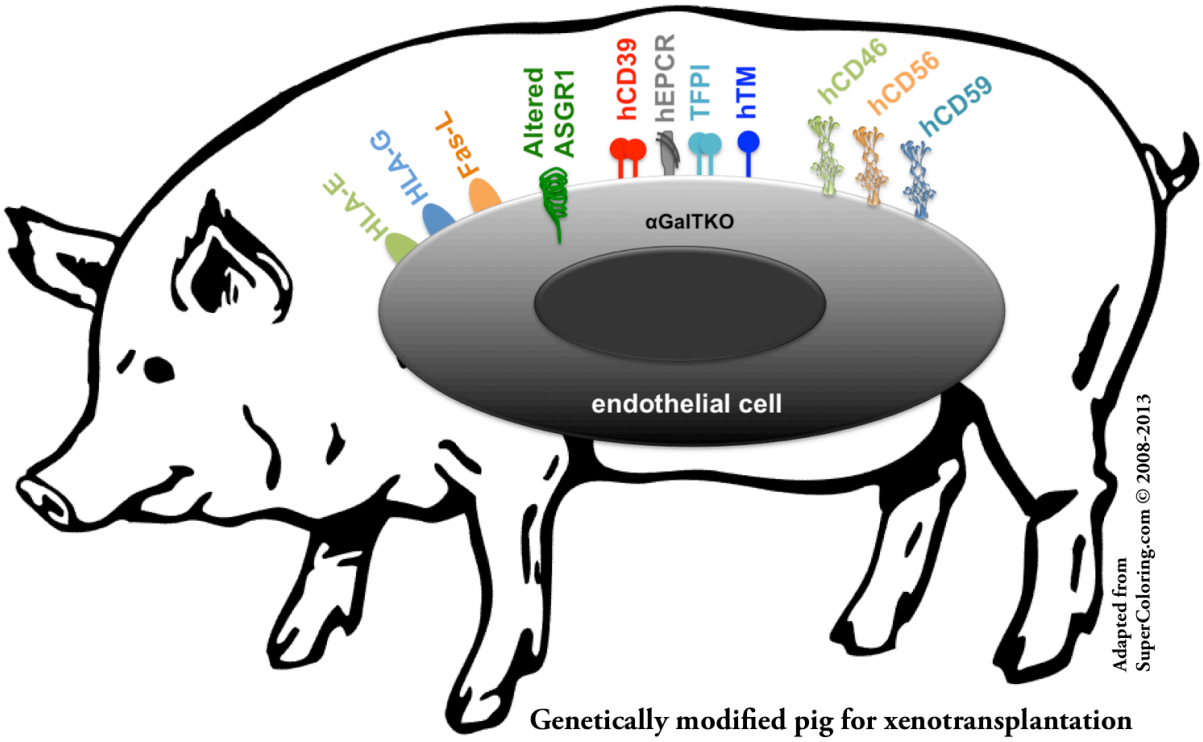
Functional evaluation of multiple human transgenes to overcome immunological and coagulation barriers associated with pig-to-human xenotransplantation



Genetically modified pig for xenotransplantation

PhD Thesis

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Functional evaluation of multiple human transgenes to overcome immunological and coagulation barriers associated with pig-to-human xenotransplantation

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Bern, Dean of the Faculty of Medicine

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To my Family

Preface

Pig-to-human xenotransplantation, particularly using genetically engineered pigs, is one of the possible alternatives to solve the critical and increasing shortage of human organs for the purpose of clinical transplantation. In the last few years, significant progress has been made in overcoming the immunological and pathophysiological barriers of xenotransplantation and thus to move one step closer to clinical trials of pig organ or cell transplantation. The generation of pigs lacking Galα1,3Gal (Gal) epitopes or overexpressing human complement regulators has largely overcome the barrier of hyperacute rejection. Though, delayed xenograft rejection remains a major hurdle, including humoral responses against non-Gal epitopes, and cellular innate immune responses. Other factors like thrombocytopenia, coagulation dysregulation and inflammation appear to play a significantly greater role in the rejection of xenografts than allografts. Numerous attempts to overcome these problems have been suggested, but the most convincing approach would, in any case, be the genetic manipulations in donor pigs.

The aim of my PhD was to study early immunological responses which contribute to xenograft rejection. In addition, expression and functional evaluation of (multi)-transgenic proteins in genetically modified pigs were performed using ex vivo and in vitro pig-to-human xenotransplantation models that are very much needed before implementing their organs into pre-clinical or clinical pig-to-(non)human primate transplantation.

In this thesis, I have included 3 accepted and published articles and two more projects are presented as manuscripts. I am the first author of 4 papers and to another one I contributed as a co-author. These manuscripts were prepared with a common xenotransplantation background, except one, the Bio-Plex paper. The thesis ends with a conclusion, acknowledgements and my curriculum vitae. In addition, I also contributed to other published studies as a co-author, and I listed them in my publications list.

At the end of this preface, I would like to thank my supervisor Prof. Robert Rieben for his expert guidance and support throughout the thesis. I would also like to thank my colleagues, friends and the other lab members for their direct and indirect contributions to this thesis. Finally, a big part of my acknowledgement goes to my wife Swathi Bongoni and my parents for their support, love and praises.

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Abstract

The transplantation of organs and cells of porcine origin into humans could overcome the critical and continuing problem of shortage of human donor organs and cells. The most profound barrier to pig-to-primate xenografting is the immunological rejection mediated by hyperacute and/or acute vascular rejection mechanisms. Hyperacute and acute vascular rejection, which are mediated by natural xenoreactive antibodies (mainly anti-Gal), can be overcome by complement- and/or antibody-modifying strategies. If hyperacute and acute vascular rejection mechanisms are prevented, the xenografts are, however, subject to cellular rejection mediated by innate immune cells, including NK cells, macrophages and neutrophils. In addition, the development of coagulation dysregulation has been identified as a formidable barrier for pig-to-primate xenotransplantation. It seems to be caused by the presence of several incompatibilities between pig and primate coagulation cascades. Use of pigs with new genetic modifications, including expression of human thrombomodulin, tissue factor pathway inhibitor or CD39, may solve this problem. These pigs are just becoming available for testing in nonhuman primates.

The main aim of this study was to examine early immunological responses that are involved in pig-to-human xenotransplantation. These include activation of the lectin pathway of complement and the genetic approaches, like alpha-Gal transferase knockout and transgenic expression of human complement regulatory proteins, to inhibit activation of the lectin pathway. The second objective was the establishment of an *ex vivo* xenoperfusion system to perfuse wildtype as well as genetically modified porcine forelimbs with heparinized, whole human blood. In this system, the protective effects of the transgenes against humoral and cellular responses were tested. Finally, an *in vitro* model was developed to explore pig-to-human molecular incompatibilities in the coagulation system and to test current genetic strategies to overcome them. This model also provides a platform for future studies to test genetic and pharmaco-therapeutic strategies aimed at overcoming xenorejection.

In conclusion, I could show that the lectin pathway is activated by human anti-Gal IgM and may play a pathophysiological role in pig-to-human xenotransplantation. Overexpression of hCD46 and HLA-E provided protection against complement- and NK cell-mediated responses, and the use of pigs with new genetic modifications, including expression of thrombomodulin, may help to overcome the incompatibilities in the coagulation systems between pig and human.

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Introduction

1 Organ transplantation

Allogeneic organ transplantation is well-established and universally accepted medical treatment for end-stage organ failure. The shortage of organs and tissues from deceased individuals for the purpose of clinical transplantation is well known, and continues to restrict allotransplantation. Although, at present, there are over 120,000 patients waiting for a donor organ in the USA, 18 individuals will die each day while waiting for an organ. During the current year, less than 30,000 of donor organs will become available (Figure 1) (1). According to NHS Blood and Transplant, there are 10,000 patients needing new organs in the UK — most commonly liver, kidneys, hearts — and three die every day while waiting (2). The gap between the number of available transplantable organs and patients on the waiting list grows to widen each year, and it remains exceedingly unlikely that human organs will meet the demand for organ transplantation. The situation is even worse for patients with diabetes mellitus, who are in need of cell transplantation, such as islet transplantation. In the USA, while many of the 2-3 million patients with Type 1 diabetes would benefit from pancreatic islet transplantation, the potential supply of islets from deceased human donors each year (<7000) will undoubtedly never be sufficient to treat the millions of patients with diabetes (1-3).

2 Xenotransplantation

Alternatives to clinical allotransplantation include stem cell therapy, artificial organs and the use of organs and cells from other species, i.e., xenotransplantation. The progress towards creation of artificial organs for permanent treatment of organ failure in humans is slow; thus, the use of living organs, tissues and/or cells from animals to humans (xenotransplantation) may still be the most promising approach to significantly expanding the pool of available donor organs for humans. This approach has the added advantage of permitting genetic modification of the donor organ/animal to improve molecular compatibility that could possibly reduce the use of anti-rejection treatment of the recipient, which is often associated with a number of adverse effects (4-7). The concept of xenotransplantation has been known for centuries, as there have been a number of clinical attempts during past 300 years or more. The first transplantation attempt was performed in the early 16th century

without any knowledge of immunological species barriers. In 1667, Jean Baptiste Denis began the first documented xenotransplantation trail, a lamb-to-human blood transfusion (8). Another xenotransplantation took place in 1682, when a physician repaired the skull of a Russian nobleman with a piece of bone from the skull of a dog. In 19th century, Serge Voronoff, a famous French physician, carried out a significant number of chimpanzee or baboon testicular transplantations to man, which was known as "human revitalization transplantation" (9-11).

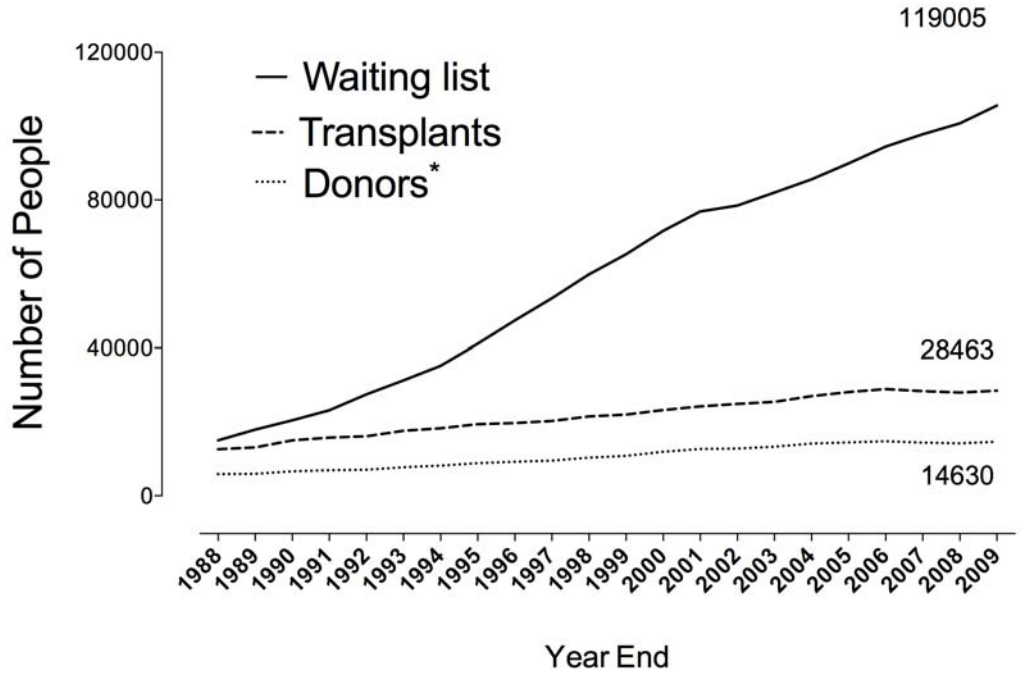

Figure 1: Over the past three decades, the gap between the number of patients waiting for a transplant and the number of patients receiving a transplant has continued to widen, and it is one of the important factors that contribute to waiting time from listing to transplant. Data from optn.transplant.hrsa.gov and OPTN/SRTR Annual Report (2009) (1). *Data include deceased and living donors.

Later, in 1963, several chimpanzee-to-human kidney transplantations were performed and the most successful was documented by Reemtsma et al. in 1964, whereby the graft lasted for 9 months without any rejection symptoms, but later perished due to a severe electrolyte imbalance (12). In 1967, Barnard realized the first heart human-to-human transplantation (13). Over the past 50 years, significant progress was made in the understanding the barriers to xenotransplantation and the possible solutions that would help overcome them (14). These achievements indicated that long-term survival and function of a xenograft might be possible in humans, even with the relatively ineffective immunosuppression available at that time (15-18).

2.1 Choice of animal as donor organ source for xenotransplantation

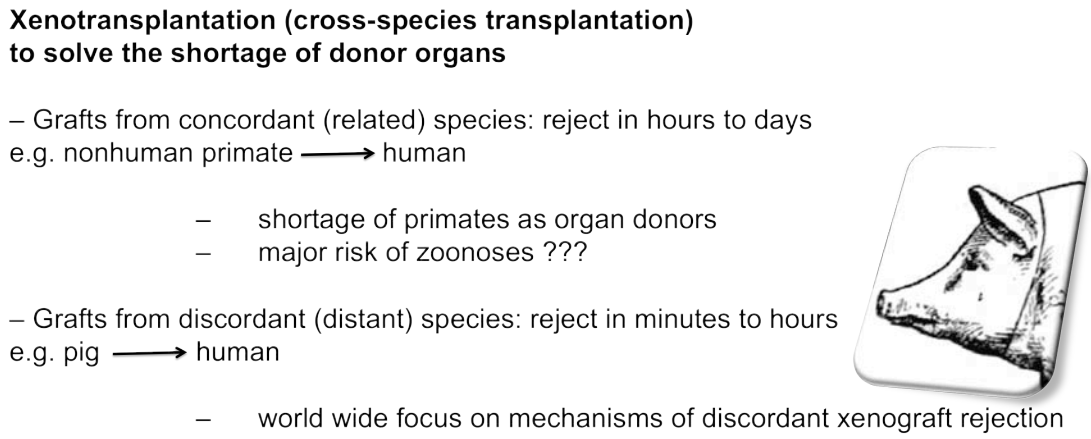

Figure 2: Key considerations in the choice of a donor species for xenografting, adapted from (19).

Two main types of xenotransplantation might be applied clinically: transplantation of primarily vascularized organs such as heart or kidney, and transplantation of tissues or cells, such as islet cells, bone marrow, and neural cells, among many others. Transplantation of vascularized xenografts will undergo immediate rejection process, as the endothelium exposed to the recipient's circulation results in rapid immune responses against xenograft. Due to complex requirements that an animal species must meet in order to be appropriate organ donor, no such an animal exists. Although non-human primates are most similar to humans anatomically and physiologically, for a number of reasons, they are not considered a suitable source of organs for clinical transplantation. These species represent the potentially high risk for infectious disease transmission, breeding difficulties, organ size differences and other impracticalities, as well as ethical issues, which have largely excluded them from further consideration (20). Pig is now generally accepted to be the species of choice for transplantation, despite it being 'discordant' or phylogenetically more distant from humans, and posing the additional immunological barrier of instantaneous rejection (21, 22), (23). Several reasons for pig still being favored include physiological similarities with human, high reproductive capacity, reduced cost of production, reduced risk of xenozoonoses, maintenance under hygienic specific pathogen-free conditions, potential for genetic modifications, and minimal

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ethical concerns. These factors make pigs, rather than non-human primates, the most promising organ donors to humans (11).

	Pig	Baboon
Availability	Unlimited	Limited
Breeding potential	Good	Poor
Period to reproductive maturity	4 – 8 months	3 – 5 years
Length of pregnancy	114 ± 2 days	173 – 193 days
Number of offspring	5 – 12	1 – 2
Growth	Rapid (adult human size Within 6 months)**	Slow (9 years to reach maximum size
Size of adult organs	Adequate	Inadequate*
Cost of maintenance	Significantly lower	High
Anatomical similarity to humans	Moderately close	Close
Physiological similarity to humans	Moderately close	Close
Relationship of immune system to humans	Distant	Close
Knowledge of tissue typing	Considerable (in selected herds)	Limited
Necessity for blood type compatibility with humans	Probably unimportant	Important
Experience with genetic engineering	Considerable	None
Risk of transfer of infection (xenozoonosis)	Low	High
Availability of specific pathogen-free animals	Yes	Yes
Public opinion	More in favor	Mixed

Notes: *The size of certain organs, eg, the heart, would be inadequate for transplantation into adult humans; **breeds of miniature swine are approximately 50% of the weight of domestic pigs at birth and sexual maturity, and reach a maximum weight of approximately 30% of standard breeds.

Table 1: Advantages and disadvantages of the pig vs. baboon as a potential source of organs and cells for humans, modified from (11, 21).

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3 Immunobiology of pig-to-human xenotransplantation

During the last century, the development of xenotransplantation has defined many challenges in terms of cross-species immunology and physiology that must be addressed before clinical implementation (20). The major barrier is that the xenografts are subjected to severe and irreversible rejection mechanisms as a result of immunological and physiological differences between pig and human (24, 25). Based on small and large animal models, the immunological barriers for discordant xenografts are hyperacute rejection and acute vascular rejection. If hyperacute and/or acute vascular rejection are averted (for example, by complement inhibition) the graft may undergo accommodation, a condition in which the graft appears to resist injury despite the presence of anti-donor antibodies and the complement of the recipient. The graft may also undergo cellular rejection and chronic rejection, as that observed in allografts, if acute vascular rejection is avoided. Another potential obstacle is the risk of transmission of infectious agents, in particular porcine endogenous retroviruses (26, 27).

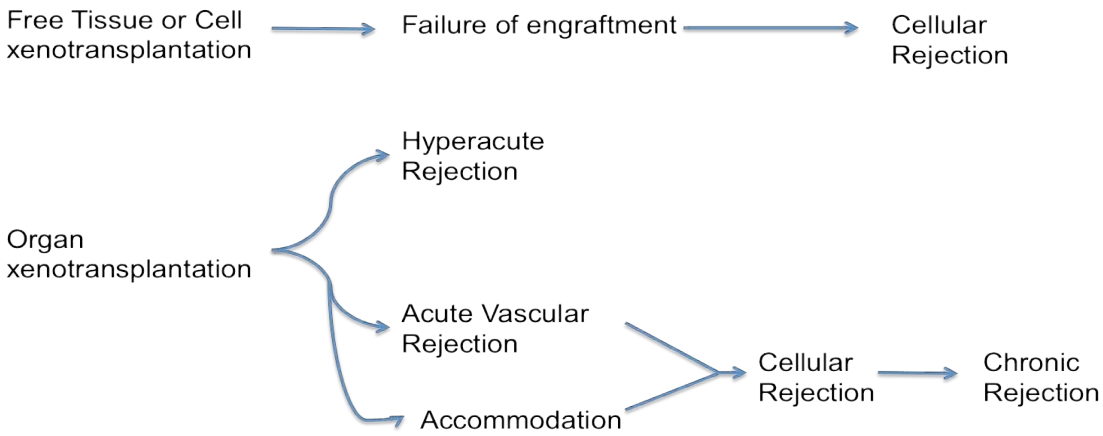

Figure 3: The immunological responses to xenotransplantation, modified from (27).

4 Hyperacute rejection

Hyperacute rejection is a very rapid event that results in irreversible graft damage and loss within minutes to hours of transplantation (28). It involves both immunological and non-immunological factors, and has been characterized historically by the rapid onset of edema, hemorrhage and vascular thrombosis (29-31). However, it is triggered by the presence of preformed xenoreactive natural antibodies, mostly IgM (as well as IgG, in the case of rodents) (32-35).

Xenoreactive natural antibodies are similar to those produced naturally against blood group antigens (36). Many studies have now conclusively demonstrated that xenoreactive antibodies that recognize the trisaccharide group, galactosyl α-(1,3)-galactosyl β-1,4-N-acetyl glucosaminyl (Gal) are the most important in the onset of hyperacute rejection (37). While these saccharides are abundantly expressed on endothelial cells of non-primate mammals and New World monkeys, they are absent in humans, apes, and Old World monkeys, as these species evolutionarily lack the α1,3-galactosyltransferase (GalT) gene to synthesize Gal (38). Upon encounter of Gal expressing microorganisms in the gastrointestinal tract in early life, they generate anti-Gal antibodies (39). They exist as IgM, IgG and IgA classes (40). It is estimated that these antibodies constitute 1% of immunoglobulins in the circulation (41). It is widely accepted that natural antibodies initiate hyperacute rejection by activating the complement cascade, as well as endothelial cell activation and damage. It leads to platelet activation, aggregation and adhesion, as well as coagulation and disruption of vascular endothelial integrity (42). The essential role of IgM anti-Gal antibodies in hyperacute rejection is well documented in pig-to-primate xenotransplantation models with a strong correlation of binding of anti-Gal antibodies to endothelium and ability to fix complement (32, 43).

Shedding of heparan sulfate proteoglycan and loss of anticoagulants, including thrombomodulin, tissue factor pathway inhibitor (TFPI) and co-adenosine diphosphatase (ectoADPase), may be pivotal for endothelial dysfunction. This changes the healthy anti-inflammatory and anticoagulant endothelial glycocalyx into a pro-inflammatory and procoagulant form (44-46). In addition, activated pig endothelial cells express von Willebrand factor (vWF) that can interact with human glycoprotein 1b on platelets, independently of shear stress (47-49).

4.1 The complement system

The complement system is a highly conserved group of proteins and glycoproteins in blood and tissue fluids, whose role is to assist antibodies in clearing pathogens and foreign organisms. It is composed of 30 different proteins, including plasma, serosal and membrane-bound proteins, working together in a highly regulated complex cascade, as part of the innate immune system. The system can be activated by at least three well-characterized pathways, namely the classical, the alternative and the lectin pathway. Each of the three complement pathways has its own mechanism of target recognition and activation that results in the activation of the central factor C3, followed by the activation of the common terminal complement pathway. It finally leads to formation of C5b-9 — the membrane attack complex (MAC) (50).

Hyperacute rejection initiated by (1) binding of xenoreactive natural antibodies that activate the classical and/or the lectin pathway of complement, (2) direct activation of host complement through the graft endothelium, and/or (3) failure of complement regulation (51). The cascade begins with the formation of the C1 complex (C1q, C1r and C1s) and MBL/MASPs complex. Activated C1r/C1s or MASPs cleave C4 and C2 to generate C3 convertase to build up C5 convertase and proceed toward the formation of the MAC. Alternative pathway can occur by directly activating C3 when exposed to xenogeneic endothelium, in the absence of immune complexes (52, 53). Complement activation leads to a number of events that can contribute to the induction of xenograft damage. The most obvious effect of membrane-inserted MAC can be direct cell lysis by pore formation and thereby loss of cell membrane integrity (54). In addition, upregulation of cell adhesion molecules and increased production of cytokines and chemokines result in subsequent leukocyte adhesion and graft infiltration by inflammatory cells (55, 56). Furthermore, the anaphylotoxins C3a and C5a act as chemoattractants, inducing endothelial cell activation and modulating vascular tone and permeability y (57-59).

Under physiological conditions, the activation of complement cascade is controlled by an array of complement regulatory proteins present in the plasma and on cell membranes. A number of membrane-bound complement regulatory factors (CRFs) are described in pertinent literature. Complement receptor 1 (CR1, CD35) is a receptor for C3b and C4b, which also acts as a cofactor for factor I mediated cleavage of C3b and C4b and inhibits C3 convertases from all pathways. Decay accelerating factors (DAF, CD55) and membrane co-factor proteins (MCP, CD46) also inhibit complement activation at the C3 convertase step, while CD59 prevents

formation of C5b-9 or MAC. Moreover, there is a series of serum phase CRFs, which include C1 inhibitor, C4 binding protein, factor H, clusterin (apo-J) and S protein (vitronectin). The fluid-phase regulators act primarily to prevent uncontrolled activation of complement in the fluid phase, whereas the membrane-bound regulators directly protect the host cell from complement attack (reviewed in (60, 61)).

The development of hyperacute rejection depends on formation of the terminal C5b-9. It was supported by the two lines of studies, hyperacute rejection does not occur in inherently C6-deficient recipients (62, 63) and complement inhibition using anti-C5 antibodies prevents some of the features of hyperacute rejection (64). On the other hand, empirical evidence indicates that organs from human CD59-transgenic pigs that significantly resist assembly of C5b-9 and some features of tissue injury still undergo hyperacute rejection (65). These observations suggest that the development of hyperacute rejection may not solely depend on the formation of the C5b-9. This may be supported by the ability of C5b67 complexes to disrupt the integrity of endothelial layers (66), as well as contribution of C3a and C5a mediated proinflammatory reactions (reviewed in (51)).

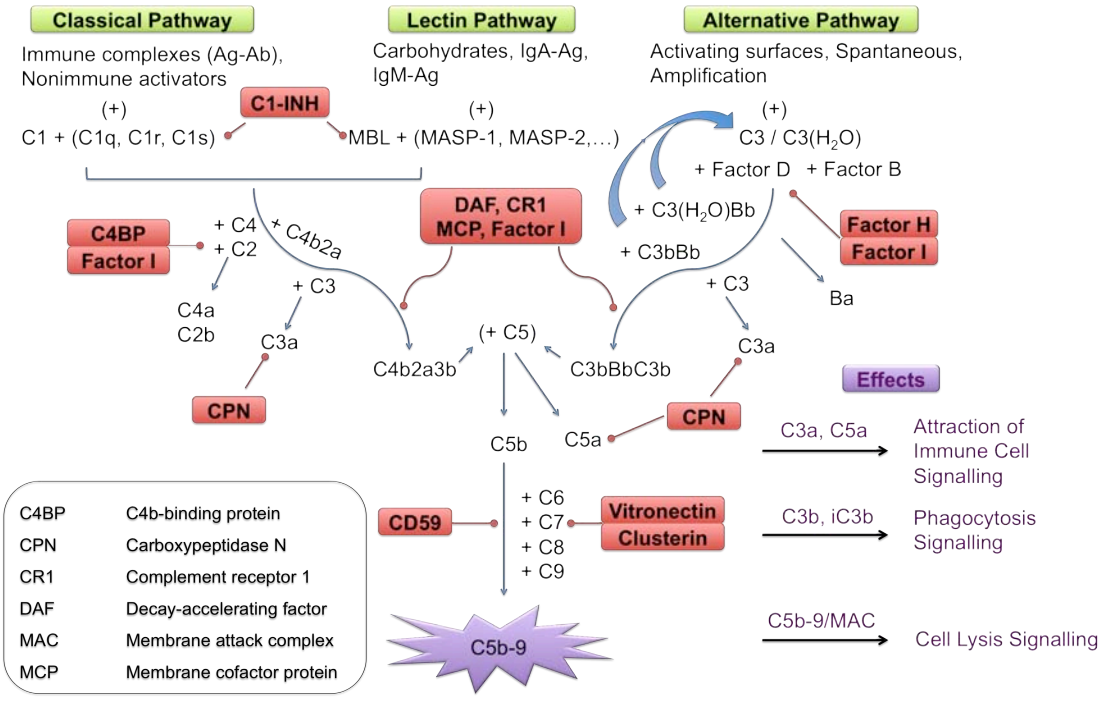

Figure 4: Overview of the complement system – activation and regulation, modified from (67, 68).

4.2 Vascular endothelium

As noted above, natural antibodies and complement systems can induce total destruction of xenograft tissue. The underlying mechanism for such damage involves mainly activation of graft endothelium, the inner layer of blood vessels. It is the first site of xenograft in contact with recipient blood and represents the initial barrier to transplantation via hyperacute rejection. Under physiological conditions, resting endothelial cells provide anti-inflammatory, anti-coagulant and anti-platelet aggregatory intravascular environment (69, 70). Essentially, most surface proteins on the endothelium coating, the glycocalyx, are proteoglycans with *O-glycosyl* linkage that are typically characterized by up to 200 sugar residue-long, un-branched side chains (71). The majority (50-90%) of proteoglycans associated with the endothelium are so-called heparan sulfate proteoglycan (HSPG) with a varying number of glycosaminoglycan (GAG) side chains (mostly HS on the endothelium) (72). Heparan sulfate, attached to the endothelial cell surface, provides anti-oxidant and anti-coagulant effects via binding and activation of anti-thrombin III and superoxide dismutase. By enhancing the activity of TFPI and antithrombin III, the glycocalyx markedly regulates the anti-coagulant and anti-inflammatory properties of the endothelium (73-75). In addition, the balance between endothelial thrombomodulin, tissue factor and plasminogen activator inhibitors (PAI) equally contribute to an anticoagulatory state (44, 76).

In response to inflammatory mediators (IL-1, TNF, C5b-9), endothelium adopts proinflammatory and procoagulant state by expressing tissue factor and by enhancing prothrombinase assembly, as well as by attenuating thrombin inhibition. These changes are also associated with the production of cytokines and the increased expression of adhesion molecules, such as E-selectin, VCAM-1 on the endothelium. Collectively, this process is defined as endothelial cell activation. Pober and Cotran classified endothelial cell (EC) activations into an early ‘Type 1’ activation that occurs independent of gene activation or protein synthesis, or a later phase, ‘Type 2’, activation that is associated with protein synthesis (69).

4.2.1 Type I endothelial activation

In hyperacute rejection, Type 1 endothelial cell activation occurs following damage by complement fixing antibody. IgM is the classically described immunoglobulin subclass in this setting (30). Type I activation involves three detrimental elements of the early EC response resulting in stagnation of blood flow, promotion of leucocyte adhesion and initiation of thrombosis. The first is the release of factors that modulate vessel tone and platelet function with the release of the prostaglandins and endothelium-derived relaxation factor-nitric oxide (77, 78). The second is rapid mobilization of P-selectin from cytoplasmic Weibel-Palade bodies to the EC surface, which enhances neutrophil adhesion (79). In vivo, upregulation of P-selectin can occur as a result of endothelial cell stimulation by thrombin, histamine or MAC. The third pertains to the development of an overwhelming pro-coagulant state, accompanied by the simultaneous loss of EC surface natural anticoagulants, including HS, antithrombin III, TFPI and thrombomodulin. Endothelial retraction, exposing the subendothelial matrix and tissue factor, as well as additional EC expression of tissue factor initiates the clotting cascade (80). Additional events include loss of membrane ectoADPase (contributing to platelet aggregation), release of platelet activating factor, and production of PAIs (44, 81), C5a-induced loss of heparan sulphate and exposure of foreign carbohydrates, such as sulphatides, which can activate the contact system of coagulation (82).

4.3 Complement regulation in xenotransplantation

Rather than by the administration of immunosuppressive agents, which are largely ineffective, the prevention of the innate immune response has been approached by the genetic engineering of the organ-source pig. There were several key reasons for initially producing genetically modified pigs by overexpressing one or more human (h) CRPs on the endothelial cell surface. First, when the addition of hCRPs to porcine tissue was proposed, it was thought that incompatibility between pig and human CRPs ("homologous restriction") resulted in uncontrolled complement activation (83, 84). Indeed, CD59 was initially known as homologous restriction factor. In vitro evidence showed that the complement regulators in pigs were inefficient or inactive against human complement (85-88). However, findings of recent studies indicate that the transgenic expression of hCD55 or hCD46, with or without hCD59, significantly abrogated hyperacute rejection of pig-to-primate vascularized organs despite the presence of high titer anti-Gal antibody. This effect was much greater than could have been expected from simply circumventing homologous restriction (reviewed in (89)). Thus, the concept of homologous restriction of CRFs has been questioned. Vandenberg and Morgan found that the analogues of CD59 from different species could inhibit complement from many different sources (90, 91). Similar results were seen with CD46 (92, 93). It has also been suggested that supraphysiological levels of CRPs could overcome reduced cross-species effect (94). In addition, Paul Morgan showed that pig CRPs were perfectly effective against human complement. Thus, quantity of CRPs expression, rather than species specificity, is important (95, 96). However, one might consider using pigs transgenic for hCRPs, as they are all receptors for human tropic viruses, such as echovirus and measles (reviewed in (89)).

Owing to the marked improvements in the efficiency of cloning strategies, the gene for the pig GalT enzyme that is required for Gal expression was successfully knocked out by homologous recombination using somatic-cell nuclear transfer technology. In 2003, pigs engineered to lock the gene for GalT (GalTKO) were first made available for experimental studies. As predicted, the transplantation of organs (heart & kidney) from GalTKO pigs into non-human primates reduced the frequency of hyperacute rejection. Thus, the GalTKO pig is now the basic standard platform for other current and future genetic solutions to xenotransplantation (reviewed in (7)). The importance of anti-nonGal antibodies in the humoral response to a GalTKO pig organ was highlighted by Cowan and d'Apice. In GalTKO pig organ xenotransplantation,

interaction of anti-nonGal antibodies and non-Gal antigens mediate complement activation via both the classical and the alternative pathway, especially after pig islet transplantation. Without complete suppression of production of these antibodies, there is progressive activation and injury of the graft endothelium, resulting in thrombotic microangiopathy, with subsequent graft loss. Five years after the first results of GalTKO pig-to-nonhuman primate organ transplantation were reported, the addition to GalTKO of one or more hCRPs has been associated with increased protection compared to either manipulation alone. Therefore, expression of multiple hCRP genes in GTKO pigs is probably preferable, rather than relying on a single gene (reviewed in (97-99)).

4.4 Coagulation system

Coagulation is vital to the normal hemostatic response to vascular injury. Various factors present in the inactive state in the blood participate in the coagulation system. These coagulation factors may be categorized into substances, cofactors and enzymes. Fibrinogen is the main substrate in the coagulation cascade, and the cofactors — including tissue factor, factor V, factor VIII, and Fitzgerald factor — accelerate the enzyme activities. When activated, all the enzymes, except factor XIII, are serine proteases. On the basis of their physical properties, the coagulation factors may also be categorized into three groups, namely (1) the contact proteins — factor XII, XI, prekallikrein and high molecular weight kininogen (HMWK); (2) the thrombin proteins — factor II, VII, IX and X; (3) and the fibrinogen or thrombin sensitive proteins — factor I, V, VIII and XIII (reviewed in (100-102)).

The main mechanism of coagulation cascade activation in inflammation is mediated by exposure of tissue factor to the circulation (103). The final aim of the coagulation cascade includes fibrin polymerization that seals the endothelial breach with a fibrin-enmeshed plug to stop bleeding. When the integrity of vessel wall is disrupted, circulating blood cells or endothelial cells start to express tissue factor in response to proinflammatory stimuli. Upon exposure, tissue factor binds to FVII and the complex tissue factor-FVIIa, as part of the extrinsic pathways, initiates the activation of the coagulation cascade via the conversion of down-stream serine protease factors FIX and FX into activated forms FIXa and FXa. The intrinsic pathway involves other serine proteases, such as FXII, FXI and FIX, which are sequentially activated upon contact with negatively charged surfaces (contact activation). The extrinsic and intrinsic pathways converge at the stage of the FXa/FVa complex, which cleaves prothrombin (FII) to thrombin (FIIa), whose key function is to convert fibrinogen to fibrin. Thrombin generated by activated coagulation cascade is a multifunctional molecule; in addition to fibrin conversion, thrombin itself is one of the strongest activators of platelets, inflammatory cells (leukocytes and endothelial cells) with consequent increased production of inflammatory mediators and increased leukocyte adhesion and chemotaxis. Moreover, it is also involved in feedback activation of coagulation factors V, VIII, XI and XIII (reviewed in (100, 104)).

Fibrinolysis is the physiological process that removes insoluble fibrin clots through enzymatic digestion of the cross-linked fibrin polymers. This system is initiated when plasminogen is converted into the potent serine protease plasmin, which leads to degradation of fibrin, fibrinogen, FV and FVIII. The main activators of this

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plasminogen are tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA), kallikrein or factor XII. It has been shown that severe trauma and hemorrhage is associated with decreased plasminogen levels, as well as increased plasma levels of plasmin and plasmin-anti plasmin complexes, indicating early fibrinolytic events. Inhibitors of fibrinolysis include α2-pasmin inhibitor, plasminogen activator inhibitor-1 (PAI-1) and -2 (PAI-2). Fibrinolysis is also initiated by activated protein C (APC) by interfering with plasmin inhibitors, PAI-1 and thrombin-activatable fibrinolysis inhibitor (TAFI) (100, 107, 108).

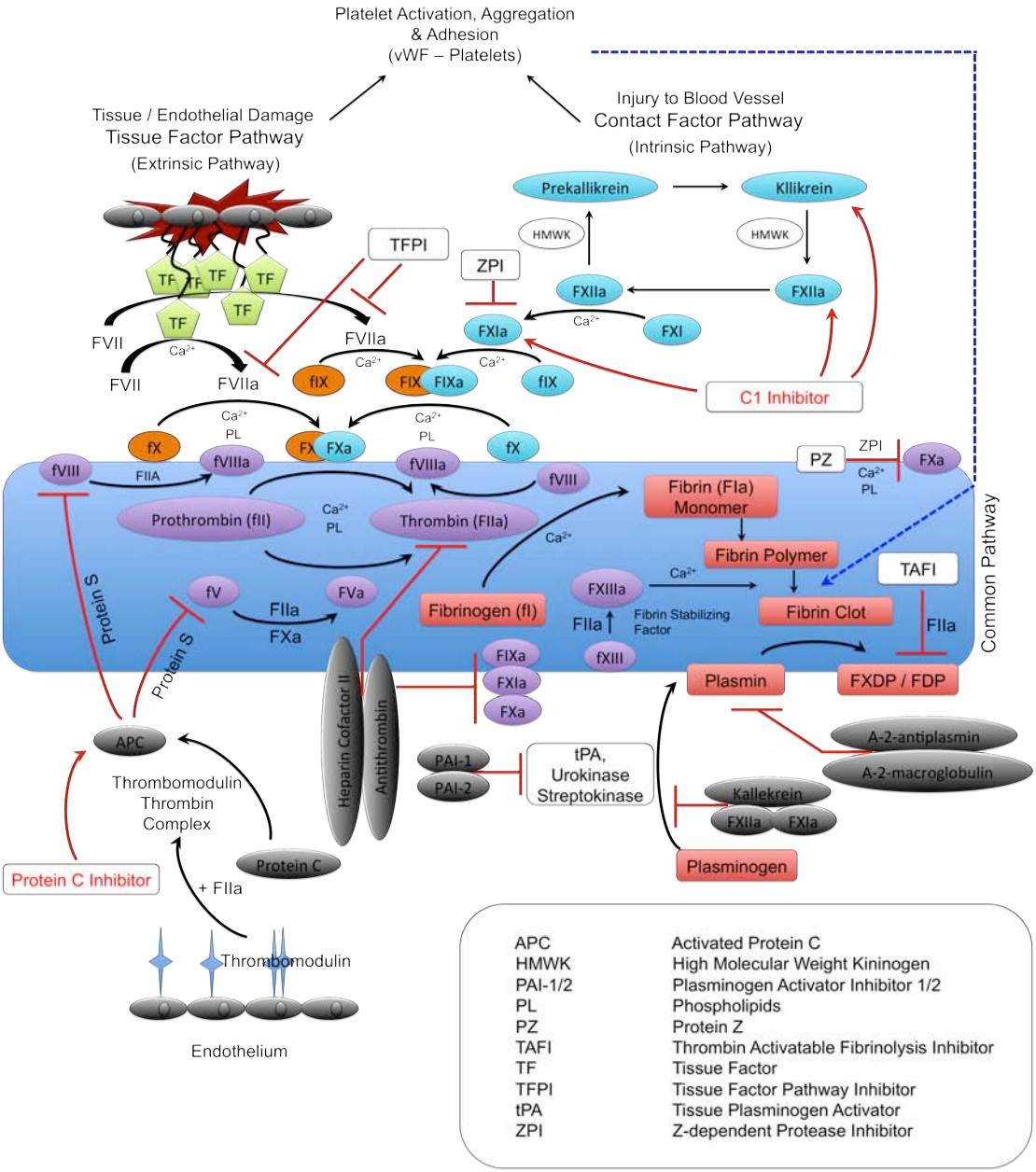

Figure 5: Schematic of the extrinsic and intrinsic coagulation cascade, adapted from (105, 106).

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4.5 Platelets

Platelets are anuclear fragments derived from the bone marrow megakaryocytes. Two major intracellular granules present in the platelets are α-granules and the dense bodies. The α-granules contain platelet thrombospondin, fibrinogen, fibronectin, platelet factor 4, vWF, platelet-derived growth factor, β-thromboglobulin, and FV and FVIII. The dense granules contain ADP, ATP, and serotonin. When platelets aggregate, they release both the α-granules and dense bodies, lose their membrane integrity, and form plug (109). In addition, platelet aggregates provide localized environment that supports plasma coagulation through the release of micro-platelet membrane particles rich phospholipids and various coagulation proteins (104). Activated platelets secrete IL-1β, which is a major activator of endothelial cells. The platelet and injured vessel wall interaction involves a series of events that include platelet adhesion to endothelial components, activation, structural change, platelet granule release and stabilization of fibrin formation and clot retraction. This interaction of platelets with endothelial cells results in secretion of IL-6 and MCP-1 from endothelial cells, as well as the expression of adhesion molecules E-selectin, VCAM-1 and ICAM-1 on endothelial cells. Ultimately, it leads to activation of platelets, whereby the exposure of negatively charged phospholipids facilitates the assembly of coagulation factors, leading to the generation of thrombin and subsequent fibrin deposition (110, 111).

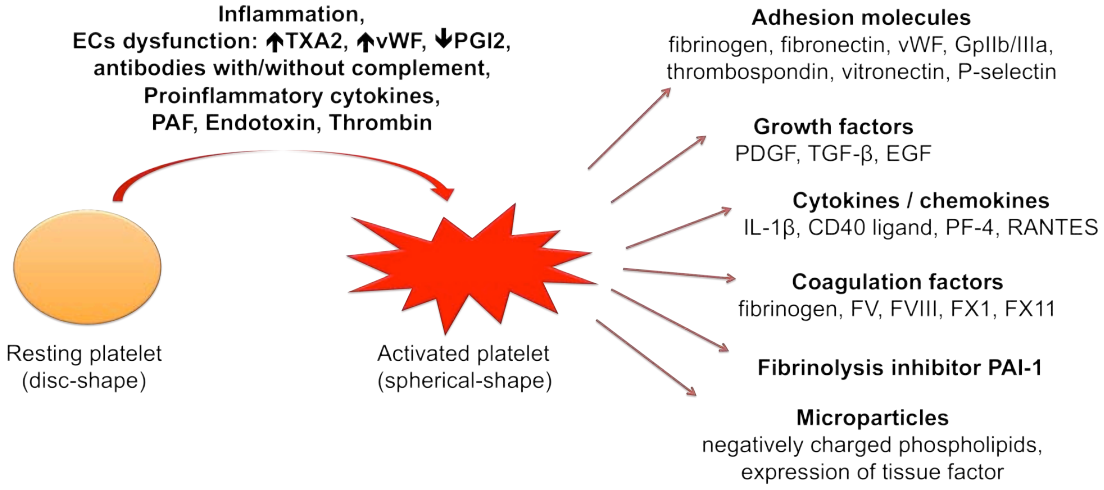

Figure 6: Platelet activation during inflammation, adapted from (107). EC – endothelial cell; vWF – von Willebrand factor; TXA2 – thromboxane A2; PGI2 – prostacyclin; PAF – platelet activating factor; GpIIb/IIIa – glycoprotein complex IIb/IIIa; PDGF – platelet derived growth factor; TGF – transforming growth factor; EGF – epidermal growth factor; PF4 – platelet factor 4; PAI – Plasminogen activator inhibitor;

In AVR, the interactions between porcine endothelial cells, platelets and other blood cells are at the nexus of a complex network that contributes to coagulation. Together with loss of anticoagulant regulators, such as TFPI, thrombomodulin, or HS, or through fgl-2 conversion, thrombin and fibrin are deposited in the graft as a result of intrinsic platelet activation (82, 112). In addition, ineffective degradation of ADP by the loss of ectonucleoside triphosphate diphosphohydrolase-1 (CD39) and eccto-5'-nucleotidases (CD73) aggravates the activation and aggregation of platelets (113, 114). Administration or transgenic expression of CD39 substitutes significantly prolonged graft survival by inhibiting platelet activation and aggregation. Furthermore, molecular incompatibilities also directly contribute to the activation of platelets. Recent findings indicate that primate platelets are activated to express tissue factor when incubated with porcine endothelial cells in the absence of the involvement of antibodies and complement. This effect is distinct from the effects of porcine vWF on human platelet aggregation (115). In addition to proinflammatory mediators, activated platelets express surface ligands, such as glycoprotein IIb/IIIa, that increase platelet aggregation in response to various stimuli. Through the expression of P-selectin, activated platelets bind avidly to leukocytes. Additionally, the secretion of ADP from dense granules recruits other platelets to the site of injury and enhances aggregation through the receptor P2Y12 (116). Activated platelets also exert a direct procoagulant effect on endothelial cells and circulating peripherally activated platelets contribute to consumptive coagulopathy (reviewed in (117)).

The fate of activated platelets in a xenograft recipient may differ depending on the organ transplanted. Thrombocytopenia can be defined as a disorder characterized by an abnormally low number of platelets (<50 x 10^3/mm^3 in humans), determined by standard assays. Following porcine liver transplantation in baboons, a propound thrombocytopenia develops within minutes, thus precluding prolonged survival of the recipient. This phenomenon has also been observed in the pig-to-baboon kidney transplantation model (118). The main mechanisms by which thrombocytopenia develops are not fully understood. Investigation of causes and kinetics of thrombocytopenia is necessary to develop appropriate therapeutic interventions. Platelet activation and increased platelet-white blood cell (WBC) aggregation and platelet-WBC subtypes occurred immediately after porcine liver xenotransplantation, indicating that a low platelet count does not necessarily indicate loss of platelets from the circulation (119). In addition, ex vivo perfusion of porcine liver with human platelets resulted in platelet phagocytosis by pig liver sinusoidal endothelial cells

(LSEC) through asialoglycoprotein receptor-1 (ASGR1) and hepatocytes. Aggregation and phagocytosis of baboon platelets by pig LSEC has also been documented. Pig LSEC mediated phagocytosis of xenogenic platelets can be prevented by blocking ASGR1 and/or integrin adhesion pathways (120, 121).

