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Assessment of the protective role of human CD46 on allogeneic complement activation in vitro

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Faculty of Science, University of Bern

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Fabian Luther
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Thesis Supervisors
Prof. Dr. R. Rieben
Dr. phil. nat. R. Sfriso
Dr. phil. nat. N. Sorvillo

1. Abstract

Organ transplantation is unarguably the first and best choice for a wide range of end-stage organ diseases. The huge lack of organ donors and available organs for transplantation calls for alternative solutions to overcome this discrepancy. Xenotransplantation is the process of transplanting an organ or cells from a different species and could be a potential solution for the lack of organs. Pigs are considered as suitable organ donors for humans despite their genetical distance to us. They are similar in organ size and physiology, simple and quick to breed. Nevertheless, introducing pigs as potential organ donors brought up new immunological barriers such as a process called hyperacute rejection that leads to an early loss of graft. Antibodies and complement are key players in early graft failure in xenotransplantation. Similarly, in the setting of ABO blood group incompatible allotransplantation, preformed alloreactive antibodies cause complement activation which subsequently leads to graft failure. CD46 is a membrane bound complement inhibitor and acts as a cofactor to cleave C3b and C4b. Donor pigs that were genetically modified and expressed human CD46 (hCD46) as well as HLA-E have been produced to impede complement activation in the xenotransplantation setting.

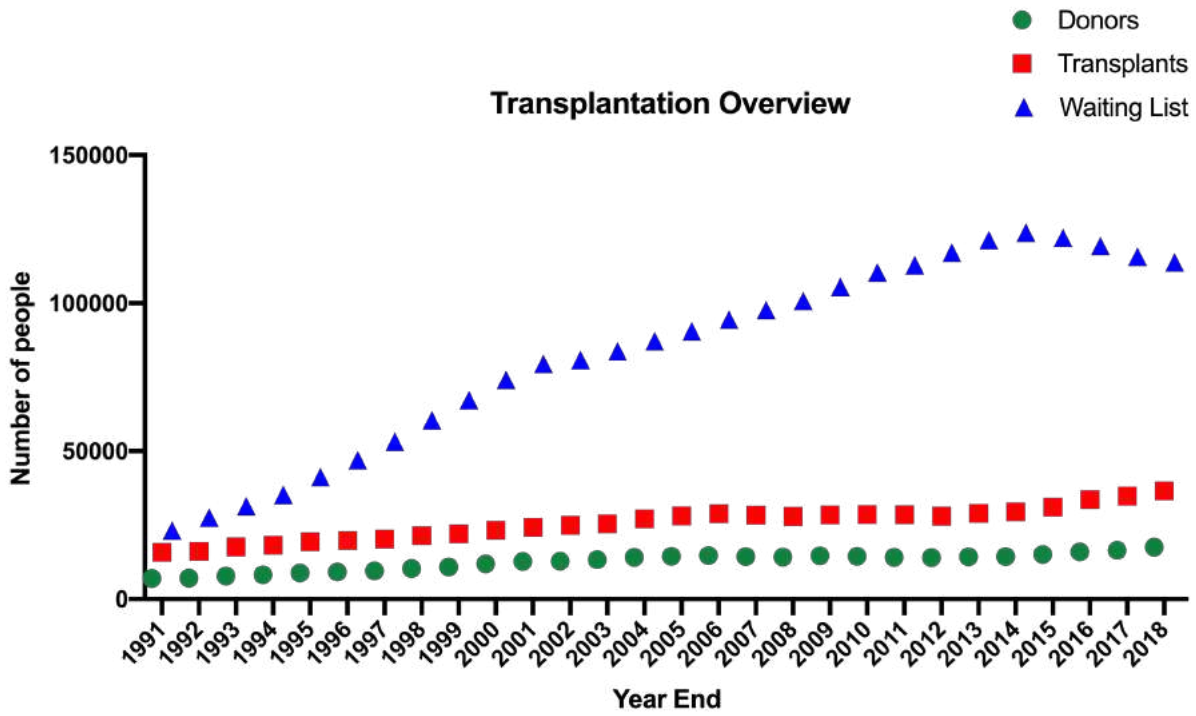
In this *in vitro* study, a microchannel lined with a monolayer of either wild type or transgenic porcine aortic endothelial cells was cultured and exposed to a physiological pulsatile flow. The channels were perfused with different perfusates (normal human serum, allogeneic pig serum, heat-inactivated allogeneic pig serum, human anti-gal antibodies or pure DMEM cell culture medium) to mimic the situation of endothelial cell activation due to pre-formed antibodies and complement. The role of hCD46 in the modulation of allogeneic complement activation was measured by immunofluorescence staining for C3b/c and C4b/c. Our novel 3D *in vitro* microfluidic system is able to overcome the limitations of standard flatbed culture *in vitro* systems by using higher amounts of allogeneic serum, thus resembling the *in vivo* situation where an unlimited amount of complement proteins is circulating.

We were able to observe allogeneic complement deposition in an *in vitro* system after perfusing the cells with human anti-gal antibodies and allogeneic pig serum. We observed a tremendous difference in the ability of wild type pig cells and pig cells transgenic for human CD46 and HLA-E to regulate the complement deposition after the previously mentioned perfusion. The transgenic cells were noticeably better at regulating the complement deposition, thus brining us to the conclusion that human CD46 participates in the cleavage of porcine complement proteins. This study shows the beneficial effect of the overexpression of hCD46 on complement deposition in an allogeneic pig setting.

2. Introduction

2.1. Organ Shortage

Organ transplantation is unarguably the first choice therapy for a wide range of end-stage organ diseases due to increased survival and quality of life compared to patients without transplantation[1–4]. With further improvements and better outcomes of transplantations the number of people waiting on the transplantation list has heavily increased[3,4]. This increase led to the major current problem of organ transplantation: the enormous gap between the number of available transplants that are performed every year and the patients waiting for an organ on the transplant waiting list[1].
As of July 2019, more than 113'000 people are on the national transplant waiting list in the U.S and only 36'529 transplants were performed in 2018. 20 people die each day waiting for a transplant, while another person is added to the waiting list every 10 minutes. (www.organdonor.gov)



Despite the effort to enlarge the organ donor pool through including more live donations, a national effort to expand deceased donor donations, split organ donations, paired donor exchange, national sharing models and a greater usage of expanded criteria donors (ECD), the donor pool mainly relies on donations after brain death (DBD)[2,5].
It is crucial to mention, that most fatalities are never even considered as potential sources for organ donation because of the severe ischemic injuries that happen following cardiac arrest. Any short duration of post mortem ischemia can give rise to the occurrence of delayed graft function (DGF) and a risk of primary nonfunction[5]. It is safe to say, that we are in need of alternative solutions to overcome the ever-widening gap between the need and supply of organs for transplantation.

It is worth mentioning, that there are also other approaches that could potentially solve the lack of donor organs. Artificial organs and mechanical devices are two options

available for very specific types of organ failures but the chances of them replacing organ transplantation in the long-term is unlikely. Stem-cell derived organs (i.e. organoids) and the stem cell field as a whole are crucial contesters and could in the future be an option to treat end stage organ failures.

2.2. Xenotransplantation

2.2.1. "General concept"

Xenotransplantation (XTx), originating from the Greek word 'xeno' meaning 'foreign', is defined as the transplantation of an organ across the species barriers, for example pig to primate[6]. The World Health Organization (WHO) defines clinical xenotransplantation as any procedure, which involves the transplantation, implantation or infusion into a human recipient of either live cells, tissues, or organs from a non-human animal source; human body fluids, cells, tissues or organs that have had ex vivo contact with live non-human animal cells, tissues or organs[7]. Modern gene editing technologies allow for the genetic modification of the donor, thus overcoming potential compatibility problems and allowing to personalize an organ. An overview of the gene editing technologies will be presented later. This modification results in an improved molecular compatibility with the recipient[8]. Xenotransplantation has numerous advantages: It could potentially provide an unlimited supply of cells and organs for clinical transplantations[6] and it allows for a better planning of a pre-treatment to augment the acceptance of the graft due to the organs being available electively.

First attempts of 'xenotransplantation' can be dated back to the 17th century, where blood transfusions across species borders were performed[9]. An important person to mention is Carrel. He pioneered the surgical work of blood vessel anastomosis allowing countless attempts at nonhuman primate (NHP) organ transplantations in the 20th century[8]. Back in 1963-64, a patient who received a pair of chimpanzee kidneys was able to go back to work for almost 9 months with the support of these kidneys and the first heart transplantation was performed in 1964 with a chimpanzee as 'donor'[8]. Recently, a research group in Munich was able to transplant pig hearts into baboons and the animals survived for up to 6 months[10].