The Ashwell–Morell asialoglycoprotein receptor (ASGR) is expressed by the LSEC, on the surface of Kupffer cells (KC) and hepatocytes. It represents one of the lectins that bind and endocytosis circulating glycoproteins. It can function as a recycling receptor for desialylated glycoproteins and other molecules with terminal galactose or N-acetyl-galactosamine residues (122, 123). Proposed functions include the clearance of asialoglycoproteins (124), antigen-antibody complexes (125) and apoptotic cells (126). Since the surface of many eukaryotic cells is rich in glycoproteins possessing terminal galactose/N-acetyl-glactosamine residues, ASGR1 may also participate in cell-cell, cell-matrix or intramatrix interactions (127). Desialylated platelets are also recognized and cleared by this receptor, which thereby demonstrates a protective effect against disseminated intravascular coagulation. The receptor also seems to be involved in vWF-linked hemostasis, as an increased vWF half-life and circulating vWF in Asgr-1-deficient mice has been shown (128).

During liver xenotransplantation, porcine LSEC mediated acute thrombocytopenia was observed. Furthermore, most human platelets (93%) bind and are taken up by LSEC and KC within 15 to 30 min following addition, based on the findings pertaining to in vitro and ex vivo wild-type liver perfusion with human platelets. Early biopsies of ex vivo perfused liver suggested phagocytosis of intact platelets by LSEC, and later biopsies showed degraded platelets within hepatocytes, suggesting that platelets were transferred from LSEC to hepatocytes via transcytosis. ASGR1 expressed by pig LSEC and macrophage antigen complex-1 (Mac-1) by KC participated in this process, as confirmed by in vitro studies using knockdown of these proteins by siRNA. A recent study revealed that, when compared to porcine platelets, human platelets express four times more β1-4 N-acetyl glucosamine and N-acetyl glucosamine β1-4 N-acetyl glucosamine ligands for ASGR1 and Mac-1 (120, 123). However, expression of ASGR1 on porcine aortic or arterial endothelial cells and its role in xenogenic platelet phagocytosis has never been studied.

5 Acute vascular rejection

If hyperacute rejection is avoided by depleting xenoreactive antibodies or by controlling complement activation, xenografts are rejected within days, instead of minutes to hours, by a process termed to as acute vascular rejection (AVR), delayed xenograft rejection, or acute humoral xenograft rejection. The pathology of AVR is not very different to that observed in hyperacute rejection, commencing with the deposition of fibrin and upregulation of tissue factor, and ultimately resulting in vascular thrombosis and edema. AVR is also characterized by progressive (over several days) infiltration of mononuclear cells (primarily monocytes and natural killer (NK) cells), gross disruption of the microvasculature with widespread activation of coagulation and intravascular thrombosis, interstitial hemorrhages, focal areas of infarction and cessation of graft function (4, 5, 129). In addition, AVR is distinguished from hyperacute rejection by its delayed kinetics following transplantation and the observed initial graft function before the onset of rejection as well as type II endothelial cell activation, swelling or disruption. Deposition of natural anti-Gal antibodies and complement are considered a hallmark for the diagnosis of AVR (130). Removal of these antibodies delays the onset of rejection and suppression of antibody synthesis further prolongs survival. Thus, antibodies still may be the main initiators of endothelial cell and monocyte activation, although AVR also occurs in the absence of natural anti-Gal antibodies. In the absence of Gal epitopes, grafts still demonstrated features of AVR (although reduced), including antibody deposition and consistent thrombotic microangiopathy, inferring that non-Gal antibodies may be also play a role in its onset (131, 132). This mechanism was indicated by the findings of the studies conducted by Kuwaki and colleagues and Chen et al., where AVR of GalTKO-/- xenografts were associated with the presence of circulating elicited anti-nonGal antibodies (133-135). However, a direct role of such antibodies in the pathogenesis of AVR was not provided. These findings, in conjunction with those of Lin and colleagues, indicate that the avoidance of AVR will require strategies that will enable controlling the antibody response against nonGal antigens and possibly lead to prevention of the coagulation system disorders (136).

5.1 Type II endothelial activation

Data from small and large animal models provided considerable evidence in support of endothelial cell activation during AVR. The association of type II endothelial cell activation and AVR was first recognized during vascularized cardiac xenotransplantation using guinea pig-to-rat model in which hyperacute rejection was prevented by complement or antibody depletion (137, 138). Type II endothelial activation involves transcription of new proteins and the resultant phenotypic change on the graft endothelial cell is a shift toward procoagulant and pro-inflammatory states. Procoagulant features of activated endothelial responses include the down-regulation of surface thrombomodulin and the increased expression of tissue factor, FVII/FVIIa and PAIs, which is consistent with dense local fibrin deposition, thereby promoting platelet aggregation and thrombosis and inhibiting clot lysis (76, 139). Pro-inflammatory characteristics include endothelial cell induction of adhesion molecules, including, progressively, E-selectin, P-selectin, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1. In addition, nuclear factor kappa B (NFκB)-dependent endothelial production of cytokines and chemokines, including interleukin (IL)-1α, IL-8 and monocyte chemoattractant protein-1 (MCP-1), have been demonstrated (76, 140, 141).

The overall significance of endothelial activation in AVR is, on one hand, reflected in their role as down-stream events, present essentially in any inflammatory response, albeit to a more florid extent than usual. On the other hand, the ability to genetically engineer the donor animal suggests the potential for modulating endothelial responses by genetic approaches — e.g., by the transgenic expression of protective genes that inhibit NFκB-dependent gene transcription and inhibit apoptosis — that can be useful in diminishing type II endothelial activation. Hence, a unifying concept for AVR therapy is that with additional targeting of complement, coagulation and host macrophage and NK cells. Modulation at the graft level by such genetic approaches will be important to the development of clinical xenotransplantation as a practical solution to the lack of organ donors.

5.2 Coagulation disorders associated with xenograft rejection

The identification of Gal as the major target antigen for human and nonhuman primate anti pig antibodies and the production of GalTKO pigs have helped to address the problem of hyperacute rejection. However, AVR, chronic rejection, and other barriers — for example, coagulation dysregulation in the form of thrombotic microangiopathy and/or consumptive coagulopathy — need to be overcome to prolong graft survival (reviewed in (4, 7, 117)). Platelet deposition and coagulation within the microvasculature are common to hyperacute rejection and AVR of vascularized xenografts (82). Exposure of endothelial cells to xenoreactive antibodies, complement, platelets, immune cells and cytokines results in loss of natural anticoagulant pathways and change to a procoagulant phenotype (reviewed in (117, 142)).

Three major anticoagulant mechanisms involved in the regulation of coagulation activation are antithrombin, protein C (PC) pathway and tissue factor pathway inhibitor (TFPI). Antithrombin inhibits coagulation at multiple levels by targeting several serine proteases, FIXa, FXa and thrombin. It is present in the circulation; however, it becomes more active when associated with the endothelial glycocalyx (143). PC pathway proteolytically cleaves activated coagulation cofactors FVa and FVIIIa (144). TFPI is the main inhibitor of the tissue factor-FVIIa complex and FX (145). The main function of the coagulation inhibitors is to prevent blood clotting under physiological conditions and to slow down the activated coagulation cascade after vascular injury (reviewed in (107, 129)).

The coagulation response to xenotransplantation varies, depending on the type of organ, and quite likely the distinct vasculature. While deletion of the GalT gene in pigs has removed the major xenoantigen, it has not eliminated the problem of coagulation dysregulation and vascular injury to xenografts and the development of coagulopathy in recipients. The tendency for graft vascular coagulation in xenotransplantation is impaired by preexisting antibodies, as well as a number of cross-species differences (146). Importantly, defects in anticoagulant mechanisms can result in an increased risk of thrombosis. In vitro and ex vivo studies suggested that APC generation in porcine xenografts may be significantly compromised by cross-species incompatibility in the protein C pathway (147). These incompatibilities were confirmed by a molecular analysis showing that, while porcine thrombomodulin binds to human thrombin, it is a poor cofactor for activation of human protein C (148). Second incompatibility is noted in the initiation stage of thrombosis, whereby porcine

von Willebrand factor (vWF) spontaneously aggregates human platelets, in the absence of shear stress, through aberrant interaction between the O-glycosylated A1 domain and human platelet glycoprotein Ib (149). Third, porcine endothelial TFPI is inefficient at neutralizing human FXa, thus regulating human tissue factor clotting initiation (113). However, Lee at al. found no molecular incompatibility of pig TAFI with its human targets in vitro (150). Recently, a novel immune-independent mechanism has been described, whereby human platelets are spontaneously activated to express tissue factor after physical contact with porcine endothelial cells, independent of xenoreactive antibodies or complement (reviewed in (117)). Solving the problem of coagulation dysregulation in xenotransplantation may require different combinatorial pharmacological and genetic strategies for different organs (142, 146).
Systemic therapy with conventional anticoagulant and anti-platelet agents, including heparin and aspirin, appears to be largely ineffective against the development of graft thrombotic microangiopathy and consumptive coagulopathy in recipients of GalTKO xenografts. These observations suggest that determination of exact mechanism by which coagulation dysregulations are initiated after xenotransplantation is important to enable further genetic modification in the pig or suggest therapy that might prevent them. The GalTKO background, and transgenic expression of one or more CRFs, has become the platform for additional genetic modifications with human thrombomodulin, tissue factor pathway inhibitor or CD39, to inhibit coagulation and inflammation (43, 146).

5.2.1 Thrombomodulin

Thrombomodulin (TM) is an endothelial anticoagulant factor that promotes thrombin-mediated activation of protein C. It also prevents cleavage of fibrinogen and activation of PAR-1 and promotes activation of TAFI, as well as cleavage of the potent proinflammatory protein HMBG1—an important effector molecule in acute allograft rejection (151). As mentioned earlier, pig thrombomodulin is a poor cofactor of human thrombin for activation of human protein C and TAFI. Several research groups have recently reported the production of human thrombomodulin (hTM)-transgenic pigs (152). Recently, a pig-to-baboon heart transplantation using a heart from a GalTKO.hCD46.hTM-transgenic pig was performed by the Pittsburg group. The study findings indicated that the recipient baboon showed no or minimal platelet activation and no consumptive coagulopathy, while it maintained a stable platelet number and fibrinogen level throughout the experiment (Esker B, Unpublished data, reviewed in (43)). Similar results were reported in another two pig-to-baboon heart

transplantations using multi-transgenic (GalTKO.hCD55.hCD59.hCD39.hTM) pig hearts (Esker B, Unpublished data, reviewed in (43)). In addition to hTM, expression of human endothelial protein C receptor may also be required to produce maximum effect (89, 153).

5.2.2 CD39 / CD73

The ectonucleotidase CD39 exhibits antiplatelet activity by first degrading the platelet ATP to AMP. AMP hydrolyzed by ecto-5'-nucleotidase (CD73) to adenosine, which is an antithrombic mediator (154). Transgenic mice overexpressing hCD39 have been reported to appear healthy and possess an anticoagulant phenotype similar to that of hTM transgenic mice (155). Recent studies confirmed the protective effects of hCD39 in pigs transgenic for hCD39 (156). However, preclinical data from pig-to-nonhuman primate kidney xenotransplantation studies using GalTKO.hCD55.hCD59.hCD39 pigs was not encouraging, although expression levels of hCD39 was not optimal in these multi-transgenic pigs (157).

5.2.3 Tissue factor pathway inhibitor

Another endothelial protein, tissue factor pathway inhibitor (TFPI), is a key physiological regulator of the initiation of coagulation by forming an inactive complex with FXa and FVIIa/tissue factor (TF). Early evidence suggested incompatibilities between pig TFPI and human (h) TF pathway (113). Recent in vitro studies showed that pig TFPI binds hFXa and inactivates hFVIIa/hTF with a similar efficacy to hTFPI (150). As TFPI rapidly shed from vasculature in response to inflammation, transgenic expression of hTAFI may provide additional benefits to pig endothelium (158). Transgenic pigs expressing a membrane-anchored form of hTFPIα showed more modest expression, which was sufficient to partially block anti-nonGal antibody mediated TF upregulation (159). Since high levels of hTAFI in the pig may not be compatible with the life of the animal, strategies were shown to possibly restrict the expression of hTAFI to vasculature endothelial cells, rather than ubiquitously (Ayares D, Unpublished data, reviewed in (43)).

Addressing the problem of coagulation dysregulation in xenotransplantation requires more pre-clinical studies (pig-to-nonhuman primates) using multi-transgenic pigs to demonstrate the unimpaired health of the organ-source pig, particularly the absence of any detrimental effects of overexpression of human anticoagulant protein in the pig.

6 Interaction between coagulation and complement

Recent studies suggest that molecular intercommunications exist between the individual components of the coagulation, fibrinolysis and complement system. The most important intercommunications of the coagulation and complement systems have emerged as evidence for cross-talk between the components of the two cascades (68). In 1987, Wiggins et al. showed that plasma kallekrein is able to release C5a from C5 (160). In addition, it has also been reported that human kallekrein directly or indirectly affect the generation of active C3 fragments (161, 162). Subsequent analysis has revealed that both C3 and C5 can be proteolytically cleaved by several coagulation components, specifically FIXa, FXa, FXIa, thrombin, and plasmin (163). In a novel mechanism, platelet activation and subsequent expression of P-selectin at the site of vascular injury have been associated with complement activation (164). The coagulation components can also initiate upstream complement activation of the C3 and C5 convertases; for instance, FXIIa is able to activate the C1 complex (165, 166). Furthermore, the interaction of fibrinogen/fibrin with recognition molecules causes initiation of the lectin pathway of complement (167).

On the other hand, the coagulation cascade is activated by complement either directly or indirectly. Findings of some in vitro studies indicate that C5a induces TF activity on endothelial cells and may thereby activate the exogenous coagulation pathway (168). Following another recent study, the authors reported that C5a induced “switch” in mast cells from a pro-fibrinolytic (t-PA release) to a pro-thrombotic phenotype upregulated PAI-1 release (169), thus modifying the balance between pro- and anticoagulation. MASP2, a component of the lectin pathway of complement, initiates the activation thrombin and subsequent generation of the fibrin mesh (170, 171).

The intercommunication of coagulation and complement has also been documented to exert negative regulatory effects on their biological functions, in addition to initiation of the activation of both cascades. Thrombin can induce the expression of complement regulator decay accelerating factor (DAF) (172) that results in significantly reduced C3 deposition and complement-mediated cell cytotoxicity. In a similar way, TAFI generated by thrombin-thrombomodulin complex can play a dual role in the inhibition of plasmin-mediated fibrinolysis and the inactivation of C3a and C5a (173, 174).

7 Cell-mediated rejection

Cell-mediated immunity to xenotransplantation leading to cellular rejection is likely to be an important obstacle to xenotransplantation. The $CD4^+$ T cells are the prime initiators of this response and $CD8^+$ T cells and macrophages are the ultimate effectors (175). Cell-mediated xenograft rejection can develop in the absence of features of humoral xenograft rejection by the deletion or restriction of the xenograft humoral components. However, the cell-mediated immune responses to xenotransplantation are likely to be more severe than those pertaining to allotransplantation, as the xenografts possess more antigens, and molecular incompatibilities between species cause disordered regulation of cell-mediated responses (176, 177). The presence of cellular infiltrates — mainly T and B cells, NK cells, macrophages and neutrophils — is directly associated with tissue damage in cell-mediated xenograft rejection. T cells alone can provoke xenograft rejection by recognizing antigen through the direct (by MHC) or the indirect pathway. While both $CD4^+$ and $CD8^+$ T cells can independently mediate rejection of xenografts (178). the $CD4^+$ T cells do so by generation of helper-dependent xenoreactive antibodies that function to contribute to rejection both by complement-dependent mechanisms and by antibody-dependent cell-mediated cytotoxicity. The $CD4^+$ T cell-mediated rejection was historically characterized by edema, hemorrhage, necrosis and thrombi. The $CD8^+$ T cells that contribute to rejection are helper-independent cytotoxic T cells that produce their own growth factors. Both of these $CD4^+$ and $CD8^+$ mechanisms involve cytokine synthesis in response to cyclosporine A, suggesting that the T cell immunosuppression will be important for achieving long-term xenograft survival (178). Another expression of human TNF alpha-related apoptosis inducing ligand (TRAIL) by porcine lymphocytes was shown to induce apoptosis of immortalized T-lymphocytes and this TRAIL-specific effect was confirmed by using anti-TRAIL antibodies. Thus, expression of human TRAIL in transgenic pigs may provide a protective strategy against cell-mediated rejection after xenotransplantation (6, 179).

7.1 Natural killer cells

Growing body of evidence indicates that natural killer (NK) cells represent another potential barrier to xenotransplantation (180, 181). NK cells have been implicated in xenograft injury in rodent and pig-to-nonhuman primate models. The recognition of pig endothelial cells by NK cells is not dependent on the expression of Gal and thus the GalTKO pig model offers no advantage in this respect (182, 183). In vitro and ex vivo xenoperfusion studies indicate that NK cells can infiltrate pig organs and to lyse porcine cells both directly and by antibody-dependent cell-mediated cytotoxicity (181, 184-186). In addition to possessing activating receptors, NK cell activity is tightly regulated by the expression of MHC class I ligands of inhibitory NK receptors. According to the missing self-principle, porcine endothelial swine leukocyte antigen major histocompatibility complex molecules fail to recognize NK cell inhibitory receptors. The inhibitory receptor CD94/NKG2A that is expressed on a majority of activated human NK cells binds specifically HLA-E (reviewed in (6, 187)). Activation and xenogeneic NK cytotoxicity could be avoided through interference with cellular interactions by (1) targeting specific recruitment processes, (2) by deleting or blocking the function of porcine ligands that activate NK cell receptors, (3) by inhibiting activating NK cell receptors using pharmacological agents or antibodies, or (4) by transgenic expression of HLA class I molecules on porcine endothelial cells. NK cell injury of porcine endothelial cell is inhibited by expression of HLA class I molecules, such as Cw3, E, or G, although this protection is only partial. Therefore, it is necessary to identify the activating NK receptors as well as their corresponding porcine ligands to overcome NK cell-mediated rejection of porcine xenografts (reviewed in (129)).

Macrophages have also been implicated in the rejection of solid organ or cellular xenografts (188, 189). Xenoreactive T cells, or direct interaction of inhibitory (signal-regulatory protein-α [SIRP-α]) or stimulatory receptors, mediate macrophage xenoreactivity (190). CD47 is a ligand for SIRP-α that prevents phagocytosis of autologous cells. Incompatibility of porcine and human CD47 leads to phagocytosis of pig cells by human macrophages. The recent observations indicate that further modifications of donor pigs for the expression of human CD47 may overcome macrophage-mediated xenograft rejection (191).

8 Chronic rejection

In allografts, chronic rejection is the result of not only immunologic but also non-immunologic mechanisms that are in large part related to the effects of chronic immunosuppressive treatment. Mainly due to the difficulties in overcoming acute forms of rejection, the phenomenon of chronic rejection of xenografts is not well understood. GalTKO pig heart xenografts, that survived for long periods prior to failure, were characterized predominantly by the presence of thrombotic microangiopathy (133). In addition, microvascular thrombosis is also a critical feature in antibody-dependent processes of acute rejection; ischemia-reperfusion injury, binding of xenoantibodies to the graft endothelium and activation of endothelial cells result in disruption of the mechanisms that maintain a protective anticoagulant/procoagulant balance of the endothelial surface (192). It has not yet been determined whether the chronic type of rejection seen with the GalTKO hearts is due to low-grade residual immune reactivity such as T-independent xenoantibody production, or rather to immune-reactivity-independent inappropriate activation of the coagulation pathway. The latter may be the result of molecular incompatibilities between pig and primate coagulation factors (192, 193). Chronic rejection is characterized by concentric arteriosclerosis of the graft blood vessels, along with fibrosis and atrophy of the tissue (21). The only currently available solution to chronic rejection is the transplantation of a new tissue or organ. As the availability of animal organs would greatly exceed the transplantation demands, chronic rejection may not be such a problem in terms of xenotransplantation (194).

9 Accommodation

Under certain circumstances, organ xenografts survive (195, 196) in the presence of high titer xenoreactive antibodies and complement (197), referred to as "accommodation" (198). It also defined to a vascularized transplant that acquired resistance to antibody-mediated rejection (199). This phenomenon was first described in ABO-incompatible kidney allografts in the 1980s (195, 200) and was postulated that a similar phenomenon might occur in vascularized xenografts (30, 195). Achievement of accommodation state has always been a goal of xenotransplantation research and a useful strategy for clinical xenotransplantation. Three potential mechanisms were originally proposed to explain accommodation. First, after transplantation, anti-donor antibodies may change in their affinity, specificity, or both. Second, change in the antigenic epitopes on the graft during transplantation may prevent anti-donor antibodies from binding. Third, endothelial cells develop a resistance to injury during the transplantation. Another potential mechanism of decreased sensitivity to injurious stimuli — continued stimulation of endothelial cells — causes the cells to develop resistance to re-stimulation. Of these, second and third mechanism refer to changes in the donor organ (“graft accommodation”) whereas the first describes a change in the recipient (“host accommodation”) and is an example of modulation of the anti-donor immune response (reviewed in (198)). Although, the occurrence of accommodation eliminates the need for continuing depletion of anti-donor antibodies from a graft recipient, it also provides further strategies that can be applied to manipulation of the recipient or donor to prevent more chronic forms of graft rejection (58).

10 Tolerance induction

Extant nonhuman primate studies indicate that AVR did not occur under the conditions of severe T- and B-cell depletion and/or suppression (133, 201, 202). Understanding the mechanisms of the T- and B-cell xenoresponses will support further improvement in immunosuppressive strategies or serve as a basis for the induction of xenotolerance. The induction of T- and B-cell tolerance is essential for the success of clinical xenotransplantation using several approaches, including co-stimulatory blockade (203), donor-specific transfusion (e.g., splenocytes from the organ or tissue donor into the transplant recipient), transplantation of donor thymus tissue (204), and the induction of mixed hematopoietic chimerism by stem cell transplantation (205). Unfortunately, some of these approaches are associated with adverse outcomes, such as thrombotic complications (3), as well as those due to immunological and non-immunological incompatibilities (206). Other approaches include the induction of tolerance to the Gal epitope and tolerance of T-cell independent B cells (3), as well as peripheral tolerance through a mechanism that does not have systemic side-effects, such as by CTLA4-Ig transgenic expression (129).

11 Physiology

Whether the transplanted pig organs or cells will function in a recipient satisfactorily is still unknown, even when long-term graft survival is achieved by successfully controlling immune, coagulative and proinflammatory responses (207). Extensive research is needed to determine whether pig organs can replace the physiological functions of human organs. In order to improve the understanding of the issues associated with transplanted organ survival, research has been performed in many pertinent fields, including anatomical design, metabolism, hormonal function, blood viscosity, and coagulation (208-210). While pig-to-primate kidney transplants function well on several levels, they lack compatibility with respect to erythropoietin (EPO) function, resin and hydroxylation of Vitamin D. Since porcine EPO and human EPO differ significantly (210), human recombinant EPO would need to be substituted in the patients. The functional characteristics of the pig cardiovascular system are also comparable to human with nearly identical atrial, ventricular, pulmonary and aortic pressures and cardiac output (20). Thus, pig has been widely used as model animal for human cardiovascular research (211, 212). Similarly, it can be predicted that pig-to-human transplants of hearts, kidneys, and lungs will be physiologically feasible. However, this is not the case for whole-organ liver transplants, where vast number of products manufactured, metabolized and detoxified in the liver may prohibit adequate function (213). In addition, thrombocytopenia associated with pig-liver xenotransplantation. Furthermore, xenotransplantation of lungs will require more research into their physiology (214). It has become clear that coagulation dysfunction between recipient and donor, as well as inflammation, contributes significantly to different survival lengths and to the loss of the xenograft (209). The induced thrombotic microangiopathy causes ischemic injury to the myocardium during heart transplantations and finally results in consumptive coagulopathy (215, 216).

12 Safety

Although the safety aspect of xenotransplantation remains a central topic in the field, the available data pertaining to the potential risk of transmission of infectious agents from animal to human are reassuring (7). Controlled breeding conditions, efficacy of anti-viral agents, possible vaccine development and carefully monitoring procedures at regular intervals can ensure that organs and cells will be free of such infectious agents, or at least pose minimal risk (217, 218). However, such measures will not affect the porcine endogenous retrovirus (PERV) as their coding sequences are a permanent part of the genome in all pig cells, even if they do not cause disease under physiological conditions (219, 220). Several strategies, such as siRNA technology, can be applied to prevent activation of PERV (221, 222). Moreover, Zinc-finger nuclease technology can be employed to delete PERV from the pig genome (223). By reducing the risk of PERV-mediated complications, the safety profile of xenotransplantation procedures is ultimately increased (7).

Summary

The increasingly rapid development of genetically engineered pigs will make clinical xenotransplantation more realistic. However, more preclinical (pig-to-nonhuman primate) studies will be needed to demonstrate the unanswered questions regarding incompatibilities of the organ-source pig, before 'bridging' trials of pig xenotransplantation can begin in patients. Genetically modified pigs currently available for xenotransplantation are listed in Table 2. It is hoped that, in the future, organ xenotransplantation will be carried out as a standard procedure, with relatively long-term graft survival. The limitless availability of organ-source pigs will enable retransplantation to be carried out whenever indicated, even if the survival of the graft is not as long as that of an allograft.

Gal antigen modification	
	α1,3-galactosyltransferase gene-knockout (GalTKO)
	Human H-transferase gene expression (expression of blood type O antigen)
	Endo-beta-galactosidase C (reduction of Gal antigen expression)
Complement regulation by human complement-regulatory gene expression	
	CD46 (membrane cofactor protein)
	CD55 (decay-accelerating factor)
	CD59 (protectin or membrane inhibitor of reactive lysis)
Anticoagulation and anti-inflammatory gene expression or deletion	
	Human tissue factor pathway inhibitor (TFPI)
	Human thrombomodulin
	Human CD39 (ectonucleoside triphosphate diphosphohydrolase-1)
	von Willebrand factor–deficient (natural mutant)
Suppression of cellular immune response by gene expression or downregulation	
	Porcine CTLA4-Ig (cytotoxic T-lymphocyte antigen 4 or CD152)
	CIITA-DN (MHC class II transactivator knockdown, resulting in swine leukocyte antigen class II knockdown)
	Human TRAIL (tumor necrosis factor-alpha-related apoptosis-inducing ligand)
	HLA-E/human β2-microglobulin (inhibits human natural killer cell cytotoxicity)
	Human CD47 (for species-specific CD47-SIRP-alpha natural interaction on macrophages)
	Human FAS ligand (CD95L)
	Human GnT-III (N-acetylglucosaminyltransferase III)
	LEA29Y (inhibition of the B7/CD28 costimulatory pathway of T-cell activation)
Antiapoptotic gene expression	
	Human A20 (tumor necrosis factor-alpha-induced protein 3)
	Human heme oxygenase-1 (HO-1)
	Human TNFRI-Fc (tumor necrosis factor-alpha receptor I-Fc)
Prevention of porcine endogenous retrovirus (PERV) activation	
	PERV siRNA

Table 2: Genetically modified pigs currently available for xenotransplantation research, modified from (7).

Objectives of this thesis

Before xenotransplantation can be introduced successfully into the clinic, the problems of innate immunity, coagulopathy, and inflammatory responses will have to be overcome. The transplantation of organs and cells from genetically modified pigs could mitigate these issues. The main aim of this thesis was to evaluate the function of human transgenes introduced into pigs to protect their organs against attack by complement, coagulation, as well as immune cell-mediated responses of the human recipient. The specific aims are stated in each chapter and summarized below.

1. The degree of involvement of the lectin pathway of complement in hyperacute rejection and later phases of xenograft rejection is not fully understood. None of the extant studies on xenotransplantation has addressed the activation of the lectin pathway of complement. Thus, the present study focused on the activation of the lectin pathway and the interaction of IgM and MBL in a pig to human *in vitro* xenotransplantation model.

2. Subsequent to hyperacute rejection, other mechanisms, such as acute vascular rejection and NK cell-mediated responses, play a crucial role in xenograft survival. However, neither GalTKO nor overexpression of hCRPs protect the xenograft against the latter. In this study, the potential of combined overexpression of human CD46 and HLA-E to prevent complement- and NK cell-mediated xenograft rejection was tested in an ex vivo pig-to-human xenoperfusion model.

3. Ex vivo porcine limb perfusion with whole, anti-coagulated human blood leads to platelet loss, observed within minutes to hours after perfusion. The asialoglycoprotein receptor-1 (ASGR1) (also known as the Ashwell receptor) mediates the capture and endocytosis of galactose- (Gal) and N-acetylgalactosamine- (GalNAc) terminating glycoproteins. More recently, it has been shown that ASGR1 on PLSEC bind and phagocytosis human platelets in an ex vivo liver xenoperfusion. In addition, human platelets have more Gal and GalNAc epitopes available compared with pig platelets. Owing to these observations, the aim of this study was to examine ASGR1 expression on porcine aortic/arterial endothelial cells (PAEC), as well as PAEC / ASGR1 mediated xenogenic platelet phagocytosis.

4. Based on comparative anatomical and physiological characteristics, pig has been used as model animal to develop and test novel therapies for the prevention of

cardiovascular diseases. Currently, no commercial test kits are available for multiplex detection of porcine markers by using xMAP technology. Therefore, the present study aimed to develop a bead-based multiplexed immunoassay to simultaneously detect porcine cytokines (IL-1β, IL-6, IL-10, TNFα), chemokines (IL-8, MCP-1), growth factors (basic FGF, VEGF, PDGF-bb) and injury marker (cardiac troponin-I), as well as complement activation markers (C5a, C5b-9) on the Bio-Plex platform.

5. Genetically manipulated pigs with GalTKO and overexpressing hCD46 were generated to overcome hyperacute rejection, the major barrier in pig-to-primate xenotransplantation. In addition to immunological barriers, coagulation incompatibilities between the human blood and the porcine vessel structure also hinder the xenotransplantation of vascularized organs. Thus, this study aimed to test the beneficial effects of transgenic expression of human thrombomodulin (hTM) on wild-type background or on multitransgenic (GalTKO/hCD46) background pigs against coagulation dysregulations.

Materials and methods used in achieving the aforementioned aims are described in detail in each manuscript.

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Results

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Paper I

Activation of the lectin pathway of complement in pig-to-human xenotransplantation models

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Background: In pig-to-human xenotransplantation, complement activation plays a pivotal role in early graft damage. Anti-Gal IgM antibodies are a major initiator of complement activation via the classical pathway. By amplifying C3b generation, the alternative pathway is also critical. However, involvement of the lectin pathway of complement in hyperacute and later phases of xenograft rejection has not been assessed so far.

Aim: The present study was therefore focused on lectin pathway as well as interaction of IgM and MBL in pig-to-human xenotransplantation models.

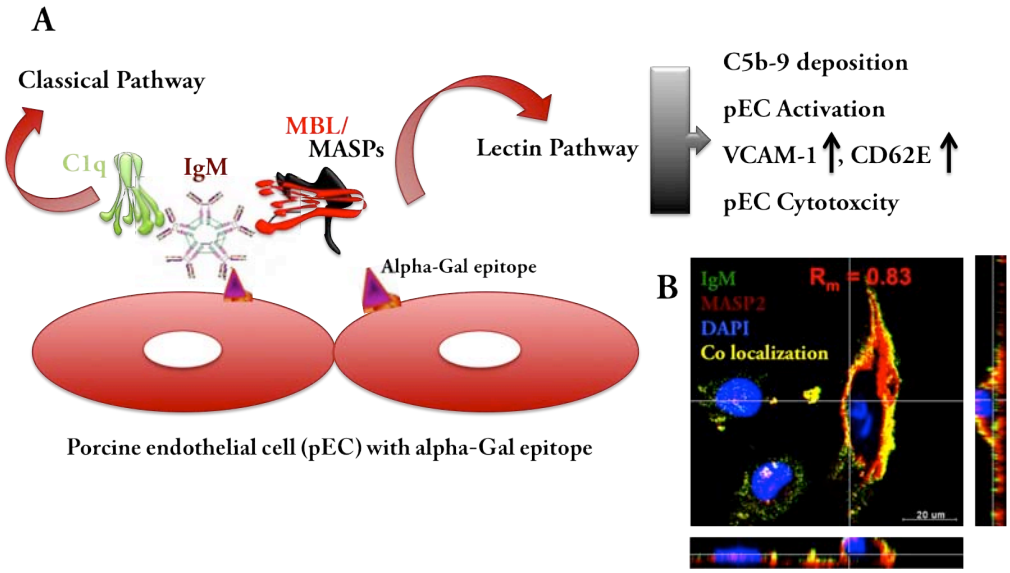

Figure: (A) Model illustrating the activation of the lectin pathway by natural anti-Gal IgM and downstream events in pig-to-human xenotransplantation. (B) In vitro porcine aortic EC treated with normal human serum and analyzed for co-localized deposition (yellow) of IgM (green) and MASP2 (red). Nuclei stained with DAPI (blue).

Conclusion: Co-localization of MBL/MASP2 with IgM and complement suggests that the lectin pathway is activated by human anti-Gal IgM and may play a pathophysiological role in pig-to-human xenotransplantation. In this study, PAEC from GalTKO/hCD46 pigs showed significantly reduced deposition of MBL and complement proteins as well as no cytotoxicity when treated with NHS. These observations suggest that GalTKO and expression hCD46 could also help to prevent lectin pathway activation during pig-to-human xenotransplantation.

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BASIC AND EXPERIMENTAL RESEARCH

Activation of the Lectin Pathway of Complement in Pig-to-Human Xenotransplantation Models

Anjan K. Bongoni,[1,2,3] David Kiermeir,[3] Hansjörg Jenni,[4] Annegret Wünsch,[5] Andrea Bähr,[5] David Ayares,[6] Jörg D. Seebach,[7] Eckhard Wolf,[5] Nikolai Klymiuk,[5] Mihai A. Constantinescu,[3] Esther Vögelin,[3] and Robert Rieben[1,3,8]

Background. Natural IgM containing anti-Gal antibodies initiates classic pathway complement activation in xenotransplantation. However, in ischemia-reperfusion injury, IgM also induces lectin pathway activation. The present study was therefore focused on lectin pathway as well as interaction of IgM and mannose-binding lectin (MBL) in pig-to-human xenotransplantation models.

Methods. Activation of the different complement pathways was assessed by cell enzyme-linked immunosorbent assay using human serum on wild-type (WT) and α-galactosyl transferase knockout (GalTKO)/hCD46-transgenic porcine aortic endothelial cells (PAEC). Colocalization of MBL/MASP2 with IgM, C3b/c, C4b/c, and C6 was investigated by immunofluorescence in vitro on PAEC and ex vivo in pig leg xenoperfusion with human blood. Influence of IgM on MBL binding to PAEC was tested using IgM depleted/repleted and anti-Gal immunoabsorbed serum.

Results. Activation of all the three complement pathways was observed in vitro as indicated by IgM, C1q, MBL, and factor Bb deposition on WT PAEC. MBL deposition colocalized with MASP2 (Manders' coefficient [3D] r^2=0.93), C3b/c (r^2=0.84), C4b/c (r^2=0.86), and C6 (r^2=0.80). IgM colocalized with MBL (r^2=0.87) and MASP2 (r^2=0.83). Human IgM led to dose-dependently increased deposition of MBL, C3b/c, and C6 on WT PAEC. Colocalization of MBL with IgM (Pearson's coefficient [2D] r_p^2=0.88), C3b/c (r_p^2=0.82), C4b/c (r_p^2=0.63), and C6 (r_p^2=0.81) was also seen in ex vivo xenoperfusion. Significantly reduced MBL deposition and complement activation was observed on GalTKO/hCD46-PAEC.

Conclusion. Colocalization of MBL/MASP2 with IgM and complement suggests that the lectin pathway is activated by human anti-Gal IgM and may play a pathophysiologic role in pig-to-human xenotransplantation.

Keywords: Mannose-binding lectin, Complement pathways, Natural antibody, Xenotransplantation, Porcine aortic endothelial cells.

(*Transplantation* 2013;96: 791–799)

The use of xenografts of pig origin may significantly expand the pool of available donor organs for humans (*1, 2*). However, the major obstacle of pig-to-human xenotransplantation is that grafts are subjected to potent xenorejection processes (*3*). Hyperacute rejection is the first and most destructive mechanism (*4, 5*), characterized by rapid loss of graft function after connecting to the host circulation. It is a consequence of the binding of human preformed xenoreactive antibodies against the disaccharide Galα1-3Gal expressed on porcine endothelium (reviewed in Ref. (*6*)). This antigen-antibody interaction activates the

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A.K.B. participated in the research design, writing of the article, performance of the research, and data analysis and contributed new reagents or analytic tools. D.K. and H.J. participated in the performance of the animal experiments and the overall design of the study. A.W, A.B., E.W., and N.K. produced GalTKO/hCD46 pigs and provided the respective endothelial cells. D.A. provided primary cells from GalTKO/hCD46 transgenic pigs for nuclear transfer experiments. J.D.S. provided scientific support and reagents and participated in the critical revision of the article. M.A.C. and E.V. participated in the concept and design of the study and carried part of the responsibility. R.R. participated in the concept and design of the study, analysis of the data, and writing of the article and carried the main responsibility for the study.
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complement cascade on the endothelial cell membrane, leading to endothelial cell activation and damage, intravascular graft thrombosis, and vessel occlusion (*7*).

The complement system is activated via three well-characterized pathways—the classic, the alternative, and the lectin pathways. Each of them has its own mechanism of activation, resulting in activation of C3, followed by generation of the membrane attack complex (*8*). In hyperacute rejection, xenoreactive antibody-mediated activation of the classic pathway is commonly accepted as the main complement activation pathway (*9, 10*). By amplifying the generation of C3b, the alternative pathway also becomes critical in xenograft rejection. However, currently available data do not answer the question whether the lectin pathway may also be involved in the initiation of xenorejection.

The lectin pathway is initiated by interactions between mannose-binding lectin (MBL) and certain carbohydrate arrays (*11*). MBL naturally exists in a complex with MBL-associated serine proteases (MASPs) (*12, 13*). When MBL binds to a target, the MASPs are activated (*14*) and cleave their respective substrates—C4 and C2 for MASP2 and C3 and C2 for MASP1 (*15*). Historically, the lectin pathway of complement activation is known as antibody-independent pathway. However, several studies using animal models of ischemia-reperfusion injury demonstrated that MBL also interacts with immune complexes to activate complement via the lectin pathway (*16–18*). In addition, a recent in vitro study suggested that 20% of human serum IgM could bind to immobilized human MBL (*19, 20*).

In a bypass activation pathway, MBL can directly activate C3 and the alternative pathway of complement, which are independent of C2 (*21*). In addition, MBL binding to polymeric IgA induces the initiation of lectin pathway of complement activation (*22*). Furthermore, myocardial and skeletal ischemia-reperfusion injury studies demonstrated that complement activation and resulting C3 deposition are mediated by IgM and MBL (*23, 24*) and independent of C1q and the classic pathway (*24*). Taken together, these observations extend the idea that activation of the lectin pathway of complement may not be completely antibody independent.

Thus far, the role of the lectin pathway of complement has not been looked at in xenotransplantation. The present study was therefore focused on lectin pathway activation in pig-to-human xenotransplantation. In particular, we investigated the interaction of MBL and IgM in pig-to-human in vitro as well as ex vivo xenotransplantation models.

RESULTS

Complement Pathways Involved in Pig-to-Human Xenorejection In Vitro (PAEC Treated with NHS)

Deposition of C1q (classic pathway), MBL (lectin pathway), and factor Bb (alternative pathway) on porcine aortic endothelial cells (PAEC) was assessed by cell enzyme-linked immunosorbent assay (ELISA). Wild-type (WT) PAEC exposed to 1:2 diluted normal human serum (NHS) showed deposition of IgM, C1q, MBL, factor Bb, C3b/c, C4b/c, and C5b-9 (Fig. 1A). In addition, MBL, C3b/c, and C6 deposition was shown on PAEC treated with 1:10 diluted, MBL-deficient, or C1q-depleted serum or NHS (Fig. 1B). Incubation of WT PAEC with MBL-deficient or C1q-depleted serum also led to expression of adhesion molecules (VCAM-1 and CD62E; Fig. 1C) as well as PAEC cytotoxicity (Fig. 1D).

Lectin Pathway Complement Activation In Vitro—Colocalization Analysis

Immunofluorescence (IF) staining was performed on WT PAEC treated with 1:10 diluted NHS for the colocalized deposition of MBL with complement proteins. Deposition of MBL on WT PAEC was colocalized with MASP2, C3b/c, C4b/c, and C6, suggesting activation of the lectin pathway in this xenotransplantation setting (Fig. 2).

Quantitative colocalization analysis was performed by Manders' correlation coefficient (R_m) using z-stack confocal images and Imaris software version 7.2.3 (Bitplane). A total overlap of both fluorescent color channels, indicating a perfect three-dimensional colocalization, is indicated by "R_m=1," and no colocalization by "R_m=0." The Manders' correlation coefficient values were 0.93 for MBL-MASP2, 0.84 for MBL-C3b/c, 0.86 for MBL-C4b/c, and 0.80 for MBL-C6, suggesting an important contribution of MBL for total complement deposition in this model.

Colocalization of IgM and MBL/MASP2 on WT PAEC

Colocalization of natural IgM with MBL and MASP2 was investigated on NHS-treated PAEC by confocal microscopy. Colocalized deposition of IgM with MBL and MASP2 was observed on PAEC (yellow staining) (Fig. 3). Manders' colocalization analysis showed near-perfect IgM colocalization with MBL (R_m=0.87) and MASP2 (R_m=0.83).

IgM-Dependent MBL and Complement Deposition on WT PAEC

Binding of MBL to the major porcine xenoantigen Galα1-3Gal on WT PAEC was assessed in anti-Gal immunoabsorbed and immunoglobulin-depleted (Ig-depleted) serum, respectively. Compared with NHS, reduced MBL deposition was observed on PAEC when incubated with anti-Gal immunoabsorbed NHS or Ig-depleted serum. Purified polyclonal human IgM was used to replete anti-Gal immunoabsorbed NHS and Ig-depleted serum, respectively, which resulted in dose-dependently increased deposition of MBL, C3b/c, and C6 on PAEC (Fig. 3C,D). In addition, WT PAEC were treated with purified human polyclonal IgM followed by incubation with purified MBL protein, resulting in a dose-dependently increased binding of purified MBL to PAEC (Fig. 3D).

Complement Regulation by GalTKO/hCD46 PAEC

WT or α-galactosyl transferase knockout (GalTKO)/hCD46 PAEC were treated with 1:10 diluted NHS and assessed for complement deposition as well as complement-mediated PAEC activation and cytotoxicity. Absence of Gal epitopes and overexpression of hCD46 (Fig. 4) significantly reduced binding of IgM, C1q, MBL, and downstream complement proteins on GalTKO/hCD46 PAEC compared with WT (Fig. 4D). This reduced complement activation on the genetically modified PAEC correlated well with reduced complement-mediated cytotoxicity (Fig. 4E). These observations were also confirmed by IF/confocal microscopy using

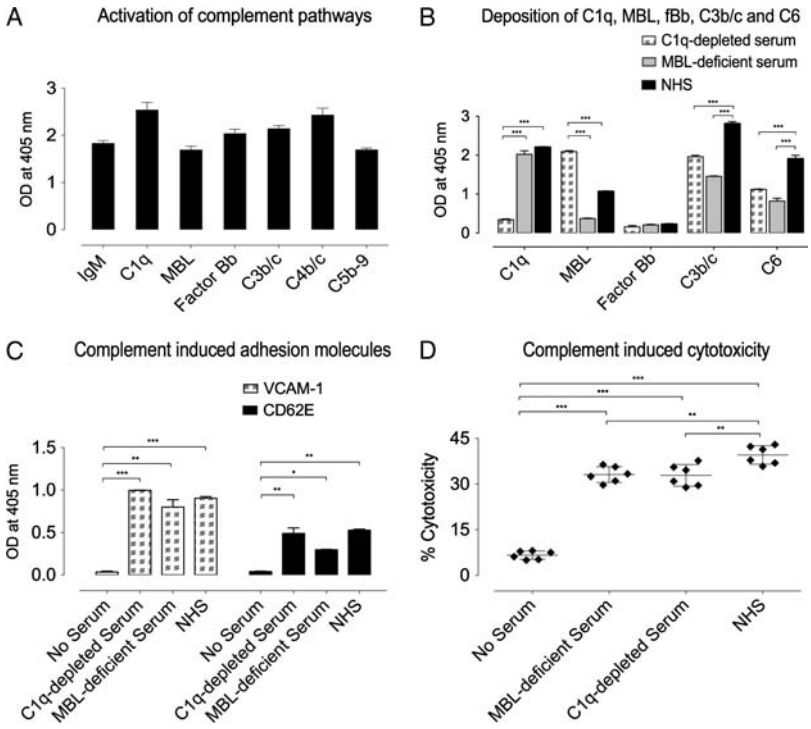

FIGURE 1. Cell ELISA, activation of classic, lectin, and alternative complement pathways in a pig-to-human in vitro xenotransplantation model. A, WT PAEC were treated with 1:2 diluted NHS at 37°C for 45 min and the deposition of human IgM as well as complement proteins was assessed by specific antibodies. B, treatment of PAEC with 1:10 diluted NHS, MBL-deficient serum and C1q-depleted serum, respectively, and assessment of deposition of C1q, MBL, factor Bb, C3b/c, and C6. C, prolonged incubation (4 hr at 37°C) of PAEC with NHS/C1q-depleted serum/MBL-deficient serum (1:10) and assessment of the expression of adhesion molecules VCAM-1 and CD62E. D, treatment of PAEC with 1:10 diluted NHS/C1q-depleted serum/MBL-deficient serum for 120 min at 37°C and assessment of cytotoxicity by calcein AM and EthD-1. Significance was tested using one-way calcein AM with Bonferroni correction (*P<0.05, **P<0.01, ***P<0.001). Data are mean±SD of three independent experiments.

WT and GalTKO/hCD46 PAEC treated with 1:10 diluted NHS (Fig. 4F).