2.2.2. Safety in xenotransplantation

The most reasonable animal of choice for xenotransplantation would be 'non-human primates' (NHP). They are phylogenetically closer than any other species to humans[11] and would also be the donor of choice from an immunological point of view [6]. NHP would be available in large numbers in the wild but bring numerous ethical and practical problems for xenotransplantation. The first issue with NHP is, that they have a potentially high risk of cross-species transmission of infections to humans. One of the main known viral transmissions was the infection of humans with HIV originating from chimpanzees in the Democratic Republic of Congo back around 1920. The time for breeding and the expenses related to breeding these animals as well as the lack of experience in genetically modifying them as well as the organ size discrepancy makes NHP less favourable for xenotransplantation[6,12].
These problematics pushed the focus for organ donor animals to the pigs as they are similar in organ size and physiology to humans and are very easy and rapid to breed. A huge concern for the use of pigs as organ donors is the risk of transmitting diseases or infections to the human recipient and possibly all those who come into contact with the patient[13–15].This transmission is termed as 'zoonosis'. This is directly linked to the characteristic of a xenograft which can not be disinfected because of the presence of living

cells[7]. Porcine endogenous retroviruses (PERVs) are the most important to bring up[12]. Nowadays, specific pathogen-free and biosecure breeding conditions together with regular monitoring of the animals make these risks neglectable[8,12,16]. Exogenous contamination of the donor pigs with viruses such as cytomegalovirus, gamma-lymphotropic herpes virus and hepatitis E virus can be bypassed by using distinct breeding techniques in a clean environment, however PERVs cannot be eliminated by breeding itself[12]. Pigs from the above mentioned breeding standards should be greatly 'cleaner' to the average deceased human donor who almost always carries agents such as Epstein-Barr virus and cytomegalovirus that can imperil the recipient's well being[8].

Shifting to pigs as organ donors for xenotransplantation resulted in the occurrence of new immunological barriers, mainly hyper acute rejection (HAR), acute vascular rejection (AVR) and delayed xenograft rejection (DXR), all caused by humoral and cellular human immune responses against endothelial cells of vascularized pig xenografts. The different types of rejections will be covered more in depth in a later chapter.

PERVs were previously mentioned as the most important transmittable infections but up to now, transmission of PERV to patients after being exposed to porcine tissues has never been observed[17]. Pig-to-human PERV transmission has so far only been observed *in vitro* in a human cell line that did not have the intracellular machinery which protects against retroviruses[18]. These retroviruses are ubiquitously abundant in the genome of every pig cell and will therefore inevitably be transplanted with the organ or the cell[15,19]. This problem was solved with the introduction of CRISPR/Cas9 technology (will be covered in detail later), which allowed for a genome-wide inactivation of the PERV copies in the pig cell lines and therefore the chance of infection was neglectable[20,21].

2.3. Gene editing

2.3.1. Gene editing technologies

The field of xenotransplantation was relying heavily on new developments in regard to gene editing technologies. Gene editing was a promising technique to overcome the main hurdles of xenotransplantation, namely molecular incompatibilities resulting in strong immune responses against the graft and potential loss of it. The first transgenic pigs were produced by microinjection of DNA into the pronuclei or nuclei of eggs from superovulated pigs back in the 1980s[22]. The next generation of transgenic pigs was created by using somatic cell nuclear transfer (SCNT) technologies on genetically modified or transfected cell lines or even on embryonic stem cells[23,24]. The methods of choice were egg electroactivation or intracytoplasmic injection[25]. A main limitation to the previously mentioned techniques to generate knock outs (KO) or transgenic animals is the reliable DNA integration at the target site[12]. A key event to overcome this issue was the discovery of homologous recombination. More recent developments in the field of genetic editing are the introduction of synthetic nucleases. This is an enzyme based method which leads to cleaving of the genome at a specific site followed by either homologous or nonhomologous repair[12].
Nowadays, three major synthetic nucleases are well established and available: zinc finger nucleases, transcription activator-like effector nucleases (TALEN) and more recently clustered-regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) nucleases. CRISPR/Cas 9 will be the main focus in this work.
CRISPR/Cas9 is based on a flexible immune mechanism, which is used by bacteria to protect themselves from foreign nucleic acids. Cas9, an endonuclease, checks the DNA for complementary 20-bp spacer region of its guide RNA. If the DNA sequence is

complementary to the guide RNA, complex formation is observed and Cas9 endonuclease activity provokes double-strand DNA cleavage at this specific site[12,26]. Multiple double-strand breaks can be implicated through the usage of multiple guide RNAs[27]. The target DNA must contain a so called protospacer adjacent motif, short PAM, which consists of the 3-nucleotide sequence NGG. PAM is recognized by the PAM-interacting domain of Cas9 before the cleavage. Gene inactivation or introduction of heterologous genes through nonhomologous or homologous recombination can be achieved through the breakage of the DNA by CRISPR/Cas9[12,26,28]. One major advantage of CRISPR/Cas9 is the targeting of multiple genes in a single reaction as well as generating pigs of one or multiple genetic strains in a single pregnancy[28]. The field of xenotransplantation heavily benefitted from these recent advances in regard to gene editing technologies, allowing to generate transgenic pigs with up to 7 genetic modifications[29].

2.3.2. Genetically modified pigs

Ethically, the use of pigs as an alternative source of organs for transplantation does not cause any concerns as roughly 100 million pigs are yearly slaughtered in the US as a source of meat and 600 million pigs are killed only in China as a source of heparin[1]. As previously mentioned, pigs are less desirable compared to nonhuman primate in regard to their genetic distance to humans as sources for organs and cells and as potential transmitters of infectious or viruses. Several molecular species incompatibilities were hindering pig-to-human xenotransplantation models to have better outcomes which is why there was a high demand for genetically modified animals to minimize xenorejection[12]. The dangerous PERVs, which could potentially infect human cells, were genome-wide completely inactivated with the help of genome editing[20].

The presence of Gal on the porcine vascular endothelium resulted in hyperacute rejection in Gal-negative recipients. By knocking out the galactosyl transferase (GalT) gene, GalT-KO pigs were generated in the early 2000s with the method of homologous recombination and somatic cell nuclear transfer and an important improvement in regards to better outcome of xenotransplantation was achieved[30–32].

Formation of the membrane attack complex (MAC) after complement activation is a part of the effector humoral response ultimately leading to organ dysfunction after xenotransplantation. This led to the introduction of human complement regulatory proteins, such as CD55 (critical for C3 activation), CD59 (MAC-inhibitory protein) and CD46 (membrane cofactor protein)[33,34]. CD46 and CD59 constructs were inserted by homologous recombination and pronuclear microinjection into porcine fertilized oocytes of CD55 transgenic background, resulting in a triple knock in transgenic pig with CD46, CD55 and CD59 working as complement regulators[33]. We will go more in depth and cover the function of them in the later chapter touching on the mechanisms of complement activation and regulation.

Overall, the introduction of GalT-KO pigs and human complement regulators have mostly solved the hurdles of hyperacute rejection in xenotransplantation[35,36]. The quick development of efficient and innovative gene editing technologies facilitate the generation of multiple transgenic pigs, which ultimately brings xenotransplantation one step closer to clinical application[12]. Having these novel gene editing technologies helps to overcome new hurdles, such as newly discovered xeno-reactive antigens that may lead to generation of new KO pigs[37].

2.4. Hurdles of transplantation

2.4.1. Hyperacute rejection

The transplantation of a pig organ into a human or non-human primate there is followed by an immediate immune response, defined as hyperacute rejection[16]. This immune reaction is directed against a terminal carbohydrate epitope Galα1-3Galβ1-4GlcNAc, short α1,3-Gal. Pigs have an enzyme called GalT (α1,3-galactosyltransferase), which covers cell surface glycoproteins and glycolipids with the blood group-like disaccharide alphaGal, short for galactose-α1,3-galactose. Old World primates and humans do not have a functional GalT gene and therefore generate a high titer of natural antibodies (nABs) specific to the alphaGal epitope[16,38,39]. These natural antibodies are produced in neonatal life and are a result of exposure to Gal-expressing viruses and microorganisms that colonize the gastrointestinal tract[38,40]. Lower species, including New World primates express the carbohydrate determinant resulting in immune tolerance to self-antigens and lack nABs against the α-gal epitope[39].

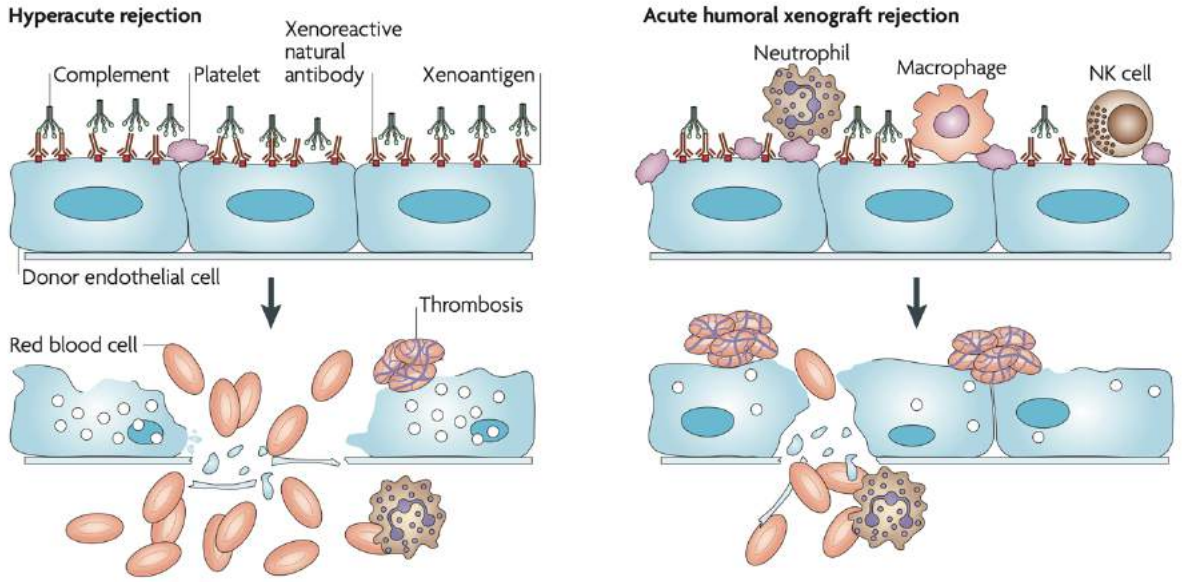


Figure 1: Overview of the hyperacute rejection and the acute humoral xenograft rejection[41].

Natural antibodies are present in the serum in absence of any antigenic stimulation. The majority of them are found in sera are of the complement-fixing IgM isotype[38,39]. Natural antibodies are prone to react with a wide variety of endogenous and exogenous antigens, including the xeno-antigens that are expressed by tissues between unrelated species[39]. It was previously shown, that the most important antibodies, namely IgG and IgM, bind to the terminal carbohydrate epitope α1,3-Gal epitope, which is ubiquitously expressed on pig vascular endothelium[16]. Preformed human natural antibodies bind to xenogeneic endothelial antigens, such as alphaGal, resulting in graft failire within minutes to hours after the transplantation[7,16]. The complement system is activated by the antibody-antigen complex formation and leads to immunoglobulin and complement deposition in the vessel wall. This deposition causes downstream formation of membrane attack complex, resulting in endothelial injury and fibrin-platelet rich thrombi[12,16,40]. Intravascular coagulation, platelet aggregation and thrombosis occur within the donor organ as well as a rapid accumulation of neutrophils within the capillaries. An accumulation of thrombi in many small vessels causes ischemia with widespread necrosis and rapid loss of the

graft[12,16,39,40]. All these events happen mainly, because of the interspecies molecular incompatibilities of membrane-bound coagulation-regulatory proteins between the pig and the human or non-human primates, namely the tissue factor pathway inhibitor, thrombomodulin and interactions with xenogeneic von Willebrand factor. A more in-depth explanation of the incompatibilities will be covered in a later chapter.

2.4.2. Acute vascular rejection

Acute vascular rejection, also known as 'Acute humoral xenograft rejection (AXHR)', is the second hurdle to overcome if the xenograft is not lost due to hyper acute rejection[16]. Acute vascular rejection is mediated through humoral and cellular immune mechanisms together with inflammation and an activated endothelium. It is often categorized as a delayed form of antibody-mediated rejection and usually happens within hours or days after transplantation[11,12]. AXHR can be induced by low level of α1,3Gal-specific natural antibodies and develop even in animals, that received an organ from an α1,3 GalT-deficient pig or in concordant xenotransplantation settings, where α1,3 Gal antibodies are missing[41]. This suggests, that xenoreactive antibodies trigger the acute humoral xenograft rejection.
A key player of AXHR are $CD4^+$ T cells – they produce interferon gamma which leads to the activation of macrophages and NK cells[42]. $CD4^+$ T cells can also cause direct cytotoxic effects mediated through the Fas-Fas ligand lytic pathway[43].
During AXHR, antibody and complement deposition as wells as graft infiltration by innate immune cells is observable[12,40]. Invaded neutrophils release inflammatory cytokines and oxygen reactive species[12]. Endothelial cell activation and injury cause thrombotic microangiopathy and disseminated intravascular coagulopathy (DIC) by impeding the anti-coagulant properties of the endothelium[44]. The main histological features of AHXR after transplantation are IgM, IgG, C4d and C5b-9 deposition, as well as the loss of capillary integrity, endothelial cell death and an extensive fibrin deposition[16].

2.4.3. T-cell mediated rejection

T-cell mediated rejection is mainly based on the differences between the major histocompatibility complex (MHC) molecules between different species or of allogeneic donor-recipient pairs[45]. Allotransplantation highlighted two pathways for antigen presentation: the direct and indirect pathway.

The direct pathway describes the principle by which recipient T cells recognize factors on intact donor major histocompatibility complex molecule-peptide complexes, which are being presented on the surface of transplanted cells[46]. There is no need for antigen processing by the recipient antigen presenting cells (APC). There is a uniquely high frequency of T cell reactivity against the alloantigens compared with normal antigens.
The direct pathway can be shown best *in vitro* by the mixed lymphocyte reaction (MLR), which allows only direct allopresentation[47]. In short, T cells from one individual are co-cultured with lymphocytes from a second individual. If the cells of one individual recognize the MHC molecules from the other cells as foreign, they proliferate and divide. The proliferation is measured usually with a ^3H-labeled thymidine uptake assay. There are two variants of this assay: the 'two-way' where both cells are free to proliferate and the 'one-way', where the cells of one individual are irradiated and are therefore unable to proliferate. *In vivo*, the direct pathway can be demonstrated through transplanted $Rag^{-/-}$ MHC class $II^{-/-}$ that have been reconstituted with syngeneic $CD4^+$ T cells[47]. This specific genotype of mice lacks $CD8^+$ T cells and the option to present antigen via the indirect

pathway but are able to reject cardiac allografts, indicating that CD4 cells from the direct pathway are sufficient to mediate graft rejection[47].
Donor dendritic cells are the main trigger of the recipient's immune response via the direct pathway. This has been observed, by depleting donor dendritic cells with an intermediate parking strategy, leading to loss of immunogenicity[46]. This immunogenicity can only be restored through adding dendritic cells of the donor strain. Transplantation leads to proinflammatory signalling, which causes the donor dendritic cells to traffic to secondary lymphoid tissues of the recipient and initiate a direct response at this site.

The indirect pathway describes the recognition of processed peptides of allogeneic histocompatibility antigens that are presented by self-MHC in a self-restricted manner and is related to the recognition of nominal antigens. Indirect alloantigen presentation leads to an alloresponse which is dominated by $CD4^+$ T cells. T cells help B cells to class switch and differentiate into antibody secreting plasma cells. $CD4^+$ T cells recognize peptides derived from antigens which have been internalized by B cell surface immunoglobulins. The presence of class-switched alloantibodies is an indicator of help that has been provided by indirect pathway T cells[46]. Antigen processing is necessary in the indirect pathway and correlates with the naturally slower response compared to the direct pathway even though there is an amplification through epitope spreading[46]. If we compare the frequency of T cells in the normal repertoire of direct vs. indirect pathway presentation the results suggest that the direct response leads the early posttransplant periods whereas the indirect pathway plays a role in the long term alloantigen presentation when the donor APCs are exhausted[46]. It is important to mention that the indirect pathway alone in absence of the direct response is sufficient to cause a rapid acute graft rejection.

A novel pathway of antigen presentation has been described, namely the 'semi-direct' pathway[46,48]. Recipient dendritic cells take up intact MHC antigen complexes from the donor dendritic and endothelial cells and present these via direct antigen presentation to alloreactive T cells[45]. The same dendritic cells are capable of presenting simultaneously antigen indirectly, resulting of stimulation of $CD4^+$ and $CD8^+$ T cells[45,46]. The cross-talk between $CD4^+$ and $CD8^+$ T cells during the beginning of an immune response is based on a 'linked' model, where both $CD4^+$ and $CD8^+$ T Cells are activated by the same antigen presenting cells. Related to transplantation, observation of cross-talk between the direct and indirect pathway, in contrast, represents a paradox to this model. It seems like the direct pathway $CD8^+$ T cells and the indirect pathway $CD4^+$ T cells are activated through different (donor vs. recipient) antigen presenting cells which would need an 'unlinked' model[46]. It is important to remember, that immunological cells have the capacity to switch surface molecules. Especially dendritic cells are able to get intact MHC-peptide complexes from other dendritic cells or endothelial cells and present these newly acquired MHC-peptide complexes to alloreactive T cells. This resolves the 'unlinked' model problem and is the basis of the semi-direct pathway of allorecognition. The semi-direct pathway proposes, that recipient APCs acquire intact allogeneic MHC-peptide complexes through MHC transfer, which would also stimulate $CD8^+$ T cells through the direct pathway. On the other hand, recipient APCs present peptides of allogeneic histocompatibility antigens from phagocytosed necrotic cell material. These peptides would be internalized, processed and therefore presentable to $CD4^+$ T cells. This links both allospecific $CD4^+$ and $CD8^+$ cell activation by the same antigen presenting cells and illustrates the three-cell model[46].

2.5. Complement System

2.5.1. Overview

The complement system was first described in the 1890s as a system which helped or 'complemented' the killing of bacteria with heat stable antibodies in normal serum[49–53]. The system involves more than 30 proteins that are either present as membrane-associated proteins or soluble proteins in the blood[49,54]. It was later shown, that this protein network is a crucial component of the innate immune system, which is essentially the first line of defense against microbial invaders[51,52]. The complement system is enhancing the humoral immune response, which depends on the production and binding of antibodies to 'foreign' substances[51,55,56]. This ultimately facilitates the elimination by the immune system. Complement activation plays also a key role in adaptive immunity, involving T and B cells which help to eliminate pathogens and maintain the immunological memory to prevent reinvasion by pathogens[49]. Activation of the complement system occurs due to changes in the microenvironment, that trigger an appropriate surface pattern, either pathogen or damage associated, and then subsequently bind complement factors[50]. The complement system has at least three pathways which are interacting together in a complex manner. The pathways are named after their respective surface recognition patterns: the classical pathway, which is initiated by antigen-antibody complexes; the lectin pathway, which is triggered by mannose containing glycoproteins or carbohydrates on microbial surfaces; and the alternative pathway, starting through