Analysis of Lectin Pathway Complement Activation during Ex Vivo Pig Limb Xenoperfusion

Extracorporeal perfusion of WT porcine forelimbs with heparin anticoagulated human blood did not reveal signs of hyperacute rejection and lasted for at least 12 hr, after which it had to be terminated due to continuous small blood losses. Blood gas analysis showed constant physiologic potassium, lactate (10.78±0.63), and pH values (7.32±0.06). All limbs showed full muscular response on neural stimulation throughout the experiments. Stable and physiologic hemodynamic perfusion parameters (pressure, flow) could be maintained throughout all perfusions. To prevent blood loss due to bleeding, biopsy samples were collected only at later time points of perfusion, except for one experiment where samples were collected 1 hr after the onset of perfusion. These biopsy samples were used to analyze deposition of MBL, C1q, and factor Bb (Fig. 5A–C).

Tissue samples taken after 12 hr perfusion were stained for deposition of human IgM, MBL, C3b/c, C4b/c, and C6. Analysis by fluorescence microscopy revealed that IgM, C3b/c, C4b/c, and C6 were codeposited with human MBL in the tissue. Quantitative two-dimensional colocalization analysis was performed by Pearson's correlation coefficient (R_p) using fluorescence microscopy images and Imaris software. A total overlap of both fluorescent color channels, represented as "R_p=+1," indicates a perfect colocalization, "R_p=0" random colocalization, and "R_p=−1" perfect exclusion. The R_p values were 0.88 for IgM-MBL, 0.82 for C3b/c-MBL, 0.63 for C4b/c-MBL, and 0.81 for C6-MBL (Fig. 5D–S).

DISCUSSION

The role of complement activation in the pathogenesis of pig-to-human xenograft rejection is well established. However, almost nothing is known to date about the activation of the lectin pathway of complement in xenorejection. We demonstrate here for the first time that all three pathways of complement are activated in a model of pig-to-human xenotransplantation. We could show full activation of complement, including formation of the terminal complement complex and complement-mediated cytotoxicity also in the absence of C1q. In addition, our data indicate that activation of the MBL route of complement is dependent on IgM, both in vitro and ex vivo, in a pig-to-human xenotransplantation model.

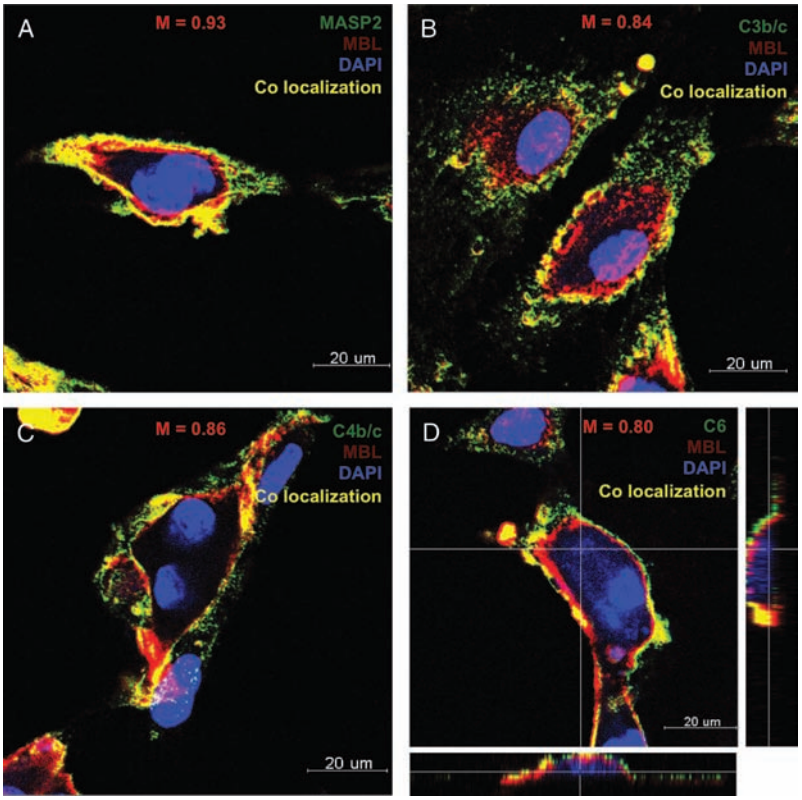

FIGURE 2. Colocalization analysis of MBL with other complement proteins. NHS (1:10) treated WT PAEC were stained for the deposition of MBL (red fluorescence) and MASP2, C3b/c, C4b/c, and C6, respectively (green fluorescence). Representative colocalization images of MBL with the respective Manders' correlation coefficients are shown: (A) MASP2 (R_m=0.93), (B) C3b/c (R_m=0.84), (C) C4b/c (R_m=0.86), and (D) C6 (R_m=0.80). Colocalization was quantified by z-series analysis using Manders' correlation coefficient. For MBL-C6 colocalization (D), also side views are shown as z-panels to the right and below the image.

Endothelium is the first cell type to make contact with the recipients' blood in xenotransplantation or xenoperfusion. We therefore chose to analyze the effects of human serum on PAEC in vitro to perfuse porcine limbs ex vivo with whole, heparin-anticoagulated human blood. PAEC treated with NHS showed deposition of MBL, C1q, and factor Bb on their surface, indicating that all three pathways are involved in complement-mediated xenograft damage. In addition, deposition of C4b/c, C3b/c, C6, and C5b-9, detected as C9 neoepitope, were also observed on NHS-treated PAEC. The same markers, indicating full activation of complement, were also found when C1q-depleted serum was used. Furthermore, C1q-depleted serum induced PAEC activation, as measured by expression of the adhesion molecules E-selectin and VCAM-1, as well as PAEC cytotoxicity in a complement-dependent manner.

Binding of MBL was colocalized with C3b/c, C4b/c, and C6 on PAEC, suggesting that deposition of MBL on the cells plays a functional role in complement activation. Because natural antibodies are the major contributors to complement activation, the involvement of IgM in lectin pathway activation was then investigated. Colocalization studies on PAEC treated with NHS revealed that MBL/MASP2 deposition was colocalized with IgM, quantitative Manders' coefficient values indicating a near 100% colocalization, suggesting that IgM and MBL may work together in a synergetic way. The importance of anti-Gal IgM in lectin pathway activation was further confirmed by depletion of anti-Gal antibodies from NHS by immunoabsorption, which led to a significantly reduced binding of IgM as well as MBL on PAEC and also to a reduction of C5b-9 deposition. Repletion of immunoabsorbed or total Ig-depleted serum with polyclonal human IgM resulted in a dose-dependently increased deposition of IgM, MBL, and further downstream complement components. In addition, a dose-dependent deposition of purified MBL was found on PAEC treated with human polyclonal IgM. Taken together, these data indicate that the activation of the lectin pathway of complement in this xenotransplantation model is dependent on IgM binding to the target cells. This finding is in line with a recent study showing MBL binding sites on IgM, containing mainly GlcNAc-terminated glycans, which are strong ligands for MBL (*20*). Also, the oligomannose structures present on IgM at Asn-402 and Asn-563 could provide binding sites for MBL (*25, 26*).

Knockout of the Gal epitope in pigs is currently being combined with overexpression of human complement regulatory proteins such as hCD46 to prevent pig-to-human xenograft rejection. In this study, PAEC from GalTKO/hCD46

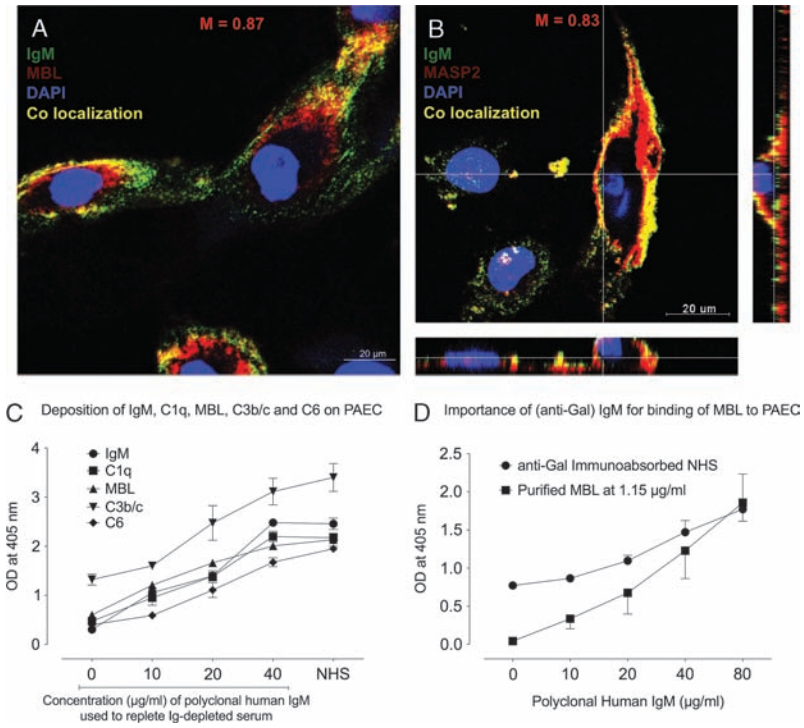

FIGURE 3. Role of IgM in the activation of lectin pathway. Colocalization (yellow) of IgM (green) with MBL and MASP2 (red), respectively. Representative colocalization images of IgM with the respective Manders' correlation coefficients are shown: (A) IgM-MBL (R_m=0.87) and (B) IgM-MASP2 (R_m=0.83) on NHS-treated WT PAEC. For IgM-MASP2 colocalization (B), also side views are shown as z-panels to the right and below the image. IgM-dependent MBL and complement deposition on PAEC. Deposition of MBL and complement components on PAEC was tested in the absence (anti-Gal immunoabsorbed NHS/Ig-depleted serum) or presence of anti-Gal IgM (repletion with polyclonal human IgM). C, deposition of IgM, C1q, MBL, C3b/c, and C6 on PAEC incubated with Ig-depleted serum repleted with increasing concentrations of purified human IgM as well as NHS. D, binding of MBL to PAEC in the presence anti-Gal immunoabsorbed NHS (1:10) or purified MBL at a fixed concentration (1.15 µg/mL). MBL-binding curves for addition of increasing concentrations of purified human IgM are shown. Data are mean±SD of three independent experiments.

pigs showed significantly reduced deposition of MBL and complement proteins as well as no cytotoxicity when treated with NHS. These observations suggest that GalTKO and expression hCD46 could also help to prevent lectin pathway activation during pig-to-human xenotransplantation.

Tissue samples from porcine forelimbs ex vivo xenoperfused with whole, heparin-anticoagulated human blood were analyzed for the involvement of IgM-mediated lectin pathway activation. Deposition of MBL, C1q, and factor Bb was observed on 1 hr perfusion samples, suggesting that all three pathways of complement are activated. In addition, MBL was colocalized with IgM in 12 hr perfusion samples and the finding of colocalized deposition of MBL with C3, C4, and C6 indicates that also in ex vivo xenoperfusion the MBL route of complement is functional, confirming the in vitro data.

In conclusion, our results demonstrate activation and pathogenic role of the lectin pathway of complement in xenotransplantation. Colocalized deposition of IgM with MBL and MASP2 indicates that activation of the lectin pathway of complement in this pig-to-human xenotransplantation model is dependent on IgM.

MATERIALS AND METHODS

Cell ELISA and Determination of Complement Activation Pathways in Xenotransplantation

A modified whole-cell ELISA was used to detect the complement pathways involved in xenotransplantation, similar to methods described previously (*27, 28*). WT PAEC as well as GalTKO/hCD46 transgenic PAEC were grown to confluence in 96-well plates and washed twice with ELISA wash buffer supplemented with or without calcium (45 mM NaCl, 10 mM HEPES, 680 mM $CaCl_2$, and 490 mM $MgCl_2$). Pooled NHS or alternatively C1q-depleted serum (A509; Quidel, Darmstadt, Germany) or MBL-deficient serum (HSR002; Statens Serum Institut, Copenhagen, Denmark) in GVB^{++} (1:2 or 1:10) was added to the cells and incubated at 37°C followed by washing with ELISA wash buffer. Cells were fixed in 1% paraformaldehyde for 15 min at room temperature (RT), washed, and blocked with phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA) for 90 min at RT. Goat anti-human IgM (fluorescein isothiocyanate [FITC] labeled; catalog number F5384; Dako, Carpinteria, CA), rabbit anti-human C1q (A0136; Dako), mouse anti-human MBL (clone 15C5; sc80598; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human factor Bb (ab72658; Abcam, Cambridge, UK), rabbit anti-human C3b/c (FITC labeled; A0062; Dako), rabbit anti-human C4b/c (FITC labeled; F0169; Dako), mouse anti-human C5b-9 (aE11; 5010; Diatec, Oslo, Norway), mouse anti-human CD62E (LS-C13922; LifeSpan,

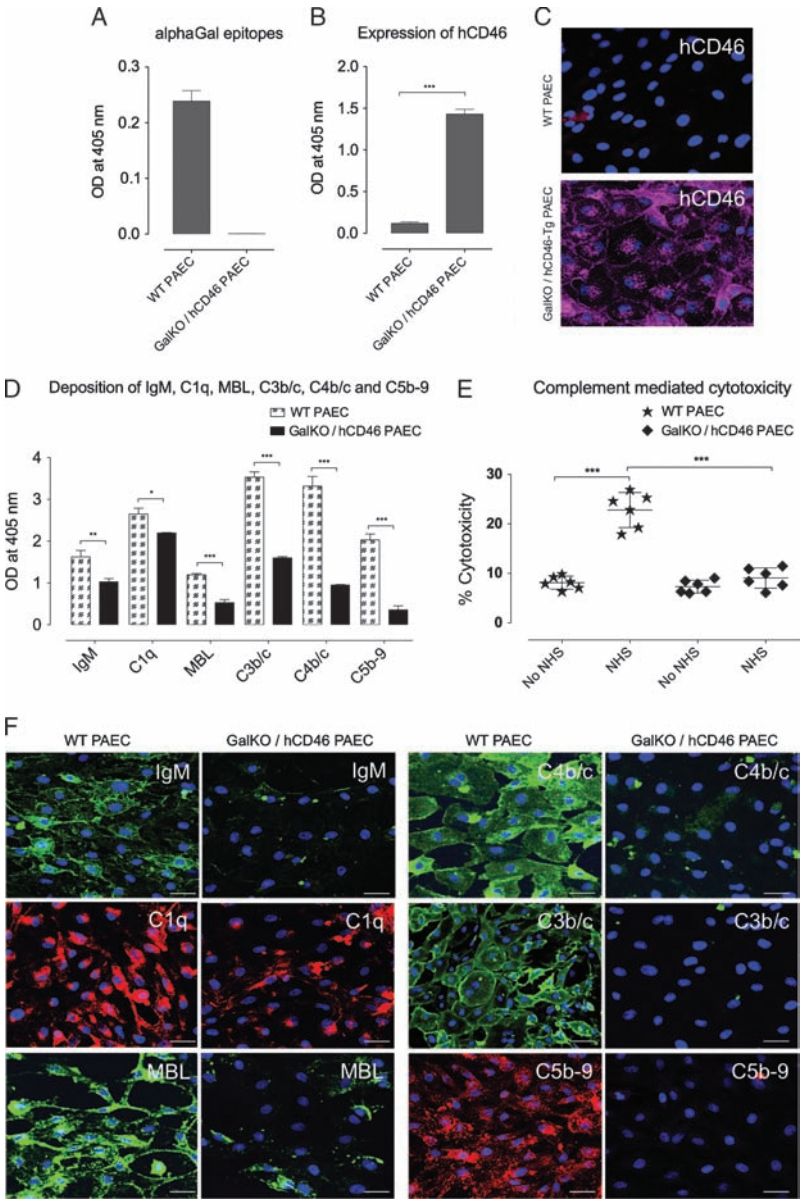

FIGURE 4. Regulation of complement activation on PAEC using genetic modification strategies. (A) Gal epitope and (B and C) hCD46 expression on WT and GalTKO/hCD46 PAEC analyzed by cell ELISA and IF staining. D, deposition of IgM, C1q, MBL, C3b/c, C4b/c, and C5b-9 on NHS (1:10)–treated WT and GalTKO/hCD46 PAEC. E, treatment of PAEC with 1:10 diluted NHS for 120 min at 37°C and assessment of cytotoxicity by calcein AM and EthD-1. F, confocal analysis of IgM, C1q, MBL, C4b/c, C3b/c, and C5b-9 on WT and GalTKO/hCD46 PAEC treated with 1:10 diluted NHS. Significance was tested using one-way analysis of variance with Bonferroni correction (*P<0.05, **P<0.01, ***P<0.001). Data are mean±SD of three independent experiments. Scale bars, 30 μm.

Seattle, WA), mouse anti-human VCAM-1 (1G11B1; 9510; Southern Biotech, Birmingham, AL), mouse anti-αGal (4F10; a gift of A. Bendelac, Howard Hughes Medical Institute [Chevy Chase, MD] and University of Chicago [Chicago, IL]), and mouse anti-human CD46 (HM2103; Hycult Biotech, Plymouth Meeting, PA) were diluted in PBS/1% BSA and incubated for 1 hr at RT followed by three washes. Subsequently, biotin-conjugated goat anti-mouse IgG (ab6788; Abcam) or goat anti-rabbit Ig (E0432; Dako) were used, diluted 1:500 in PBS/1% BSA, and incubated for 1 hr at RT. After washing, alkaline phosphatase–conjugated rabbit anti-FITC (ab49368; Abcam) or streptavidin–alkaline phosphatase (RPN1234V; GE Healthcare, Cleveland, OH) diluted 1:1000 in PBS/1% BSA was incubated for 30 min at RT. After washing, 1 mg/mL *p*-nitrophenyl phosphate substrate (Sigma, St. Louis, MO) was added. Color development was quantified at 405 nm (reference 490 nm; Infinite M1000 microplate reader; Tecan, Männedorf, Switzerland).

Complement-Mediated PAEC cytotoxicity

Complement-induced PAEC cytotoxicity was measured by calcein AM/ethidium homodimer-1 (EthD-1) staining. A Live/Dead Kit (Molecular Probes, Eugene, OR) was used and protocols provided by the manufacturer

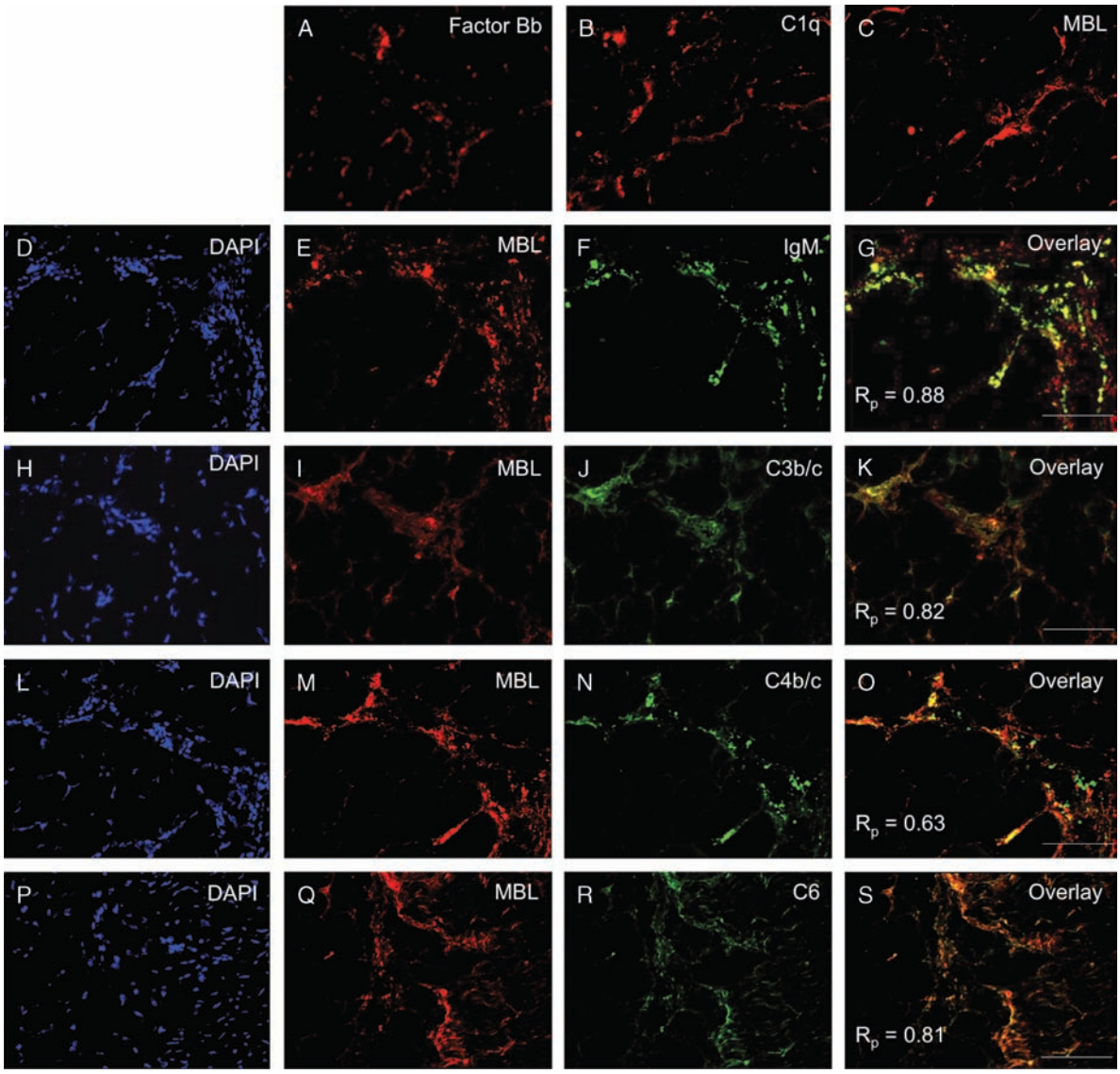

FIGURE 5. Colocalization of MBL with IgM and complement in an ex vivo xenoperfusion model. Tissue samples from ex vivo xenoperfused pig limbs with heparinized, whole human blood for 1 hr were assessed for deposition of (A) MBL, (B) C1q, and (C) factor Bb. Biopsy samples of 12 hr perfusion were analyzed for IgM-mediated lectin pathway activation by using immunofluorescence staining. D–G, deposition of MBL (red), IgM (green), and codeposition (yellow). H–K, deposition of MBL (red), C3b/c (green), and codeposition (yellow). L–O, Deposition of MBL (red), C4b/c (green), and codeposition (yellow). P–S, deposition of MBL (red), C6 (green), and codeposition (yellow). D, H, L, and P, 4′,6-diamidino-2-phenylindole staining of nuclei. The R_p values represent Pearson's colocalization coefficient. Representative images of six independent experiments are shown. Scale bars, 100 μm.

were adopted. Briefly, confluent WT and GalTKO/hCD46 PAEC in a 96-well plate were exposed to 1:10 diluted NHS, C1q-depleted or MBL-deficient serum, for 120 min at 37°C. After washing with PBS, 100 μL of 1 μM calcein AM and 2 μM EthD-1 was added to the cells and incubated in dark for 15 min. The fluorescence excitation and emissions of calcein AM and EthD-1 were acquired at 485/535 and 530/635 nm, respectively, using the Infinite M1000 microplate reader (Tecan).

IF Staining

Activation of the lectin pathway of complement was further investigated by immunofluorescence staining using confocal microscopy. Briefly, WT and GalTKO/hCD46 PAEC grown to confluence on eight-well Lab-Tek chamber slides (Milian) were washed twice with PBS^{++} (PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$) and treated with 1:10 dilution NHS for 45 to 60 min at RT. Slides were washed thrice and fixed with parapicric acid for 15 min at RT. After washing and blocking with PBS/3% BSA for 30 min at RT, slides were incubated for 60 min with primary antibodies for the following antigens: IgM (FITC labeled; F5380; Sigma), MBL (15C5; Santa Cruz Biotechnology), MASP2 (sc-17905; Santa Cruz Biotechnology), C4b/c-FITC (Dako), C3b/c-FITC (Dako), and C6 (A307; Quidel) diluted in PBS/1% BSA. After washing, fluorescence-labeled specific secondary antibodies diluted in

PBS/1% BSA were incubated for 60 min and the slides were mounted with glycergel (C0563; Dako). The stained slides were then analyzed using a Zeiss LSM5 confocal laser scanning microscope.

Immunoabsorption of Human Serum on PAA-Bdi Sepharose

To investigate the importance of anti-Galα1-3Gal IgM for MBL binding to PAEC, immunoabsorption of NHS was carried out to eliminate anti-Gal antibodies from NHS. Conjugates of the B-disaccharide Galα1-3Gal (Bdi) and poly-*N*-hydroxyethylacrylamide (PAA-Bdi), covalently linked to Sepharose 6FF (PAA-Bdi Sepharose), were obtained from Dr. Nicolai Bovin (Moscow, Russia). Mini-spin columns were packed with 100 or 200 μL PAA-Bdi Sepharose and rinsed with PBS (10–15 min, 200 rpm, 0°C). NHS (450 μL) was absorbed over the columns. Thereafter, immunoabsorbed NHS aliquots were prepared and stored at −80°C until use.

Role of IgM on MBL Binding to PAEC

The influence of IgM on binding of MBL to PAEC was investigated by cell ELISA using immunoabsorbed NHS and Ig-depleted serum (SunnyLab, Kent, UK). Human polyclonal IgM (A50168H; Meridian Life Science, Cincinnati, OH) was used to replete immunoabsorbed NHS/Ig-depleted serum. Binding of purified MBL (HSR008; Statens Serum Institut) on PAEC in the presence of human polyclonal IgM was also tested.

Ex vivo Xenoperfusion of Pig Limbs with Whole, Anticoagulated Human Blood

Six forelimbs of WT pigs were used to perfuse with whole, heparin-anticoagulated human blood (*29, 30*). Large white pigs were premedicated and anesthetized with ketamine, xylazine, midazolam, and atropine and mechanically ventilated (O_2/air 1:3, isoflurane 1–1.5 vol.%). Forelimbs were amputated by dissection of the shoulder girdle muscles using an electrocautery device (ICC 350; ERBE Elektromedizin, Tübingen, Germany). The neurovascular bundle in the axillar region was laid open and two veins and one artery of the amputated forelimb were cannulated using 10 French cannulas. All animal experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and Swiss national guidelines. The study was approved by the local animal experimentation committee of the Canton of Bern.

Five hundred milliliters of blood each were withdrawn from individual human donors into standard transfusion bags to which 10,000 IU heparin were added. The porcine forelimbs were attached to extracorporeal perfusion circuits as described previously (*29*). Xenogeneic perfusion of the porcine limbs was then performed with the whole, heparin-anticoagulated human blood. The perfusion parameters (arterial blood gas, response to nerve stimulation, etc.) were monitored throughout the experiment.

Skeletal muscle biopsies were collected before perfusion from the contralateral extremity as baseline samples. Different time-point biopsies were collected after 1 hr (n=1) and 12 hr (n=6) of perfusions. Samples were fixed in 2% buffered formaldehyde solution for 24 hr and then transferred into 18% sucrose for 15 hr. Then, the samples were embedded in Shandon M1 embedding matrix (Thermo Scientific, Waltham, MA) and stored at −20°C until sectioned.

IF Staining for IgM-Dependent Lectin Pathway Activation on Ex Vivo Xenoperfusion Samples

IF staining was performed on ex vivo xenoperfusion biopsy samples for the deposition of MBL and its colocalization with IgM, C3b/c, C4b/c, and C6. In brief, 20-μm-thick sections were cut, fixed on slide, and treated with TBS-Triton X-100 for 15 min. Deposition of MBL and its colocalization with complement proteins was assessed by using specific antibodies as mentioned above. Stained slides were observed using fluorescence microscopy (DMI 4000B; Leica, Wetzlar, Germany).

ACKNOWLEDGMENTS

The authors thank Prof. Nicolai Bovin and Dr. Elena Korchagina (Moscow, Russia) for the kind gift of PAA-Bdi Sepharose for immunoabsorption of anti-Gal antibodies. Expert technical support was provided by Dr. Daniel Mettler, Mrs. Olgica Beslac, and Mr. Daniel Zalokar (Experimental Surgery Laboratory, Department of Clinical Research, University of Bern).

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Paper II

Complement dependent early immunological responses during ex vivo xenoperfusion of wild-type and hCD46/HLA-E double transgenic porcine forelimbs with whole, heparin anti-coagulated human blood

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Status: Submitted in *Xenotransplantation*

Aim: Using a new founder line, the potential of combined overexpression of human CD46 and HLA-E to prevent hyperacute and acute vascular xenograft rejection was tested in an *ex vivo* pig-to-human xenoperfusion model.

Method: Alpha-Gal heterozygous, HLA-E/hCD46 double transgenic as well as wild-type pig forelimbs were *ex vivo* perfused with whole, heparinized human blood and autologous blood, respectively.

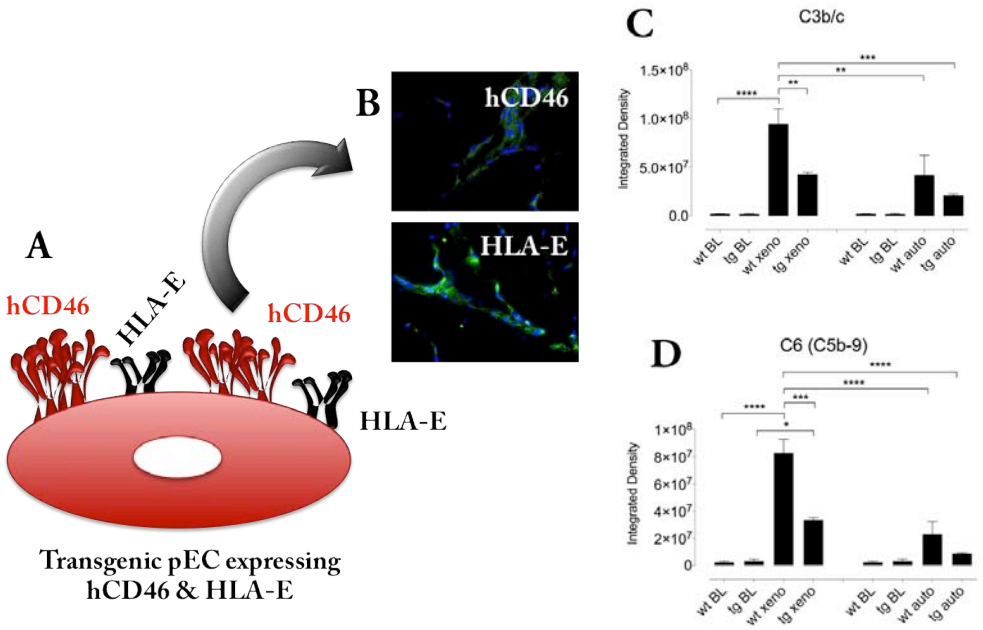

Figure: (A) Transgenic porcine EC expressing human CD46 and HLA-E. (B) Analysis of transgenic pig limb tissue by immunofluorescence staining showed expression of both transgenes. In transgenic limbs, reduced complement (C) C3b/c and (D) C5b-9 deposition was observed as compared to wild-type limbs, in both xenogenic (heparinized human blood) and autologous (heparinized pig blood) ex vivo perfusions. pEC = porcine endothelial cell; tg = transgenic; wt = wild-type; auto = autologous; xeno = xenoperfusion.

Conclusion: Transgenic hCD46/HLA-E expression clearly reduced humoral xenoresponses since the terminal pathway of complement, endothelial cell activation, and inflammatory cytokine production as well as tissue apoptosis was down-regulated. Overall, this model represents a useful tool to study early immunological responses during pig-to-human vascularized xenotransplantation.

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Complement dependent early immunological responses during ex vivo xenoperfusion of wild-type and hCD46/HLA-E double transgenic porcine forelimbs with whole, heparin anti-coagulated human blood

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Author's contributions

A.K.B. participated in the research design, writing of the article, performance of the research, and data analysis.

D.K. and H.J. participated in performing the animal experiments and the overall design of the study.

A.B. and N.K. participated in performing the animal experiments, the overall design of the study and participated in production of hCD46/HLA-E pigs.

D.A. provided primary cells from hCD46 transgenic pigs for nuclear transfer experiments.

E.W. participated in production of hCD46/HLA-E pigs.

E.V. participated in the concept and design of the study and carried part of the responsibility.

M.A.C. participated in performing the animal experiments, in the concept and design of the study and carried part of the responsibility.

J.D.S. provided scientific support and reagents and participated in the critical revision of the article.

R.R. participated in the concept and design of the study, performance of experiments, analyzing the data, writing the manuscript, and carried the main responsibility for the study.

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Abstract

Background: Besides α1,3 galactosyltransferase gene (GGTA1) knockout several transgene combinations to prevent pig-to-human xenograft rejection are currently being investigated. In this study, the potential of combined overexpression of human CD46 and HLA-E to prevent complement- and NK-cell mediated xenograft rejection was tested in an ex vivo pig-to-human xenoperfusion model.

Methods: Alpha-Gal heterozygous, hCD46/HLA-E -double transgenic as well as wild-type pig forelimbs were ex vivo perfused with whole, heparinized human and autologous pig blood, respectively. Blood samples were analyzed for production of porcine and/or human inflammatory cytokines. Biopsy samples were examined for deposition of human and porcine C3b/c, C4b/c, and C6 as well as CD62E and CD106 expression. Finally, apoptosis was measured in the porcine muscle tissue using TUNEL assays.

Results: No hyperacute rejection was seen in this model. Extremity perfusions lasted for up to 12 h without increase of vascular resistance and were terminated due to continuous small blood losses. Plasma levels of porcine cytokines IL1β, IL-6, IL-8, IL-10, TNF-α and MCP-1 as well as soluble C5b-9 were lower in blood perfused through transgenic as compared to wild-type limbs. Human C3b/c, C4b/c, and C6 as well as CD62E and CD106 were deposited in tissue of wild-type limbs, but no C6 deposition and significantly lower levels of CD62E and CD106 expression were detected in transgenic limbs perfused with human blood. Finally, transgenic porcine tissue was protected from xenoperfusion-induced apoptosis.

Conclusion: Transgenic hCD46/HLA-E expression clearly reduced humoral xenoresponses since all, the terminal pathway of complement activation, endothelial cell activation and muscle apoptosis, and inflammatory cytokine production were all down-regulated. Overall, this model represents a useful tool to study early immunological responses during pig-to-human vascularized xenotransplantation in the absence of hyperacute rejection.

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Background

With advances in the development of genetic modifications strategies, transgenic pigs are currently the preferred species for future organ xenotransplantation. The generation of alpha(1,3)-galactosyltransferase gene (GGTA1) knockout (GalTKO) pigs has largely overcome the barrier of hyperacute rejection and is considered to be the basis for further genetic modifications (1-3). In addition to GalTKO, overexpression of human complement regulatory proteins (hCRPs) CD55 (decay accelerating factor [DAF]) (4), CD46 (membrane cofactor protein [MCP]) (5-7), and/or CD59 (8) are necessary to block activation of the complement system by non-anti-Gal antibodies (9, 10). While CD59 limits the final terminal polymerization of the membrane-attack complex at the C8 to C9 stage, it has no regulatory function on C3 and C5. Therefore, CD55 and CD46 which also prevent the production of the proinflammatory chemotactic peptides C3a and C5a may be more suitable to prevent complement activation in the setting of xenotransplantation (reviewed in Ref. (11)). Although CD55 accelerates the decay of assembled C3bBb and C4b2a, but cannot cleave C4b and C3b, once deposited to the surface of pig cells (12). Moreover, CD55 is more effective in preventing the classical pathway of complement activation, whereas CD46 is effective both the classical and alternative pathways (7, 13, 14). In conclusion, CD46 may be preferred over CD55 and CD59 unless a combination of CRPs is used to achieve an even more complete complement inhibition (7, 15).

Rejection mechanisms subsequent to hyperacute rejection, such as acute vascular rejection and cellular rejection mediated by various leukocyte subsets, including natural killer (NK) cells, play a crucial role in xenograft survival (16). However, neither GalTKO nor overexpression of hCRPs protects the xenograft against the latter (17, 18). Human NK cells are able to adhere to porcine endothelial cells in vitro, infiltrate pig organs perfused with human blood ex vivo and mediate lysis of porcine cells directly or via antibody-dependent cell-mediated mechanisms (18-21). The functionality of NK cells is regulated by a balance between inhibitory and activating receptors (22). In particular, inhibitory NK receptors recognize classical and non-classical MHC class I molecules and thereby prevent autoreactivity (23). A majority of NK cells expresses the inhibitory receptor CD94/NKG2A that specifically binds to human leukocyte antigen (HLA)-E, a non-classical MHC molecule (24). The mechanism of human anti-pig xenogeneic NK cytotoxicity is based on the lack of recognition of porcine MHC molecules by human inhibitory NK receptors (25, 26).

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Consequently, stable surface expression of HLA-E on porcine cells has been shown to provide partial protection against NK cytotoxicity (27-29).

Taken together, the generation of double transgenic pigs expressing hCRPs and HLA-E on the cell surface might help to overcome human anti-pig xenograft rejection. In the present study, a new line of double transgenic pigs overexpressing hCD46 and HLA-E was achieved by crossing hCD46 transgenic pig (7) provided by Revivicor, Inc., with HLA-E/beta2-microglobulin (in short: HLA-E) transgenic pig (28). To study the effect of hCD46 and HLA-E expression on complement-mediated xenograft rejection, forelimbs of wild-type and hCD46/HLA-E-double transgenic pigs were ex vivo perfused with whole, heparinized human blood or autologous pig blood.

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Materials and methods

Ex vivo perfusion model

Six double transgenic (hCD46/HLA-E; transgenic) and 6 wild-type forelimbs of large white pigs (39.3 ± 4.1 kg) were used to perform ex vivo xenoperfusions with heparinized whole human blood. In addition, 4 transgenic and 4 wild-type pig forelimbs were used for perfusion with autologous pig blood as controls. Animal experiments in this study were performed according to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and Swiss National Guidelines. Pigs were pre-medicated with ketamine (20 mg/kg), midazolam (1 mg/kg), and atropine (0.05 mg/kg), before intubation, induction of anesthesia by isoflurane (1-1.5 vol. %) and mechanical ventilation with a Draeger respirator (O_2/air 1:3). Using an electrocautery device (ERBE ICC 350, ERBE Electromedizin), forelimbs were amputated by dissection of the girdle muscles. The neurovascular bundle in the axilla was laid open and 10 French cannulas inserted into the two veins and one artery. Five hundred ml whole human blood and 500 ml autologous pig blood, respectively, were withdrawn into standard transfusion bags containing 10,000 IU of heparin (Liquemin, Drossapharm). The amputated porcine forelimbs were connected to extracorporeal circuits (ECC) as described previously (30, 31). Hydroxyethyl starch perfusion was used for the first 5 min in order to wash out porcine blood containing cells and metabolic products accumulated in the extremity during the amputation procedure. Perfusions were performed for 12 hrs. The flow rate was maintained at 100-150 ml/min (MEDOS DataStream blood pump, model DP2; Medos Medizintechnik AG). Temperature was kept at 32°C (Heater-Cooler Unit HCU30; Maquet GmbH & Co KG), and O_2 at 21% by membrane oxygenator (MEDOS Hilite 800 LT; Medos Medizintechnik AG). The blood lost during perfusion from the open wound surface was collected into a sterile reservoir bag and drained back into the perfusion system. Throughout the experiment, perfusion parameters, including arterial blood gas analysis and perfusion pressure, were monitored. Potassium and pH levels were controlled to physiological levels by adding insulin and/or 40% glucose solution.

Tissue and blood sampling

Biopsies of skeletal muscle as well as serum and EDTA-plasma samples were taken at baseline and 12 h (end) of perfusion. Snap-frozen tissue samples were used for analysis by immunofluorescence and tissue fixed in 4% buffered formaldehyde was used for paraffin-embedding and standard histological evaluation. Blood samples

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were centrifuged at 3000 rpm for 7 min at 4°C, aliquoted and stored at -80°C for further analysis.

Histopathology

Formaldehyde fixed and paraffin embedded muscle biopsies of were transversally cut to 3 µm thick sections and stained with hematoxylin-eosin (H&E). Every sample was evaluated in a blinded manner for the following histological changes: perivascular edema, erythrocyte extravasation, leukocyte recruitment, intraluminal thrombi, and loss of endothelial layer in medium-sized vessels as indicators of endothelial dysfunction and vascular leakage. Hypereosinophilia and nuclear changes (such as pyknosis, karyorrhexis, or karyolysis as signs of necrosis and apoptosis) were also quantified. In addition, tissue-specific alterations were subsumed as loss of normal histologic architecture of the parenchyma. Disrupted muscle fibers, disturbed or lost cross striations and decomposed endomysium and epimysium were distinguished from hypothermia- and fixative-induced fiber shortening and thus gap formation as indicator of muscle edema. Fragmentation of the lamina elastica interna or externa and morphology of the vasa vasorum was assessed. Every criterion observed on H&E samples was scored from 0 to 3 (0 = absence, 1 = scarce, 2 = intermediate, and 3 = maximal presence) and summed up for a histologic injury severity score (HISS, min. = 0, max. = 21) as reported previously (31).

Immunofluorescence

Snap-frozen biopsy samples were cut into 5 µm thick sections, air dried for 30-60 min and either processed immediately or stored at -80°C until further analysis. Following fixation with acetone and hydration, the sectioned were stained using either one step direct or two-step indirect immunofluorescence techniques. The following antibodies were used: goat-anti human C3b/c-FITC (Dako), goat anti-human C4b/c-FITC (Dako), goat anti-human C6 (Quidel) / mouse anti-human C5b-9 (Diatec), mouse anti-human CD62E (LifeSpan Biosciences), mouse anti-pig VCAM-1 (CD106), goat anti-human VE-cadherin (Santa Cruz Biotechnologies), and rabbit anti-human HSPG (Abcam). Cross-reactivity with respective porcine antigen was verified. Secondary antibodies were goat anti-mouse IgG1 / IgG (Alexa488, -546 or -633), donkey anti-goat IgG Alexa488, rabbit anti-sheep IgG Alexa488 (Molecular Probes), and sheep anti-rabbit IgG-Cy3 (Sigma). Nuclear staining was done by using 4',6-diamidino-2-phenylindole (DAPI, Boehringer). The slides were analyzed using a fluorescence

microscope (DMI4000B, Leica). Quantification of fluorescence intensity was performed using Image J software, version 10.2 (http://rsb.info.nih.gov/ij/), on unmanipulated TIFF images. All pictures were taken under the same conditions to allow for correct quantification and comparison of fluorescence intensities.

Inflammatory markers

Human- or porcine-specific cytokines (IL-1β, IL-6, IL-8, IL-10, TNF-α), porcine C5a and species unspecific (human/porcine) soluble C5b-9 and MCP-1 were measured by multiplex xMAP technology (Luminex) using custom-made (32) or commercially available (Bio-Rad) reagents (33). Briefly, microbeads (Luminex) labeled with different fluorochrome codes were coupled with capture antibodies specific for the respective analyte using Bio-Plex amine coupling kit (Bio-Rad). Coupled beads were then incubated with samples, followed by biotinylated detection antibodies and Streptavidin-R-PE (Qiagen). Measurement and data analysis were performed with a Bio-Plex 100 array reader and the Bio-Plex Manager software version 6.1.

Human C5a (DuoSet kit, R&D), human C3a (reagents from Abcam) and porcine C3a (reagents from MBM ScienceBridge) were measured in EDTA plasma using ELISA according to the manufacturer's protocol. Analysis of species-specificity of these reagents using purified human and porcine antigens, respectively, revealed no cross-reactivity.

Assessment of apoptosis by TUNEL assay

Apoptosis was analyzed on 5 µm thick cryosections in high-power fields by terminal deoxynucleotidyl transferase-mediated (dUTP) nick end-labeling (TUNEL) according to the manufacturer's instructions (In Situ Cell Death Kit; Roche Diagnostics). As a positive control, slides were incubated with DNAse1 (Sigma) to induce DNA strand breaks. For every TUNEL stained section, a subsequent section was stained with DAPI to visualize total number of nuclei. Apoptosis was expressed by normalizing the results to the total number of nuclei per high-power field. Four high power fields per experiment and section were analyzed.

Statistical analysis

Data are shown as mean ± standard deviation. They were analyzed using GraphPad Prism 6 (GraphPad Software). Significance was tested using One-Way ANOVA with Bonferroni's correction or Student's t-test (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001).

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Results

Expression of hCD46 and HLA-E in transgenic pigs

Freshly frozen forelimb muscle tissue biopsies, obtained from transgenic and wild-type pigs, respectively, were stained for hCD46 and HLA-E as the protective effects of these proteins in pig-to-human xenotransplantation depend on their expression levels. Immunofluorescence staining using the specific monoclonal antibodies showed no staining of hCD46 and HLA-E on wild-type tissue whereas transgenic tissue showed strong staining, mainly on the vascular endothelium (Figure 1).

Histopathological evaluation

Histological assessment of H&E stained tissue sections revealed intermediate damage of muscle and blood vessels in both xeno- and autologous perfusions of wild-type pig limbs. Perivascular and muscular tissue edema, erythrocyte extravasation, leukocyte infiltration as well as disrupted muscle fibers and lost cross striations were the most prominent findings. Histological damage scores for xenogeneic and autologous perfusions of transgenic limbs were apparently lower than the damage seen in wild-type limbs, but the difference between groups ($p>0.2711$ to $p>0.9999$, data not shown) did not reach statistical significance (Figure 2).

Regulation of complement activation in transgenic limbs

Deposition of the complement proteins, C3, C4 and C6 on tissue samples was investigated by immunofluorescence (IF) staining. Human C3b/c, C4b/c and C6 deposition was significantly higher in wild-type xenoperfusion samples than in all other perfusions. Quantitative analysis of IF images using Image-J software revealed a significant reduction ($p<0.0001$) of C3b/c, C4b/c and C6 deposition on both transgenic xenoperfusion and transgenic autologous perfusion samples as compared to wild-type xenoperfusion samples. In wild-type autologous perfusion samples, minimal complement deposition was noted. However, this deposition was focal and may be due to minor mechanical or ischemic damage (Figure 3). In line with the reduced levels of complement deposition in the histological analysis, the plasma levels of the anaphylatoxins human C3a and human C5a as well as soluble C5b-9 were significantly reduced in xenoperfusions of transgenic limbs ($p<0.05$ to $p<0.0001$) as compared to wild-type limbs (Figure 4).

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Endothelial cell integrity and activation

We next assessed the protective effects of transgenic hCD46/HLA-E on the endothelium, focusing on cell integrity (by staining for heparan sulfate proteoglycan [HSPG] and VE-cadherin) and activation (staining for CD62E [E-selectin] and CD106 [VCAM-1]). Expression of the endothelial cell integrity markers HSPG and VE-cadherin was essentially maintained in all perfusion samples with a circumferentially staining pattern of the cells, except in wild-type xenoperfusion limbs where a reduced staining for HSPG was found (Figure 5). Endothelial activation markers CD62E and CD106 were significantly ($p<0.0001$) upregulated as compared to baseline in wild-type xenoperfusion samples and correlated well with complement deposition (r^2 = 0.89 to 0.98, data not shown). In contrast, expression of CD62E and CD106 were significantly ($p<0.0001$) reduced in transgenic xenoperfusion samples as compared with wild-type perfusions (Figure 6). Taken together these data indicated that inhibition of complement-mediated endothelial damage by transgenic expression of hCD46 and HLA-E, respectively, preserved endothelial integrity and reduced endothelial activation in transgenic xenoperfused samples.

Cytokine patterns in plasma

Concentrations of plasma cytokines at baseline were not different between groups. In wild-type xenoperfusions, porcine IL-1β, IL-6, IL-8, and IL-10 as well as human/porcine MCP-1 levels were all significantly increased over baseline levels after 12 h of perfusion ($p<0.05$ to $p<0.0001$), but this was not the case for porcine TNF-α ($p=0.247$) (Figure 7A-F). Comparison of wild-type vs. transgenic xenoperfusion revealed increased porcine IL-1β ($p<0.0001$) and IL-8 ($p=0.0190$) levels in wild-type after 12 h perfusion, but porcine IL-6 ($p=0.056$) were not significantly elevated. Similarly, the levels of porcine IL-10 ($p=0.754$), porcine TNF-α ($p>0.999$) and human/porcine MCP-1 ($p>0.999$) were not significantly different between wild-type and transgenic xenoperfusions. In addition, human cytokines in plasma samples of wild-type and transgenic xenoperfusions were measured using human-specific Bio-Plex kits. Human IL-8 levels were significantly ($p=0.046$) higher than baseline after wild-type xenoperfusions, but the difference to transgenic xenoperfusions was not significant. Levels of human IL-1β, IL-6, IL-10 and TNF-α were not significantly elevated with respect to baseline and there was no significant difference between wild-type and xenoperfusions either.

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Porcine cytokines measured in plasma samples of autologous perfusion of wild-type and transgenic limbs were used as control. Porcine IL-6 ($p=0.030$ and $p=0.052$), TNF-α ($p=0.020$ and $p=0.002$) and MCP-1 ($p<0.0001$ for both) were significantly higher at 12 h of wild-type and transgenic limb perfusions, respectively, as compared to respective baseline values. In contrast, IL-1β, IL-8, and IL-10 were not elevated as compared to baseline ($p>0.9999$). In addition, no significant differences ($p>0.9999$) of porcine cytokine levels was observed between wild-type and transgenic autologous perfusions.

TUNEL staining

To analyze apoptotic changes on muscle biopsies sections TUNEL-stainings were performed for the different experimental groups and viewed at high-power magnification. The proportion of apoptosis expressed as percentage of TUNEL positive nuclei per total nuclei was significantly greater in samples from wild-type xenoperfused limbs (70.5 ± 11.0%, $p<0.0001$) compared with transgenic xenoperfusions (45.3 ± 8.7%), wild-type autologous perfusions (29.8 ± 15.0%), and transgenic autologous perfusions (19.6 ± 8.7%) (Figure 8).

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Discussion

Ex vivo organ xenoperfusion is accepted as a useful tool to study and evaluate strategies to prevent rejection in pig-to-human xenotransplantation (34, 35). We here describe the functional potential of combined overexpression of hCD46 and HLA-E to prevent xenograft rejection in a novel ex vivo xenoperfusion model using extracorporeal perfusion of amputated limbs (30). We previously showed that prolonged autologous limb perfusion of up to 12 h using an extracorporeal circuit does not cause any significant tissue damage (31). Here we used the same system to xenoperfuse pig forelimbs with human, heparin anticoagulated whole blood. We studied the effect of transgenic expression of hCD46/HLA-E on complement activation, endothelial activation and graft damage. Surprisingly, and in contrast to organ xenoperfusion settings, no hyperacute rejection was seen in this model and perfusions lasted for at least 12 h, irrespective of the presence of transgenes. The absence of hyperacute rejection – which cannot be fully explained based on the available data – makes this model unique and suitable to study delayed immunological responses, which normally require in vivo experiments.

Histological analysis of tissue samples showed no significant damage in both wild-type autologous and wild-type xenoperfusions, but a – yet insignificant – trend towards a higher histological damage in wild-type xenoperfusions as compared to transgenic xenoperfusions was observed. These data are thus in line with the observation that complement activation may be critically influence xenograft rejection (36-38). Analysis of wild-type xenoperfusion by immunofluorescence staining showed significantly higher complement C3b/c, C4b/c and C6 deposition than detected in samples of transgenic xenoperfusions. Transgenic expression of hCD46/HLA-E significantly reduced terminal complement pathway formation, as indicated by reduced C3b/c, C4b/c and C6 deposition in transgenic xenoperfusion samples as compared with wild-type. Plasma concentrations of anaphylatoxins C3a and C5a were also higher in wild-type than in transgenic xenoperfusion samples. It was initially believed that complement regulatory proteins are species specific, but more recent work suggests that quantity of expression is more important in protecting the endothelium than species specificity (39). Indeed our data show a trend, however not statistically significant, to reduced porcine complement deposition in autologous perfusions of transgenic as compared to wild-typet tissue. Overall, our results suggest that overexpression of hCD46/HLA-E significantly reduced complement deposition and preserved endothelial integrity in xenoperfused limbs.

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Complement deposition can leads to shedding of heparan sulfate proteoglycans (40), a predominant component of the glycocalyx, which may be pivotal for endothelial dysfunction by turning the healthy anti-inflammatory and anticoagulant endothelial glycocalyx into a pro-inflammatory and procoagulant endothelial surface (41, 42). Indeed, HSPG expression was reduced following wild-type but to a lower degree in transgenic xenoperfusions. In addition, significant differences in xenoperfusion-induced endothelial activation were observed between wild-type and transgenic limbs. The graft endothelial damage leads to the release of pro-inflammatory cytokines, which in turn causes further activation of the endothelium. Adhesion molecules, including CD62E and VCAM-1, are among the first factors expressed by activated endothelium (43). Furthermore, endothelial activation also causes upregulation of vWF, tissue factor and reduced CD39 expression, and proceeds to cell loss by apoptosis and necrosis (44). Here, we showed that the overexpression of hCD46/HLA-E significantly reduced complement induced endothelial CD62E and VCAM-1 expression. Graft endothelial damage leads to the release of pro-inflammatory cytokines, including IL-6 which in turn causes further activation of the endothelium. The release of pro-inflammatory cytokines has also been associated with xenograft rejection mechanisms (33). In general, we found significantly increased porcine cytokine levels during wild-type xenoperfusion as compared to baseline levels, but this was not the case in transgenic xenoperfusions. No significant difference in the levels of porcine cytokines was observed between wild-type and transgenic xenoperfusion samples (12 h), with the exception of IL-1β. The release of porcine cytokines may be largely dependent on complement mediated porcine limb endothelium activation, in contrast to the human response, which was mediated by activated human polymorphonuclear cells, NK cells and monocytes in these perfusion settings (33).

Finally, transgenic expression of hCD46 and HLA-E clearly protected against apoptosis as well as histological damage as shown in muscle biopsies. Myocyte death in xenografts occurs via apoptotic pathway and directly correlates with the severity of graft rejection and can be induced by numerous stimuli, such as cytotoxic T lymphocytes, NK cells, xenogeneic serum and inflammatory cytokines like TNF-α (45, 46). Our study did not evaluate defined causes for apoptosis induction in limb tissue, but we show that apoptosis does occur in wild-type xenoperfusions and is associated with increased complement deposition and endothelial activation in wild-type xenoperfused limbs. In addition, the data on apoptosis correlate well with the

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observed histological features in wild-type xenoperfusions, including disrupted muscle fibers and loss of cross striations.

In conclusion, this study using ex vivo xenoperfusion of amputated pig limbs with human blood, provides evidence for a positive effect of the combined overexpression of hCD46 and HLA-E on early rejection mechanisms. Protection against xenograft tissue damage and apoptosis was determined in particular with respect to complement-mediated effects, vascular endothelial cell activation, and cytokine release.

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Figure legends

Figure 1: Expression of transgenic hCD46 and HLA-E proteins on muscle tissue samples from hCD46/HLA-E-double transgenic pigs. Freshly frozen tissue sections were stained with anti-CD46 (M177) and anti-HLA-E (3D12). Immunostaining revealed hCD46 and HLA-E expression, mainly on the vascular endothelium (indicated by asterisk) on samples stemming from transgenic pigs, but not in wild-type pig tissue. Scale bar: 50 µm.

Figure 2: Histologic evaluation of muscle tissue from ex vivo perfused porcine forelimbs: Representative H&E stained tissue samples (12h perfusion) of wild-type and transgenic porcine forelimbs perfused with whole, anti-coagulated human blood (wt xeno and tg xeno, respectively) or autologous pig blood (wt auto and tg auto, respectively) are shown. Scale bar: 100 µm. The most obvious findings in both wild-type perfusions are: perivascular and muscular edema, erythrocyte and leukocyte extravasation (indicated by solid line arrow), loss of endothelial layer (asterisk), disrupted muscle fibers (dotted line arrow), and lost cross striations (A). Histologic injury severity scores (HISS): Every of the above criteria was scored from 0–3 (0=absence, 1=scarce, 2=intermediate, and 3=maximal presence) and summed up to a HISS (minimum = 0, maximum = 21). Values are mean ± SD of HISS in hematoxylin-eosin stained limb biopsies. One-Way ANOVA with Bonferroni's post test revealed no significant difference ($p \geq 0.13$) between all limb perfusions (B). n=4 for autologous and n=6 for xenoperfusions. auto = autolohous perfusion; xeno = xenoperfusion; tg = hCD46/HLA-E double transgenic; wt = wild-type; BL = baseline;

Figure 3: Effect of transgenic expression of hCD46 and HLA-E on complement deposition. Freshly frozen tissue samples from ex vivo perfused pig limbs were analyzed for deposition of human and/or porcine complement proteins C3b/c, C4b/c and C6 using one-step direct or two-step indirect immunofluorescence staining. Representative images of C3b/c (A), C4b/c (C) and C6 (E) deposition on 12h perfused tissue samples. Scale bar: 100 µm. Wild-type xenogeneic and autologous perfusion samples showed increased deposition of complement proteins, whereas transgenic hCD46 expression resulted in significantly reduced complement deposition. Quantitative analysis of C3b/c (B), C4b/c (D) and C6 (F) deposition was performed using Image J software and statistical analysis was done by One-Way ANOVA testing with Bonferroni's correction (B). Data are mean ± SD, *$p \leq 0.05$,

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**p≤0.01, ***p≤0.001, ****p≤0.0001. auto = autologous perfusion; xeno = xenoperfusion; tg = hCD46/HLA-E double transgenic; wt = wild-type; BL = baseline;

Figure 4: Generation of human (in xenoperfusion) and porcine (in autologous perfusion) anaphylatoxins C3a and C5a and species unspecific (human/porcine) soluble (s)C5b-9. EDTA plasma samples collected after 12 h perfusion from ex vivo wild-type and transgenic limb perfusions were analyzed for the generation of complement activation products using ELISA (human C3a, human C5a and porcine C3a) and Bio-Plex (porcine C5a and sC5b-9). Complement activation at baseline (BL) and after 12 h of wild-type xenogeneic and autologous perfusions resulted in elevated levels of human and porcine C3a (A) and C5a (B) as well as soluble C5b-9 (C). Transgenic hCD46 expression inhibited complement activation upon perfusion and thus was associated with significantly reduced C3a, C5a and sC5b-9 formation. Data are expressed as mean ± SD; significance was tested using One-Way ANOVA with Bonferroni's correction (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001). auto = autologous perfusion; xeno = xenoperfusion; tg = hCD46/HLA-E double transgenic; wt = wild-type; BL = baseline;

Figure 5: Preservation of endothelial integrity after xenoperfusion of porcine limbs with transgenic expression of hCD46 and HLA-E. Depicted are representative images of HSPG (red) and VE-cadherin (green) staining of muscle biopsies (mainly on the vascular endothelium indicated by dotted lines) at baseline and after 12 h of perfusion. Staining of HSPG and VE-cadherin showed no changes in transgenic xenoperfusions as compared to baseline, whereas in wild-type xenoperfusion samples, a reduced HSPG staining was seen. Scale bar: 100 µm. auto = autologous perfusion; xeno = xenoperfusion; tg = hCD46/HLA-E double transgenic; wt = wild-type; BL = baseline;

Figure 6: Inhibition of complement-induced xenograft endothelial activation by transgenic expression of hCD46 and HLA-E. Xenoperfusion-induced endothelial activation was evaluated by IF staining on freshly frozen tissue samples for the expression of adhesion molecules CD62E (E-selectin) and CD106 (VCAM-1) as activation markers. Representative images of IF staining for CD62E (A) and CD106 (C) on sections of muscle biopsies after 12 h of perfusion are shown. Scale bar: 100 µm. Expression of CD62E and CD106 were significantly lower in samples from transgenic xenoperfusions as compared to wild-type xenoperfusion limbs. IF staining

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intensities of CD62E (B) and CD106 (D) were quantitatively measured using Image J software and One-Way ANOVA with Bonferroni's correction. Data are mean ± SD, *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001. auto = autologous perfusion; xeno = xenoperfusion; tg = hCD46/HLA-E double transgenic; wt = wild-type; BL = baseline;

Figure 7: Effect of transgenic expression of hCD46 and HLA-E on human and porcine cytokine release. Pro-inflammatory (IL-1β, IL-6, TNF-α), anti-inflammatory cytokine (IL-10) and chemokine (IL-8, MCP-1) levels were analyzed at baseline (BL) and after 12 h perfusion. Custom-made multiplex kits were used for porcine cytokines and commercially available multiplex kits for human cytokines. Data are expressed as mean ± SD; significance was tested using One-Way ANOVA with Bonferroni's correction (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001). auto = autologous perfusion; xeno = xenoperfusion; tg = hCD46/HLA-E double transgenic; wt = wild-type; BL = baseline;

Figure 8: Transgenic hCD46 and HLA-E expression protects porcine tissue from xenoperfusion-induced apoptosis. Terminal deoxynucleotidyl transferase-mediated (dUTP) nick-end labeling (TUNEL) assay was performed to detect apoptotic cells that undergo extensive DNA degradation during the last stage of apoptosis. Representative images of TUNEL staining of wild-type xeno, transgenic xeno, wild-type auto and transgenic auto perfusion samples are depicted (A). TUNEL-positive cells are shown in red and corresponding DAPI staining of all nuclei in blue. Scale bar: 100 µm. Significantly reduced apoptosis was observed in transgenic limbs as compared to wild-type limbs. The amount of apoptosis was expressed as % TUNEL by normalizing the results to the total number of nuclei per high-power field (HPE) (B). Significance was tested using One-Way ANOVA with Bonferroni's correction (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001). Data expressed as mean ± SD. auto = autologous perfusion; xeno = xenoperfusion; tg = hCD46/HLA-E double transgenic; wt = wild-type; BL = baseline;

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Figure 1

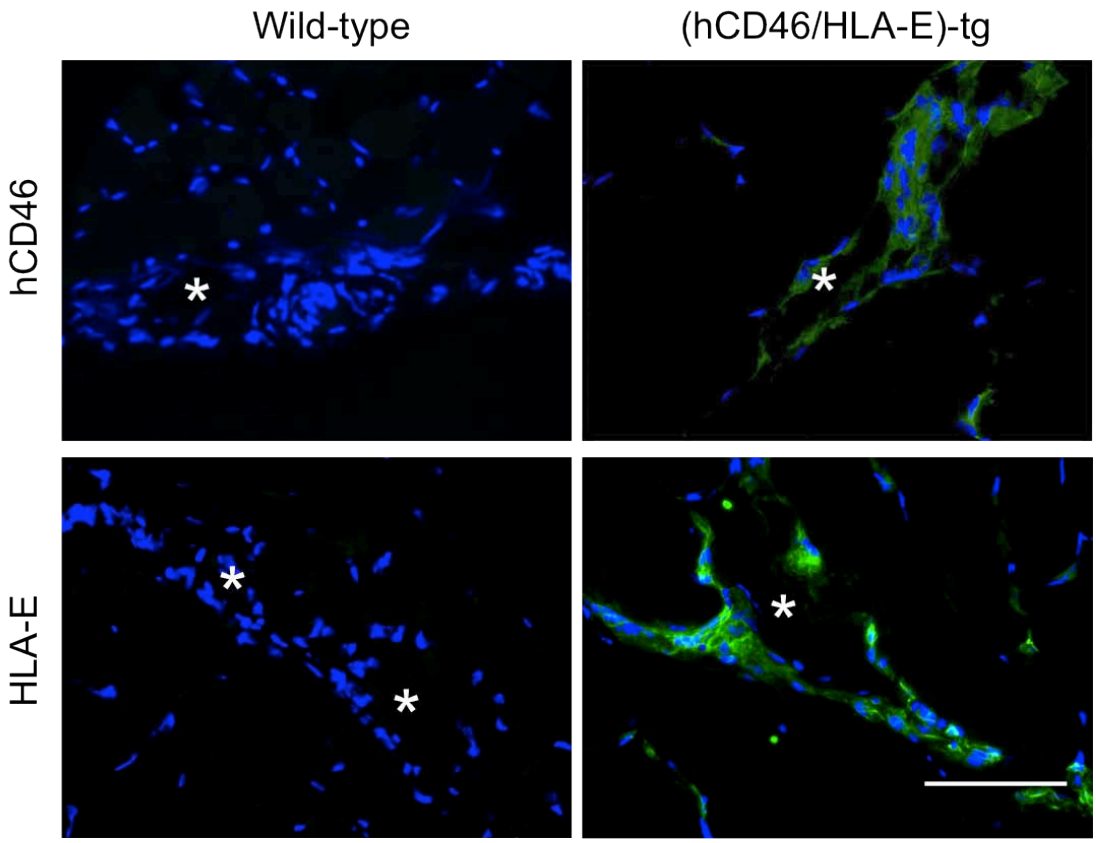

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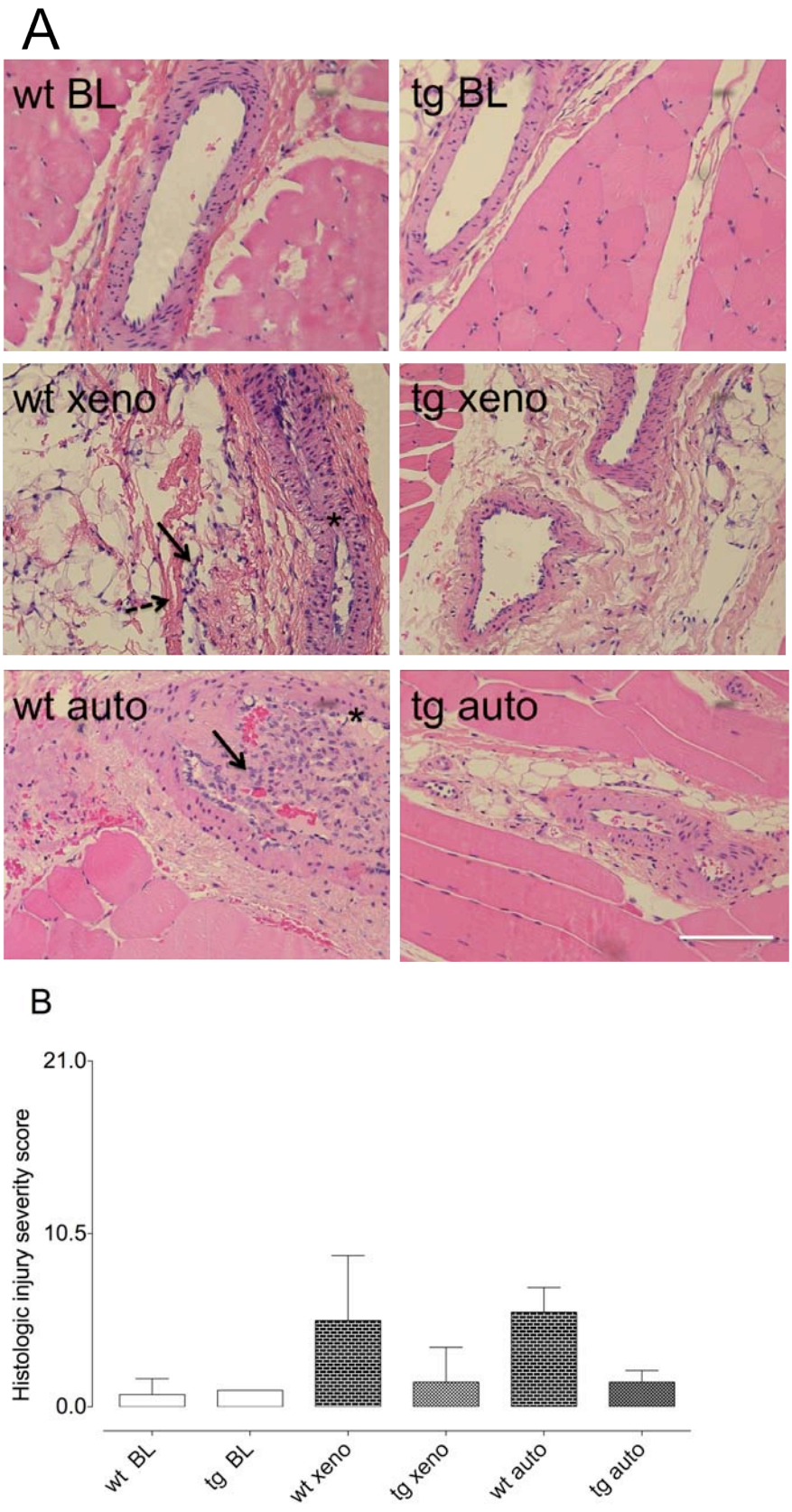
Figure 2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wt xeno
tg xeno
wt auto
tg auto
B
21.0
10.5
0.0
Histologic injury severity score
wt BL
tg BL
wt xeno
tg xeno
wt auto
tg auto

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Figure 3

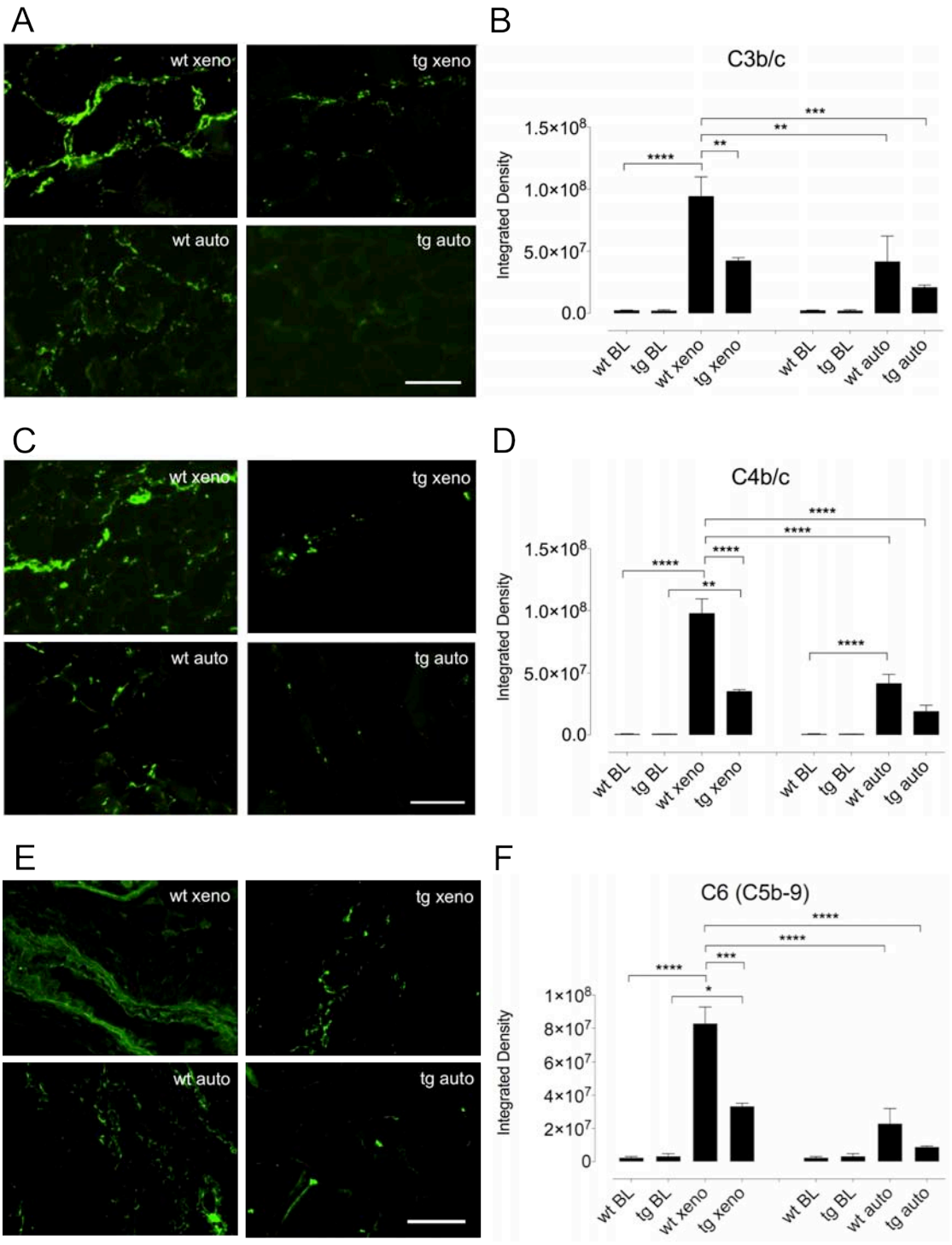
A
wt xeno
tg xeno
wt auto
tg auto
B
C3b/c
Integrated Density
1.5×10^8
1.0×10^8
5.0×10^7
0.0
wt BL
tg BL
wt xeno
tg xeno
wt auto
tg auto
C
D
C4b/c
E
F
C6 (C5b-9)
1×10^8
8×10^7
6×10^7
4×10^7
2×10^7
0

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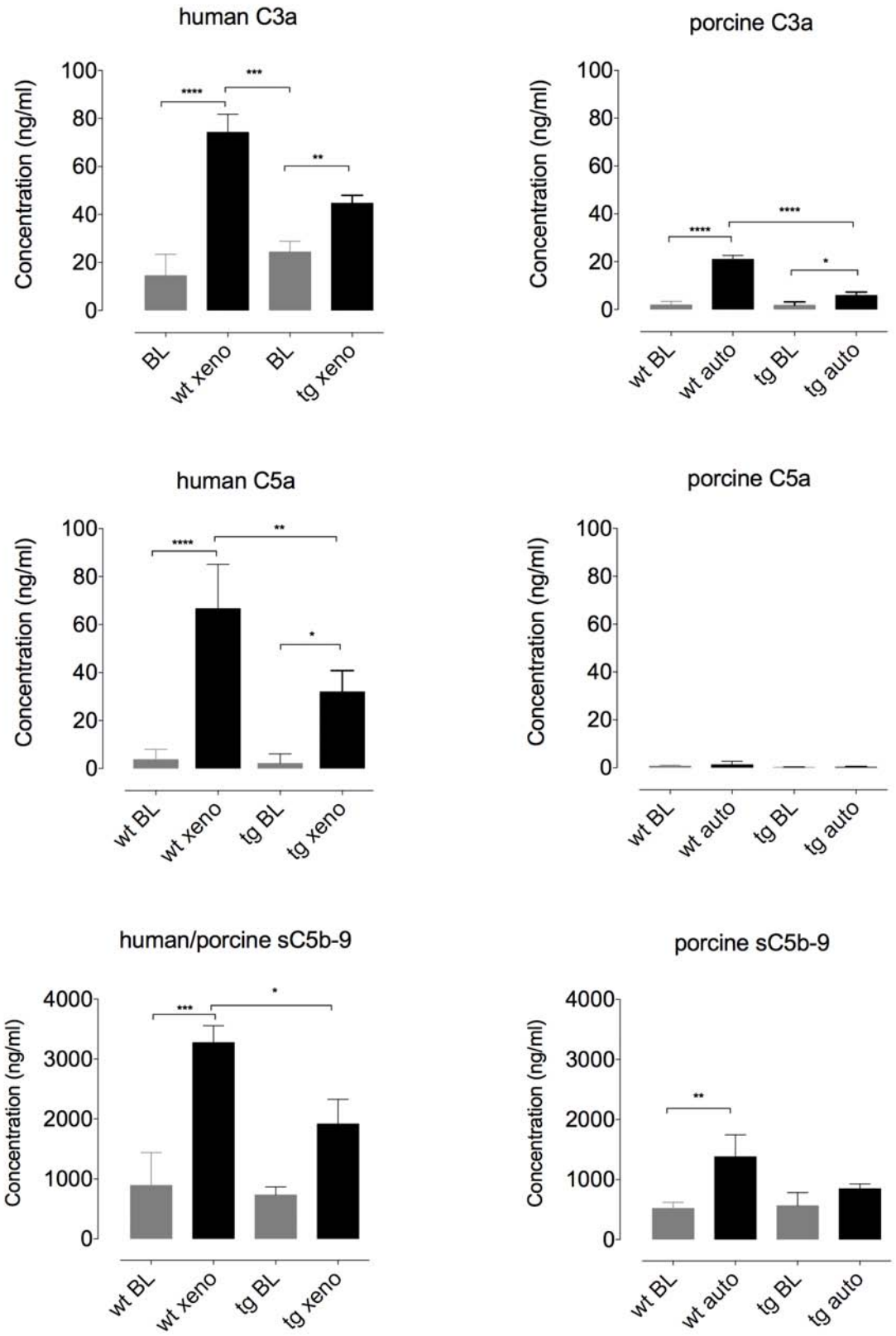
Figure 4


human C3a
Concentration (ng/ml)
100
80
60
40
20
0
****
***
**
BL
wt xeno
BL
tg xeno
porcine C3a
Concentration (ng/ml)
100
80
60
40
20
0
****
****
*
wt BL
wt auto
tg BL
tg auto
human C5a
Concentration (ng/ml)
100
80
60
40
20
0
****
**
*
wt BL
wt xeno
tg BL
tg xeno
porcine C5a
Concentration (ng/ml)
100
80
60
40
20
0
wt BL
wt auto
tg BL
tg auto
human/porcine sC5b-9
Concentration (ng/ml)
4000
3000
2000
1000
0
***
*
wt BL
wt xeno
tg BL
tg xeno
porcine sC5b-9
Concentration (ng/ml)
4000
3000
2000
1000
0
**
wt BL
wt auto
tg BL
tg auto

▪

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Figure 5

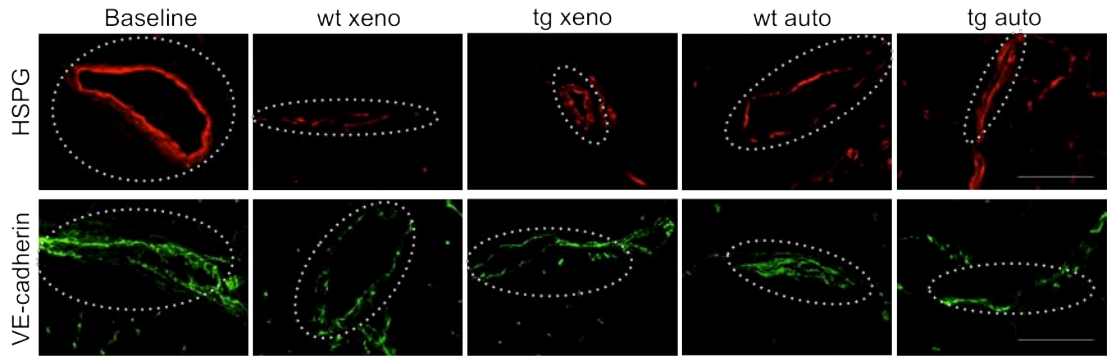

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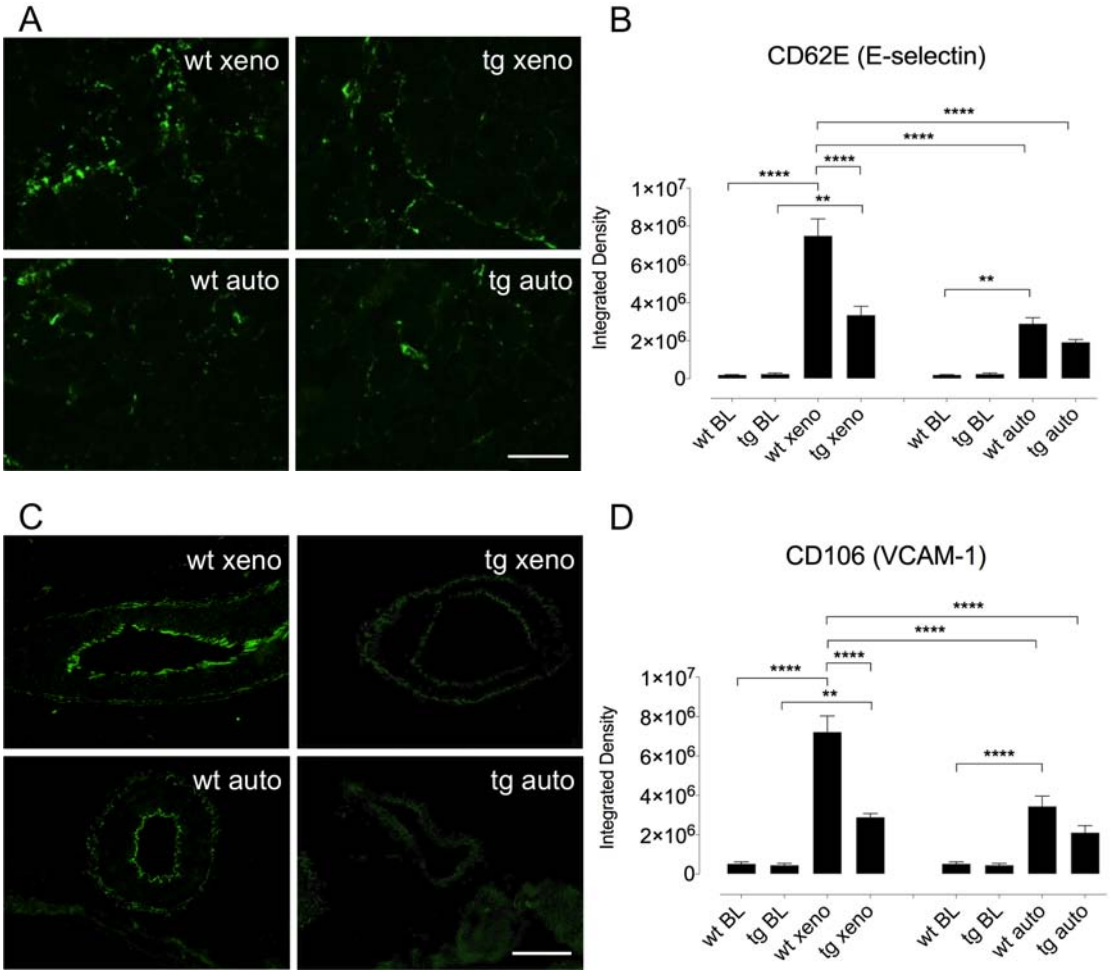
Figure 6


A
wt xeno
tg xeno
wt auto
tg auto
B
CD62E (E-selectin)
Integrated Density
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8×10^6
6×10^6
4×10^6
2×10^6
0
wt BL
tg BL
wt xeno
tg xeno
wt auto
tg auto
C
wt xeno
tg xeno
wt auto
tg auto
D
CD106 (VCAM-1)
Integrated Density
wt BL
tg BL
wt xeno
tg xeno
wt auto
tg auto

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Figure 7

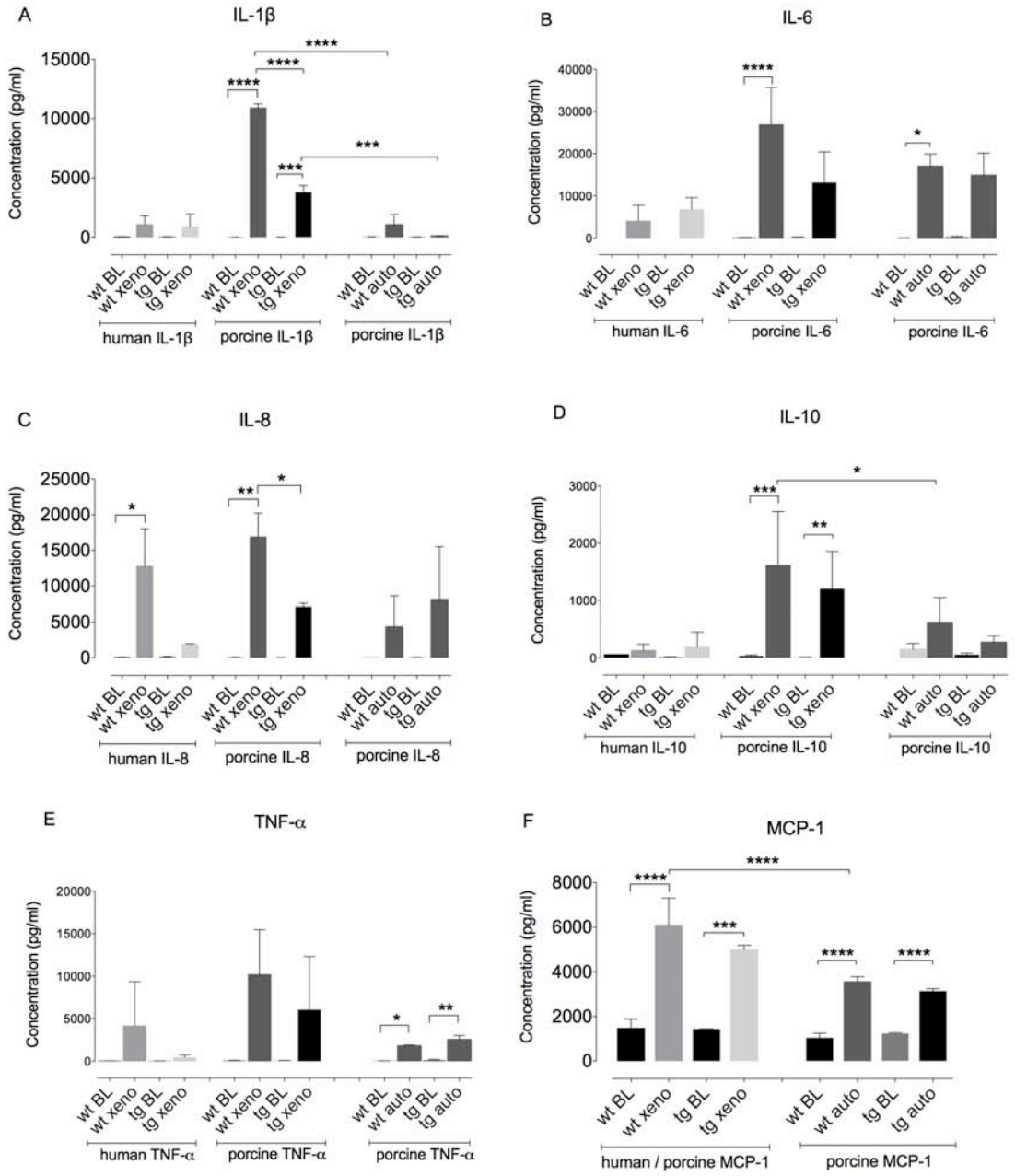
A
IL-1β
Concentration (pg/ml)
15000
10000
5000
0
****
****
****
***
***
wt BL
wt xeno
tg BL
tg xeno
wt BL
wt xeno
tg BL
tg xeno
wt BL
wt auto
tg BL
tg auto
human IL-1β
porcine IL-1β
porcine IL-1β
B
IL-6
Concentration (pg/ml)
40000
30000
20000
10000
0
****
*
wt BL
wt xeno
tg BL
tg xeno
wt BL
wt xeno
tg BL
tg xeno
wt BL
wt auto
tg BL
tg auto
human IL-6
porcine IL-6
porcine IL-6
C
IL-8
Concentration (pg/ml)
25000
20000
15000
10000
5000
0
*
**
*
wt BL
wt xeno
tg BL
tg xeno
wt BL
wt xeno
tg BL
tg xeno
wt BL
wt auto
tg BL
tg auto
human IL-8
porcine IL-8
porcine IL-8
D
IL-10
Concentration (pg/ml)
3000
2000
1000
0
***
**
*
wt BL
wt xeno
tg BL
tg xeno
wt BL
wt xeno
tg BL
tg xeno
wt BL
wt auto
tg BL
tg auto
human IL-10
porcine IL-10
porcine IL-10
E
TNF-α
Concentration (pg/ml)
20000
15000
10000
5000
0
*
**
wt BL
wt xeno
tg BL
tg xeno
wt BL
wt xeno
tg BL
tg xeno
wt BL
wt auto
tg BL
tg auto
human TNF-α
porcine TNF-α
porcine TNF-α
F
MCP-1
Concentration (pg/ml)
8000
6000
4000
2000
0
****
****
***
****
****
wt BL
wt xeno
tg BL
tg xeno
wt BL
wt auto
tg BL
tg auto
human / porcine MCP-1
porcine MCP-1

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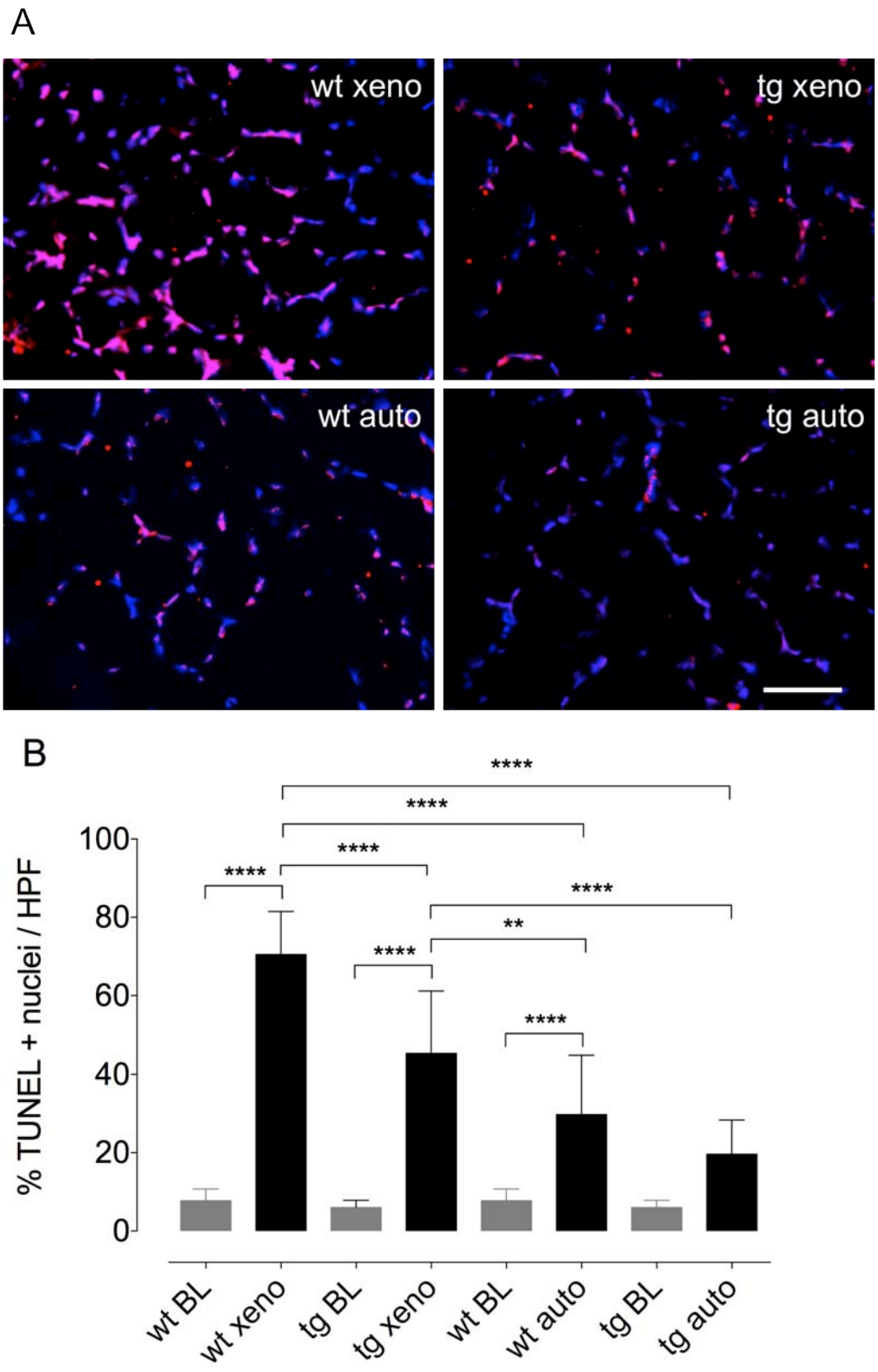
Figure 8


A
wt xeno
tg xeno
wt auto
tg auto
B
% TUNEL + nuclei / HPF
100
80
60
40
20
0
****
****
****
****
****
**
****
****
wt BL
wt xeno
tg BL
tg xeno
wt BL
wt auto
tg BL
tg auto

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Paper III

ASGR1 expressed by porcine vascular endothelial cells mediates human platelet phagocytosis in pig-to-human xenotransplantation models

Anjan K. Bongoni[1,2,3], David Kiermeir[3], Thusitha Gajanayake[3], Hansjörg Jenni[4], Andrea Bähr[5], Annegret Wünsch[5], David Ayares[6], Jörg D. Seebach[7], Eckhard Wolf[5], Nikolai Klymiuk[5], Esther Vögelin[3], Mihai A. Constantinescu[3], Robert Rieben[1,3,*]

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Status: in preparation for submit to *Xenotransplantation*

Background: Ex vivo porcine limb perfusion with whole, anti-coagulated human blood results in platelet loss observed within minutes to hours after perfusion. More recently, it has been shown that the asialoglycoprotein receptor-1 (ASGR1) on sinusoidal endothelial cells bind and phagocytosis human platelets in an ex vivo liver xenoperfusion. Human platelets have more galactose- and N-acetylgalactosamine-terminating epitopes available as compared with pig platelets for ASGR1.