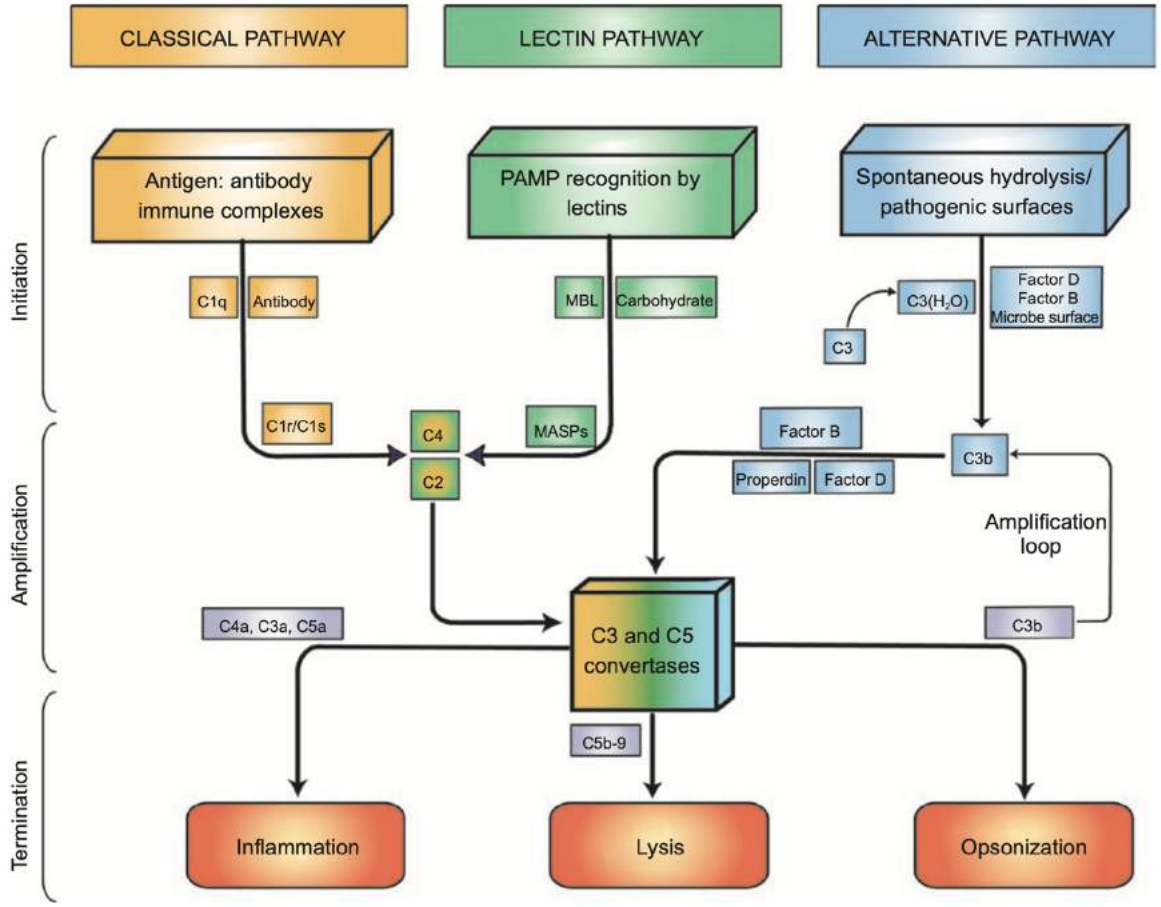


Figure 2: Schematic illustration of the three different complement activation pathways[56].

spontaneous activation of complement component 'C3' by hydrolysis or by binding of C3b molecules to properdin[49,50,54]. The three pathways converge at C3, the most abundant complement protein found in blood, ultimately resulting in the formation of the activation products C3a, C3b, C5a and the membrane attack complex (C5b-9). C3a and C5a are anaphylatoxins that cause a vast quantity of physiological responses that range from apoptosis to chemoattraction. Inappropriate activation of the complement system and deficiencies in complement are the main cause of the pathophysiology of many diseases such as systemic lupus erythematosus and asthma[57]. We will go further into detail for each specific pathway.

2.5.2. The classical pathway

The classical pathway is dependent on IgG or IgM binding to pathogens or to other foreign and non-self antigens, causing the formation of immune complexes. The C1 complex, which is a multimeric complexes that consists of C1q, C1r and C1s molecules, binds the Fc portion of the IgG or IgM immune complex[49,58]. C1q is hexameric and its affinity to IgG increases greatly when IgG is aggregated or when the Fc regions of IgG are packed in a hexamer conformation[59]. The relative affinity of C1q for a single IgG is low, even though the C1q binding sites on free IgG are normally exposed[60]. Ab isotype, Ag density and conformation of the aggregated IgG are all influencing C1q binding affinity and classical pathway activation[54,59].
C1s and C1r are being activated as a result of C1q binding to exposed Fc portions of IgG or IgM. After that, C1s cleaves C4 and C2 to form the classical pathway C3 convertase, C4bC2a. C4bC2a, the C3 convertase of the classical pathway and lectin pathway and C3bBb of the alternative pathway continue to cleave C3 to release C3a and C3b. The C3b acts as an opsin that assists to further amplify the complement activation and to help with phagocytosis. Additionally, C3b complexes with the C3 convertase to build the C5 convertase C3bBbC3b and C4bC2aC3b. The C5 convertases cleave C5, which results in the formation of C5a and C5b. The membrane attack complex C5b-9 (MAC), also called terminal complement complex is then initiated by C6 and C7 binding to C5b and then C8 and several molecules of C9 binding to the C5bC6C7 complex. The membrane attack complex inserts himself through forming a pore which then leads to cell lysis.
Pentraxins (PTX) are able to recognize pathogens and eliminate them through direct binding to C1q. Pentraxins are separated into two subfamilies based on their subunit structure: The acute phase proteins SAP and CRP belong to the short PTX family; The prototype proteins PTX3 belong to the long PTX family. The liver and other tissues synthesize Pentraxins in response to an infection.

2.5.3. The lectin pathway

The lectin pathway (LP) works immunoglobulin independent. The pathway is activated through mannose binding lectin (MBL) or Ficolin, which binds to carbohydrate moieties on the surface of pathogens including bacteria, parasites, yeast and viruses[61]. The targeted structures belong to the same family and are better known as pattern-recognition receptors (PRR). PRRs are being used as tools to perform non-self recognition. They are very specific to detect highly conserved structures expressed in large groups of microorganisms called pathogen-associated molecular patterns (PAMPs). Ficolin and MBL are circulating in the serum as complexes with MBL-associated proteins (MASPs)[62]. There are four structurally related MASPs: 1,2 and 3 as well as a truncated MASP2 better known as MAP19[49]. After binding to a pathogen a conformational change is induced, which results in autoactivation of MASP2. MASP2 cleaves C4 to form C4a and C4b[63]. The latter binds to surfaces of pathogens and forces C2 to bind to ich. C2 is cleaved by MASP2

to form C2b and C2a – C4b together with the attached C2a has an enzymatic activity and builds the lectin pathways C3 convertase C4bC2a. The role of the other mentioned MASPs is not fully understood, although MASP1 can cleave C2 but not C4 and has an important role in enhancing the complement activation by the bound complexes[49].

2.5.4. The alternative pathway

The third pathway is called alternative pathway (AP) and is activated through carbohydrates, lipids and proteins that are on foreign and non-self surfaces. The AP works as an individual complement pathway and has a crucial function as an amplification loop of both the classical and lectin pathway. There is a constant hydrolysis of C3 at a very low level to form C3b. C3b binds to the target, for example bacteria. The bound C3b recruits Factor B followed by Factor D[64]. The latter cleaves the former to generate the C3 convertase C3bBb[49]. This convertase is stabilized in the presence of plasma properdin. Properdin belongs to the family of proteins and is released by activated neutrophils or can be found in macrophages and T cells. The protein stabilizes the convertase by binding to C3b and preventing the cleavage of the convertase by Factor H and I. Properdin can directly bind to necrotic and apoptotic cells and initiate complement activation[49].

2.5.5. C3 independent pathways

Complement can also be activated through so called C3 independent pathways. Proteases that have been released by neutrophils and macrophages, factors such as Kallikrein, plasmin and Factor XIIa (Hageman factor) are able to generate complement activation products[49,63]. Recent studies have shown, that Thrombin, a member of the coagulation pathway, is able to locally generate C5a in an in vivo C3 deficient mice which are unable to generate the conventional C5 convertase[65]. This study highlights the complex interconnections between the plasma cascade systems and these connections will be further discussed later.

2.5.6. Regulation of complement

The complement system is a very responsive and highly instable system that can exert severe reactions in a short amount of time and is potentially very destructive to the organism[66]. This highlights the need for a strict and rigorous regulation of this potent system. Given these impactful consequences that this system can trigger, there are several mechanisms in place to limit the complement activation both locally and time dependent[49]. One example is the phlogistic potential of C3a and C5a that gets quickly controlled by the plasma carboxypeptidase. This peptidase cleaves the C-terminal Arginine resulting in a C3a des-Arg and C5a des-Arg, both having less than 10% of their original biological potential[49,63]. Another example is the inactivation of C3b and C4b by proteolytic cleavage into the fragments iC3b, C3dg, C3c, C4c and C4d by serine protease Factor I in the presence of cofactors. The cofactors necessary for this cleavage are: membrane cofactor protein (MCP, CD46) and complement receptor 1 (CR1, CD35) which are both membrane bound and Factor H, which is bound to the host surface[67]. Complement receptor I enhances phagocytosis, which helps to clear immune complexes. Failure of clearing these immune complexes can lead to the deposition of the immune complexes and to severe tissue injury. Furthermore, C1 inhibitor (C1-INH) is able to inactivate C1r, C1s and MASP2. The complement activation is also prevented by inhibiting the assembly of C3 convertase or once it is formed, by inhibiting the activity with the help of decay acceleration factor (DAF, CD55), C4 binding protein (C4BP) and Factor H[49]. The negative regulation of MAC complexes formation is done with the help of S protein (a plasma glycoprotein synthesized by endothelial cells), vimentin (a cytoskeletal protein)

and CD59 by interfering with the assembly of MAC. We will emphasize on the function and structure of the membrane cofactor protein CD46, since we were utilizing a transgenic cell type with this gene introduced.