Aim: These observations led us to examine ASGR1 expression on porcine aortic endothelial cells (PAEC) as well as PAEC / ASGR1 mediated xenogenic platelet phagocytosis.

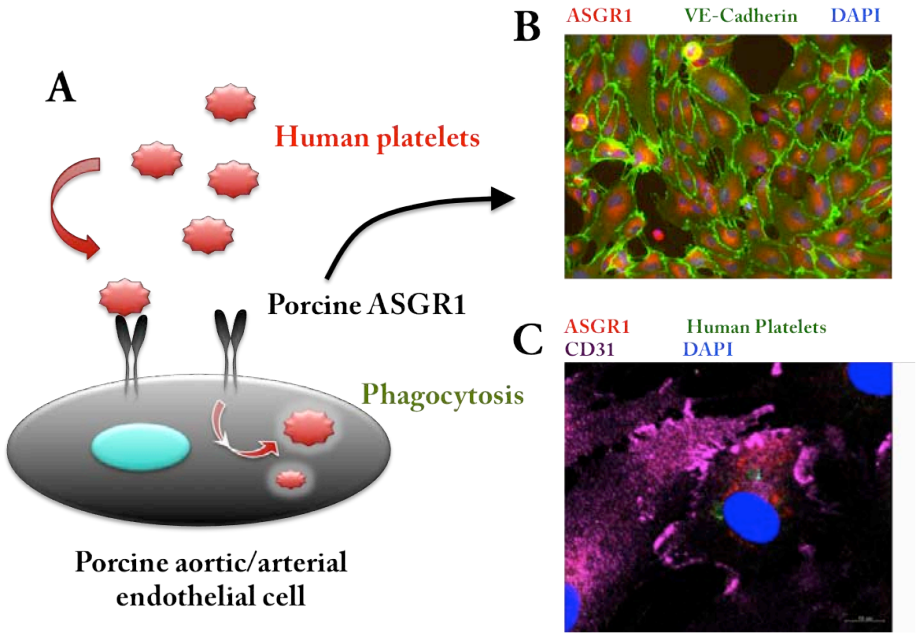

Figure: (A) Diagrammatic illustration of porcine endothelial and ASGR1 mediated human platelet phagocytosis in pig-to-human xenotransplantation. (B) Immunofluorescence staining of porcine aortic/arterial endothelial cells showed ASGR1 expression (red) and the endothelial marker VE-cadherin (green). (C) In vitro analysis of ASGR1 (red) mediated phagocytosis of freshly isolated CFSE-labeled human platelet (green). Endothelial cells stained positive for CD31 (purple). Endothelial nuclei stained with DAPI (blue).

Conclusion: In xenoperfusion of porcine tissue with human blood, ASGR1 expressed on pig vascular endothelium is involved, at least in part, in binding and phagocytosis of human platelets.

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ASGR1 expressed by porcine vascular endothelial cells mediates human platelet phagocytosis in pig-to-human xenotransplantation models

Anjan K. Bongoni[1,2,3], David Kiermeir[3], Thusitha Gajanayake[3], Hansjörg Jenni[4], Andrea Bähr[5], Annegret Wünsch[5], David Ayares[6], Jörg D. Seebach[7], Eckhard Wolf[5], Nikolai Klymiuk[5], Esther Vögelin[3], Mihai A. Constantinescu[3], Robert Rieben[1,3,*]

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Key words: Porcine vascular endothelial cells, ASGR1, platelets, phagocytosis, xenotransplantation

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Author's contributions

A.K.B. participated in the research design, writing of the article, performance of the experiments, data analysis.

D.K. and H.J. participated in performing the animal experiments and the overall design of the study.

A.B., A.W., E.W., N.K. produced HLA-E/hCD46 pigs and multi-modified pigs and provided the respective endothelial cells.

D.A. provided primary cells from hCD46 transgenic pigs for nuclear transfer experiments.

J.D.S. provided scientific support and reagents and participated in the critical revision of the article.

T.G., E.V. and M.A.C. participated in the concept and design of the study and carried part of the responsibility.

R.R. participated in the concept and design of the study, analyzing the data, writing the manuscript, and carried the main responsibility for the study.

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Abstract

Background: The asialoglycoprotein receptor-1 (ASGR1) mediates capture and endocytosis of Gal(beta1-4) and GalNAc(beta1-4) terminating glycoproteins. Human platelets, carrying more of these epitopes than pig platelets are phagocytosed by porcine liver sinusoidal endothelial cells (LSEC) following binding to ASGR1. Here we assessed ASGR1 expression on porcine aortic / limb arterial endothelial cells (PAEC/PLAEC) as well as PAEC/ASGR1 mediated xenogeneic platelet phagocytosis in vitro and ex vivo.

Methods: Wild-type and HLA-E/hCD46 double transgenic porcine forelimbs were perfused with whole, heparinized human or autologous pig blood. Platelets were counted at regular intervals during perfusion. Ex vivo pig limb biopsies as well as in vitro cultured PAEC/PLAEC were characterized for ASGR1 expression. Human platelets were exposed to PAEC and analyzed for ASGR1-mediated xenogeneic platelet phagocytosis including inhibition assays using anti-ASGR1 antibodies.

Results: Human platelet numbers decreased from 121 ± 31 at beginning to 14 ± 6 x 10^3/µl ($P<0.0001$) after 10 min of perfusion, whereas no significant decrease of pig platelets was seen during autologous perfusions (176 ± 35 to 161 ± 54 x 10^3/µl). Porcine endothelial cells on muscle biopsies and PAEC/PLAEC in vitro showed ASGR1 expression. Phagocytosis of human CFSE-labeled platelets by PAEC peaked at 15 min and was inhibited by rabbit anti-ASGR1 antibody and asialofetuin, a competitive ligand for ASGR1.

Conclusion: ASGR1 expressed on aortic and limb arterial pig vascular endothelium plays a role in binding and phagocytosis of human platelets. Therefore, ASGR1 may represent a novel therapeutic target to overcome thrombocytopenia associated with vascularized pig-to-human xenotransplantation.

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Background

Xenotransplantation of porcine organs and cells may help to expand the pool of available donor organs for clinical transplantation. However, despite considerable progress over the past decades immune responses and other barriers prevent clinical application. The use of organs stemming from genetically modified pigs has indeed prevented hyperacute rejection, antibody-mediated or cellular acute rejection, at least in animal models (1-3). Nevertheless, other issues related to physiological and immunological incompatibilities, including thrombotic microangiopathy and systemic consumptive coagulopathy, need to be solved (4-6). The latter are characterized by platelet activation, aggregation and sequestration leading to a dysregulation of the coagulation system and eventually to organ dysfunction (7).

Thrombocytopenia has been observed previously in pig-to-primate xenotransplantation models, especially in pig-to-baboon liver transplantation (8) and during ex vivo porcine liver perfusion with human blood (9). Although hepatic function, including coagulation, has proved to be satisfactory, the immediate development of thrombocytopenia is very limiting for pig liver xenotransplantation even as a 'bridge' to allotransplantation (10). Recently, it has been shown that porcine Kupffer cells and liver sinusoidal endothelial cells (LSEC) bind and phagocytose human platelets in ex vivo perfusion systems (11). Many scavenger receptors have been reported on LSEC that facilitate the clearance of particulate, molecular and cellular ‘waste’ from the circulation (12), one of them being the asialoglycoprotein receptor-1 (ASGR1), a C-type lectin. It functions as a recycling receptor, which mediates capture and phagocytosis of Galβ1-4 and GalNAcβ1-4 terminating glycoproteins (13, 14). Other functions of ASGR1 that have been put forward include clearance of asialoglycoproteins (15) and apoptotic cells (16). ASGR1 may also participate in cell-cell, cell-matrix or intramatrix interactions (17). The receptor is expressed on porcine Kupffer cells, hepatocytes (18) and on porcine LSEC. Finally, ASGR1 on porcine LSEC mediates phagocytosis of human platelets (19). ASGR1-mediated human platelet binding depends on the availability of carbohydrate binding sites on ASGR1 as well as exposed ligand concentrations on platelets such as Galβ1-4GalNAc. Recently, it has been shown that, compared to porcine platelets, human platelets have four times more Galβ1-4GlcNAc and GalNAcβ1-4GalNAc ligands for ASGR1 (20).

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In summary, thrombocytopenia following liver xenotransplantation and ex vivo xenoperfusion is a well recognized phenomenon. In contrast, little is known on platelet phagocytosis mediated by other endothelial sites. Here we report the observation that ex vivo xenoperfusion of porcine forelimbs with human blood leads to an immediate loss of human platelets from circulation. We therefore examined ASGR1 expression on porcine vascular endothelial cells (PAEC/PLAEC), as well as its role for binding and phagocytosis of human platelets.

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Materials and methods

Ex vivo perfusion model

HLA-E (32)/hCD46 (33) double transgenic (tg) as well as wild-type (wt) pig forelimbs were used to perform ex vivo perfusion with whole, heparinized anti-coagulated human blood (xenoperfusion) or autologous blood (autologous perfusion), respectively. Care and use of animals in this study were according to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and Swiss National Guidelines. Pigs (wt and tg) were pre-medicated with ketamine (20 mg/kg), midazolam (1 mg/kg), and atropine (0.05 mg/kg) before intubation, induction of anesthesia by isoflurane (1-1.5 vol. %) and mechanical ventilation with a Draeger respirator (O_2/air 1:3) were maintained till the end of experiment. Using an electrocautery device (ERBE ICC 350, ERBE Electromedizin), forelimbs were amputated by dissection of the girdle muscles. In the axillar region, the neurovascular bundle was laid open and two veins and one artery were cannulated using 10 French cannulas (34, 35).

From human donors, 500 ml blood was withdrawn into standard transfusion bags to which 10,000 IU of heparin were added. The amputated porcine forelimbs were connected to extracorporeal circuits as described previously (34) and HAES-perfusion was used for the first 5 min in order to wash out porcine blood and cells as well as metabolic products accumulated in the extremity during the amputation operation. Porcine forelimbs were used for either xenogeneic or autologous perfusion with whole, heparinized anticoagulated human or autologous blood, respectively, for up to 12 hrs. Throughout the experiment, the perfusion parameters (arterial blood gas, nerve stimulation etc.) were monitored. Blood samples were collected at regular time intervals and blood cell counts performed using an automated hematology analyzer (Sysmex KX21N) to measure changes in platelet counts.

Endothelial cell isolation and culture

Porcine aortic endothelial cells (PAEC) and porcine limb arterial endothelial cells (PLAEC) were isolated from porcine aortas and femoral arteries using a previously described method (36). Briefly, excised blood vessels were rinsed with PBS and filled with 0.1% collagenase in PBS for 30 minutes. Endothelial cells in the collagenase perfusate were collected, centrifuged and the cell pellets resuspended in DMEM cell culture medium (Dulbecco's Modified Eagle's Medium) containing 10% fetal calf serum (FBS), 100 U/ml Penicillin, 100 µ g/ml Streptomycin (Invitrogen) and

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endothelial cell growth medium II supplement mix (C-39216, PromoCell). Cells were plated in cell culture flasks and grown until confluence, and were then used between passages two and eight in all experiments.

Platelet isolation and CFSE labeling

10 ml blood was drawn freshly from a healthy volunteer into citrate tubes and centrifuged at 150 x g for 15 min at 22°C. The supernatant, consisting of platelet-rich plasma (PRP), was collected and centrifuged at 1500 x g for 15 min at 22°C to obtain purified platelets. Washed platelets were resuspended in 15 ml carboxyfluorescein diacetate succinimidyl ester (CFSE, C34554, Invitrogen) work solution (5 µM) for 30 min at 37°C and then 15 ml PBS was added. The platelet solution was centrifuged at 300 x g for 15 min at 22°C. The resulting pellet was washed in 25 ml PBS solution, counted using a hemocytometer, and stored at room temperature for a maximum of 3 h. Samples were diluted to a final concentration of $4x10^8$ platelets/ml with PBS for experiments.

Immunofluorescence

Endothelial cells (PAEC and PLAEC) originating from aorta and femoral arteries, respectively, were grown as described above and seeded on 8-well Lab-Tek chamber slides (Milian SA). Cultured cells were fixed with 3.7% formaldehyde for 20 minutes, washed with PBS-1% BSA-0.05% Tween 20 and blocked with 3% BSA in PBS for 30 min at room temperature (RT). For analysis of tissue biopsies, slides with 5 µm cryosections of porcine limb muscle were fixed in acetone for 5 min. The slides were then incubated with endothelial specific antibodies: rat anti-pig CD31 (PECAM-1, clone 377537, #MAB33871, R&D systems), goat anti-human VE-cadherin (#sc-6458, crossreacting with porcine VE-cadherin, Santa Cruz Biotechnologies), rabbit anti-human ASGR1 (#18-003-42438, crossreacting with porcine ASGR1(19), GenWay Biotech) and mouse anti-human CD41 (FITC-labeled, #F7088, Dako) for 60 min at RT or overnight at 4°C. After primary antibody incubation, slides were washed and exposed to fluorescence conjugated secondary antibodies: donkey anti-rat IgG Cy3 (#712-166-150, Jackson Immunoresearch), donkey anti-goat IgG Alexa 488 (#A11055, Invitrogen) and sheep anti-rabbit IgG Cy3, (#C2306, Sigma) for 60 min at RT. After the immunostaining process, slides were washed, stained with 4', 6-diamidino-2-phenylindole (DAPI, #236 276, Boehringer Mannheim) and then mounted with Glycergel (#C0563, Dako). The slides were analyzed using a confocal laser-scanning microscope (LSM5, Zeiss).

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Western blot

Endothelial cells (PAEC) were lysed in radio-immunoprecipitation buffer (#R0278, Sigma) with protease inhibitor cocktail (#I3911, Sigma). Samples were analyzed with 4-20% mini-Protean TGX precast gels (#456-1095, Bio-Rad) and immunoblot analysis. Membranes were blocked with 50% Odyssey blocking buffer (#927-40000, LI-COR Biosciences) in PBS for 30 min followed by incubation with anti-ASGR1, and anti-beta actin (Rockland Immunochemicals for Research) according to manufacturer recommended specifications. Following primary antibody, membranes were probed with the appropriate secondary antibody conjugated to IRDye 800CW or IRDye 680CW (LI-COR Biosciences). Membranes were scanned with a LI-COR near-infrared Odyssey scanner (LI-COR Biosciences).

Flow Cytometry

Cells (PAEC and PLAEC) were harvested using EDTA-0.05% trypsin at 37°C, washed with PBS-1% BSA-0.02% sodium azide. Pellets were fixed by 2% formaldehyde in PBS for 15 min. Cells were then washed and stained with rabbit anti-ASGR1 for 30 min on ice in the dark. Following washing, the appropriate secondary antibody, sheep anti-rabbit IgG Cy3 was added for 30 min at 4°C in the dark. Cells were washed and resuspended in 1% formaldehyde. Data were collected with a BD LSR II Flow-Cytometer (BD Biosciences).

Cell ELISA

Confluent PAEC cultured in 96-well plate ($1x10^6$/well) were treated with pooled normal human serum (1:10) for 60 min at 37°C. After washing, cells were fixed in 3.7% formalin for 20 min, washed and blocked using PBS-1%BSA for 30 min at RT. Subsequently, rabbit anti-ASGR1 antibody was incubated for 60 min at RT followed by washing. Biotinylated goat anti-rabbit Ig (#E0432, Dako) was added for 60 min at RT, washed and incubated with alkaline phosphatase conjugated streptavidin for 30 min at RT. After washing, p-nitrophenyl phosphate substrate (#S0942, Sigma) was added. Color development was quantified at 405/490 nm using an Infinite M1000 microplate reader (Tecan).

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In vitro platelet phagocytosis – Cell ELISA

Analysis of phagocytosis of human platelets by PAEC was performed based on the assay described by Paris et al. (19). In brief, PAEC were cultured in 96-well plates ($1x10^6$/well), grown to confluence. $4x10^7$ CFSE-labeled human platelets were added to wells on the 96-well plates containing confluent endothelial cells. For inhibition assay, PAEC were pre-incubated with polyclonal rabbit anti-ASGR1 antibody, polyclonal rabbit IgG as isotype control (Sigma), or asialofetuin (#A4781, Sigma) for 30 min prior to addition of human platelets. The plates were placed on a shaker for 30 sec at 900 rpm to initiate the platelet-endothelial interaction. The plates were washed 3x with PBS, and then fixed in 3.7% formaldehyde-PBS for 20 min at RT. After fixation, the wells were washed and 50 µl PBS added. The fluorescence excitation and emissions of CFSE were acquired at 492/517 nm using a Tecan Infinite M1000 microplate reader.

In vitro platelet phagocytosis – Immunofluorescence staining

$4x10^7$ CFSE labeled human platelets were added to confluent endothelial cells on 8-well chamber slides for 60 min at 37°C. After incubation, the slides were washed with PBS^{++} (PBS containing 1mM $CaCl_2$ and 1mM $MgCl_2$) and fixed with 3.7% formaldehyde for 20 min. Following washing, slides were incubated with the following primary antibodies: anti-CD31, anti-VE-cadherin and anti-ASGR1 for 60 min at RT. After washing, fluorescence labeled secondary antibodies were incubated for 60 min, washed and mounted with glycergel. Slides were analyzed using a Zeiss LSM5 confocal laser-scanning microscope.

Statistical analysis

Data are shown as mean ± standard deviation. Data were analyzed using GraphPad Prism 6 (GraphPad Software). Significance was tested using One-Way ANOVA with Bonferroni's correction (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001).

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Results

Ex vivo perfusion model of xenotransplantation-induced platelet loss

A rapid loss of circulating human platelets was observed during xenoperfusion of pig forelimbs with whole, heparinized human blood, whereas in autologous pig blood perfusions only a slow decrease of platelet numbers was seen. The average platelet counts at baseline were 236.3 ± 28.9 $x10^3$/µl (human blood) and 301 ± 67.2 $x10^3$/µl (pig blood). The counts dropped about 2-fold to 121.4 ± 31.3 $x10^3$/µl and 171.3 ± 26.37 $x10^3$/µl, respectively, after the first run through the perfusion system before even getting into contact with porcine tissue. These reductions in platelet counts are explained by the dilution of the blood with hydroxyethyl starch solution (HAES; Voluven, Fresenius) which was used for priming of the extracorporeal circuit. After 10 min of xenoperfusion with human blood, platelet counts dropped approximately 10-fold, from 121.4 ± 31.3 $x10^3$/µl at baseline to 14.7 ± 6.1 $x10^3$/µl in wild-type (wt) ($p<0.0001$), and to 13.0 ± 5.6 $x10^3$/µl in hCD46/HLA-E transgenic (tg) pig limbs ($p<0.0002$), respectively. There were no significant differences in platelet counts between wt and tg pig limbs perfused with human blood throughout the experiments ($p=0.77$), whereas a lower platelet drop was noted in the tg compared to wt pig limbs perfused with autologous blood ($p=0.028$). In autologous pig blood perfusions the respective values after 10 min were not significantly lower than at onset (171.3 ± 26.37 $x10^3$/µl), namely 135.3 ± 25.6 $x10^3$/µl for wt and 188.3 ± 65.7 $x10^3$/µl for tg. These values were significantly ($p<0.0001$) higher than the ones of the respective xenoperfusions. After 60 min of perfusion, platelet counts were 11.3 ± 4.5 $x10^3$/µl (wt, xeno), 22.0 ± 9.4 $x10^3$/µl (tg, xeno), 65.3 ± 12.1 $x10^3$/µl (wt, auto) and 139.0 ± 51.0 $x10^3$/µl (tg, auto), respectively, and stayed at these levels throughout the remaining perfusion time (720 min in total, Figure 1).

ASGR1 expressed by porcine vascular endothelial cells

As shown by immunofluorescence (IF) and confocal microscopy cultured PAEC/PLAEC were positive for CD31, VE-cadherin, and ASGR1 (Figure 2A). Expression of ASGR1 on PAEC was further confirmed using flow cytometry (Figure 2B) and cell ELISA (Figure 2C). Treatment with NHS, which activates porcine endothelial cells had no effect on ASGR1 expression levels on PAEC (Figure 2C). Western blot analysis of whole cell lysates using PAEC cultured from wild-type as well as from GalTKO/hCD46/human thrombomodulin (hTM), HLA-E/hCD46, and

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GalTKO/hCD46 transgenic pigs all showed ASGR1 protein expression as a band at 50 kDa with no significant differences in expression levels (Figure 2D).

ASGR1 protein expression on pig limb biopsy samples

Wt and tg porcine limb muscle biopsy samples, collected at different time points, were all positive for ASGR1 expression as assessed by immunofluorescence. The fact that staining for ASGR1 co-localized with VE-cadherin/CD31 in all biopsy sections (Figure 3) indicated that ASGR1 is indeed expressed on endothelial cells, corresponding to the observations made in vitro using cultured PAEC/PLAEC. Moreover, muscle tissue biopsies from xenoperfusions were also analyzed for human platelet infiltration/sequestration by staining for CD41, a human platelet marker. As shown in Figure 3B, a higher number of human platelets was found in the muscle tissue after 1 h of perfusion as compared with 9 h and 12 h perfusion, respectively. Furthermore, fluorescence microscopy analysis showed that sequestered platelets (CD41) were co-localized with ASGR1 (Figure 3C) indicating binding of platelet aggregates to the endothelium via ASGR1.

Phagocytosis of CFSE-labeled human platelets by porcine endothelial cells

Freshly isolated human platelets were labeled with CFSE (carboxyfluorescein diacetate succinimidyl ester) and incubated with cultured PAEC in vitro. Within minutes human platelets uniformly bound to PAEC, with a peak of fluorescence intensity at 15 min. At 30 to 60 min, the fluorescence declined to ~ 50% (Figure 4A) as reported previously by Paris LL et al using LSEC and CFSE-labeled human platelets in vitro (19, 20). As shown by overlay immunofluorescence staining for CD31/ASGR1 (Figure 4B) CFSE-labeled human platelets adhered to the surface of PAEC after 30 min incubation. After 45 min however, CFSE-labeled human platelets were internalized by PAEC, and positive for double staining by ASGR1 and the lysosomal marker CD107a (Figure 4C).

Platelet phagocytosis is inhibited by blocking of the ASGR1 receptor

In order to confirm involvement of ASGR1 in human platelet phagocytosis, we examined the effect of blocking ASGR1 antibodies. Pre-incubation of PAEC with polyclonal rabbit anti-ASGR1 antibody (Figure 5A) at 2 µg/ml, (*$p<0.05$) or 1-2 µg/ml asialofetuin (Figure 5B), a competitive ligand for ASGR1, for 30 min, significantly reduced phagocytosis of CFSE-labeled human platelets, whereas a non-specific isotype control (polyclonal rabbit IgG) had no effect (Figure 5).

Discussion

During ex vivo xenoperfusion of porcine limbs with heparinized human blood, human platelets disappeared within minutes from the circulating blood. This phenomenon is in line with the observation of xenograft-induced thrombocytopenia and represents a major problem in pig-to-primate xenotransplantation (8). Determination of the mechanisms responsible for this thrombocytopenia is necessary for the development of preventive strategies. In this study, we have shown the expression of ASGR1, a known platelet binding C-type lectin receptor, in PAEC as well as PLAEC. In addition, we show by specific inhibition using polyclonal anti-ASGR1 antibody that human platelet binding and uptake by PAEC/PLAEC is mediated via ASGR1.

ASGR1, a component of the Ashwell-Morell asialoglycoprotein receptor, is located on hepatocytes, macrophages and LSEC, and mediates binding and removal of circulating glycoproteins carrying Gal and GalNAc terminating epitopes (20, 21). ASGR1 was previously considered a hepatocyte-specific protein (22), but has also been shown to occur extrahepatically in thyroid (23), small and large intestines (24, 25), and testis (26). Its existence in renal proximal tubular epithelial cells has also been studied (27), whereas it was never been described to occur on endothelial cells outside the liver. Our data now show for the first time that in addition to LSEC, extrahepatic vascular endothelial cells can also express ASGR1. In vitro, porcine vascular endothelial cells (from aorta as well as from the femoral artery), characterized by expression of the endothelial specific markers VE-cadherin and CD31, were shown to express ASGR1 by immunofluorescence staining, flow cytometry, immunoblot, and cell ELISA. Taken together, these assays demonstrate that the ASGR1 protein is present on PAEC as well as PLAEC.

The asialoglycoprotein receptor is composed of two subunits, a major subunit (ASGR1) and a minor subunit (ASGR2) (28, 29), both of which are necessary to form a functional trimer of two ASGR1 and one ASGR2 (30, 31). Staining of limb muscle tissue sections confirmed the presence of ASGR1 in vascular endothelial cells. In addition, muscle tissue biopsies from porcine limbs, ex vivo xenoperfused for 1 h with human blood, stained positive for CD41, indicating human platelet sequestration. Staining for CD41 co-localized with ASGR1 protein, suggesting ASGR1-dependent human platelet binding and sequestration.

ASGR1-mediated human platelet binding and phagocytosis was further investigated using PAEC incubated with CFSE-labeled human platelets in vitro. Binding of human platelets to PAEC was observed and peaked after 15 min incubation. Thereafter, the

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CFSE fluorescence elicited by the labeled platelets gradually declined and at 45 min was co-localized with CD107a, a marker for lysosomes. We conclude that CFSE-labeled platelets first attached to the surface of PAEC via ASGR1, followed by uptake and phagocytosis. Indeed, pre-incubation of PAEC with polyclonal anti-ASGR1 antibody significantly reduced human platelet binding. Also blocking of ASGR1 using asialofetuin, a competitive ligand for ASGR1, significantly reduced platelet uptake in a dose-dependent manner.

Our results demonstrate that receptor/ligand interactions are involved in the clearance of human platelets during ex vivo pig-to-human limb xenoperfusion. The presence of higher Galβ1-4 or GalNAcβ1-4 ligand concentrations on human as compared to porcine platelets may facilitate porcine ASGR1 mediated human platelet phagocytosis (20). Generation of genetically modified pigs carrying human ASGR1, with or without deletion of the porcine ASGR1, may therefore help to overcome this important hurdle in pig-to-human xenotransplantation.

In conclusion, we found expression of functional ASGR1 in porcine vascular endothelial cells. ASGR1 on the vascular endothelium of porcine organs may therefore mediate binding and phagocytosis of human platelets during pig-to-human xenotransplantation.

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Figure legends

Figure 1. Platelet count during ex vivo perfusion of porcine forelimbs with heparinized autologous or human blood. Amputated forelimbs from wt and hCD46/HLA-E transgenic (tg)pigs were connected to extracorporeal perfusion circuit, and then xenogeneic or autologous perfusions were performed. Perfusate blood samples were collected at regular time intervals and platelets counted using a Sysmex analyzer. A rapid loss of platelet counts was observed in wt and tg xenogeneic perfusions, but not in autologous perfusions. Autologous perfusions n=3, xenogeneic perfusions n=6. Significance was tested for area under the curve using One-Way ANOVA with Bonferroni's correction (*p0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001).

Figure 2. Expression of ASGR1 on PAEC/PLAEC in vitro. (A) Cultured PAEC (upper row) or PLAEC (lower row) were analyzed for expression of ASGR1 as well as the endothelial specific markers CD31 and VE-cadherin by IF staining and fluorescence microscopy. Both PAEC and PLAEC were positive for CD31, VE-cadherin and ASGR1. Nuclei stained with DAPI. One representative of at least three independent experiments is shown. (B) PAEC cultures were analyzed for expression of ASGR1 by flow cytometry. One representative of three independent experiments is shown. (C) Incubation of PAEC with NHS has no effect on expression of ASGR1 as analyzed by cell ELISA, n=3, shown are mean ± SD. Negative control indicates no anti-ASGR1 antibody, but only secondary antibody. (D) Western blot analysis of ASGR1 protein and beta-actin from whole cell lysate using PAEC of different genetic backgrounds (1 = wild-type; 2 = GalTKO/hCD46/hTM transgenic; 3 = HLA-E/hCD46 transgenic; 4 = GalTKO/hCD46 transgenic). Representative data of two independent experiments are shown.

Figure 3. ASGR1 expression on biopsy samples from ex vivo perfusions. (A) Pig limb skeletal muscle samples were collected before perfusion from the contralateral extremity as baseline sample and were stained for expression of ASGR1 as well as endothelial markers (CD31 and VE-cadherin). Results show that ASGR1 protein is expressed in vascular endothelial cells that are positive for CD31 or VE-cadherin. Representative data of three independent experiments are shown. (B) Biopsy samples, collected at different xenoperfusion time points, were stained for platelets using anti-CD41 antibody to study binding and sequestration. Human

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platelet binding and sequestration was high at 1 h perfusion and then declined after 9 h and 12 h perfusion. (C) Staining for co-localization of human platelets with pig ASGR1 on 1 h xenoperfused biopsy samples. Human platelets stained with CD41 antibody co-localized with porcine ASGR1.

Figure 4. Human platelet phagocytosis and degradation by PAEC. (A) CFSE-labeled human platelets were incubated with PAEC for different time points as shown on the x-axis, followed by detection of CFSE fluorescence intensity on a plate reader. PAEC without CFSE-labeled platelets were also used to detect the autofluorescence of PAEC. Data are expressed as mean ± SD of three independent experiments. (B, C) Confocal microscopy analysis of CFSE-labeled human platelets by PAEC in vitro. (B) CFSE-labeled platelets (green fluorescence) were added to PAEC. After 30 min incubation, platelets adhered to PAEC that were positive for CD31 (red fluorescence). Side views are shown as z-panels to the right and below the image. (C) Prolonged incubation of CFSE platelets for 45 min resulted in internalization into PAEC. Shown are PAEC stained for ASGR1 (red) and the lysosomal marker CD107a (violet). Side views are shown as z-panels to the right and below the overlay image. In all fluorescence staining experiments, nuclei stained with DAPI. One representative of three independent experiments is shown.

Figure 5. Inhibition of human platelet binding and phagocytosis. PAEC were pre-incubated with increasing concentrations of either rabbit polyclonal anti-ASGR1 antibody or polyclonal rabbit IgG as isotype control (A), or asialofetuin (B) for 30 min, followed by incubation with CFSE-labeled human platelets. After 15 min incubation, human platelet binding and phagocytosis was measured using a fluorescence plate reader. Significance was tested using One-Way ANOVA with Bonferroni's correction (*$p \leq 0.05$, **$p \leq 0.01$). Data are expressed as mean ± SD of three independent experiments.

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Figure 1

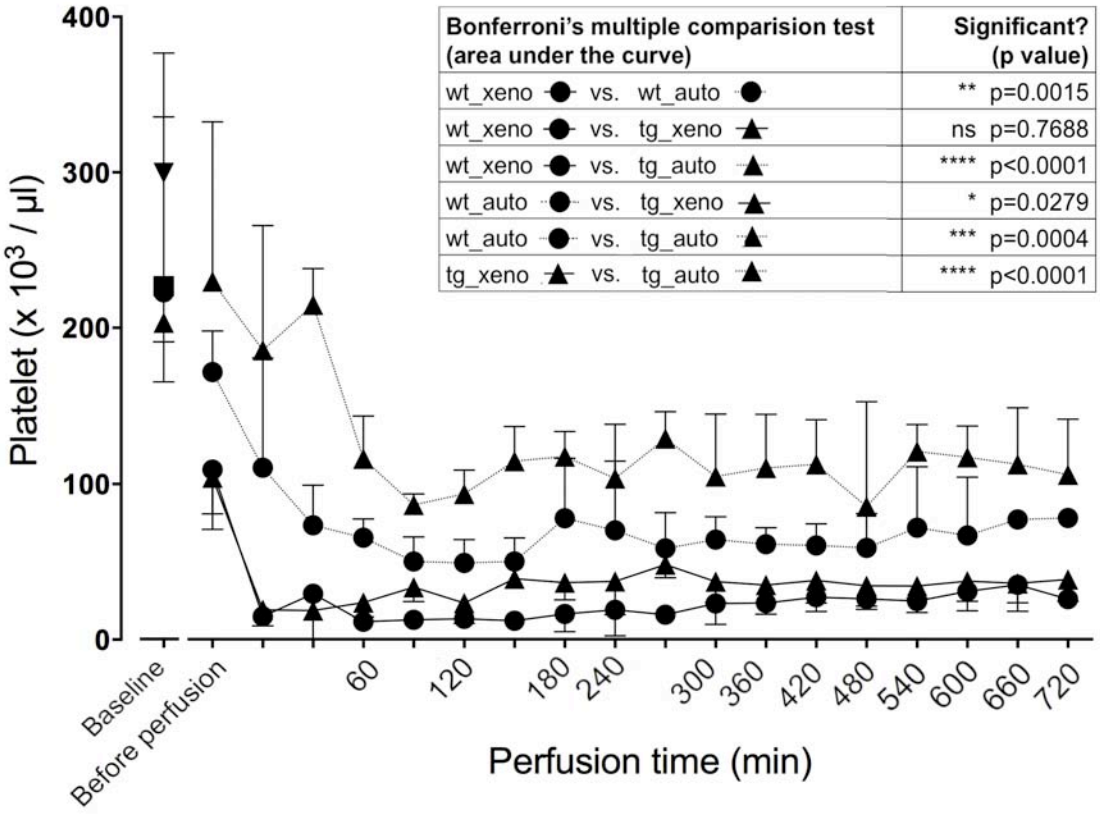
Bonferroni's multiple comparision test (area under the curve)
Significant? (p value)
wt_xeno vs. wt_auto
** p=0.0015
wt_xeno vs. tg_xeno
ns p=0.7688
wt_xeno vs. tg_auto
**** p<0.0001
wt_auto vs. tg_xeno
* p=0.0279
wt_auto vs. tg_auto
*** p=0.0004
tg_xeno vs. tg_auto
**** p<0.0001
Platelet (x 10³ / µl)
400
300
200
100
0
Baseline
Before perfusion
60
120
180
240
300
360
420
480
540
600
660
720
Perfusion time (min)

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Figure 2

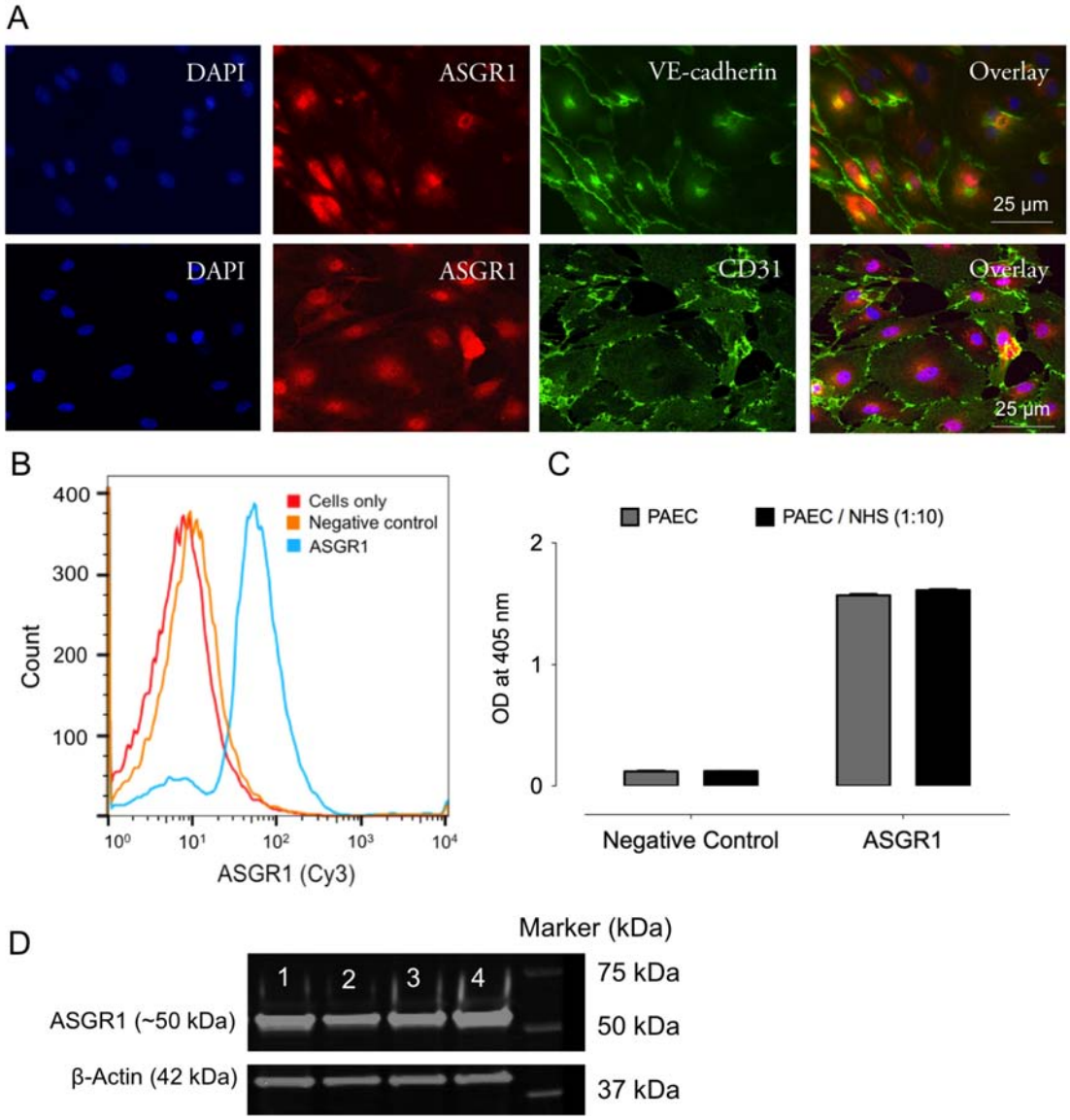
A
DAPI
ASGR1
VE-cadherin
Overlay
25 µm
DAPI
ASGR1
CD31
Overlay
25 µm
B
Cells only
Negative control
ASGR1
Count
400
300
200
100
10^0
10^1
10^2
10^3
10^4
ASGR1 (Cy3)
C
PAEC
PAEC / NHS (1:10)
OD at 405 nm
2
1
0
Negative Control
ASGR1
D
Marker (kDa)
1
2
3
4
75 kDa
50 kDa
37 kDa
ASGR1 (~50 kDa)
β-Actin (42 kDa)

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Figure 3

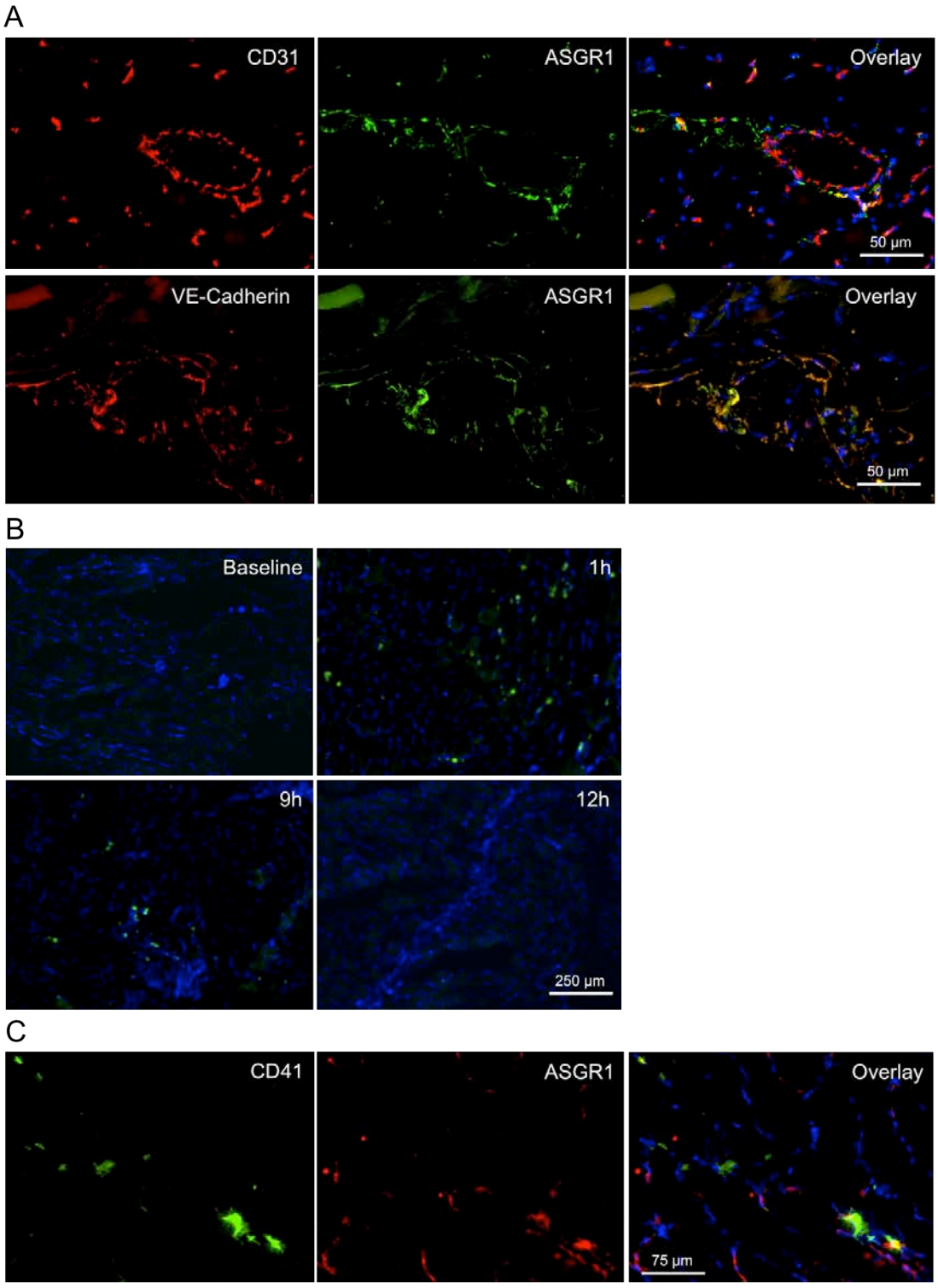
A
CD31
ASGR1
Overlay
50 µm
VE-Cadherin
ASGR1
Overlay
50 µm
B
Baseline
1h
9h
12h
250 µm
C
CD41
ASGR1
Overlay
75 µm

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Figure 4

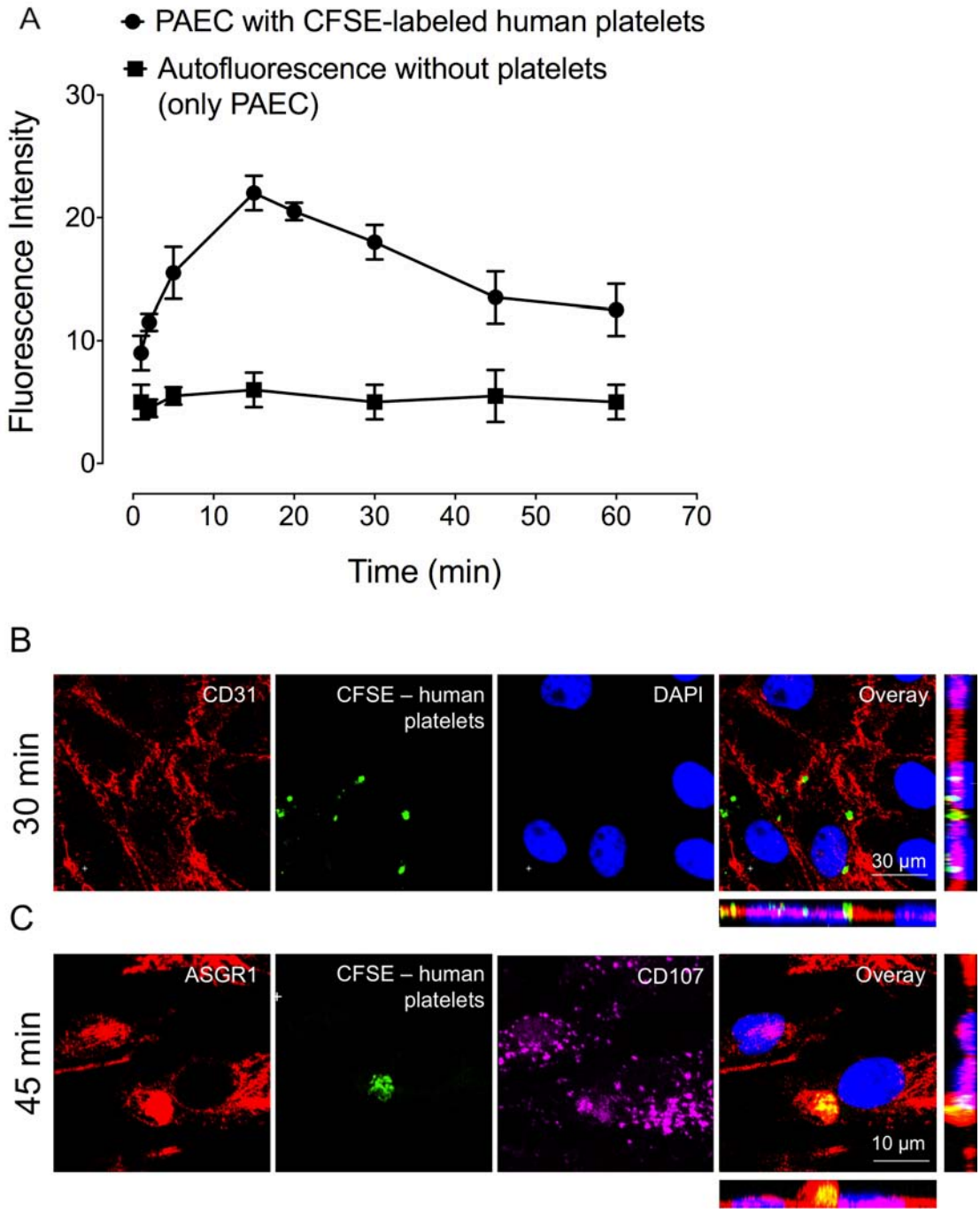
A
PAEC with CFSE-labeled human platelets
Autofluorescence without platelets
(only PAEC)
Fluorescence Intensity
30
20
10
0
0
10
20
30
40
50
60
70
Time (min)
B
30 min
CD31
CFSE – human platelets
DAPI
Overay
30 µm
C
45 min
ASGR1
CFSE – human platelets
CD107
Overay
10 µm

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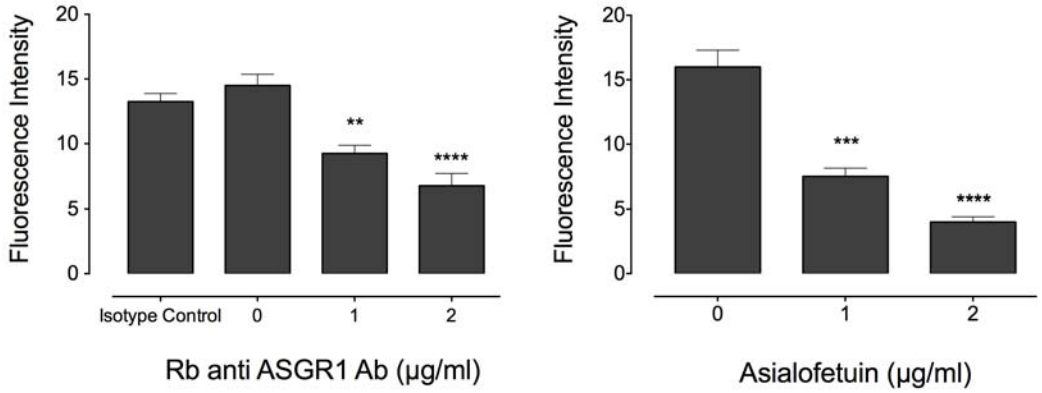
Figure 5


Fluorescence Intensity
20
15
10
5
0
**
****
Isotype Control
0
1
2
Rb anti ASGR1 Ab (µg/ml)
Fluorescence Intensity
20
15
10
5
0
***
****
0
1
2
Asialofetuin (µg/ml)

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Paper IV

Development of a Bead-Based Multiplex Assay for the Simultaneous Detection of Porcine Inflammation Markers Using xMAP Technology

Anjan K Bongoni[1,2], Jonas Lanz[1], Robert Rieben[1], Yara Banz*[1,3]

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Background: Based on comparative anatomic and physiologic characteristics, pigs have been used as model animal to develop and test novel therapies for the prevention of cardiovascular diseases. Currently no commercial test kits are available for multiplex detection of porcine markers by using xMAP technology.

Aim: Therefore, we aimed at developing a bead-based multiplexed immunoassay to detect simultaneously porcine cytokines (IL-1β, IL-6, IL-10, TNFα), chemokines (IL-8, MCP-1), growth factors (basic (b)FGF, VEGF, PDGF-bb), and cardiac injury marker troponin-I, as well as complement activation markers (C5a, soluble (s)C5b-9) on the Bio-Plex platform.

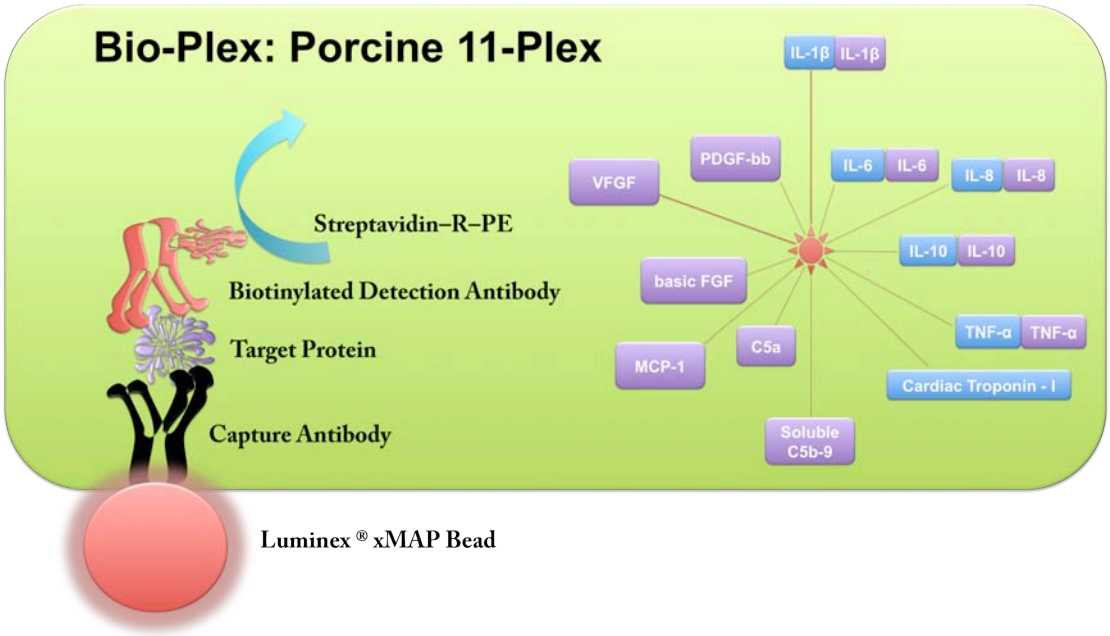

Figure: Diagrammatic illustration of the basic principle of the Bio-Plex assay and the analytes list of the developed porcine 11-plex (purple boxes: IL-1β, IL-6, IL-10, TNFα, C5a, sC5b-9, C5a, MCP-1, bFGF, VEGF and PDGF-bb) and 6-plex (blue boxes: IL-1β, IL-6, IL-10, TNFα, and cardiac troponin-I) assays.

Conclusion: We were successful to establish bead based 11-plex and 6-plex assay to detect porcine markers with a good assay sensitivity, broad dynamic range, low intra- & inter-assay variance and cross-reactivity. This assay therefore represents a new, useful tool for the analysis of serum samples from experiments with pigs.

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ORIGINAL ARTICLE

Cytometry
PART A
Journal of the International Society for Advancement of Cytometry

Development of a Bead-Based Multiplex Assay for the Simultaneous Detection of Porcine Inflammation Markers Using xMAP Technology

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Additional Supporting Information may be found in the online version of this article.

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Abstract

Commercially available assays for the simultaneous detection of multiple inflammatory and cardiac markers in porcine blood samples are currently lacking. Therefore, this study was aimed at developing a bead-based, multiplexed flow cytometric assay to simultaneously detect porcine cytokines [interleukin (IL)-1β, IL-6, IL-10, and tumor necrosis factor alpha], chemokines (IL-8 and monocyte chemotactic protein 1), growth factors [basic fibroblast growth factor (bFGF), vascular endothelial growth factor, and platelet-derived growth factor-bb], and injury markers (cardiac troponin-I) as well as complement activation markers (C5a and sC5b-9). The method was based on the Luminex xMAP technology, resulting in the assembly of a 6- and 11-plex from the respective individual singleplex situation. The assay was evaluated for dynamic range, sensitivity, cross-reactivity, intra-assay and interassay variance, spike recovery, and correlation between multiplex and commercially available enzyme-linked immunosorbent assay as well as the respective singleplex. The limit of detection ranged from 2.5 to 30,000 pg/ml for all analytes (6- and 11-plex assays), except for soluble C5b-9 with a detection range of 2–10,000 ng/ml (11-plex). Typically, very low cross-reactivity (<3% and <1.4% by 11- and 6-plex, respectively) between analytes was found. Intra-assay variances ranged from 4.9 to 7.4% (6-plex) and 5.3 to 12.9% (11-plex). Interassay variances for cytokines were between 8.1 and 28.8% (6-plex) and 10.1 and 26.4% (11-plex). Correlation coefficients with singleplex assays for 6-plex as well as for 11-plex were high, ranging from 0.988 to 0.997 and 0.913 to 0.999, respectively. In this study, a bead-based porcine 11-plex and 6-plex assay with a good assay sensitivity, broad dynamic range, and low intra-assay variance and cross-reactivity was established. These assays therefore represent a new, useful tool for the analysis of samples generated from experiments with pigs.

Key terms

xMAP technology; multiplex flow cytometry; bead-based immunoassay; cytokines; porcine

Pigs have been widely used as biomedical research models over the past decades (1). Based on their comparative anatomic and physiologic characteristics (2), they represent a suitable model species for investigation of a large number of human diseases and for technical developments in surgery or anesthesia. Animal experiments using pigs have made valuable contributions not only in the field of human medicine, including research of the cardiovascular (3,4) system, but also in the field of critical and intensive care medicine (5,6).

For the evaluation of such experiments, analyses of blood samples for the detection of cytokine profiles or organ-specific markers are indispensable tools. Cytokine levels are traditionally measured by enzyme-linked immunosorbent assay (ELISA) allowing analysis of only a single marker at a time. These tests may be rather expensive and time consuming. In particular, the assessment of multiple markers may require a considerable volume of serum or plasma. This may be a limiting factor in

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Table 1. Overview of all the reagents and diluents used for the multiplex immunoassay to detect porcine cytokines, chemokines, growth factors, and cTn-I

		CAPTURE ANTIBODIES				DETECTION ANTIBODIES			RECOMBINANT PROTEIN	
MARKER		CATALOG NUMBER	CLONE	SOURCE	COUPLING CONCENTRATION (μG/ML)	CATALOG NUMBER	CLONE	SOURCE	CATALOG NUMBER	SOURCE
1	IL-1β	MAB6811	77724	R&D	20	BAF681	pAb	R&D	681-PI	R&D
2	IL-6	AF686	pAb	R&D	20	BAF686	pAb	R&D	686-PI	R&D
3	IL-8	MAB5351	105105	R&D	20	BAF535	pAb	R&D	535-PI	R&D
4	IL-10	MAB6931	148801	R&D	20	ASC9109	mAb	Invitrogen	693-PI	R&D
5	TNF-α	MAB6902	103304	R&D	20	BAF690	103302	R&D	690-PT	R&D
6	cTn-I	Ab19615	19C7	Abcam	15	4T21/2B	pAb	HyTest	8T53P	HyTest
7	C5a		T13/9	MBM	20		pAb	MBM		MBM
8	sC5b-9	5010	aE11	Diatec	25	A706	mAb	Quidel	A127	CompTech
9	MCP-1	500-P34	pAb	PeproTech	20	PBB0089S	pAb	King Biotech	RP0017S	King Biotech
10	bFGF	500-P18	pAb	PeproTech	20	500-18Bt	pAb	PeproTech	100-18B	PeproTech
11	VEGF	500-P10	pAb	PeproTech	20	BAF293	pAb	R&D	293-VE	R&D
12	PDGF-bb	500-P47	pAb	PeproTech	20	500-P47Bt	pAb	PeproTech	100-14B	PeproTech

mAb, monoclonal antibody; pAb, polyclonal antibody; coupling concentration, antibody concentration for coupling assay.
Abcam: Abcam (Cambridge, MA); CompTech: Complement Technology (Tyler, TX); Diatec: Diatec Monoclonals AS (Oslo, Norway); HyTest: HyTest (Turku, Finland); Invitrogen: Invitrogen (Carlsbad, CA); King Biotech: Kingfisher Biotech (Saint Paul, MN); MBM: MBM Sciencebridge GmbH (Göttingen, Germany); PepriTech: PeproTech EC (London, UK); R&D: R&D Systems (Minneapolis, MN).

which minimal sample volume is available as is the case from microdialysis studies or long experiments that require serial testing of multiple parameters. Besides, cytokines themselves may alter each other's function and regulate the production of other cytokines (7), rendering cytokine profiles or ratios more valuable than single cytokine measurements (8). For these reasons, multiplex flow cytometry by the xMAP Technology has rapidly established itself (9,10).

Multiplexed bead-based immunoassays for quantitation of human cytokines have been described in the past years (11–13), and several multiplexed cytokine detection assays are now commercially available to detect human, mouse, and rat cytokines based on xMAP technology. Multiplex kits for porcine cytokines are commercially available. However, no such kits for porcine markers combining cytokines, complement activation markers, and growth factors/angiogenesis factors are currently available. A major limitation for the development and use of porcine multiplex assays has been the lack of specific antibody pairs. Only relatively few reagents are currently available commercially for the detection of selected markers. In addition, microsphere-based multiplex assays for the detection of porcine cytokines have been recently reported (14–16).

In this article, we describe the development of a novel assay to detect porcine proinflammatory cytokines [interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor alpha (TNF-α), monocyte chemotactic protein (MCP)-1], the anti-inflammatory cytokine IL-10, growth factors [basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor-bb (PDGF-bb)], complement activation markers [C5a, soluble (s)C5b-9], and a cardiac marker [cardiac troponin (cTn)-I]. We have developed an 11-plex assay including all parameters besides cTn-I (IL-1β, IL-6, IL-8, IL-10, TNF-α, C5a, sC5b-9, MCP-1, bFGF, VEGF, and PDGF-bb) and a 6-plex assay with a subset of the markers of the 11-plex assay plus cTn-I (IL-1β, IL-6, IL-8, IL-10, TNF-α, and cTn-I). As a multiplex for cytokine detection, this assay may serve as a tool to determine the "inflammation fingerprint" in a given pig experiment, and as a 6- or 11-plex may be particularly useful in, for example, the context of studying cardiac, limb, or general ischemia/reperfusion injury or models of shock.

Materials and Methods

Technology

The described assays are based on Luminex xMAP technology, a multiplexed sandwich immunoassay technique performed on the surface of 5.5-μm polystyrene beads. Details of the assay principle have been published elsewhere (17) and are available online (Bio-Plex Suspension Array System by Bio-Rad, Hercules, CA). The system was primarily set up as a single plex and assembled stepwise to a multiplex assay (see below).

Bead Coupling

Carboxylated polystyrene beads [catalog no. 171-5060(24), (25), (27), (28), (35), (36), (38), (43), (44), (46), (50), (54), (56)] and the Bio-Plex Amine Coupling Kit (catalog no. 171-406001) were purchased from Bio-Rad, and coupling procedures were performed according to the manufacturer's instructions. All traces of sodium azide were removed prior to use by dialysis (Slide-A-Lyzer MINI Dialysis Units, Pierce, Rockford, IL).

With the exception of polyclonal antibodies to detect porcine IL-6, MCP-1, bFGF, VEGF, PDGF-bb, and cTn-I, monoclonal mouse antibodies were used in the first step of the sandwich immunoassay (Table 1). The anti-human bFGF, VEGF, PDGF-bb, sC5b-9, and cTn-I antibodies were found to cross-react with porcine antigen; all other antibodies were

described by the manufacturers to be specific for pig. The amount of antibody needed for a successful coupling reaction and assay performance was between 15 and 25 μg per 1.25 million beads. The efficiency of antibody coupling was validated using a biotin-conjugated antibody directed against the Fc region of the respective coupled antibody followed by streptavidin-PE by measuring the mean fluorescence intensity (MFI) on the Bio-Plex platform. MFI > 2000 was taken to indicate successful coupling against a reaction using coupled beads with streptavidin-PE only as background. An example of the original sampling is shown in a histogram of double discriminator gating and a bead map with regions for the 11-plex setting is shown in the supplementary figure.

Multiplex Assay

All details concerning origin of reagents used for the assay are listed in Table 1. Antibody-coupled beads were diluted in assay diluent [a mixture (1:1) of PBS and 0.5% BSA pH 7.2 and Tris pH 7.6] at a concentration of 2500 beads per 50 μl. Bead suspension (50 μl per well) was added to a microplate (MultiScreen HTS 1.2-μm Durapore Membrane, Millipore Corporate, Billerica, MA). Following washing with wash buffer [a mixture (1:1) of PBS 0.1 M pH 7.2 and Tris 0.5 M pH 7.2], 50 μl of standards/samples diluted in assay buffer containing 0.5% polyvinyl alcohol (PVA) and 0.8% polyvinylpyrrolidone (PVP) were added to the wells. Incubation was performed for 30–60 min at room temperature, protected from light under continuous gentle shaking, and followed by three washing steps. The biotinylated detection antibodies were diluted in assay buffer to the concentration of 1 μg/ml and 25 μl per well. The detection antibody solution was added and incubated on the shaker for another 30 min in the dark. After three washing steps, streptavidin-R-PE (Qiagen, Hilden, Germany) was added (at 1 μg/ml). After 10 min of incubation and washing, the plate was subsequently analyzed by the Bio-Plex reader.

Dynamic Range and Limit of Detection

Standard curves with large ranges were set up to determine the dynamic range of the assay: concentrations from 2.5 to 10,000 pg/ml (PDGF-bb), 2.5–15,000 pg/ml (IL-8), 5–15,000 pg/ml (MCP-1, bFGF, and VEGF), 5–30,000 pg/ml (IL-1β, IL-6, and IL-10), 10–25,000 pg/ml (C5a), 5–24,000 pg/ml (TNF-α), 10–10,000 pg/ml (cTn-I), and 2–10,000 ng/ml (sC5b-9) (11-plex, Table 2; 6-plex, Fig. 1).

Determination of the "standard recovery" (known as back calculation of standards) is used to assess the quality of a curve fit by comparing calculated concentrations, which are based on the standard curve calculated by a five-parameter logistic equation, to expected concentrations (ratio of observed to expected known amount, expressed as a percentage) (18). By definition, the part of the standard curve showing standard recovery percentages between 70 and 130% was considered acceptable and defined as the dynamic range (Bio-Rad Principles of Curve Fitting for Multiplex Sandwich Immunoassays, Rev B). Ten replicates of the zero standard were run to identify the average fluorescence intensity and standard deviation. The lowest limit of detection (LLOD) was defined as the lowest concentration of the standard dilution

Table 2. LLOD and dynamic ranges

	IL-1β (pg/ml)	IL-6 (pg/ml)	IL-8 (pg/ml)	IL-10 (pg/ml)	TNF-α (pg/ml)	MCP-1 (pg/ml)	bFGF (pg/ml)	VEGF (pg/ml)	PDGF-bb (pg/ml)	C5a (pg/ml)	sC5b-9 (ng/ml)
11-plex											
LLOD[a] (pg/ml)	5	5	5	5	10	10	10	10	2.5	10	2
Range[b] (pg/ml)	5–30,000	5–30,000	5–15,000	5–30,000	5–24,000	5–15,000	5–15,000	5–15,000	5–10,000	10–25,000	2–10,000
	IL-1β (pg/ml)	IL-6 (pg/ml)	IL-8 (pg/ml)	IL-10 (pg/ml)	TNF-α (pg/ml)	cTn-I (pg/ml)					
6-plex											
LLOD[a] (pg/ml)	5	5	5	5	10	10					
Range[b] (pg/ml)	5–30,000	2.5–30,000	5–15,000	5–30,000	5–24,000	10–10,000					

[a] Lowest limit of detection (LLOD) is defined as two standard deviations above the mean fluorescence intensity of 10 replicates of the zero standard in the 11- and 6-plex assays.
[b] Range is defined as part of the standard curve where the ratio of observed to expected, known amount of standard, expressed as a percentage [(observed concentration/expected concentration) × 100] lies between 70 and 130%.

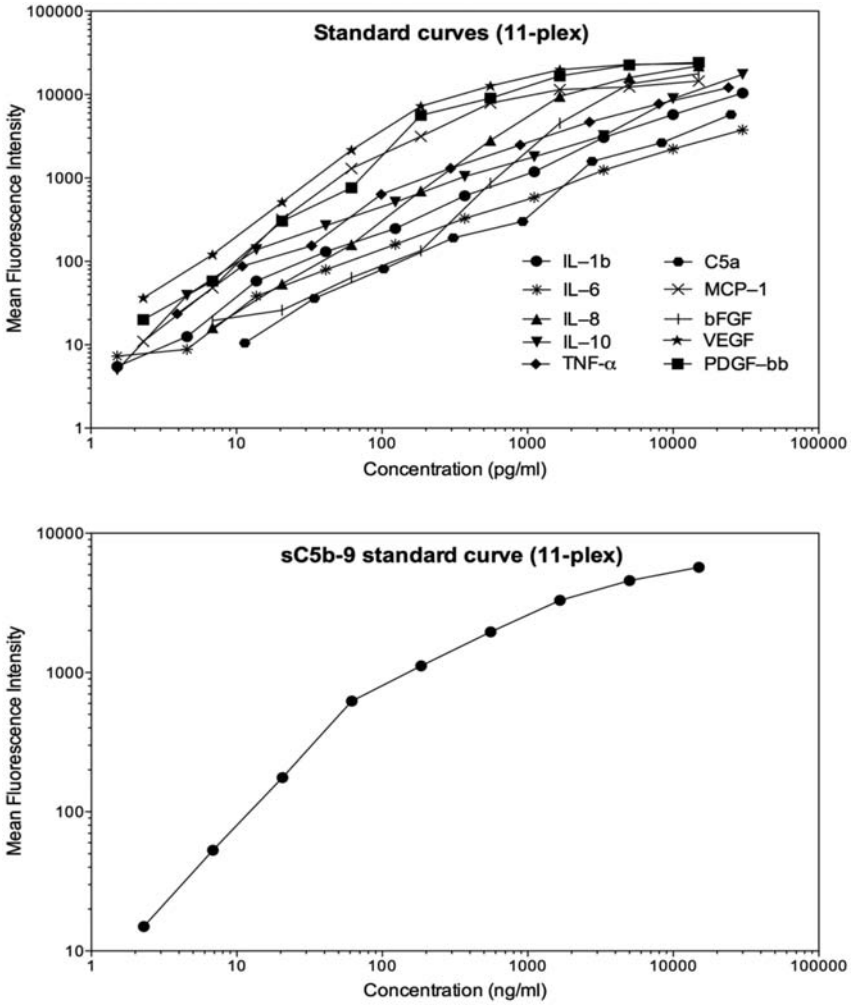

Figure 1. Standards' recovery curves. Standard dilution curves for recombinant proteins from 11-plex (A and B) showing the range of the curves with a standards' recovery value between 70 and 130%. Data were generated with a five-parametric regression formula using the Bio-Plex Manager software and plotted in a log—log curve. A representative curve for each parameter is shown. Each presented point corresponds to an average fluorescence value of at least 100 analyzed beads for the individual parameter.

with an average fluorescence intensity of two standard deviations above the average fluorescence intensity of the zero standard. It provides a reasonable limit to differentiate between an actual minimal amount of sample and background noise and provides more valuable information concerning the minimal detectable amount of a specific protein than the dynamic range, which expresses the range of the detectable concentration in relation to the quality of curve fit. Data were obtained and analyzed using the Bio-Plex-manager Software 6.1 (Bio-Rad).

Cross-Reactivity

To analyze cross-reactivity, two tests were performed:

1. Standard cross-reactivity: Single standards containing one of the recombinant proteins at a known concentration were run in the presence of all capture beads and all biotinylated reporters. Obtained fluorescence intensities were compared with the average background values of five replicates of the zero standard. In case of cross-reactivity, the concentration of a particular analyte was obtained from a standard curve, and the detection by nonrelevant antibodies was expressed as a percentage of the actual analyte concentration [(observed concentration/actual concentration) × 100].
2. Reporter cross-reactivity: Standards containing all recombinant proteins at known concentration were run in the presence of all capture beads and single biotinylated reporter. Percentage of cross-reactivity was calculated according to the same formula as for standard cross-reactivity.

Reproducibility

Porcine plasma samples/standard diluent-only were spiked with a known concentration of recombinant protein, aliquoted, and stored at −80°C until use. To determine intra-assay and interassay variance, four to five replicates of each spiked sample

Table 3. Analysis of cross-reactivity between reagents (11-plex)

	RECOMBINANT PROTEINS										
BEADS	IL-1β (PG/ML) 8554	IL-6 (PG/ML) 8000	IL-8 (PG/ML) 8554	IL-10 (PG/ML) 4000	TNF-α (PG/ML) 9000	MCP-1 (PG/ML) 10,000	BFGF (PG/ML) 10,000	VEGF (PG/ML) 10,000	PDGF-BB (PG/ML) 10,000	C5A (PG/ML) 27,200	SC5B-9 (NG/ML) 3333
IL-1β	–	N	0.5%	N	N	1.5%	1%	1%	2.7%	<0.1%	0.1%
IL-6	<0.1%	–	<0.1%	N	N	N	0.6%	1.1%	2.5%	<0.1%	<0.1%
IL-8	1%	N	–	N	N	N	1.5%	N	1.2%	0.2%	<0.1%
IL-10	<0.1%	N	<0.1%	–	<0.1%	0.9%	0.7%	1.5%	2%	N	N
TNF-α	0.4%	N	N	0.1%	–	0.9%	0.5%	0.9%	0.8%	N	0.5%
C5a	N	<0.1%	0.3%	<0.1%	N	2%	1.9%	1%	1.9%	–	0.2%
MCP-1	2%	0.4%	0.6%	1.6%	1.5%	–	1.1%	0.3%	0.5%	<0.1%	0.3%
bFGF	0.4%	N	N	N	<0.1%	N	–	1%	2.5%	N	0.1%
VEGF	<0.1%	N	<0.1%	N	<0.1%	0.1%	0.3%	–	1%	N	<0.1%
PDGF-bb	0.4%	0.3%	0.5%	N	<0.1%	N	0.4%	0.3%	–	<0.1%	<0.1%
sC5b-9	<0.1%	N	<0.1%	N	N	N	1.1%	2.6%	3%	N	–

N = below detectable range.

(50 μl) were either read on the same plate or on different plates. The obtained values were compared, and the respective coefficient of variation was calculated by the following formula: coefficient of variation = (standard deviation/average) × 100.

Spike Recovery

Ethylenediaminetetraacetic acid (EDTA) plasma samples or EDTA plasma prediluted in standard/sample diluent were spiked with known amounts of recombinant proteins and analyzed to determine the proximity of the expected value to the actual value measured in the spiked plasma. This method assessed variability due to assay preparation, the interference of substances present in the sample or sample matrix, and the regression analysis (19). In addition, sample diluent-only was spiked with recombinant proteins and tested accordingly.

Correlation Between 6-plex/11-plex with Respective Singleplex

Standard/sample diluent-only was spiked with different amounts of recombinant proteins (IL-1β, IL-6, IL-8, IL-10, and TNF-α) and analyzed with 6-plex and 11-plex assays as well as singleplex assays. The results were evaluated by correlation with the respective cytokine from 6-plex with corresponding singleplex assays. Similarly, all parameters from the 11-plex assay were evaluated by correlation with the respective singleplex.

Sample Testing

To evaluate how applicable the developed multiplex assay is for the analysis of actual experimental porcine samples, for instance, from an experimental setting of cardiopulmonary bypass (CPB) or extracorporeal limb perfusion, EDTA plasma samples were analyzed at different time points [baseline, during reperfusion (120 min), and during euthanasia or off CPB]. For the 11-plex, samples were obtained from extracorporeal porcine limb perfusion experiments. This study was aimed at assessment of ischemia/reperfusion (I/R)-induced endothelial activation/injury in amputated pig limbs following prolonged preservation by the use of modified extracorporeal perfusion (20). Briefly, porcine forelimbs were perfused with autologous

Table 4. Intra-assay and interassay variance of the multiplex setup

	IL-1β	IL-6	IL-8	IL-10	TNF-α	MCP-1	bFGF	VEGF	PDGF-bb	C5a	sC5b-9
11-plex											
Intra-assay (%) ($n = 8$)	7.1	12.1	11.5	9.5	5.3	8.1	7.7	12.9	9.4	9.1	12.1
Interassay (%) ($n = 3$)	18.4	14.4	24.0	16.6	15.2	10.1	15.1	17.3	26.4	15.2	14.0
	IL-1β	IL-6	IL-8	IL-10	TNF-α	cTn-I[a]					
6-plex											
Intra-assay (%) ($n = 6$)	5.0	5.5	5.9	7.4	5.1	4.9					
Interassay (%) ($n = 3$)	10.6	8.1	11.9	10.1	12.6	28.8					

Intra-assay variance: eight replicates of spiked samples at different concentrations measured on the same plate. Coefficient of variation = [(standard deviation/average) × 100]. Interassay variation: the coefficient of variation for three replicates of distinct concentrations measured on different plates at different time points.

[a] For each assay (by 6-plex), standard dilutions for cTn-I were newly prepared, whereas cytokines were spiked and stored at −80°C.

blood using extracorporeal circulation (ECC) for 12 h following 6 h of cold ischemia (4°C). The limbs were subsequently replanted with a 7-day follow-up. EDTA plasma samples were analyzed for the extent of reperfusion injury. Care and use of animals in the study were in compliance with the European Convention on Animal Care and the respective Swiss National Guidelines. The study was approved by the Animal Experimentation Committee of the Canton of Bern, Switzerland.

Statistical Analysis

All data obtained from the multiplex assays were generated with a five-parametric regression formula using the Bio-Plex Manager software 6.1 (Bio-Rad), and standard curves were constructed by a five-parameter regression formula. Prism version 5.0b (Graphpad Software, La Jolla, CA) software was used for all analyses.

Results

Setting up multiplex assays requires identifying the specific antibody pairs, the appropriate diluents as well as establishing optimized dilution factors. Different diluents were tested for their potential to reduce nonspecific binding or so-called matrix effects. In the case of experimental samples, these effects may be mediated by heterophilic antibodies, although these have previously only been described in human sera (21). In the optimized assay, 0.8% PVP and 0.5% PVA were applied as adjuvants to the standard/sample diluent, as previously described (22). Commercially available high-performance ELISA buffer/assay diluent was chosen as a diluent for the detection antibodies.

Lowest Limit of Detection

The assay achieved a LLOD of 5 pg/ml for IL-1β, IL-6, and IL-8, 10 pg/ml for TNF-α, cTn-I, C5a, MCP-1, bFGF, and VEGF, and 2 ng/ml for sC5b-9, respectively (6-plex and 11-plex; Table 2).

Cross-Reactivity

1. Standard cross-reactivity: Tests with nonrelevant cytokine standards showed no fluorescence intensity greater than two standard deviations above the average background value of five replicates of the zero standard. Recombinant cTn-I appeared to interact with all nonrelevant capture antibodies to a certain degree, showing a cross-reactivity of 0.8% with the TNF-α antibody pair and between 1.1 and 1.4% with IL-1β, IL-6, IL-8, and IL-10 antibodies (6-plex setting).

Recombinant MCP-1, bFGF, VEGF, and PDGF-bb showed interaction with all nonrelevant capture antibodies to a certain degree, with a cross-reactivity of 0.5–3% (11-plex; Table 3).

2. Tests for reporter cross-reactivity showed minimal detection using the cTn-I-specific antibody as a single biotinylated reporter. Cross-reactivity was 0.8% for IL-1β, 0.9% for IL-8, and 0.7% for IL-10 (6-plex). However, there was no significant recognition of recombinant proteins by nonrelevant detection antibodies (6- and 11-plex).

Table 5. Spike recovery

		STD. DILUENTS		EDTA PLASMA UNDILUTED		EDTA-P + STD. DILUENT (1:5)		EDTA-P + STD. DILUENT (1:10)	
MARKER	EXPECTED CONCENTRATION	OBSERVED CONCENTRATION	% RECOVERY	OBSERVED CONCENTRATION	% RECOVERY	OBSERVED CONCENTRATION	% RECOVERY	OBSERVED CONCENTRATION	% RECOVERY
IL-1β	2000 pg/ml	2006.0 ± 101.6	100.3 ± 5.1	695.9 ± 20.5	34.8 ± 1.0	1363.0 ± 43.7	68.1 ± 2.2	2015.0 ± 237.1	100.8 ± 11.8
IL-6	4000 pg/ml	4031.0 ± 95.1	100.8 ± 2.4	523.0 ± 112.1	13.1 ± 2.8	2310.0 ± 151.9	57.8 ± 3.8	3967.0 ± 10.9	99.2 ± 0.3
IL-8	5000 pg/ml	5358.0 ± 808.4	107.2 ± 16.2	1490.0 ± 56.6	29.8 ± 1.1	3691.0 ± 513.1	73.8 ± 10.3	5002.0 ± 1197.0	100.0 ± 23.9
IL-10	2000 pg/ml	1973.0 ± 118.0	98.6 ± 5.9	268.9 ± 13.8	13.5 ± 0.7	1284.0 ± 40.3	64.2 ± 2.0	1563.0 ± 13.5	78.2 ± 0.7
TNF-α	1300 pg/ml	1320.0 ± 19.7	101.6 ± 1.5	551.5 ± 12.2	42.4 ± 0.9	1088.0 ± 35.5	83.7 ± 2.7	1327.0 ± 18.6	102.1 ± 1.4
MCP-1	5000 pg/ml	5530.0 ± 601.4	110.6 ± 12.0	1263.0 ± 60.9	25.3 ± 1.2	3838.0 ± 110.5	76.8 ± 2.2	4861.0 ± 54.2	97.2 ± 1.1
bFGF	2000 pg/ml	2168.0 ± 59.9	108.4 ± 3.0	605.9 ± 41.9	30.3 ± 2.1	1190.0 ± 43.1	59.5 ± 2.2	1525.0 ± 185.2	76.3 ± 9.3
VEGF	5000 pg/ml	5263.0 ± 156.4	105.3 ± 3.1	1443.0 ± 60.0	28.9 ± 1.2	2983.0 ± 125.1	59.6 ± 2.5	4171.0 ± 193.3	83.4 ± 3.9
PDGF-bb	2000 pg/ml	1999.0 ± 30.6	99.9 ± 1.5	510.2 ± 24.7	25.5 ± 1.2	1290.0 ± 45.4	64.5 ± 2.3	1539.0 ± 122.7	76.9 ±6.1
C5a	3400 pg/ml	3653.0 ± 150.9	107.4 ± 4.4	1153.0 ± 107.3	33.9 ± 3.2	2010.0 ± 296.5	59.1 ± 8.7	2680.0 ± 288.2	78.8 ± 8.5
C5b-9	1000 ng/ml	1107.0 ± 56.4	110.7 ± 5.6	227.2 ± 28.8	22.7 ± 2.9	644.8 ± 56.3	64.5 ± 5.6	963.7 ± 136.0	96.4 ± 13.6

Std. diluent = standard/sample diluent; EDTA-P + Std. diluent (1:5/1:10) = porcine EDTA plasma prediluted in standard/sample diluent to 1:5 or 1:10.

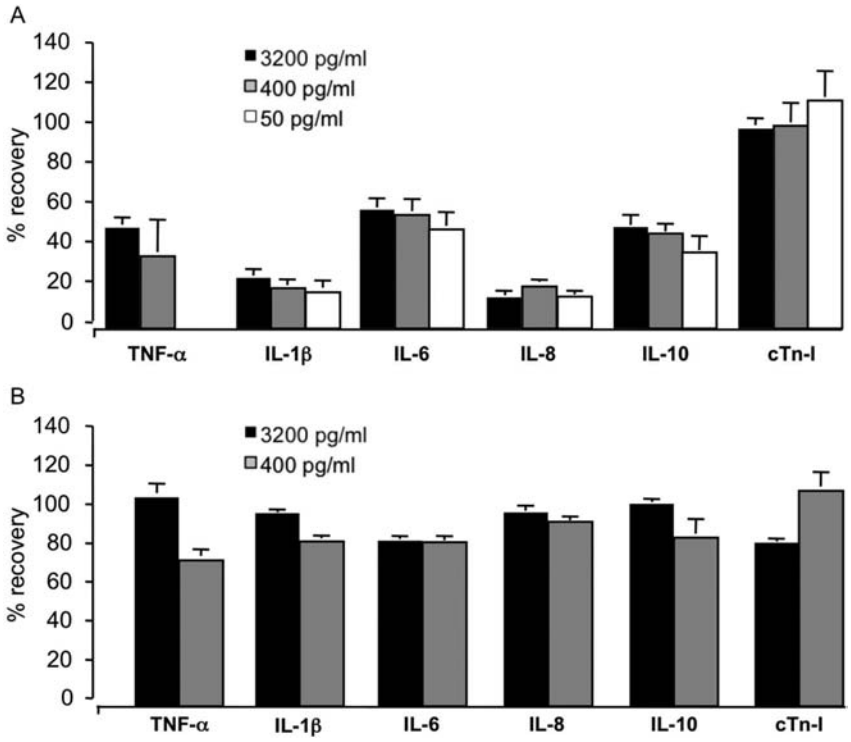

Figure 2. Spike recovery in plasma samples and diluent-only by 6-plex. Spike recovery to determine the proximity of the expected value to the actual value measured (6-plex): EDTA plasma samples were spiked with different amounts of recombinant protein (3200, 400, and 50 pg/ml) and analyzed to determine the proximity of the measured values to the expected ones [% recovery = (observed − neat)/expected) × 100] (A). Although spike recovery was consistent for the individual parameter measured, recovery percentages between different parameters varied for spiked plasma samples. In addition, diluent-only was spiked with the respective recombinant proteins (B). Recovery percentages were consistent for spiked diluent-only samples (between 75 and 115%). Data are expressed as average ± standard deviation.

Reproducibility

The coefficients of variation for intra-assay replicates lay between 5.3–12.9% (mean: 9.5%, 11-plex) and 4.9–7.4% (mean: 5.6%, 6-plex) for all samples. Interassay replicates showed coefficients of variation, which ranged from 10.1 to 26.4% (mean: 10.8%, 11-plex) and 8.1 to 28.8% (mean: 13.7%, 6-plex; Table 4).

Spike Recovery

Depending on the parameter tested, recovery values for spiked plasma samples varied from 13.1 to 42.4% and 15.8 to 114.7% for the 11- and 6-plex, respectively (Table 5 and Fig. 2). For the individual parameter, however, the percentage of recovery was fairly consistent within the different concentrations. To test for matrix effects, EDTA plasma was prediluted (1:5 or 1:10) in a dose-dependent manner with standard/sample diluent and spiked with a known concentration of the recombinant protein. The percent recovery of spiked, prediluted EDTA plasma samples showed increased recovery (% recovery between 57.8 to 83.0% and 76.3 to 102.1% for 1:5 and 1:10 predilution, respectively) when compared with undiluted plasma, suggesting a relevant matrix on spiking. Furthermore, spiking of recombinant proteins in standard/sample diluent-only without plasma showed far less variation between parameters and consistent recovery values between 90 and 118% and 75 and 115% for 11- and 6-plex, respectively.

Correlation Between Multiplex Assay (6-plex or 11-plex) and Respective Singleplex

The concentration of the majority of samples measured with multiplex correlated well with the concentrations measured in the respective singleplex. Correlation coefficients (r^2) were 0.9888 for TNF-α, 0.9945 for IL-1β, 0.9965 for cTn-I, 0.9967 for IL-8, 0.9968 for IL-6, and 0.9976 for IL-10 by 6-plex and singleplex (Fig. 3A). Similarly, high correlation coefficients (r^2) between 11-plex and the respective singleplex assays ranging from 0.913 for sC5b-9 to 0.999 for bFGF were found (Fig. 3B).

Sample Testing

The course of cytokines, growth factors, and complement activation markers in EDTA plasma samples of a model of ECC limb perfusion was analyzed by 11-plex assay at different time intervals of perfusion as shown in Figure 4. Increased concentrations of cytokines (IL-1β, IL-6, IL-8, and MCP-1), growth factors (VEGF), and complement activation markers

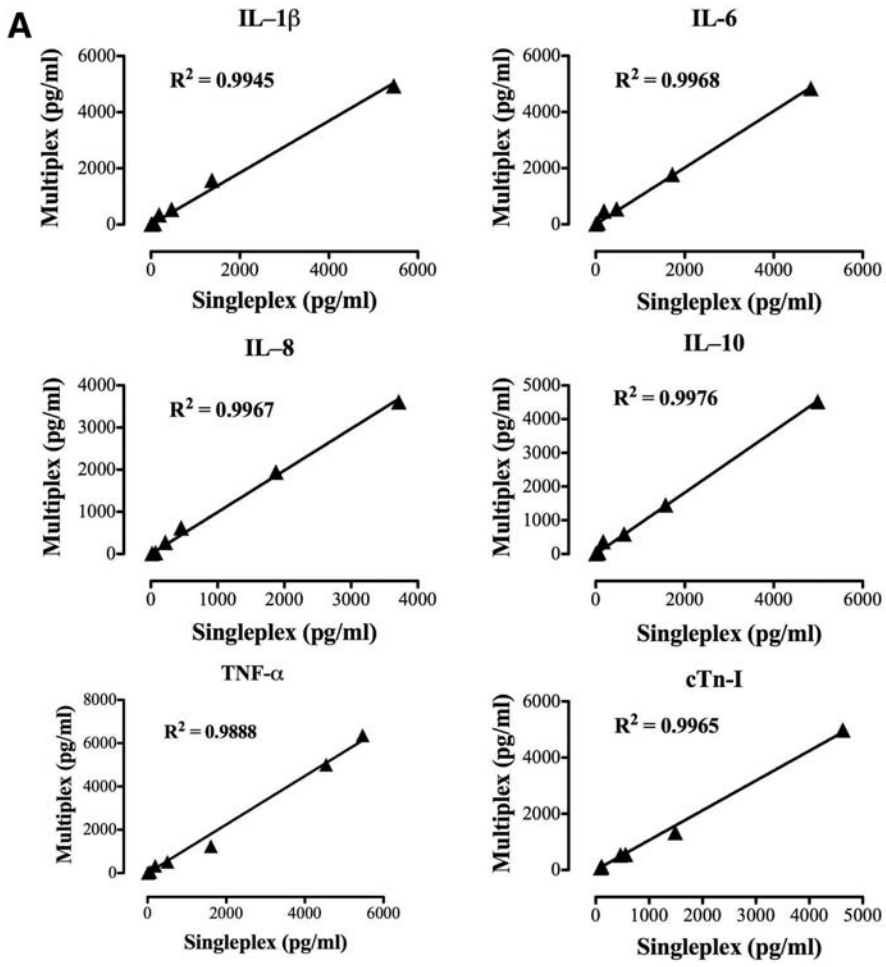

Figure 3. Correlation between 6-plex and singleplex as well as 11-plex and singleplex. Standard/sample diluent-only spiked with different amounts of recombinant cytokines were measured in the 6-plex assay as well as in the singleplex. Correlation between 6-plex and corresponding singleplex was good with coefficients (r^2) of 0.9888 for TNF-α, 0.9945 for IL-1β, 0.9965 for cTn-I, 0.9967 for IL-8, 0.9968 for IL-6, and 0.9976 for IL-10 (A). The 11-plex assay also correlated well with corresponding singleplex assays, r^2 ranging from 0.9133 (sC5b-9) to 0.9990 for bFGF (B).

(C5a and sC5b-9) were observed during ECC. IL-10, TNF-α, bFGF, and PDGF-bb showed only a minimal increase during ECC. However, the I/R injury-induced inflammatory response and complement activation appear to be of limited duration in this model as activation markers dropped back down to baseline levels by the end of the 7-day replantation period.

Discussion

In this study, an 11-plex assay for the simultaneous detection of porcine IL-1β, IL-6, IL-8, IL-10, TNF-α, C5a, sC5b-9, MCP-1, bFGF, VEGF, and PDGF-bb as well as a 6-plex assay including five cytokines and cTn-I was set up following initial optimization of all individual parameters in a singleplex setting.

Although various studies have described the setup of multiplex assays for the detection of human neutralizing monoclonal antibodies (23) or human cytokines (13,24,25), only few studies have been currently published describing multiplex detection of porcine cytokines (14–16). The potential advantage of the current assay, apart from including a larger number of cytokines, is the addition of complement activation markers and growth factors as well as tissue-specific injury markers (cTn-I). The multiplex assay may be used in more general porcine models of tissue injury or, with the addition of cTn-I, in particular for the investigation of cardiac injury, for example, in myocardial infarction. However, the assay sensitivity for the complement markers (C5a and sC5b-9) as well as growth factors (VEGF and PDGF-bb) was compromised when combined with cTn-I. In addition, with the increase of background values, the LLOD of these markers was high in the presence of cTn-I antibodies. Thus, two multiplex panels were developed as an 11-plex and an overlapping 6-plex with the addition of cTn-I.