2.5.7. Membrane cofactor protein CD46

CD46 was first discovered and described in 1986 as one of the essential membrane-bound complement regulators[68]. It acts as a cofactor in the factor I-mediated proteolytic cleavage and inactivation of opsonins C3b and C4b[69]. This regulator is expressed on all nucleated cells as a type-I transmembrane protein, so only erythrocytes do not have CD46 expression[70]. The gene is located in the 'regulators of complement activity (RCA) gene cluster on the chromosome 1 (1q32.2). The human RCA gene cluster consists of more than 60 different genes of which 15 are related to complement[70]. There are four distinct isoforms of CD46, that arise through alternative mRNA splicing of the transcript of a single gene. The four isoforms have four N-terminal conserved complement control protein (CCP) domains, where the C3b and C4b binding sites are located[68]. This region is then followed by differently glycosylated extracellular domains 'BC' and C'. They are generated through splicing of gene regions 'A', 'B' and 'C', coding for serine, threonine and a proline-rich (STP) region[68,71]. After the STP region, there is a region of 12 amino acids with unknown function, a transmembrane anchor and one of the two distinct cytoplasmic domains CYT-1 and CYT-2. Both these cytoplasmic tails have shown to mediate distinct cellular functions. The four isoforms are named CD46 C1, BC1, C2 and BC2. The expression patterns of these four isoforms are tissue specific, while most of the cells express all four isoforms[72]. The cofactor activity of the CD46 involves proteolytically inactivation of C3b and C4b by FI, a plasma serine protease. CD46 binds to the substrate (C4b or C3b) before FI is able to cleave the two fragments.

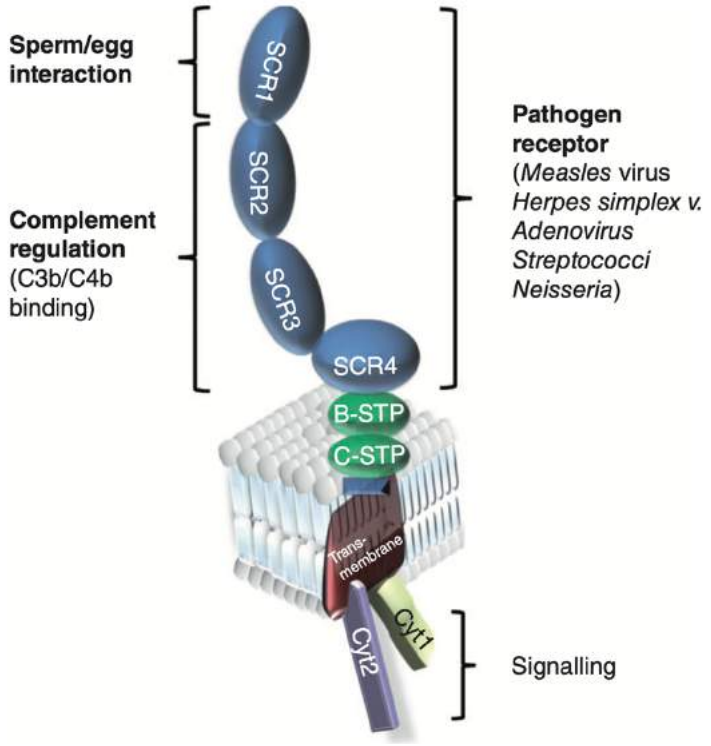


Figure 3: Structure and function of CD46[71].

The necessity of CD46 as a complement regulator is shown by the fact that CD46 deficiency or a genetic defect in CD46 results in significantly lowered surface expression and a reduction in C3b and/or C4b inactivating capacity. This disruption leads to uncontrolled complement activation and C3b deposition on the host endothelium and systemic microthrombi formation. In a clinical setting this manifests as atypical hemolytic uremic syndrome (aHUS) which is characterized by microangiopathic haemolytic anaemia, thrombocytopenia and acute renal failure[57,71].
CD46 is also involved in other biological processes, including sperm-egg fusion during fertilization, T cell regulation and infection processes due to its pathogen receptor

properties[71]. CD46 is a universally expressed receptor for vaccine strains of the measles virus, as well as a huge variety of other pathogens that together contribute heavily to the morbidity and mortality worldwide. This complement regulator is not only a passive entry site for pathogens, but also influences different cellular activities in response to pathogen or complement binding, and thus plays a major role in the host response to infection[69].

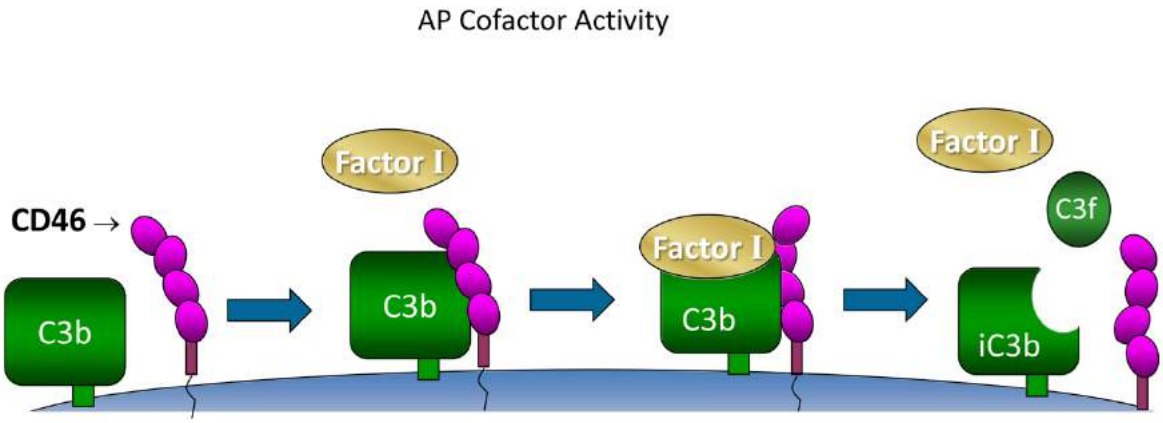


Figure 4:The cofactor activity of CD46 in the alternative pathway[70].

2.5.8. Coagulation and Complement

The coagulation system and the complement cascade are two different systems with unique pathophysiological roles. Despite all their differences, these two systems have a lot in common and are interconnected at various points (see figure 5)[50,73]. Both systems help to set up the innate defence against microbial invasion. Furthermore, the coagulation and the complement system rely on the presence of foreign or altered cellular surfaces to initiate the pathways. This necessity ensures the organization of a quick but controlled start of the cascade in terms of the spatiotemporal localization. A localization for example in close proximity of the vascular endothelium lowers the kinetic requirements for this reaction to happen because it starts a local increase in normally minimal concentrations of the respective trigger.

Both cascades reactions can be grouped into three phases: the initiation, the amplification and the propagation reaction. All these reactions in both cascades happen in a self-reinforcing manner. The organization of the coagulation and the complement pathways allow for multiple points of amplification, negative or positive regulation and interaction with other systems[50].

Natural inhibitors or cofactors that work as regulatory molecules in the systems are usually present at the same setting and physiologically impact both systems. The inhibition of both systems normally happens at two levels: inhibition of the actual enzyme activity and/or restriction of the binding capacity of a component of the cascade[50,74].

Single components of the complement or coagulation cascade interact with receptors on the cell surface and mediate downstream biological effects. This is a common characteristic of the coagulation and complement system, since this allows for cross-talk in multiple instances and could explain the connection of both systems with countless clinical inflammatory and thrombotic conditions[50,57].

In the setting of a systemic inflammation, the activation of the coagulation cascade is accompanied by an activation of the complement system. This activation results in the

generation of C3a and C5a. The C5a is able to induce tissue factor activity in human endothelial cells and could potentially be involved in the activation of the extrinsic coagulation pathway[73].