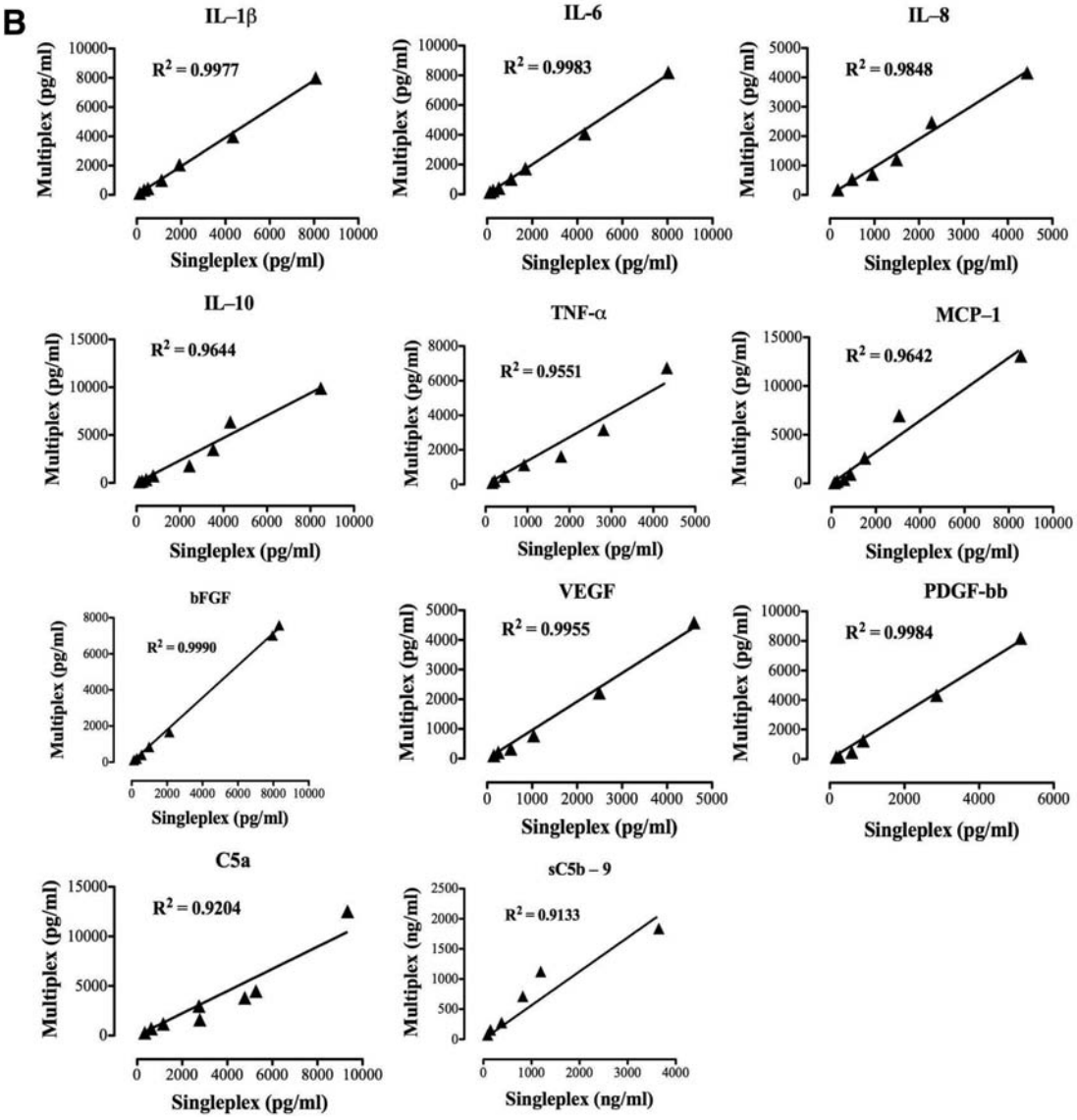

Figure 3. (Continued)

Sensitivity of the assay (for cytokines) in terms of the lowest level of detection was comparable with commercial ELISAs. In addition, the dynamic detection range for all analytes was significantly broader in our bead-based assays. For cytokines, the range was higher than in commercial ELISAs, eliminating the need for multiple dilutions of high-concentration samples. Moreover, the simultaneous detection of analytes insured an internal consistency.

Standard cross-reactivity tests showed some recognition of recombinant PDGF-bb protein by all beads in the multiplex setting. As a certain amount of cross-reactivity was observed with all beads, this phenomenon is most likely due to nonspecific binding of MCP-1, bFGF, VEGF, and PDGF-bb to the beads than due to recognition of the protein by other capture antibodies. This may also explain the minimal cross-reactivity found in reporter cross-reactivity tests when analyzing all beads with detection antibodies. As far as intra-assay and interassay variance is concerned, the presented multiplex assay showed values comparable with and within an acceptable range when compared with those observed by others (13,26).

Although recovery values for spiked plasma samples varied to a certain extent, the percentage of recovery was stable within each tested parameter. By prediluting the EDTA plasma in standard/sample diluent, the percent recovery of each parameter following spiking was dose dependently increased to a maximum. This indicates that the low recovery values were due to absorption of the respective recombinant protein following spiking into the plasma samples. Furthermore, there

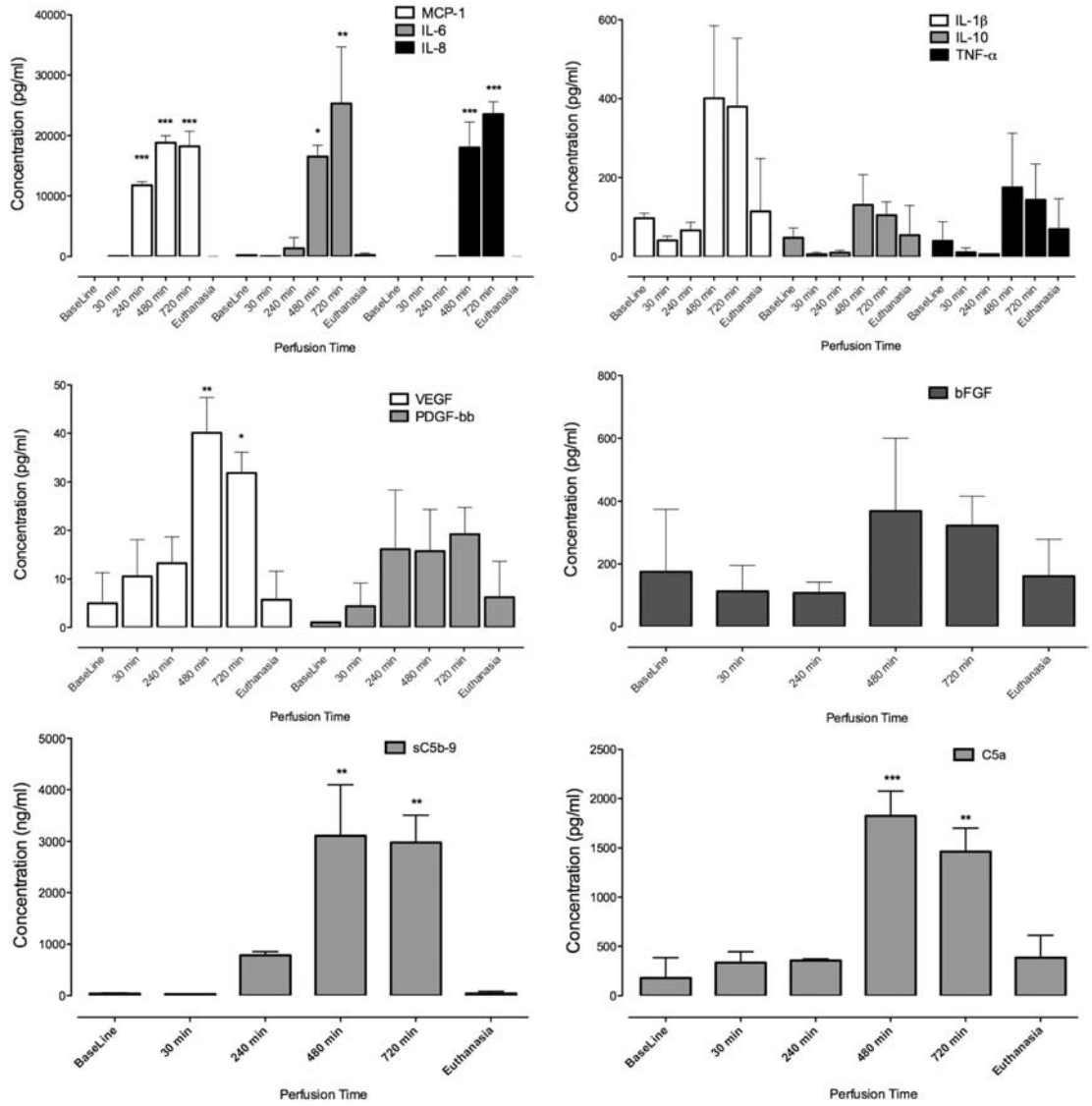

Figure 4. Plasma cytokine levels measured during extracorporeal circulation. Samples from porcine forelimb perfusion experiments in ECC models were used to validate the established 11-plex assay. EDTA plasma levels of cytokines, MCP-1, VEGF, PDGF-bb, bFGF, C5a, and sC5b-9 were reanalyzed by 11-plex at different time points as shown in the figure. During perfusion, IL-1β, IL-6, IL-8, MCP-1, and VEGF as well as complement activation marker, C5a and sC5b-9, levels were significantly higher at perfusion time points than baseline, as shown by one-way ANOVA with Bonferroni correction (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). Concentrations of all measured markers dropped to normal after replantation.

was a good correlation between the results obtained in singleplex versus 6-plex or singleplex versus 11-plex. Additionally, cytokine concentrations measured by 6-plex and 11-plex assays showed correlation coefficients (r^2), 0.9720 for IL-8, 0.9736 for TNF-α, 0.9873 for IL-6, 0.9904 for IL-1β, and 0.9995 for IL-10, indicating for a good sensitivity of both assays. In contrast, recovery percentages in diluent-only spiked samples were more consistent and generally higher, most likely due to significantly reduced matrix effects. Taken together, interassay variance and spike recovery values of some analytes showed deviations from expected values; however, relative concentrations of each parameter were consistent.

Cytokine concentrations in the multiplex assay correlated well with those measured using commercial ELISAs. Although in certain longitudinal studies, such as in the illustrated model, relative cytokine values may be adequate to evaluate the follow-up, some experimental setups require exact values. In general, however, absolute concentrations of antigens as measured by ELISA

or similar technique have to be regarded with caution as values may differ significantly among commercially available kits (27). In multiplex analysis, at least the issue of repeated testing (on different plates and different days) may be reduced as up to 80 samples, and in the current assay, up to 11 parameters can be measured simultaneously on one single plate.

The established 6-plex and 11-plex assays were validated using samples from in vivo porcine experiments in CPB (data not shown) and ECC models, respectively (28). The increase in IL-1β, IL-6, IL-8, TNF-α, and cTn-I on reperfusion post-CPB fit in with data from other authors. The course of IL-10 post-CPB has, in part, been shown to be biphasic, with an initial drop in early reperfusion (29). However, an early increase has been described by others (30). In the limb perfusion model with ECC, plasma samples were measured using 11-plex. IL-1β, IL-6, IL-8, MCP-1, C5a, and sC5b-9 as well as VEGF levels were increased significantly from baseline during ECC, and all markers were again back to normal at the end of the replantation period. Endothelial activation due to I/R injury induces inflammatory reactions, for example, shedding of endothelial glycocalyx, activation of complement and coagulation cascades, and increased cytokine release, causing endothelial dysfunction (31–34). In this study, we observed that prolonged preservation of amputated pig limbs using ECC has no effects on I/R-induced injury as the results fit in with the expected course of inflammatory markers within this experimental setting (20).

In conclusion, we were able to establish a bead-based multiplex assay to detect porcine cytokines (IL-1β, IL-6, IL-10, and TNF-α), chemokines (IL-8 and MCP-1), growth factors (bFGF, VEGF, and PDGF-bb), injury markers (cTn-I), and complement activation markers (C5a and sC5b-9) in porcine blood samples with a good assay sensitivity, broad dynamic range, and low intra-assay variance. The simultaneous detection of analytes allows for an internal consistency and greatly increases the amount of information obtained from a single volume-limited sample. This method should prove a valuable tool in the analysis of porcine samples obtained from experimental setups using pig as a model animal to study various states of disease, including cardiovascular disease and shock.

Acknowledgments

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Author Contributions

A.K.B. and J.L. performed most of the experimental work (data collection), analyzed and interpreted the data, performed statistical analyses, and wrote and critically revised the manuscript. R.R. participated in the concept and design of the study, helped to write and critically edit the manuscript, and carried a part of the overall responsibility. Y.B. participated in the concept and design of the study, helped to write and critically edit the manuscript, and carried a part of the overall responsibility. All authors read and approved the final version of the submitted manuscript.

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Paper V

Regulatory sequences of the porcine *THBD* gene facilitate endothelial-specific expression of bioactive human thrombomodulin in single- and multitransgenic pigs

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Background: Beside immunological barriers, coagulation incompatibilities between the human blood and the porcine vessel obstruct the xenotransplantation of vascularized organs. Therefore, genetically engineered pigs lacking alpha1,3 galactosyl-galactose transferase gene (GalTKO) and overexpressing human CD46 and human thrombomodulin (hTM) were generated to overcome hyperacute rejection and acute vascular rejection.

Aim: We generated and tested (multi-) genetically modified pigs expressing hTM against the problem of impaired activation of coagulation cascade.

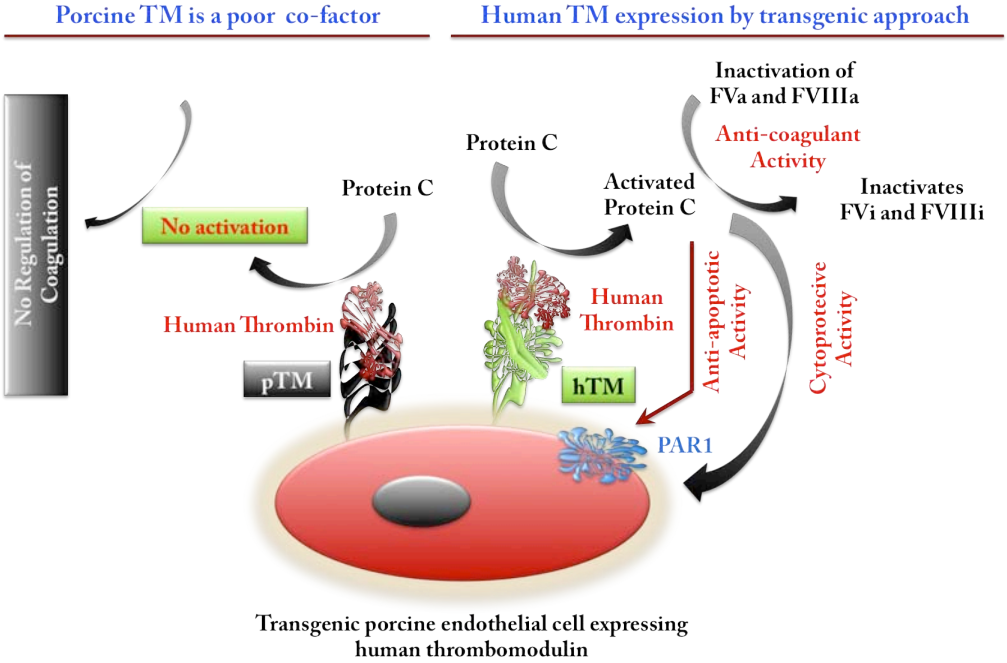

Figure: Schematic of the interaction of porcine and transgenic human thrombomodulin with human thrombin on porcine endothelial surface. Porcine TM-thrombin complex is a poor activator of human protein C and that results in unregulated coagulation activation. Transgenic expression of human TM on porcine endothelial cells could provide beneficial effects, including protein C activation (APC), APC-mediated alteration of gene expression, anti-inflammatory activities, anti-apoptotic activities, and protection of endothelial barrier functions.

Conclusion: Our results give an indication that (multi-) genetically modified donor pigs described in this study have the potential to overcome coagulation incompatibilities in pig-to-primate xenotransplantation.

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BASIC AND EXPERIMENTAL RESEARCH

Regulatory Sequences of the Porcine *THBD* Gene Facilitate Endothelial-Specific Expression of Bioactive Human Thrombomodulin in Single- and Multitransgenic Pigs

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Background. Among other mismatches between human and pig, incompatibilities in the blood coagulation systems hamper the xenotransplantation of vascularized organs. The provision of the porcine endothelium with human thrombomodulin (hTM) is hypothesized to overcome the impaired activation of protein C by a heterodimer consisting of human thrombin and porcine TM.

Methods. We evaluated regulatory regions of the *THBD* gene, optimized vectors for transgene expression, and generated hTM expressing pigs by somatic cell nuclear transfer. Genetically modified pigs were characterized at the molecular, cellular, histological, and physiological levels.

Results. A 7.6-kb fragment containing the entire upstream region of the porcine *THBD* gene was found to drive a high expression in a porcine endothelial cell line and was therefore used to control hTM expression in transgenic pigs. The abundance of hTM was restricted to the endothelium, according to the predicted pattern, and the transgene expression of hTM was stably inherited to the offspring. When endothelial cells from pigs carrying the hTM transgene—either alone or in combination with an aGalTKO and a transgene encoding the human CD46—were tested in a coagulation assay with human whole blood, the clotting time was increased three- to four-fold ($P<0.001$) compared to wild-type and aGalTKO/CD46 transgenic endothelial cells. This, for the first time, demonstrated the anticoagulant properties of hTM on porcine endothelial cells in a human whole blood assay.

Conclusions. The biological efficacy of hTM suggests that the (multi-)transgenic donor pigs described here have the potential to overcome coagulation incompatibilities in pig-to-primate xenotransplantation.

Keywords: Xenotransplantation, Coagulation incompatibility, Thrombomodulin, Transgenic donors, Human thrombomodulin, Transgenic donor pigs.

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A.W. participated in the design of the study, performed characterization of endothelial cells and participated in data interpretation and writing of the article. A.Ba. performed genomic analysis of the pigs and managed pig housing. A.K.B. performed analysis of biological function. E.K., A.Bl., and R.W. performed expression analysis of the pigs. W.B. performed evaluation studies of vectors. S.H. performed flow cytometric analysis. M.K., V.Z., and B.K. performed nuclear transfer and embryo transfer. C.F. participated in the analysis of founder pigs. J.M.A. and B.R. participated in the design of the study and data interpretation. R.S. designed evaluation studies of the vectors. H.N. provided scientific support in nuclear transfer. R.R. designed analysis of biological function. D.A. provided multitransgenic pigs. E.W. participated in the design of the study and in data interpretation and writing of the article. N.K. participated in the design of the study, performed bioinformatics analysis and vector construction, and participated in data interpretation and writing of the article.
E.W. and N.K. equally contributed to this study.
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The use of multitransgenic donor pigs, as well as the advanced preoperative and postoperative care of nonhuman primates, has led to impressive progress in the xenotransplantation of vascularized organs over the last decade, tempting one to think about its clinical implementation in the near future (*1*). In particular, the survival of pig-to-baboon heart transplants for up to 8 months inspires these ambitions (*2*). Yet, such feats are still exceptional, and the immunosuppressive regimens that have led to these promising data are far from clinical application owing to the severe side effects that result from systemic and high-dose administration of drugs. An appealing approach to circumvent these complications would be the genetic tailoring of donors because this allows the local but sustained application of an active agent. In a recent article, we demonstrated the applicability of such a strategy by using islet transplants from transgenic pigs that sufficiently suppressed T-cell–mediated rejection at the transplant site in humanized mice but reduced the systemic burden of immunosuppression in the recipient by two orders of magnitude (*3*). Similarly, the coagulation problems that occur in xenotransplantation of vascularized organs (*4, 5*) might be resolved by using genetically modified donors. Coagulation issues in xenografts have been supposed to occur due to incompatibilities between human blood and the porcine vessel wall, with the main obstacle being the impaired activation of protein C by a heterodimer consisting of human thrombin and porcine thrombomodulin (*6*). Numerous attempts to overcome this problem have been suggested (reviewed in Cowan et al. (*7*)), but the most convincing approach would, in any case, be the transgenic expression of the human variant of thrombomodulin (hTM) in donor pigs, in particular as the molecule not only has anticoagulant but also anti-inflammatory properties (*8*). The beneficial effect of hTM has been demonstrated in early xenoperfusion studies (*9, 10*), and two models of hTM expressing pigs have been presented recently (*11, 12*). In contrast to these studies that used ubiquitously active promoters to drive hTM expression, other studies aimed at the identification of regulatory elements to restrict the abundance of a transgene to the endothelial wall (*13–15*) as the correct balance of the blood coagulation system might require not only the presence of functionally compatible key players but also their tightly controlled expression (*16, 17*). The multifaceted regulation of thrombomodulin on the transcriptional as well as post-translational level seems to involve numerous stimulators such as shear stress, hypoxia, reactive oxygen species, free fatty acids, or inflammation (reviewed in Conway (*8*)) and suggests a *THBD* promoter to control hTM expression. Although regulatory sequences from different species have been successfully used in numerous transgenic mouse models, species-specific transcriptional initiation has been described in defined physiological processes such as the regulation of pluripotency (*18*), embryo-maternal communication (*19*) or immunology (*20*). Moreover, increasing evidence for a more significant divergence in the transcriptional regulation even between closely related species has been found (*21, 22*) and the role of specific regulatory elements has been postulated to be more relevant for expression control compared to other parameters such as epigenetic pattern, cellular environment, or the abundance of transcription factors (*23*). Consequently, transgene expression in the pig might be more efficiently initiated by porcine regulatory elements than by orthologous sequences from human or mouse.

Thus, we (i) developed and tested an expression vector for reliable transgene expression in porcine endothelial cells, (ii) generated single- and multitransgenic pigs that exhibit and inherit strong and endothelial-specific expression of hTM, and (iii), for the first time, demonstrated the potential of hTM on transgenic porcine endothelial cells to prolong clotting time in a human whole-blood coagulation assay.

RESULTS

Interspecies Comparison of THBD Regulatory Elements

As fundamental characteristics of promoter regions should be conserved among related species, we examined the thrombomodulin locus of human, macaque, mouse, rat, dog, horse, cattle, dolphin, and pig to identify the most essential regulatory elements (Fig. 1A; **Figure S1, SDC,** http://links.lww.com/TP/A885). For the region between the thrombomodulin encoding *THBD* gene and the upstream *CD93* gene, multispecies alignments revealed huge differences in the intergenic length, ranging from less than 7 kb to almost 18 kb between the respective species. Nonetheless, three regions were identified in the alignment, which showed high homology between all species, whereas the fragments in between were characterized by large gaps in one or more sequences and a reduced similarity between the remaining species. One region located closely to the 3′-UTR of *CD93* was predicted to contain another potential polyadenylation site of the gene. At the very other end of the alignment, the presumed core promoter of the *THBD* gene contained commonly conserved binding sites for numerous transcription factors (TFs) and core promoter elements. The third region 8.5 kb upstream of the *THBD* transcription start might represent an enhancer element because it contained conserved binding sites for diverse TFs as well. A fourth region 14.5 kb upstream of the *THBD* transcription start is of particular interest because eight species showed sequence homologies similar to those in the other conserved regions as well as potential TF binding sites, whereas this fragment was completely absent in the pig. Another interesting feature of the porcine sequence was the unique presence of a potential insulator segment adjacent to the novel potential polyadenylation site of *CD93*.

Design of an Endothelial-Specific hTM Expression Vector

Based on a genomic fragment containing the human *THBD* locus, we introduced two modifications (Fig. 1B). First, we replaced the 3′-UTR and downstream region of *THBD* by a widely used polyadenylation cassette from the bovine growth hormone (*GH*) gene because the 3′-UTR of *THBD* has been described to cause mRNA instability in the presence of cytokines (*24*). Second, we replaced the 5′-UTR and the upstream region of human *THBD* by a porcine fragment containing the complete intergenic region between *CD93* and *THBD*, the transcription start, as well as the 5′-UTR. In an in vitro assay, we transfected immortalized porcine endothelial cells with the respective vectors, determined the

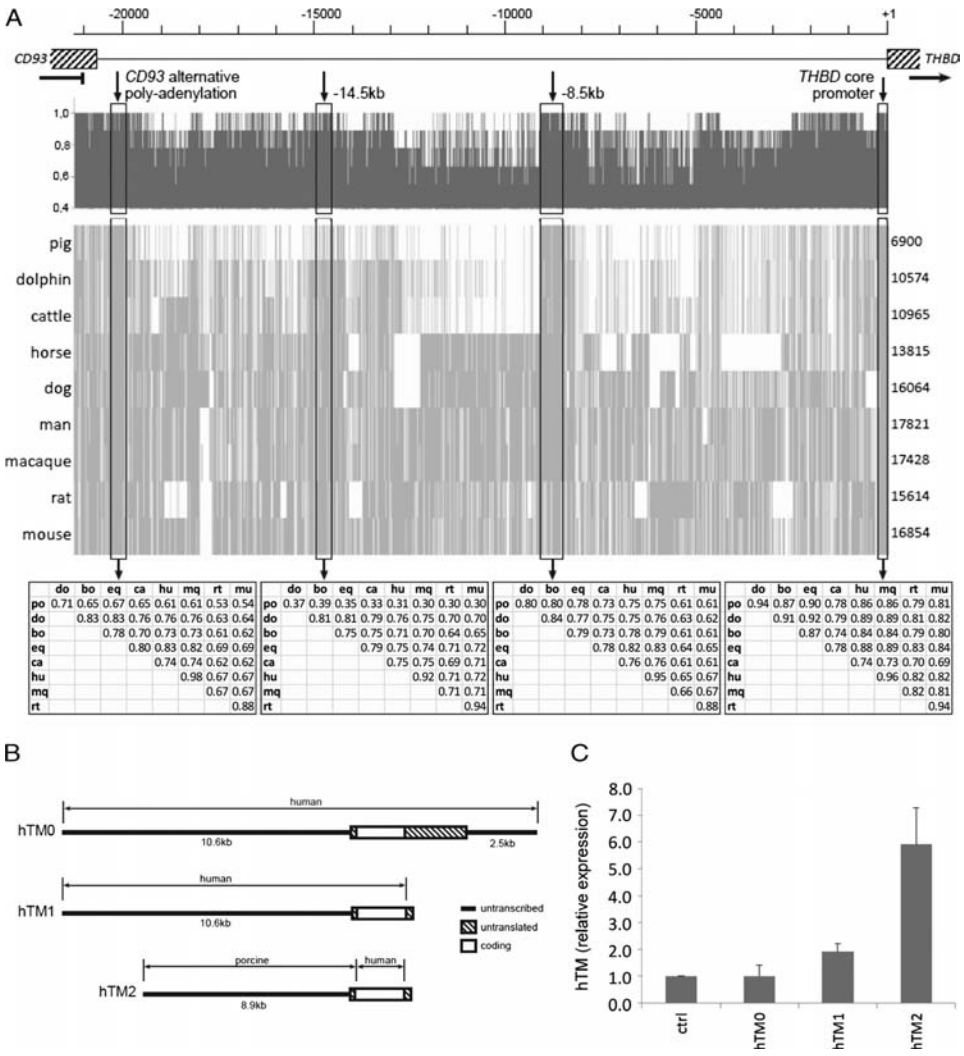
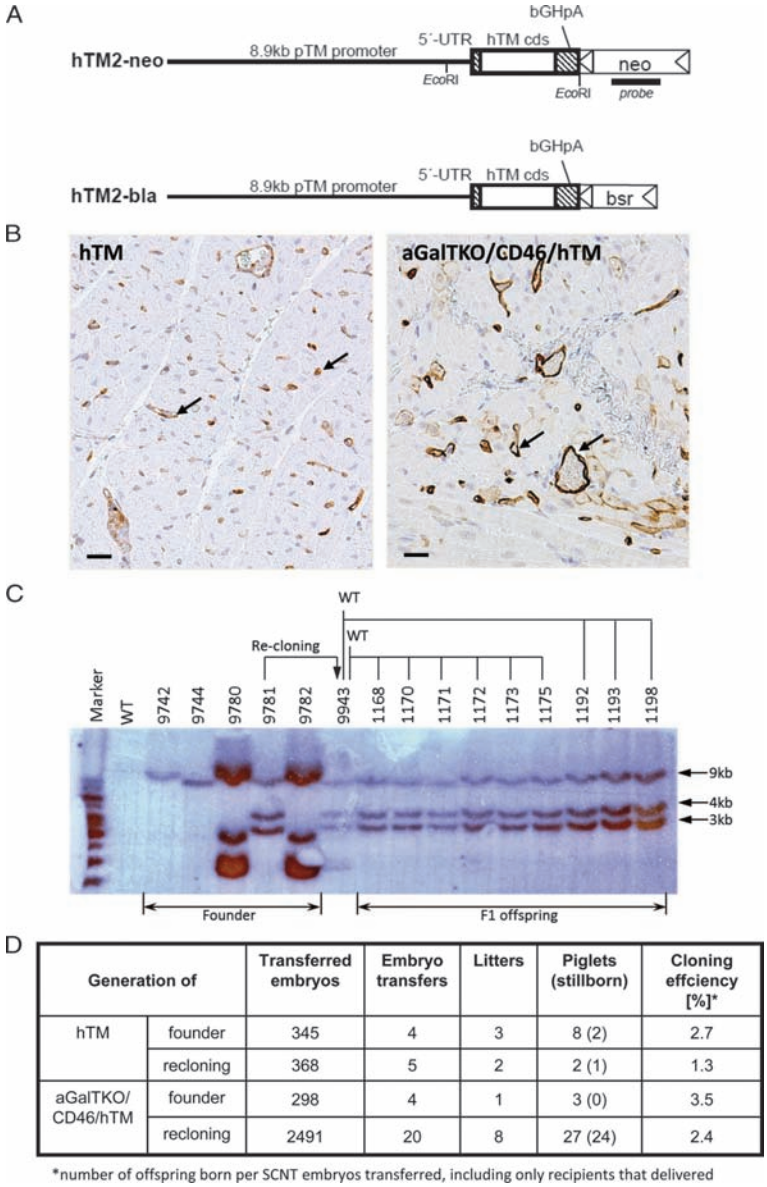


FIGURE 1. Evaluation of *THBD* regulatory sequences. (A) From nine mammalian species, a 21-kb alignment of the intergenic regions between the *CD93* and *THBD* genes was generated. Four conserved regions (*boxed*) have been identified by a homology plot (*upper panel*), a density plot (*middle panel*, with *white bars* indicating gaps in the respective species), as well as the accumulation of potential conserved TF binding sites (see **SDC,** http://links.lww.com/TP/A885). The calculation of similarity matrices (*lower panel*) for these regions indicated strong evolutionary conservation among the examined species with the exception of the −14.5-kb region that was lacking in pig but strongly conserved among the other species. The shown homology values between pig and the other species for this region result from alignment artifacts. (B) Three different hTM constructs were compared. hTM0 consisted of a completely human genomic fragment; in hTM1, the *THBD* 3′-UTR and downstream region were replaced by a polyadenylation cassette from the bovine *GH* gene and hTM2 contained the modified 3′-end as well as the regulatory region upstream of the porcine *THBD* gene. (C) PEDSV.15 cells transfected with the constructs hTM0, hTM1, and hTM2 were stained with the anti-hTM antibody 6980-100 and analyzed by flow cytometry. Relative expression was determined by comparing the number of hTM-positive cells obtained with each construct with background staining found in mock transfected PEDSV.15 cells (ctrl). Data represent mean values ±SD obtained in three independent experiments.

proportion of hTM expressing cells by FACS analysis, and clearly found the porcine promoter to drive a stronger expression than the human fragment (Fig. 1C; **Figure S2, SDC,** http://links.lww.com/TP/A885).

Establishment of hTM Expressing Pigs

Primary fibroblasts from a wild-type pig and kidney cells from an alpha1,3-galactosyl transferase–deficient (aGalTKO) (*25*) and CD46 transgenic (*26*) pig were nucleofected with



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Generation of		Transferred embryos	Embryo transfers	Litters	Piglets (stillborn)	Cloning effciency [%]*
hTM	founder	345	4	3	8 (2)	2.7
	recloning	368	5	2	2 (1)	1.3
aGalTKO/ CD46/hTM	founder	298	4	1	3 (0)	3.5
	recloning	2491	20	8	27 (24)	2.4

*number of offspring born per SCNT embryos transferred, including only recipients that delivered

FIGURE 2. Generation of hTM-transgenic pigs by SCNT. (A) The hTM2 construct was linked to a neomycin (neo) resistance cassette for transfection into wild-type cells and to a blasticidin S (bsr) resistance cassette for transfection into aGalTKO/CD46 cells. Localizations of the *Eco*RI restriction sites and the neo-specific probe for Southern blotting are indicated. (B) Transgenic founders were examined for hTM expression by immunohistochemical detection of hTM (*brown color*) in vascular endothelial cells in the cardiac tissue of hTM and aGalTKO/CD46/hTM founder pigs. Bars=20 μm. (C) Southern blot examination of transgenic pigs carrying the hTM2-neo vector with the neo-specific probe on *Eco*RI-digested genomic DNA. Marker is 1 kb ladder (Thermo Scientific); WT is wild-type control; 9742, 9744, 9780–9782 are founder animals; 9943 was recloned from primary cells of founder 9781; and 1168–1198 are transgenic offspring that were delivered from two independent litters after mating 9943 to wild-type sows. The approximate sizes of the transgene-carrying fragments are indicated on the right side. (D) The cloning and recloning efficiencies of SCNT experiments are within our long-term experience.

the hTM2 expression vector that has been linked to a neomycin or a blasticidin S resistance cassette, respectively (Fig. 2A). After growing cells under selection, stable cell clones were pooled and used for somatic cell nuclear transfer (SCNT) to generate hTM or aGalTKO/CD46/hTM pigs according to Richter et al. (*27*). First, a total of eight hTM transgenic founder pigs were generated and five were examined for hTM expression in various organs either at the day of birth or at the age of 1 month. All founders showed strong and endothelial-specific expression of the transgene in heart and kidney, and three of them showed additionally strong expression on vascular endothelial cells in the liver and lung. Southern blot analysis suggested that the five founders represent four different integration patterns of the construct (Fig. 2C). Primary kidney cells from an animal with a high and endothelial-specific expression in the heart (Fig. 2B, left panel) were chosen to reproduce the founder by SCNT. Two delivered animals were raised to fertility, and one of them was mated to wild-type sows. The inheritance of the transgene integration pattern (Fig. 2C) and its expression pattern to vital and fertile offspring over, as of now, two generations indicates that the transgene does not interfere with animal health or reproductive traits. Second, a total of three aGalTKO/CD46/hTM founders were generated and examined for hTM expression at an age of either a few days or 1 month. Corresponding to the findings for the hTM transgenic pigs, all aGalTKO/CD46/hTM animals showed strong and endothelial-specific hTM expression in the heart and kidney, and two of them also revealed high hTM abundance on endothelial cells of the lung and liver. A total of nine aGalTKO/CD46/hTM animals have been reproduced by SCNT from primary cells of a highly expressing founder with positive hTM immunoreactivity on vascular endothelial cells of cardiac tissue specimens (Fig. 2B, right panel). The efficiencies of cloning and recloning hTM-transgenic animals (Fig. 2D) reflect our long-years' experience (*28*).

For a more detailed analysis of transgene expression and its biological function, individuals of both transgenic lines were examined at the histological, cellular, molecular and physiological levels. For both the hTM and the aGalTKO/CD46/hTM lines, several animals were examined. As the results were consistent within the lines, data from no. 1198 are shown as representative for the hTM line and data from no. 1259 are shown as representative for the aGalTKO/CD46/hTM line.

Expression Analysis of hTM

The expression of hTM in tissue specimens and cultured endothelial cells was investigated using immunochemical methods.

In cardiac tissue sections of both lines, hTM immunoreactivity was exclusively present on the endocardium and in vessels of large as well as small caliber, displaying congruent abundance patterns with the endothelial cell marker von Willebrand factor (Fig. 3). In the kidney, hTM expression was present in the endothelia of intertubular capillaries and larger vessels but was not detectable in glomerular endothelia. The lungs of hTM and aGalTKO/CD46/hTM pigs displayed diffuse endothelial hTM immunoreactivity, whereas in the liver, endothelial hTM expression was detected on the endothelia of large vessels but not on those of the sinusoids (see **Figure S3, SDC,** http://links.lww.com/TP/A885).

The expression of the transgene on cultured aortic endothelial cells was investigated in more detail with FACS, immunofluorescence, and Western blot analyses. Immunocytochemistry verified the endothelial nature of the cultivated cells by positive CD31 staining and showed broad although unevenly distributed hTM expression (Fig. 4A). The finding of prevalent hTM expression was confirmed in flow cytometric analysis as at least 90% of the cells coexpressed CD31 and hTM in both transgenic lines (Fig. 4B). The presence of different expression levels was verified at a more quantitative level, as CD31-positive cells from aGalTKO/CD46/hTM pigs revealed two clearly distinct populations regarding the expression levels of hTM. In contrast, cells from hTM pigs showed a more homogenous population of cells that coexpressed CD31 and hTM. Cultivated endothelial cells were also used to extract protein for Western blot analysis. A single and distinct band corresponding to the expected size of hTM (116 kDa) was detected with the hTM-specific antibody (Fig. 4C).

Biological Function of hTM on Porcine Endothelial Cells

In a first approach we tested whether hTM facilitated the cleavage of protein C in the presence of human thrombin in a biochemical assay (Fig. 5A). We found clear evidence that the amount of activated protein C (APC) in the supernatant of hTM or aGalTKO/CD46/hTM cells increased with the concentration of protein C in a dose-dependent manner, whereas the turnover of protein C remained at the basal level for any of the examined concentrations upon exposure to control cells lacking hTM.

In a further experiment, we tested whether hTM also possesses the ability to prevent blood coagulation/clotting in a more physiologic assay that models the processes at the interface of human blood and porcine vessel wall (*29, 30*). Aortic endothelial cells from wild-type, hTM, aGalTKO/CD46, and aGalTKO/CD46/hTM pigs were grown on microcarrier beads and tested for their potential to prolong the clotting time of freshly withdrawn, non-anticoagulated human blood. While clotting time in the presence of aGalTKO/CD46 aortic endothelial cells was slightly but significantly prolonged (100.9±20.5 min, $P<0.01$) as compared to the wild-type control (62.3±15.5 min), the effect of hTM either on the wild-type background or in the triple-transgenic combination (aGalTKO/CD46/hTM) was more pronounced, resulting in a three- to four-fold increase in clotting time (176.1±13.5 min, $P<0.001$ for hTM and 190.8±14.0 min, $P<0.001$ for aGalTKO/CD46/hTM) compared to wild-type endothelial cells (Fig. 5B). As the increased coagulation time in the presence of hTM-expressing cells might also result from the cytoprotective properties of the transgene product, we conducted another set of whole blood coagulation experiments and took samples at distinct time points to evaluate the coverage of the beads with endothelial cells (see **Figure S4, SDC,** http://links.lww.com/TP/A885). Albeit cells being lost during the experiment, we did not observe differences between cells that express hTM and those that do not and, thus, conclude that the main course of action of the hTM transgene was by its control of thrombin regulation. The effective anticoagulatory properties of hTM were further illustrated by the formation of thrombin–antithrombin III

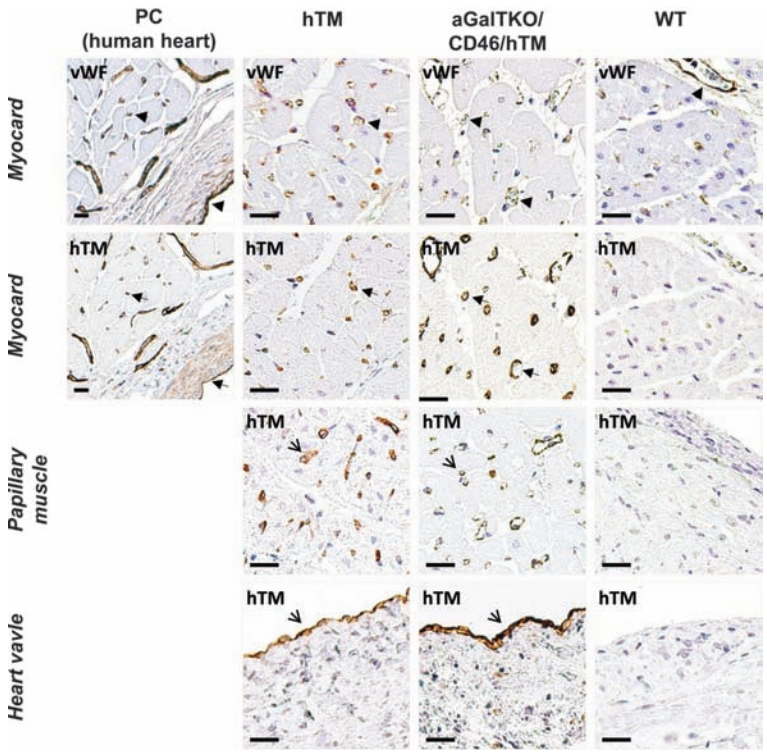

FIGURE 3. Immunohistochemical detection of hTM in the cardiac tissue of a wild-type control pig (WT), an hTM pig, a GalTKO/CD46/hTM pig, and in human positive control tissue (PC). Sequential sections of left ventricular myocardia were stained for the endothelial-specific von Willebrand factor (vWF) and hTM. In human and porcine cardiac tissue specimen, vWF was restricted to endocardial and vascular endothelial cells (*brown color*, *arrowheads*, *top row*). Staining of hTM corresponded to that of vWF in hTM and aGalKO/CD46/hTM pigs (*brown color*, *arrows*, *second top row*), whereas no staining was seen in WT pigs. Positive hTM immunoreactivity (*brown color*) was also detected in the capillary endothelia in the papillary muscle and in endocardial cells of the left atrioventricular heart valve of hTM and aGalTKO/CD46/hTM pigs but not in WT pigs. Paraffin sections. Bars=20 μm.

(TAT) complexes (Fig. 5C). While wild-type and aGalTKO/CD46 cells caused an immediate and steady increase of TAT until coagulation occurred, hTM transgenic cells kept TAT levels constantly low in the first phase of the experiment. Moreover, although slightly increasing afterward, in these groups, TAT did not reach the maximum levels that were seen in the wild-type or aGalTKO/CD46 groups.

DISCUSSION

Advancements in the production of genetically modified donor pigs are a major reason for the progress that has been made in xenotransplantation research in recent years. Many different transgenic pigs have been described (*31, 32*) and many more will be produced in the future. The biotechnological production process itself does not represent a main hurdle any more, but the relatively long generation time of the pig and economic aspects of housing are still challenging factors. Thus, the detailed design of transgenic approaches and their thorough evaluation is of importance for efficient production of novel donor pig models. Consequently, we made use of state-of-the-art in silico analysis tools to estimate the potential of regulatory elements of the *THBD* gene and compared different vector systems in vitro to determine the most promising strategy for the generation of transgenic pigs and, later on, put effort in comprehensive characterization at the molecular, cellular, histological, and physiological levels.

Regarding the regulatory properties of *THBD*, the conserved regions found in multispecies alignments are indicative of common regulatory mechanisms of TM expression in mammals, but the diverse length of the examined sequences as well as the lack of certain segments in distinct species in otherwise highly conserved regions might also indicate unique properties in individual species. In the absence of detailed promoter studies in the literature, the regulative properties of *THBD* remain unclear, but the relatively short intergenic region between *THBD* and the upstream *CD93* in the pig allowed the usage of its entire length to control transgene expression.

The fact that hTM is not expressed on all endothelial cells and the distinct expression pattern of hTM in heart, kidney, lung, and liver strictly mirrors the data that have been published on a mouse model with a *lacZ* reporter

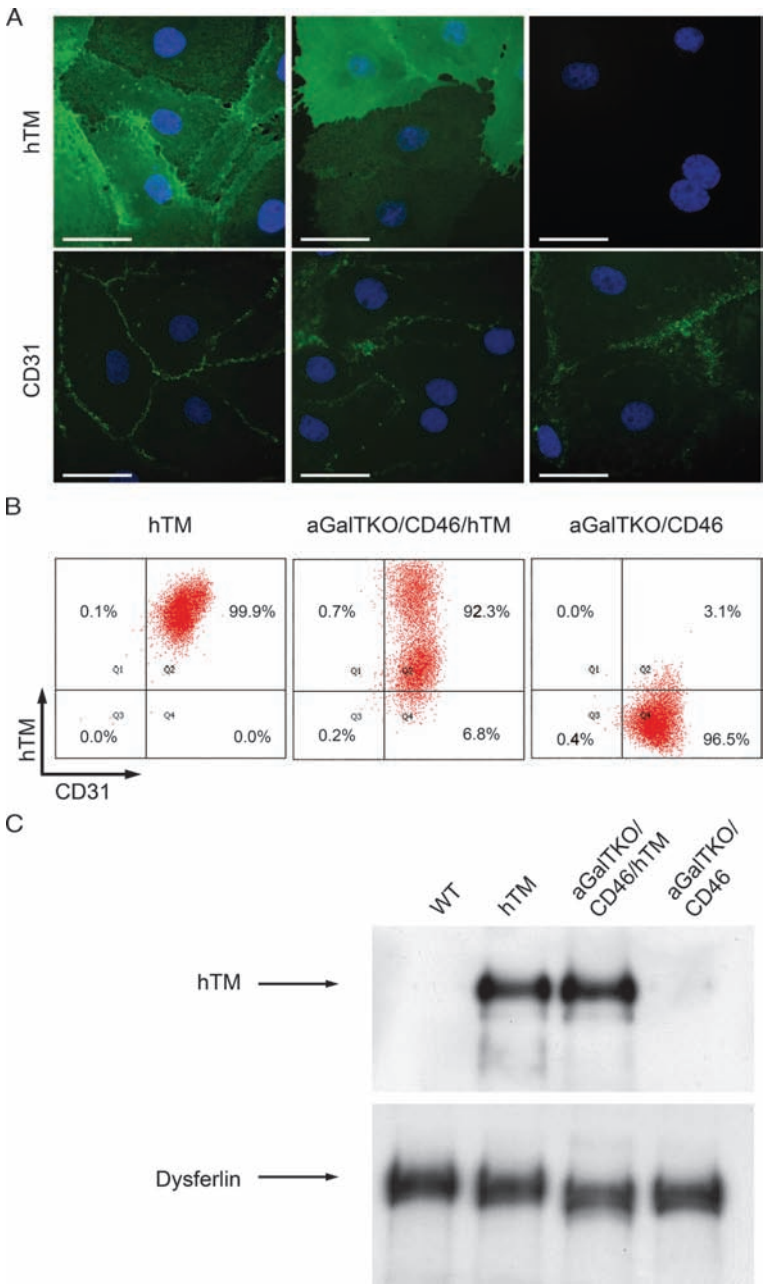

FIGURE 4. Expression of hTM on endothelial cells from hTM and aGalTKO/CD46/hTM pigs. (A) Immunocytochemistry showed hTM on paraformaldehyde-fixed endothelial cells from hTM and aGalTKO/CD46/hTM pigs, whereas hTM expression was absent on aGalTKO/CD46 cells. CD31 staining confirmed the endothelial origin of the cells. Bars=20 μm. (B) FACS analysis revealed a strong coexpression of CD31 and hTM on hTM and aGalTKO/CD46/hTM endothelial cells. (C) Immunoblot analysis showed a band with the correct size (116 kDa) demonstrating the expression of hTM in hTM and aGalTKO/CD46/hTM endothelial cells but not in wild-type or aGalTKO/CD46 cells.

knockin in the murine *Thbd* locus (*33*), suggesting that TM regulation is similar among mammalian species and further indicating that the transgene expression resembles the entire physiologic regulation of TM. This would be of relevance as one might assume that species-specific incompatibilities in blood coagulation have to be restored to a tightly regulated

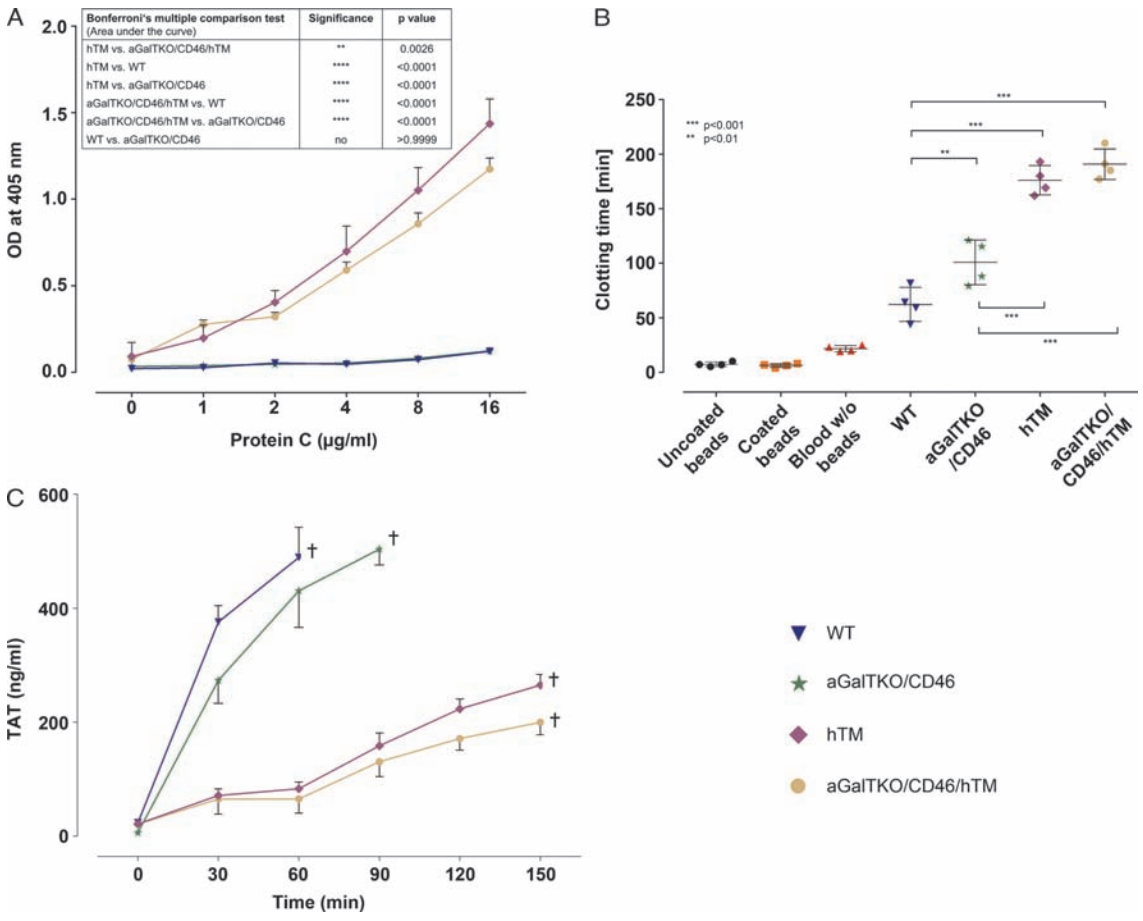

FIGURE 5. Biological function of the hTM transgene. (A) The ability of porcine endothelial cells to activate protein C in the presence of human thrombin was evaluated in a biochemical assay. The abundance of APC was detected colorimetrically. hTM transgenic endothelial cells revealed clear and concentration-dependent APC production when the amount of protein C was increased, whereas control cells (aGalTKO and WT) did not show any protein C turnover. Data are plotted as mean values, and standard deviations are indicated when they were above 0.025 OD_{405}. The inset shows the significance of area under the curve differences between the groups. Four independent experiments were performed for each group. Significance test was performed using one-way analysis of variance with Bonferroni correction. (B) Measurement of the clotting time after incubation of freshly withdrawn, non-anticoagulated whole human blood with wild-type or genetically modified endothelial cells cultivated on microcarrier beads. Microcarriers with or without collagen coating, incubated with blood, showed a strong procoagulant property with a short clotting time. Microcarriers covered by confluent hTM or aGalTKO/CD46/hTM endothelial cells showed a strong anticoagulant effect compared to wild-type and aGalTKO/CD46 PAECs. Four independent experiments were performed for each group. Significance test was performed using one-way analysis of variance with Bonferroni correction. (C) Formation of thrombin–antithrombin III (TAT) complexes was determined as a fluid phase coagulation parameter (thrombin generation) in EDTA plasma samples taken from whole blood coagulation assays at regular time intervals of 30 min until coagulation occurred (indicated by "†"). TAT is indicated as mean value (± standard deviation when it was >10.0 ng/mL). Three independent experiments were performed for each group.

physiological level, which is in contrast to other transgenic donor pigs for xenotransplantation that aim at immunological rejection processes, where the functional properties of a transgene may be less subject to transcriptional regulation. Ongoing transplantation studies using aGalTKO/CD46/hTM donors (B. Reichart and D. K. C. Cooper, personal communication) will have to prove the relevance of our strategy in nonhuman primate (NHP) models.

Albeit such transplantation experiments into primates being the most stringent tool for the evaluation of genetically modified donor organs, they suffer from their tremendous costs and the challenging experimental setup that is attributed to the complexity of the NHP system. Thus, the comprehensive examination of given transgenes under constant regimens in vivo at experimental numbers that are sufficient for scientific conclusions is limited to a small

number of transgenic combinations, and thus, they have to be selected by preevaluation in less complex systems. Here, we used an in vitro assay that exploits the natural anticoagulant properties of endothelial cells in combination with whole, non-anticoagulated human blood to test the efficacy of the hTM transgene product in coagulation control, either alone or in combination with the aGalTKO/CD46 transgenic background. Our coagulation assay confirmed the beneficial effect of hTM either on the wild-type or on the aGalTKO/CD46 background. These findings illustrate the suitability of the whole blood coagulation assay for the evaluation of clotting mechanisms in xenotransplantation approaches because it facilitates the analysis of several transgene combinations and, moreover, resembles the immediate interaction of porcine endothelium and human blood, also potentially avoiding wrong conclusions from in vivo transplantation models due to differences in the coagulation systems between NHP and man (*34*).

In summary, we developed a novel genetically modified pig for xenotransplantation and present evidence for its potential to overcome the incompatibilities in the coagulation systems between pig and man.

MATERIALS AND METHODS

Pig Housing

Animal experiments have been carried out according to the guidelines of the responsible authority (Regierung von Oberbayern, approval no. 55.2-1-54-2531-54).

Bioinformatic Analysis

Sequences were fetched from the Ensembl genome browser (www.ensembl.org) and prepared in BioEdit (*35*). Mobile genetic elements were removed by RepeatMasker (*36*), and multiple alignments were done by ClustalW2 (*37*) and by using the Genomatix ElDorado/Gene2Promoter and GEMS Launcher software packages (Genomatix, Munich, Germany). The latter was also used to assign binding sites of common transcription factors.

Vector Construction

Genomic fragments of the human and porcine *THBD* genes were excised from the BACs RP4-753D10 and CH242-263H13, respectively. The fragments were combined with other genetic elements using a two-step polymerase chain reaction, Cre-mediated recombination, and other conventional cloning procedures.

Vector Evaluation

The immortalized porcine endothelial cell line PEDSV.15 (kindly provided by Prof. J. Seebach, Geneva (*38*)) was used for transfection and analyzed for hTM expression by flow cytometry.

Founder Pig Generation

Transgenic pigs were established according to Richter et al. (*27*) and Klymiuk et al. (*39*) using the hTM2 expression vector and raised up to an age of 2.5 months. Genotyping was carried out as described elsewhere (*40*). Founders with a high-level expression of hTM were reestablished by SCNT and raised for breeding purposes.

Necropsy, Histopathology, and Immunohistochemistry

For histological examination, cardiac tissue samples (including left and right heart ventricles, papillary muscles, and heart valves) and specimens of liver, kidney, and lung were taken from hTM-transgenic pigs and aGalTKO/CD46/hTM pigs. Control tissues were obtained from age-matched male wild-type pigs.

Expression Analysis of Aortic Endothelial Cells

Porcine aortic endothelial cells (PAECs) were isolated from the aorta of transgenic pigs as described earlier (*38*) with minor modifications. Colocalization studies of hTM were performed with the endothelial-specific marker PECAM-1 (CD31). Cultivated cells were then analyzed either in a multicolor flow cytometric assay, immunocytochemistry, or Western blot analysis.

APC Assay

The measurement of APC was performed with confluent PAECs on a 96-well plate.

Coagulation Assay

The coagulation-inhibiting effects of the different genetically modified porcine endothelial cells were monitored in vitro using PAECs grown on microcarrier beads and whole, non-anticoagulated human blood as previously described (*29, 30*). Details about endothelial cell culture on microcarriers and the coagulation assay are described in the Supplemental Material. (see **SDC,** http://links.lww.com/TP/A885). Human blood was drawn from healthy volunteers.

For further details on materials and methods, see the Supplemental Material (see **SDC,** http://links.lww.com/TP/A885).

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Supplemental Digital Content (SDC)

Materials and Methods

Vector construction. For the hTM0 vector, a 17 kb fragment was excised by *Bst*BI-*Eco*RI from RP4-753D10, cloned into a *Cla*I/*Eco*RI linearized pBSK vector and equipped with a floxed neomycin resistance cassette by making use of the *Not*I and *Eco*RI sites. The plasmid backbone was excised via *Sal*I sites and the vector was purified on low melting agarose (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's protocols. For the hTM1 vector, the 3´-part of the hTM coding region was amplified by the primers 5´-cccttcccaccaggcacttc-3´ and 5´-agctggAAGCTTccagctaaggtgctttggtag-3´, cloned into pGEM Teasy (Promega, Madison, WI, USA), sequenced and cloned as a *Xba*I/*Spe*I fragment into a *Spe*I linearized vector containing a bGH poly-adenylation cassette (a gift from M.R. Schneider, Gene Center, LMU Munich). The combined sequence was used to replace the 3´-UTR and downstream region of the 17 kb human fragment that has been cloned into the pBSK vector via the *Rsr*II/*Sal*I sites. hTM1 was finalized by linking the modified hTM sequence to a neomycin resistance cassette via *Eco*RI sites and processed as described for hTM0 for genetic modification of porcine cells. For the hTM2 vector, the hTM-bGHpA fragment that was made for hTM1 was subcloned into pBSK via *Eco*RI and linearized with *Bgl*II/*Bam*HI to insert a 6.5kb *Bgl*II fragment from the BAC CH242-263H13. This plasmid was re-opened with *Bgl*II to insert a synthetic fragment that linked the genomic sequence of pTM (amplified by 5´-cacgctctcctgtttctcc-3´ and 5´-caaggaccaggaccccaagCATggtgtcagcacgccaag-3´) and hTM (amplified by 5´-cttggcgtgctgacaccATGcttggggtcctggtccttg-3´ and 5´-gagcgcactgtcattaggtg-3´) at the translational start codon by 2-step PCR. Eventually, a *Hpa*I/*Sal*I neomycin resistance cassette was inserted into the *Eco*RV/*Sal*I sites and hTM2 was excised from the plasmid backbone via *Not*I/*Sal*I.

Vector evaluation. Two µg of vector DNA was transfected into the immortalized porcine endothelial cell line PEDSV.15 using the Amaxa™ Cell Line Nucleofector™ Kit (Lonza, Cologne, Germany). Control cells were treated with the Nucleofector™ Kit without administration of cDNA (mock transfection). The cells were cultivated in DMEM medium supplemented with 10% FCS, 50 U/ml penicillin, 4 mM L-glutamin, 50 µg/ml streptomycin, and 0.05 mM β-mercaptoethanol). After 16 h, the cells were incubated with the hTM-specific

antibody 6980-100 (mouse IgG1, Abcam, Cambridge, UK) followed by a second incubation with Phycoerythrin (PE)-conjugated rat anti-mouse Ig (BD Biosciences; San Jose, CA, USA). Stained cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Founder pig generation. 0.5×10^6 porcine fetal fibroblasts (wild-type) and 1.0×10^6 kidney cells (aGalTKO/CD46) were nucleofected with 3.0-4.5 µg linearized plasmid DNA using the program U12 of the Nucleofector™II device (Lonza) and the Amaxa™ Basic Nucleofector™ Kit Primary Fibroblasts (Lonza). Selection was conducted for 7-10 days either with 0.6 mg/ml G418 (Life Technologies, Carlsbad, CA, USA) for the fetal fibroblasts and 10 µg/ml blasticidin S (PAA, Pasching, Austria) for the kidney cells. The growing cell clones were mixed during the selection process in a passaging step.

Genomic characterization. Genotyping of hTM transgenic pigs was done on genomic DNA isolated from ear tissue samples by a genomic DNA isolation kit (Nexttec, Leverkusen, Germany) with the primers 5´-cgacgcagtcctgcaacga-3´ and 5´-ccggagtcacagtcggtgccaa-3´. Southern blotting was done on *Eco*RI digested genomic DNA that was isolated from ear-tissue by EasyDNA™ (Life Technologies) and hybridization was done with a α^{32}P-dCTP (Perkin-Elmer, Waltham, MA, USA) labelled neo-specific probe that has been amplified by the primers 5´-ccattgaacaagatggattgcac-3´ and 5´-gaagaactcgtcaagaaggcgatag-3´.

Culture of endothelial cells. After isolation of endothelial cells with 0.1% Collagenase II (Life Technologies) from the aorta (PAEC), they were seeded on 0.1% gelatine-coated culture dishes in M199 medium supplemented with L-glutamin (PAA), 100 units/ml penicillin, 100 µg/ml streptomycin (PAA) and 20 % FCS (Life Technologies). After 24 h the medium was replaced by endothelial cell medium containing endothelial medium supplements (PAA), 100 units/ml penicillin, 100 µg/ml streptomycin, 293 mg/l L-glutamine (PAA) and 5% FCS.
Biosilon microcarrier beads (polystyrene, 160-300 µm) were coated with bovine collagen I (100 µg/ml, sc-29009, Santa Cruz Biotechnology, Dallas, TX, USA) in 0.2% acetic acid (v/v) for 60 min at room temperature. Endothelial cells, harvested from one 175-cm^2 flask, were mixed with 5-7 ml collagen-coated beads in 100 ml medium M 199 (Sigma-Aldrich, St. Louis, MO, USA) plus supplements (10% heat inactivated fetal bovine serum (Life Technologies), 1% penicillin/streptomycin (Life Technologies), 1% L-glutamine (Life Technologies), 1.2% supplement mix-II (C-39216, PromoCell, Heidelberg, Germany),

5000 U/ml heparin (Heparinum natricum, Liquemin®) into a 500 ml stirred culture flask. The beads were stirred for 3 min at 100 rpm with intervals of 60 min at 37°C. After overnight incubation, 75 ml of medium M 199 plus supplements was added to the flask and incubated for another 24 h. Then, the volume of the medium was maintained to 320 ml with RPMI medium 1640 (Sigma-Aldrich) plus supplements. For every 48 h, 100 ml of the culture medium was exchanged with RPMI medium 1640 plus supplements. Cells reached confluence after 6 to 8 days, estimated by fluorescence microscopy after staining with DAPI (4', 6'-diamidino-2-phenylindole; Roche Diagnostics, Indianapolis, IN, USA).

Flow cytometry. Porcine endothelial cells from hTM, wild-type or aGalTKO/CD46 animals were grown until 80-100% confluence. Cells were detached with accutase (PAA) and washed with staining buffer (PBS pH 7.2, 1% BSA, 0.1% NaN_3). All incubation steps were performed in staining buffer for 20 min in the dark and on ice and were followed by two washing steps. 1×10^5 cells were resuspended in 100 µl of the appropriate working solution in the following sequence: (1) rat-anti-pig-CD31 (1:200, R&D Systems, Minneapolis, MN, USA) or isotype control rat IgG1 (1:400, AbD Serotec, Kidlington, UK), (2) followed by donkey-anti-rat IgG-FITC (1:300; Jackson ImmunoResearch Laboratories, Suffolk, UK), (3) a blocking step with normal rat serum (1:20 in PBS), (4) mouse-anti-hTM (1:500, Abcam, Cambridge, UK) or mouse IgG1 (1:75, Becton Dickinson, BD, Franklin Lakes, NJ, USA), (5) rat-anti-mouse-kappa-light-chain-biotin (1:100, AbD Serotec) and (6) Streptavidin-RPE (1:300, Southern Biotech, Birmingham, AL, USA). After staining the cells were fixed with 1% paraformaldehyde and stored at 4°C until measurement. Measurements were performed on a BD FACSCanto II (Becton Dickinson) and analyzed with BD FACS DIVA or FlowJo (Tree Star Inc., Ashland, OR, USA) software.

Immunocytochemistry. hTM and aGalTKO/CD46/hTM endothelial cells, grown in endothelial cell medium (see above) on µ slides (ibidi, Planegg, Germany) coated with 0.1% gelatine until 80-100% confluence, were fixed with 4% paraformaldehyde in PBS pH 7.4 for 20 min. After washing two times with ice cold PBS, the cells were incubated with PBS containing 1% BSA (Carl Roth, Karlsruhe, Germany) for 30 min. The following antibodies were diluted with 1% BSA-PBS: rat-anti-pig-CD31 (R&D Systems) 1:200, mouse-anti-human-TM (Abcam) 1:400, goat anti mouse (Abcam) 1:200. First and second antibodies were incubated for 1 h at room temperature with washing steps in between. A "no primary antibody" control was performed. Finally the cells were mounted with VECTASHIELD® mounting medium with

Dapi (Vectorlabs, Burlingame, CA, USA) and evaluated with an epifluorescence microscope Axiovert 200 (Zeiss, Oberkochen, Germany).

Immunoblot analysis. Endothelial cells were lysed in lysis buffer [150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1% NP-40, 5 mM EDTA, 1 mM PMSF, 10 mM NaF, Complete® protease inhibitor (Roche, Basel, Schweiz)], and protein concentration was determined by BCA assay. Equal amounts of total protein were separated by 8% SDS-PAGE under reducing conditions and blotted to PVDF membrane. Human thrombomodulin and dysferlin were detected using mouse monoclonal antibodies (sc-13164, Santa Cruz Biotechnology, and NCL-Hamlet, Novocastra, Newcastle upon Tyne, UK) and horseradish peroxidase-coupled polyclonal goat anti-mouse antibodies (Jackson ImmunoResearch). Bound antibodies were visualized using ECL reagent (RPN2106; GE Healthcare, Chalfont St Giles, Germany).

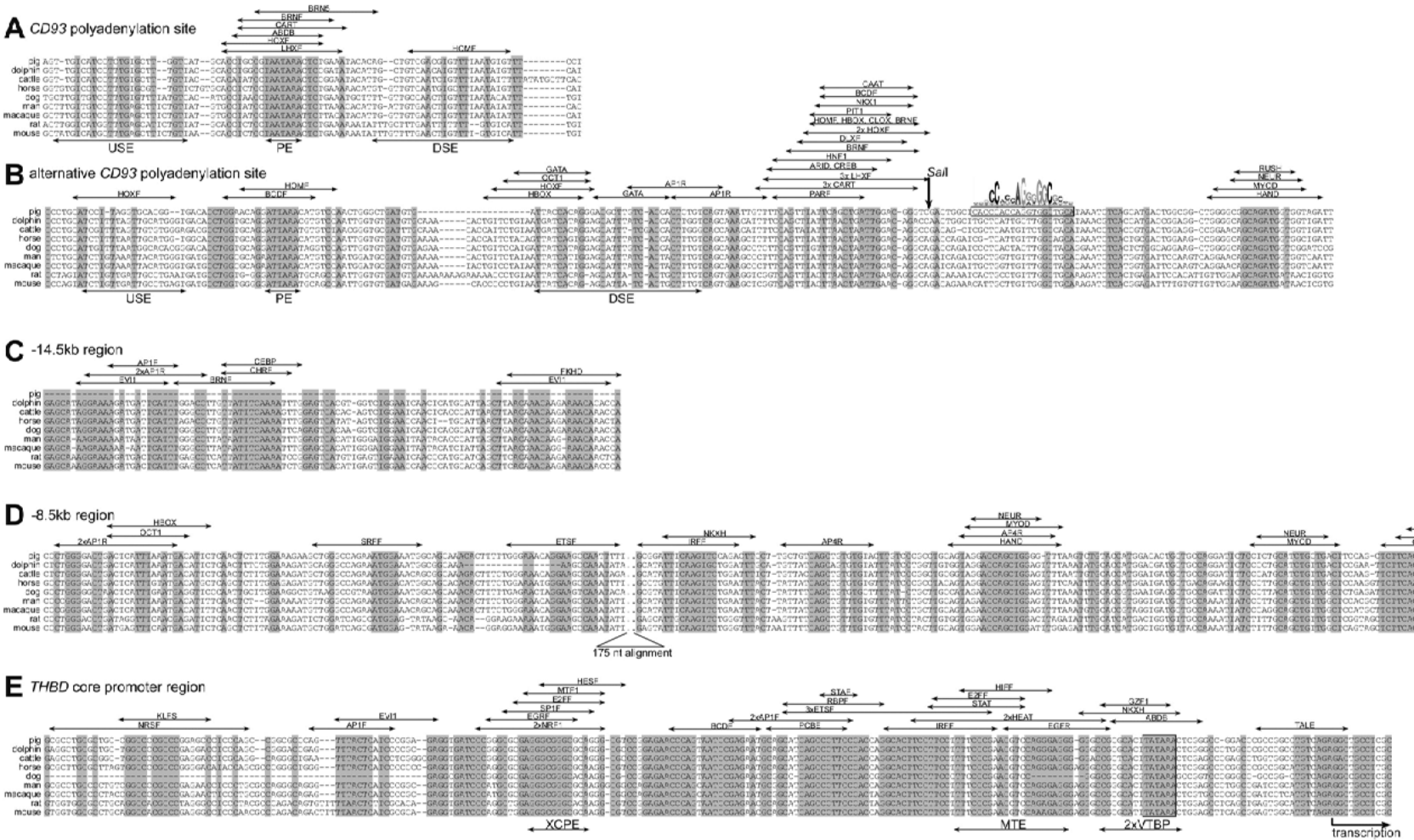
Necropsy, histopathology and immunohistochemistry. Histological sections of formalin-fixed and paraffin embedded tissue samples were routinely prepared. Immunohistochemistry was performed using antibodies specific for thrombomodulin (sc-13164, Santa Cruz Biotechnology) and for the endothelial cell marker von Willebrand factor, (vWF, polyclonal rabbit anti-human vWF, A0082; Dako, Glostrup, Denmark). For detection of vWF and hTM, the standard avidin-biotin peroxidase complex method was employed (secondary antibody for vWF: Biotinylated goat anti-rabbit IgG, E0432; secondary antibody for hTM: goat anti-mouse IgG, E0433; both from Dako). Diaminobenzidine was used as the final chromogen and hemalum as nuclear counterstain. Positive control slides consisted of human heart muscle. For negative controls, slides containing human myocardium, and heart tissues of hTM-tg and of WT-pigs were stained with buffer instead of the primary antibody.

Activated protein C (APC) assay.

Aortic endothelial cells were incubated with 0.125 U/ml of human thrombin (Sigma) and 0-16 µg/ml human PC (EMD Milipore/Calbiochem®, Billerica, MA, USA) at 37°C for 60 min in a final volume of 100 µl. The reaction was terminated by addition of 10 µl of 20 µg/ml hirudin (Refludan Celgene, Summit, NJ, USA), and 100 µl of supernatant was then transferred to another 96-well plate. One hundred microliters of 2 mmol/l S-2366 (Chromogenix, Milano, Italy) was added and the change in absorbance was measured using an Infinite M1000 microplate reader (Tecan, Maennedorf, Switzerland).

Coagulation assay. 2 ml collagen-coated biosilon microcarrier beads with confluent endothelial cells were taken into a 10 ml polypropylene tube and washed twice with RPMI medium 1640 without supplements, to remove medium that contains heparin. Blood was withdrawn from healthy volunteers, who did not have any medication at least for 2 weeks, into 10 ml polypropylene syringes. Washed, heparin free beads are then be incubated with freshly withdrawn, non-anticoagulated whole human blood at a volume ratio 1:4 of bead-to-blood i.e., 2 ml beads and 8 ml whole blood. Samples of the bead-blood mixture are incubated at 37°C with a gentle rocking, and tubes were observed at regular intervals for clotting time. As a minor modification of the published protocol, agitation of the blood/bead mixture ran uninterrupted until coagulation occurred.

The formation of thrombin-antithrombin III (TAT) complexes as well as the loss of endothelial cells from silica beads was measured similarly, but experiments were interrupted to sample 2 ml of blood-bead mixture after 30, 60, 90, 120 and 150 min to retrieve PAEC-covered beads as well as EDTA-plasma samples. The formation of TAT complexes was measured in EDTA plasma by ELISA (TAT-EIA 270R1, Enzyme Research Laboratories, Swansea, UK) according to the manufacturer's protocol. Baseline values of TAT were measured in EDTA plasma from blood samples taken prior to incubation with beads. Sampled beads from the different time points of incubation were washed 3x with PBS containing $MgCl_2$ and $CaCl_2$ and then fixed for 20 min in parapicric acid solution. Following washing, the beads were stained with DAPI. The coverage of beads with endothelial cells was determined by counting the number of cell nuclei on 20 randomly selected beads, on a defined rounded bead surface using fluorescence microscopy (DMI 4000B; Leica, Wetzlar, Germany). Baseline values were determined by counting cell nuclei before incubation with whole blood. Significance test was performed using One-Way ANOVA with Bonferroni correlation.


A CD93 polyadenylation site
BRNF
ERNF
CART
ABCB
HOXF
LHXF
HOME
pig
dolphin
cattle
horse
dog
rhesus
macaque
rat
mouse
USE
PE
DSE
B alternative CD93 polyadenylation site
HOXF
HOME
BCDF
GATA
CCT1
HOXF
HBOX
GATA
AP1R
AP1R
CAAT
BCDF
NKX1
PIT1
HOME, HBOX, CLOX, BRNF
2x HOXF
D_XF
BRNF
HNF1
ARID, CREB
3x LI-XF
3x CART
PARF
SalI
RUSH
NEUR
MyCD
HAND
USE
PE
DSE
C -14.5kb region
AP1F
2xAP1R
EVI1
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BRNF
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DCT1
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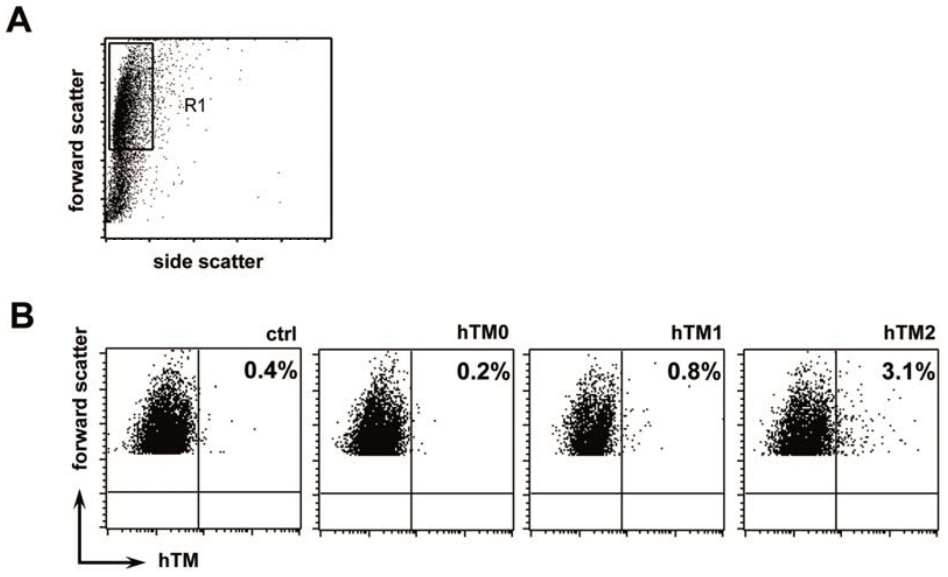
NKXH
IRF2
AP4R
NEUR
MYOD
AP4R
HAND
NEUR
MyCD
NEUR
MYOD
HAND
CAAT
175 nt alignment
E THBD core promoter region
HESF
MTF1
E2FF
SP1F
EGRF
2xNRF1
KLFS
NRSF
EVI1
AP1F
STAF
RBPF
3xETSF
2x6P1F
BCDF
PCBF
HIFF
E2FF
STAT
2xHEAT
IRFF
EGRF
OZF1
NKXH
ABDF
TALE
XCPE
MTE
2xVTBP
transcription

Figure S1: Conserved regions in the multi-species alignment of hTM regulatory regions. (A) The poly-adenylation (pA) site adjacently to the *CD93* gene clearly showed conserved upstream sequence element (USE), a polyadenylation element (PE) and a downstream sequence element (DSE). Different pA signals seem to be used in the examined species as there was no conserved (C/T)A in the alignment. (B) An alternative pA signal is located 475 nt downstream of the annotated CD93 pA signal. The consensus is conserved among all species examined (ATTAAA); its potential to terminate mRNA synthesis was predicted by PolyAPred (http://www.imtech.res.in/raghava/polyapred/help.html) and is illustrated by the presence of USE and DSE elements in the vicinity. A high degree of sequence conservation was also observed downstream of this putative pA signal, resembling numerous putative transcription factor (TF) binding sites (TFB). Interestingly, an insulator element (boxed) was proposed for the pig, but not for other species. Albeit many of such potential binding sites are identified by the used algorithms (e.g. a total of 17 potential insulators in the examined pig sequence of 6900 nt, not shown), the high incidence for this particular site is remarkable, as the similarity of this element to the position weight matrix (consensus is shown above) was significantly higher (0.885) as those of the other putative binding elements (0.777±0.044). The *Sal*I site that represents the 5´-end of the used porcine promoter element is indicated by an arrow. (C) A region that was conserved in 8 species but not in the pig was identified 14.5 kb upstream of the *THBD* gene. The potential enhancer function is illustrated by the high degree of sequence conservation and the presumed binding of several TF. (D) Another potential enhancer element was found 8.5 kb upstream of the *THBD* gene and is conserved among all 9 examined species. Again, numerous TFB have been predicted, located mainly in two conserved sections that are separated by a 175 bp segment of lower similarity. (E) The core promoter of *THBD* turned out to be of relatively short length (approximately 250 bp in the alignment) and is, hence, characterized by a high density of potential TFB and promoter core elements. Again, evidence for presumed unique regulatory properties in distinct species

was found as the dog lacks remarkable parts of promoter region that are highly conserved among the other species. Classical core promoter elements such as the X-core promoter element (XPCE), motif ten element (MTE) and vertebrate TATA binding protein factor (VTBP) were found to be conserved among all species examined.



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Exp.	ctrl	hTM0	hTM1	hTM2
#1	0.4	0.6	0.9	1.8
#2	0.2	0.2	0.3	1.1
#3	0.4	0.2	0.8	3.1

Figure S2: Evaluation of hTM constructs by flow cytometry. (A) Correlated analysis of forward/side scatter properties of PEDSV.15 cells. Viable cells (gate R1) were identified on the basis of scatter characteristics. (B) hTM expression in gated in PEDSV.15 cells. The cells were stained with an anti-hTM specific antibody (6980-100) 16h after transfection with 2µg of the respective vectors or without DNA. (C) Three independent experiments confirmed that only hTM2 resulted in transgene expression that was considerably above the control (ctrl) values.

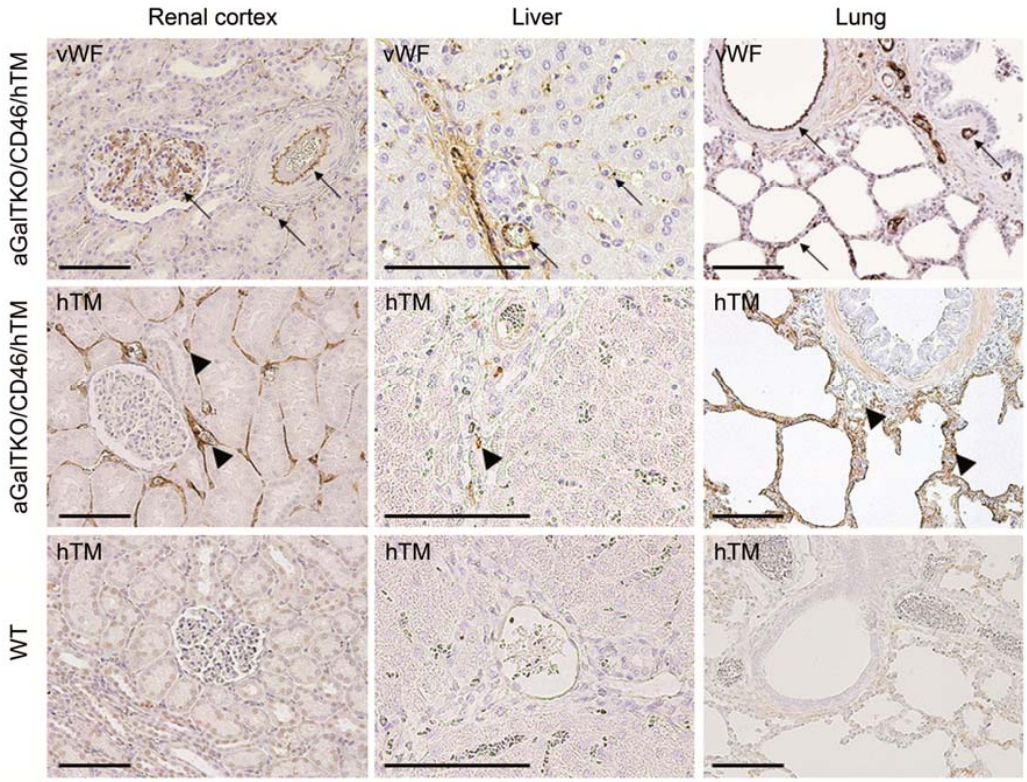

Figure S3: Immunohistochemical detection of the endothelial-specific *von-Willebrand-Factor* (vWF) and hTM in kidney (left column), liver (middle column), and lung (right column) tissue of an aGalTKO/CD46/hTM pig and a wild-type control pig (WT). In the renal cortex, the vascular endothelia of the glomerula, intubular capillaries and larger caliber renal vessels are marked by vWF expression (brown color, arrows) whereas hTM-immunoreactivity was restricted to non-glomerular endothelia (brown color, arrowheads). In the liver, vWF is expressed in both, vascular and sinusoidal endothelial cells (brown color, arrows), whereas hTM expression was only detected on the endothelia of larger blood vessels, but absent in sinusoids. In the lung, vWF is expressed in endothelial cells of pulmonary vessels and alveolar capillaries (brown color, arrows), while hTM displayed diffuse immunoreactivity on the endothelia. Tissue sections from the respective organs of control pig (WT) showed that there was no un-specific staining of the hTM antibody. Paraffin sections. Bars = 100 µm.

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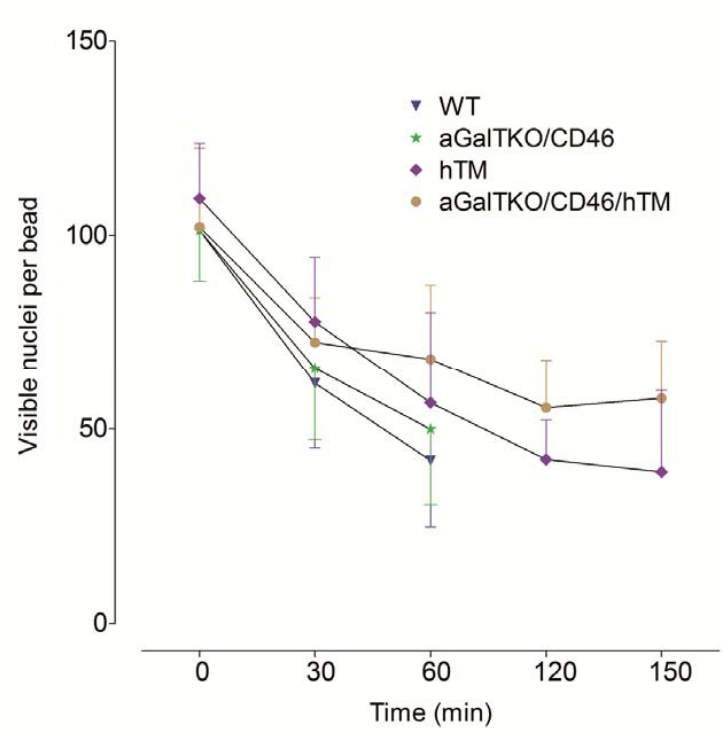

Figure S4: Loss of endothelial cells during coagulation assay. Samples from whole blood coagulation assays were taken at regular time points and the number of endothelial cells on silica beads was determined by counting cell nuclei after DAPI staining. Mean values ± standard deviation are depicted. Significance test was performed using One-Way ANOVA with Bonferroni correlation.

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General Conclusions

Pig-to-human xenotransplantation has immense potential to solve the critical need for organs, tissues and cells for clinical transplantation. However, this requires understanding and overcoming the immunological and other barriers that are associated with xenotransplantation, for which advances in genetic technologies are necessary. In recent years, considerable advancement has been achieved in overcoming the major barriers.

During my PhD, I was involved in studying various immunological responses and genetic manipulations that enabled exploring cross-species incompatibilities with the aim of reducing xenotransplantation-induced graft damage. Complement activation plays a pivotal role in xenograft rejection. It can be activated predominantly via the classical pathway and in some models via the alternative pathway. However, the degree of involvement of the lectin pathway of complement in xenotransplantation had not been studied so far. Our results (Paper I) demonstrate activation and a pathologic role of the lectin pathway of complement in xenotransplantation. Co-localized deposition of IgM with MBL and MASP2 indicates that activation of the lectin pathway of complement may not be antibody independent.

Ex vivo xenoperfusion of hCD46/HLA-E double transgenic pig limbs with heparinized, whole human blood (Paper II) showed positive effects of the combined overexpression of these transgenes. These effects include reduced xenoperfusion induced complement deposition and endothelial activation and protected the xenograft against tissue damage and apoptosis. In addition, as no hyperacute rejection was noted, the model may thus represent a useful tool to study delayed immunological responses during pig-to-human xenotransplantation in the absence of hyperacute rejection.

Porcine asialoglycoprotein receptor 1 (ASGR1) mediates human platelet phagocytosis (Paper III) during ex vivo xenoperfusion of wild-type and hCD46/HLA-E double transgenic pig limbs with human blood. While it was previously believed that expression of ASGR1 is liver-specific, the results obtained in this work indicate that porcine arterial/aortic endothelial cells (PAEC) also express ASGR1. The results of in vitro experiments demonstrate that human platelets, when incubated with PAEC, are captured by the ASGR1 and subsequently processed in to phagocytosis. It can therefore be assumed that ASGR1 may represent a novel therapeutic target to overcome xenotransplantation-induced thrombocytopenia.

In another study (Paper IV), a bead-based Bio-Plex assay was developed to simultaneously measure 11-porcine inflammation markers (multiplex) using xMAP technology. Multiplex assays for testing samples and probes from the most common research model animals — mice and rats — are available. While pig is a common large animal used in biomedical research, comprehensive commercial assays are often lacking. This study describes the establishment and validation of a 6- and 11-plex assay for detection of porcine cytokines, chemokines, and growth factors, as well as injury and complement activation markers. The importance of extensive evaluation of dynamic range, sensitivity, cross-reactivity, intra- and inter-assay variance, spike recovery and correlation between multiplex and commercially available assays and the respective singleplex is highlighted. Thus, this assay represents a new tool for analyzing samples generated from experiments with pigs.

The current understanding of immunology and the advancements in genetic manipulation have made xenotransplantation in clinical settings more realistic. Nonetheless, there are other formidable barriers that still need to be overcome. The immune responses are complicated by factors that appear to be significantly greater in xenotransplantation than allotransplantation. These responses include coagulation dysregulation due to molecular incompatibilities between the coagulation-anticoagulation systems of humans and pigs, which contribute to the development of thrombotic microangiopathy in the graft and/or a consumptive coagulopathy in the recipient. In collaboration with Munich, pigs with new genetic modifications, including expression of hTM, are just becoming available for testing strategies that can be applied in order to overcome coagulation dysregulation in vitro using microcarrier model (Paper V). In this study, wild-type and hTM transgenic PAEC were cultured on micro carries and incubated with freshly withdrawn non-anticoagulated human blood to study the effects of hTM on blood coagulation (clotting time). The findings demonstrate for the first time that the transgenic hTM expression on PAEC resulted in three-to-four fold increase of clotting time, as well as reduced thrombin generation, compared to wild-type PAEC. This serves as evidence that (multi)-genetically modified pigs expressing hTM has potential to overcome the incompatibilities of the coagulation system between pig and primate.

The increasingly rapid development of genetically engineered pigs will bring clinical xenotransplantation closer to reality. However, it is hard to estimate the exact number of genetic modifications that may be required in the organ-source pig for clinical xenotransplantation.

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Curriculum Vitae

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2009 – 2013	PhD in immunology, University of Bern
2008 – 2009	Masters in Biomedicine, University of Skövde
2002 – 2004	Masters in Biotechnology, Bangalore University
1999 – 2002	BSc Biotechnology, Kakatiya University

Awards

IXA-TTS 2013 Young Investigator Travel Grant to attend the International Xenotransplantation Association 2013 Congress, Osaka, Japan

Travel Award, 13th European Meeting on Complement in Human Disease 2011, Leiden, The Netherland

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List of publications, abstracts and presentations

Publications in peer-reviewed journals

Anjan K. Bongoni, David Kiermeir, Hansjörg Jenni, Annegret Wuensch, Andrea Bähr, David Ayares, Jörg Seebach, Eckhard Wolf, Nikolai Klymiuk, Mihai A. Constantinescu, Esther Vögelin, Robert Rieben: Activation of the lectin pathway of complement in a pig-to-human xenotransplantation model. ***Transplantation. 2013 Nov 15;96(9):791-9***

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Anjan K. Bongoni, David Kiermeir, Thusitha Gajanayake, Hansjörg Jenni, Andrea Bähr, Annegret Wünsch, David Ayares, Jörg D. Seebach, Eckhard Wolf, Nikolai Klymiuk, Esther Vögelin, Mihai A. Constantinescu, Robert Rieben: ASGR1 expressed by porcine vascular endothelial cells mediates human platelet phagocytosis in pig-to-human xenotransplantation model. *Manuscript In preparation*

Anjan K. Bongoni, David Kiermeir, Hansjörg Jenni, Andrea Bähr, David Ayares, Jörg D. Seebach, Nikolai Klymiuk, Esther Vögelin, Mihai A. Constantinescu, Robert Rieben: Coagulation and fibrinolytic activity during ex vivo xenoperfusion of wild-type and (HLA-E/hCD46)-double transgenic porcine forelimbs with whole, anticoagulated human blood. *Manuscript In preparation*

Thusitha Gajanayake, Radu Olariu, Franck Leclère, Praveen Vemula, Zijiang Yang, **Anjan K Bongoni**, Yara Banz, Mihai A Constantinescu, Robert Rieben, Esther Vögelin: A Single, Local Injection of Tacrolimus Encapsulated Hydrogel Leads to Long-Term Vascularized Composite Allograft Survival. *Manuscript in preparation*

Thusitha Gajanayake, **Anjan K Bongoni**, Radu Olariu, Praveen Vemula, Mihai A Constantinescu, Robert Rieben, Esther Vögelin, Franck M Leclère: Analysis of plasma and intragraft cytokine profiles in a rat model of vascularized composite allotransplantation. *Manuscript In preparation*

Benedikt K. Kislinger, Hendrik Tevaearai, Hans-Jörg Jenni, Erich Gygax, **Anjan K. Bongoni**, Jan A. Plock, Thierry P. Carrel, Robert Rieben, Mario Stalder: Improved Myocardial Protection during Minimized Extracorporeal Circulation (MECC) using Crystalloid Single-shot Cardioplegia. *Manuscript In preparation*

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Ex vivo xenoperfusion of CD46&HLA-E doubling transgenic pig limbs: Effect of transgenic expression of human CD46. 13th European Meeting on Complement in human diseases in Leiden, The Netherland, August 2011.

ASGR1 expressed by porcine aortic endothelial cells mediates human platelet phagocytosis in *in vitro* as well as during *ex vivo* pig-to-human porcine forelimb xenoperfusion ***presented*** at the 12th International Xenotransplantation Association, *Nov 10-13, 2013, Osaka, Japan*

Activation of the lectin pathway of complement in pig-to-human xenotransplantation models ***presented*** at the 12th International Xenotransplantation Association, *Nov 10-13, 2013, Osaka, Japan*

Ex vivo xenoperfusion of (hCD46 & HLA-E)-double transgenic porcine forelimbs with whole, heparinized human blood: Study of early immunological responses ***presented*** at the 12th International Xenotransplantation Association, *Nov 10-13, 2013, Osaka, Japan*

Poster presentations at International conferences

Activation of the lectin pathway of complement in pig to human xenotransplantation: Role of natural IgM. The XXIV International Complement Workshop 2012, Chania, Greece

Effect of transgenic hCD46 expression during ex vivo xenoperfusion of hCD46 / HLA-E double transgenic pig limbs with human blood at the 24th The Transplantation Society in Berlin, Germany, July 15-19, 2012

IgM-Dependent Activation of the Lectin Pathway of Complement in Xenotransplantation at the 15th Berlin Symposium Xenotransplantation in Berlin, July 2012

- Study of early immunological responses during ex vivo xenoperfusion of (hCD46 & HLA-E)-double transgenic porcine forelimbs with whole, heparinized human blood at the 14th European Meeting on Complement in human diseases 2013 in Jena, Germany

IgM-mediated activation of the lectin pathway of complement in pig-to-human xenotransplantation models at the 14th European Meeting on Complement in human diseases 2013 in Jena, Germany

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Declaration of Originality

Last Name, First Name: **Anjan Kumar BONGONI**
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I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.

All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.
I am aware that in case of non-compliance, the Senate is entitled to withdraw the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 69, of 7 June 2011.

Place, date

Bern, 15.10.2013

Signature