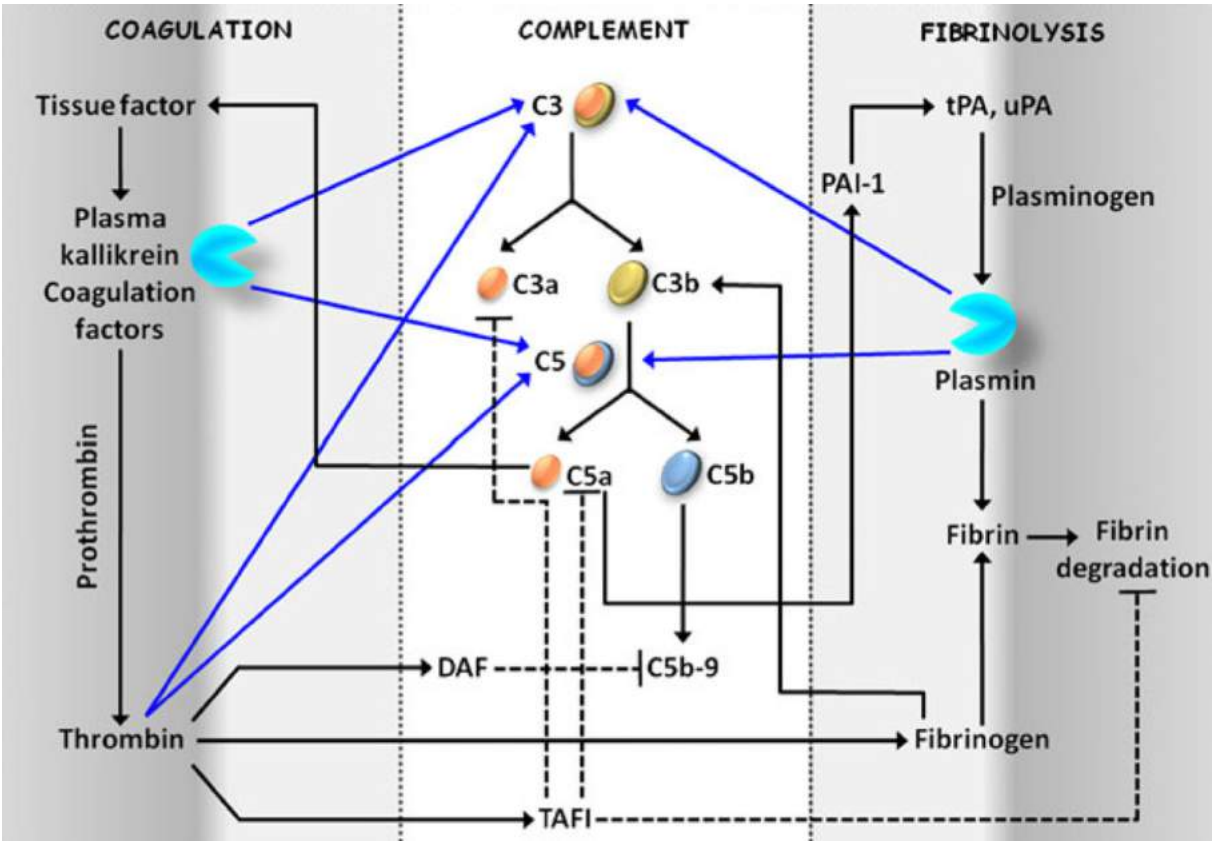


Figure 5: Overview of the interconnectivity between coagulation, complement and fibrinolysis[50].

2.6. Coagulation

The word haemostasis originated from the Greek word 'haeme' meaning blood and 'stasis' meaning stop[74]. It is defined as the cessation of bleeding in the body[50]. This equilibrium is maintained in the body through complex interactions between the fibrinolytic system and coagulation as well as platelets and the vessel wall, always favouring a balanced anticoagulant state with procoagulant factors only becoming activated under thoroughly controlled conditions. This equilibrium is disturbed once the procoagulant activity overshoots the anticoagulant state by higher activity of coagulation factors and decreased activity of naturally occurring inhibitors. To accomplish a normal haemostasis and endothelial cell function after a xenotransplantation it is inevitable to overcome the known molecular incompatibilities so that one can prevent the disarray of coagulation and endothelial cell activation[75].

2.6.1. Primary haemostasis

The complex interplay between platelets, vessel wall and adhesive proteins results in the primary haemostasis and ends in the formation of the initial platelet plug. In the normal state, the endothelial cells that line the vascular wall have different antithrombotic properties such as the negatively charged heparin-like glycosaminoglycans, neutral phospholipids, the synthesis and secretion of platelet inhibitors, coagulation inhibitors as

well as fibrinolysis activators[74]. When this protective layer of endothelial cells is disrupted, the highly thrombogenic subendothelial layer is exposed. This layer contains collagen, Von Willebrand factor (vWF) and proteins that are involved in platelet adhesion such as laminin, thrombospondin and vitronectin.
Platelets derive from megakaryocytes and are disc shaped anucleate cellular fragments that play a fundamental role in haemostasis. They form the initial haemostatic plug, providing a surface for assembly of activated coagulation factors. This process completes with the formation of a fibrin stabilized platelet aggregate and subsequent clot retraction[76].
In the normal physiological state of the vascular endothelium, platelets can not adhere to the endothelium due to previously mentioned antithrombotic properties. After vascular injury, vWF and collagen from the subendothelial tissue are exposed and the protective layer of the vascular endothelium is no longer existing. Platelets are able to adhere to the collagen and the vWF and undergo a morphological change. The platelets form pseudopods and have an irregular surface, thus increasing their surface area significantly. vWF acts as a connector between the endothelial collagen and the platelet surface receptors GpIb and promotes platelet adhesion after vascular injury. GpIb is the glycoprotein complex I receptor and the main receptor for vWF[74,76]. Following adhesion, degranulation leads to release of different factors. Calcium is being released at this step, binding to the phospholipids that appear after platelet activation, providing a surface to assemble coagulation factors.
Activated platelets generate Thromboxane A2 (TxA2), which results in more stimuli for further platelet aggregation. Together with ADP, TxA2 expands the platelet aggregate, which then leads to the formation of a platelet plug that seals the vascular injury temporarily[73,77]. A conformational change is initiated through ADP binding in the GpIIb/IIIa receptors that are distributed on the surface of the platelet leading to deposition of fibrinogen. A conversion of this fibrinogen to fibrin is catalysed through the thrombin generation, increasing the stability of the platelet plug.

2.6.2. Coagulation proteins

An overview of coagulation proteins and their respective nomenclature and physiological concentration in blood plasma can be seen in table 3. The main part of coagulation proteins are precursor of proteolytic enzymes, so called zymogens, that are in circulation in their inactive form[74].
A crucial mediator of coagulation is 'Von Willebrand factor' (vWF), which is produced in the endothelium and the subendothelial connective tissue. It is a glycoprotein present in the blood plasma. The job of vWF is to facilitate the platelet adhesion to the subendothelial surface. It works as a carrier protein for Factor VIII and its coagulant activity.
Another coagulation protein is prothrombin, which is an unstable protein. Activated factor X cleaves the prothrombin into two proteins, one of them is thrombin which has inflammatory effects. This cleaving process is augmented when the co-factor V is in complex with the activated factor X. The combination of co-factor V and activated factor X is termed as prothrombinase-complex and is typical for a 'procoagulant state' of the endothelium[78].
A coagulation protein that is synthesized in the liver is fibrin, the precursor of fibrinogen. It is a key player for determining the strength of the blood clot.
Factor III is a membrane bound glycoprotein and better known as tissue factor. It has procoagulant abilities and is usually ubiquitously distributed in the subendothelial tissues and in fibroblasts. Tissue factor gets triggered by several stimuli, such as inflammation, physical injury, direct vascular injury as well as hypoxia[74,77].

Clotting factor number	Clotting factor name	Function	Plasma half-life (h)	Plasma concentration (mg/L)
I	Fibrinogen	Clot formation	90	3000
II	Prothrombin	Activation of I, V, VII, VIII, XI, XIII, protein C, platelets	65	100
III	TF	Co factor of VIIa	-	-
IV	Calcium	Facilitates coagulation factor binding to phospholipids	-	-
V	Proacclerin, labile factor	Co-factor of X-prothrombinase complex	15	10
VI	Unassigned			
VII	Stable factor, proconvertin	Activates factors IX, X	5	0.5
VIII	Antihaemophilic factor A	Co-factor of IX-tenase complex	10	0.1
IX	Antihaemophilic factor B or Christmas factor	Activates X: Forms tenase complex with factor VIII	25	5
X	Stuart-Prower factor	Prothrombinase complex with factor V: Activates factor II	40	10
XI	Plasma thromboplastin antecedent	Activates factor IX	45	5
XII	Hageman factor	Activates factor XI, VII and prekallikrein		-
XIII	Fibrin-stabilising factor	Crosslinks fibrin	200	30
XIV	Prekallikerin (F Fletcher)	Serine protease zymogen	35	
XV	HMWK- (F Fitzgerald)	Co factor	150	
XVI	vWf	Binds to VIII, mediates platelet adhesion	12	10 µg/mL
XVII	Antithrombin III	Inhibits IIa, Xa, and other proteases	72	0.15-0.2 mg/mL
XVIII	Heparin cofactor II	Inhibits IIa	60	-
XIX	Protein C	Inactivates Va and VIIIa	0.4	-
XX	Protein S	Cofactor for activated protein C		-

HMWK – High molecular weight kininogen; vWf – Von Willebrand factor; TF – Tissue factor

Figure 6: An overview of the coagulation proteins / clotting factors and their nomenclature[74].

2.6.3. Coagulation cascade

The coagulation cascade is a system with two different pathways, called extrinsic and intrinsic pathway, both converging at the factor X activation and leading to the proteolytic activation and conversion of prothrombin to thrombin[50,74]. Depending on the factor that acts as an initiator of the cascade, the respective pathway will be triggered. The starting point of both pathways happens at the cell surface, more specifically the platelets, foreign microparticles, biomaterials or activated endothelium. This results in a proteolytic activation of soluble circulating coagulation factors, also known as serine proteinases, by other components of the coagulation cascade[50].

The extrinsic pathway is considered as the initial step in the plasma mediated haemostasis and is activated through tissue factor that is expressed in the subendothelial tissue. Under physiological conditions, the normal vascular endothelium is intact and diminishes the contact between tissue factor and the plasma procoagulants. A vascular injury can disrupt this quiescent equilibrium and expose tissue factor, that then binds to factor VIIa and calcium and enhances the conversion of factor X to Xa[74,79].

The intrinsic pathway is a parallel pathway with the ultimate goal of thrombin activation by factor XII. It starts with factor XII, high molecular weight (HMW) kininogen, prekallikrein and factor XI, resulting in the activation of factor XI. The activated factor XI further activates factor IX. A tenase complex on a phospholipid surface which activates factor X is produced through the activated factor IX and its cofactor factor VIII.

The common pathway forms the prothrombinase complex through the activated factor X along with its cofactor factor V, tissue phospholipids, platelet phospholipids and calcium[74]. This prothrombinase complex is able to convert prothrombin to thrombin – the thrombin is able to cleave circulating fibrinogen to insoluble fibrin and activate factor XIII. This activation results in a covalent crosslinking of fibrin polymers that are incorporated

in the platelet plug, thus creating a fibrin network that stabilizes the clot and forms a definitive secondary haemostatic plug[73,74].
The initiation phase of the coagulation pathway is activated by the binding of circulating activated factor VII (VIIa) to tissue factor on activated endothelial cells, monocytes, platelets or microparticles. A small amount of thrombin is generated that is insufficient to stimulate significant clotting. The thrombin feedback-activates numerous coagulation

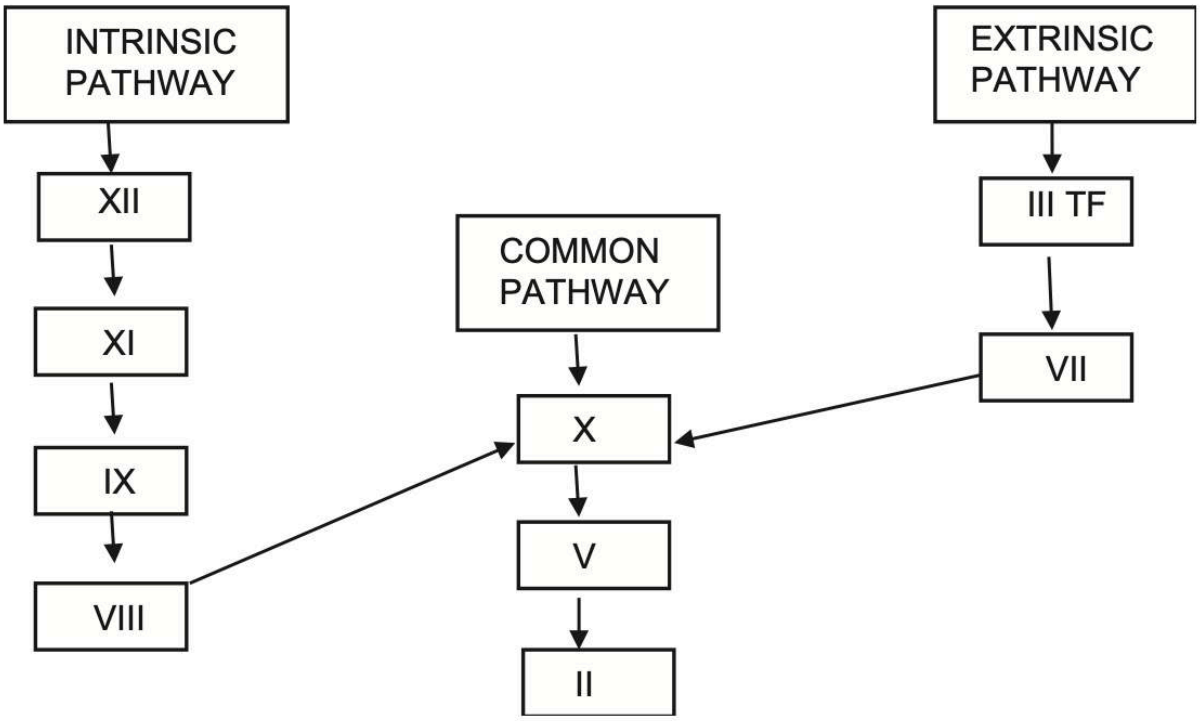


Figure 7: Illustration of the common, extrinsic and intrinsic pathway of the coagulation cascade[74].

zymogens, which further amplifies the tissue factor independent thrombin production (the propagation phase)[38,79]. Thrombin additionally activates platelets through the proteinase activated receptor 1 (PAR-1). This intends to provide a surface for the assembly of the coagulation complexes and a burst of thrombin which is responsible for the main fibrin production.

2.6.4. Regulating and resolving coagulation

Endothelial-bound and circulating molecules are able to control coagulation on a multi-level scale. Tissue factor pathway inhibitor (TFPI) controls the initiation phase by forming an active complex with factor Xa and VIIa/TF. There are different forms of TFPI on the surface of endothelial cells, either bound by direct membrane anchoring or by loose association with other proteins or glycosaminoglycans. By inactivating several serine proteases including thrombin and FXa, Antithrombin (AT) is an important regulator of the propagation phase. AT is present in the serum and has a higher activity when it is associated with heparan sulfate glycosaminoglycans that are attached to the endothelial cell surface[38,74,80].
The protein C pathway is a crucial regulator of the propagation phase with the help of the endothelial membrane proteins thrombomodulin (TBM) and endothelial protein C receptor (EPCR). TBM can bind thrombin and modify the activity by changing the preferred substrate range. This precludes the cleavage of fibrinogen and activation of

PAR-1 and therefore promotes the activation of protein C and thrombin-activated fibrinolysis inhibitor. Inactivation of factor Va and VIIIa is achieved through the interaction of protein C and its cofactor protein S. EPCR is able to enhance activated protein C generation by approximately 20-fold in vivo through presenting protein C to the TBM/thrombin complex[38]. The protein C pathway has important anti-inflammatory effects, worth mentioning are the cleavage of the potent proinflammatory protein high mobility group protein B1 (HMGB1) by TBM/thrombin and anti-inflammatory signalling through PAR-1 mediated by EPCR/activated protein C[38,74].

Resolving the coagulation is achieved through fibrinolysis. This is a process which involves a specific enzymatic cascade that ultimately leads to the removal of fibrin deposits. The key player of the fibrinolytic system is plasminogen, a zymogen of the serine proteinase plasmin. Plasmin is generated through two other serine peptidases, namely the tissue plasminogen activator (tPA) and the urokinase plasminogen activator (uPA). The former is the main activator of fibrinolysis, in which active plasmin is generated on the fibrin surface while the latter is responsible for generating soluble plasmin.
The coagulation and fibrinolytic system have a direct role in activating downstream components of the enzymatic cascade. Other than that, they can be seen as systems that are able to convert a mechanical information, such as a fibrin deposit or a blood clot, from a damaged tissue or a leaky vessel into complex biochemical signals to trigger the cell response. A lot of the effector serine proteinases of the two systems act as ligands to transmit pro- or anti-inflammatory properties on cells with the help of G-protein-coupled receptors, the proteinase activated receptors (PARs). One example is the signalling that is found in thrombin. This signalling activates PAR resulting in vascular biological and inflammatory responses with a main focus on platelet aggregation[50].

2.7. The vascular endothelium

The vascular endothelium plays a crucial role in transplantation, as it is the interface between the recipient and the donor and the first tissue, that comes in contact with the recipient's blood[38,81]. It is the primary target of the immune response and can immediately get activated through antibody binding and activation of complement if pre-formed anti-donor antibodies are present in the recipient's blood.
The endothelium itself plays an important role for survival as it preserves the anti-coagulant properties and facilitates the physiological control of vasoregulation and modulation of the vascular permeability[82]. Pathological consequences of acute and chronic inflammation as well as protective responses to acute and chronic inflammation, wound healing and major cardiovascular disorders are mediated through the endothelium. The endothelial cells that are lining every blood vessel wall are constantly exposed to mechanical forces that are generated through blood flow[83]. This blood flow is translated into endothelial mechanotransduction and the response of endothelial cells to hemodynamic forces generated through blood flow is critical to the homeostasis of the circulatory system[82,84]. A modulation of endothelial cell morphology and function happens due to activation of mechano-sensing, signalling pathway and changes in gene and protein expression. Endothelial cells have many functions, such as forming a transport barrier between the blood and vessel wall, regulating circulation functions, transmitting biochemical stimuli and reacting to blood flow-induced mechanical stimuli such as shear stress, pressure and circumferential stretch just to name a few.

2.7.1. Flow and shear stress

Shear stress is defined as the force per unit area created when a tangential force (blood flow) acts on a surface (endothelium)[82]. The vascular endothelial cells that cover the inner surface of blood vessel are persistently exposed to shear stress due to the frictional force that is created by blood flow[85]. The shear stress ranges from 10 to 40 dyn/cm^2 for arterial endothelial cells and 1-6 dyn/cm^2 for venous endothelial cells[84]. There are three primarily mechanical forces caused by blood flow: the pressure caused by hydrostatic forces within the vessel, hoop stresses resulting due to the balance between the cell-cell contacts and vasomotion of the vessel as well as the shear stress caused by the friction of the blood flow against the vessel wall[86]. The shear stress acts on endothelial cells and produces a multitude of cellular responses, including elongation and alignment of cell in the flow direction; enhanced or suppressed production of molecules associated with homeostasis, such as nitric oxide, prostacyclin and calcium; gene expression; and structural remodelling[85,87].

An *in vitro* study has shown, that shear stress applied to endothelial cells triggers multiple mechanosensors located at the cell membrane[85]. Mechanosensors are biomolecules that work as initial responders to changes in the mechanical environment to trigger mechanotransduction. To the family of mechanosensors belong integrins, tyrosine kinase receptors (particularly vascular endothelial growth factor receptor-2, VEGFR-2), G proteins and G protein-coupled receptors, ion channels and intercellular junction proteins. Possible mechanosensors are the local membrane structures such as gap junctions, membrane lipids, caveolae and the glycocalyx. The endothelial glycocalyx is directly localized on the luminal surface of the endothelium and interacts with the blood flow, therefore playing a crucial role in endothelial mechanotransduction, modulation of vascular permeability and the mediation of leukocyte adhesion[88,89]. We will go more into detail about the composition and function of the glycocalyx in a later chapter. The glycocalyx is exposed to the mechanical forces of the blood flow and transmits this fluid stress to the cell through the core proteins of the glycocalyx[90,91]. The connections of these proteins to the actin cytoskeleton (syndecans) and the plasma membrane (glypicans) is mediating cell signalling (e.g. NO production, cytoskeletal reorganization)[92]. This stress is also transmitted to other regions of the cell, especially to intercellular junctions and the basal adhesion plaques, where transduction to intracellular biochemical signals is mediated[91].

The major evidence that highlights the importance of the endothelial glycocalyx layer in mechanotransduction originates from enzyme degradation experiments. Removing specific components of the glycocalyx enables studying of the role of these removed parts. A study demonstrated, that after selective enzymatic removal of glycosaminoglycan components(an important molecule in the glycocalyx), especially heparan sulfate, resulted in the complete inhibition of shear stress-induced nitric oxide (NO) production and that the glycocalyx is shear sensitive and closely connected to membrane rafts and transmembrane structures[88].

Shear stress plays an important role in the synthesis of different components of the endothelial glycocalyx. In an experiment with human umbilical vein endothelial cells (HUVECs), heparan sulfate components of the glycocalyx increased by 1.4-fold after high shear stress and this increase was not seen in static cultured cells[93]. A different study showed an increased mean fluorescence intensity (MFI) after 24 hours of shear, concluding an increased synthesis of glycocalyx components[88]. A third study concluded that their results showed shear stress stimulated heparan sulfate and chondroitin/dermatan sulfate synthesis and heparan sulfate secretion on the cell surface and into the extracellular matrix[80].

Knockdown of glypican-1, a cell membrane heparan sulfate proteoglycan (HSPG) inhibits the activation of endothelial nitric oxide synthase (eNOS) under shear stress. Endothelial nitric oxide synthase is a central regulator of cellular function and is essential for endothelial homeostasis. This includes adaption of flow to tissue's demand and responses to injury such as re-endothelialisation and the sprouting of endothelial cells to repair damaged endothelium[94]. eNOS is also essential to release and synthesize the potent vasodilator, anti-oxidant and anti-inflammatory mediator nitric oxide. Reduced expression of eNOS is an important element for fluid shear stress regulation of site-specific endothelial functional phenotype[85]. It was shown in two different studies, that shear stress induces clustering of the major components of the endothelial glycocalyx, especially glypican-1 during the initial shear exposure[88,95]. A degradation of heparan sulfate led to significant inhibition of the motility and proliferative response of endothelial cells to shear stress and enhanced the adhesion of leucocytes to the endothelium[95].
Shear stress regulates the alignment and remodelling of endothelial cells by activating the Rho family GTPases (Cdc42, Rho and Rac) that enhance the formation of stress fibers and focal adhesion and regulate cytoskeleton reorganization[85]. In large arteries, endothelial cells are responsive to shear stress environment and adapt to it by elongating and changing their morphology. In contrast, endothelial cells from atheroprone regions show a cobble-stone, non-aligned morphology[88]. Cell morphology experiments showed that after 12 hours, elongation starts to happen but only after 36 hours, maximal alignment to the flow vector is reached[93].
The adaption of the endothelium to fluid shear stress is controlled by the transformation in the actin cytoskeleton. This results in a rearrangement of the filamentous actin (F-actin) into bundles of stress fibres. These bundles are aligned in the direction of flow and into a diffuse network of short microfilaments that include lamellipodia and filopedia[88]. These stress fibres function as cellular cytoskeleton-contractile elements and consist of actin filaments in a parallel alignment. Up on exposure to shear stress, endothelial cell movement is reduced while the polymerization of filamentous actin is enhanced and actin stress fibres form in alignment with the direction of flow[96].

2.7.2. Cell activation

The endothelial cell activation plays a crucial role in the pathophysiology of sepsis, vascular rejection of transplanted organs, ischemia/reperfusion injury and other diseases that are linked to the vascular system[81]. Endothelial cells can be in two different states: either the quiescent state or the activated state[97]. In a healthy blood vessel, endothelial cells remain quiescent, showing an antithrombotic, anti-inflammatory and non-adhesive surface to the circulating blood in the vascular lumen[86,98,99]. The most important anti-coagulant properties of a quiescent endothelial are the expression of tissue factor pathway inhibitors (TFPIs) able to block the initiation of coagulation; the expression of heparan sulfate proteoglycans that are capable of binding anti-thrombin III and inactivate thrombin; and the expression of thrombomodulin, which is a membrane protein with the ability to change specificity of thrombin from a pro-coagulant converter of fibrinogen to fibrin to an anti-coagulant activator of protein C[100]. Protein C together with protein S inactivates different components of the clotting cascade, as mentioned in a previous chapter. Quiescent endothelial cells express the ectoenzyme called nucleoside triphosphate diphosphohydrolase (NTPDase, CD39). This enzyme is able to catalyse the reaction which degrades platelet-derived ATP and ADP thus inhibiting amplification pathway resulting in a platelet plug formation[98]. Endothelial cells produce nitric oxide (NO) and prostaglandin I_2, better known as prostacyclin, which both prevent platelet

adhesion and aggregation. The HSPG, a main component of the endothelial glycocalyx binds superoxide dismutase, which degrades reactive oxygen species and helps to keep the endothelial cells in a quiescence state.

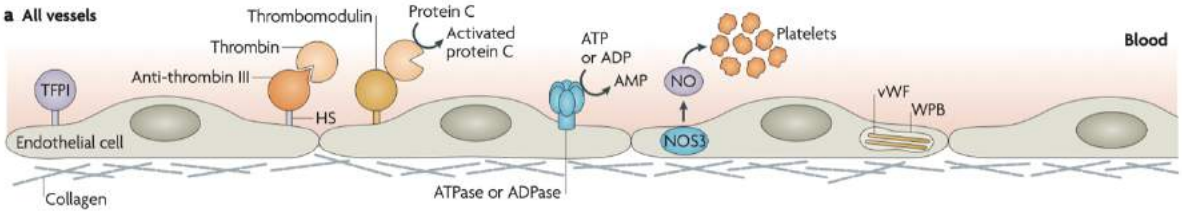


Figure 8: A schematic illustration of the functions of resting endothelial cells[100].

Many different factors, such as antibody binding, complement activation or ischemia/reperfusion injury, are able to disrupt this equilibrium and shift the endothelial cells state from quiescent to activated[38]. In a pathological setting, for example hyper acute rejection, anti-endothelial cell antibodies and complement can lead to a shedding of HSPG, thus exposing a procoagulant and pro-inflammatory surface which is very likely to play an essential role in the pathogenesis of vascular rejection in xenotransplantation[98]. Cytokines, such as tumor necrosis factor (TNF-α) or interleukin-1B (IL-1B) or other stimuli can lead to an upregulation of a number of genes, including E-selectin, intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), monocyte chemoattractant protein-1 (MCP-1), interleukin-1 and interleukin-8, plasminogen activator inhibitor-1 (OAI-1) and tissue factor[86,101]. There are two forms of endothelial cell activation: The protein synthesis-independent type I endothelial cell activation and the type II endothelial cell activation. Type I activation is characterized by endothelial cell stimulation and HSPG release and is accompanied by gap formation between the endothelial cell and the exposure of von Willebrand factor, collagen and subendothelial tissue factor[98]. Macrophages and natural killer (NK) cells infiltrate the tissue and lead to tissue damage and induce a procoagulant state of the endothelial cells. The type II endothelial cell activation is mainly induced by Gal alpha 1,3Gal binding lectins and involves the progressive stimulation of the transcription of pro-inflammatory genes, including interleukin-8, ICAM-1 and E- and P-Selectin[98]. The prothrombotic event of endothelial cell activations involve the upregulation of tissue factor, downregulation of thrombomodulin and endothelial protein C receptor, as well as loss of heparan sulfate and the associated anticoagulant proteins and the loss of CD39[38]. A chronic activation of endothelial cells is associated to chronic inflammatory diseases such as atherosclerosis[86].

2.8. Endothelial glycocalyx

2.8.1. Composition of the endothelial glycocalyx

The endothelial glycocalyx lines the luminal side of the vascular endothelial cells and interacts with the blood flow and is a key player for endothelial mechanotransduction, modulation of vascular permeability and mediation of leukocyte adhesion[88,93]. The main components of the glycocalyx are a fine coating of glycoproteins bearing acidic oligosaccharides and terminal sialic acids and proteoglycans with their associated glycosaminoglycan side chains[87,98]. The composition and dimension of the endothelial glycocalyx is very dynamic, changing continuously and replacing the by plasma sheared material[102]. Disturbed flow exposure in large vessels, protease degradation and removal of plasma components, particularly albumin modify the endothelial glycocalyx[95]. The

glycocalyx works as a platform to provide endothelial cells with a structure to bind plasma proteins and soluble glycosaminoglycans[103]. This sugar layer provides a force-sensitive area between the arterial wall and the flowing blood[93]. High shear stress directly enhances the rate of glycocalyx synthesis compared to cells experiencing no shear stress.

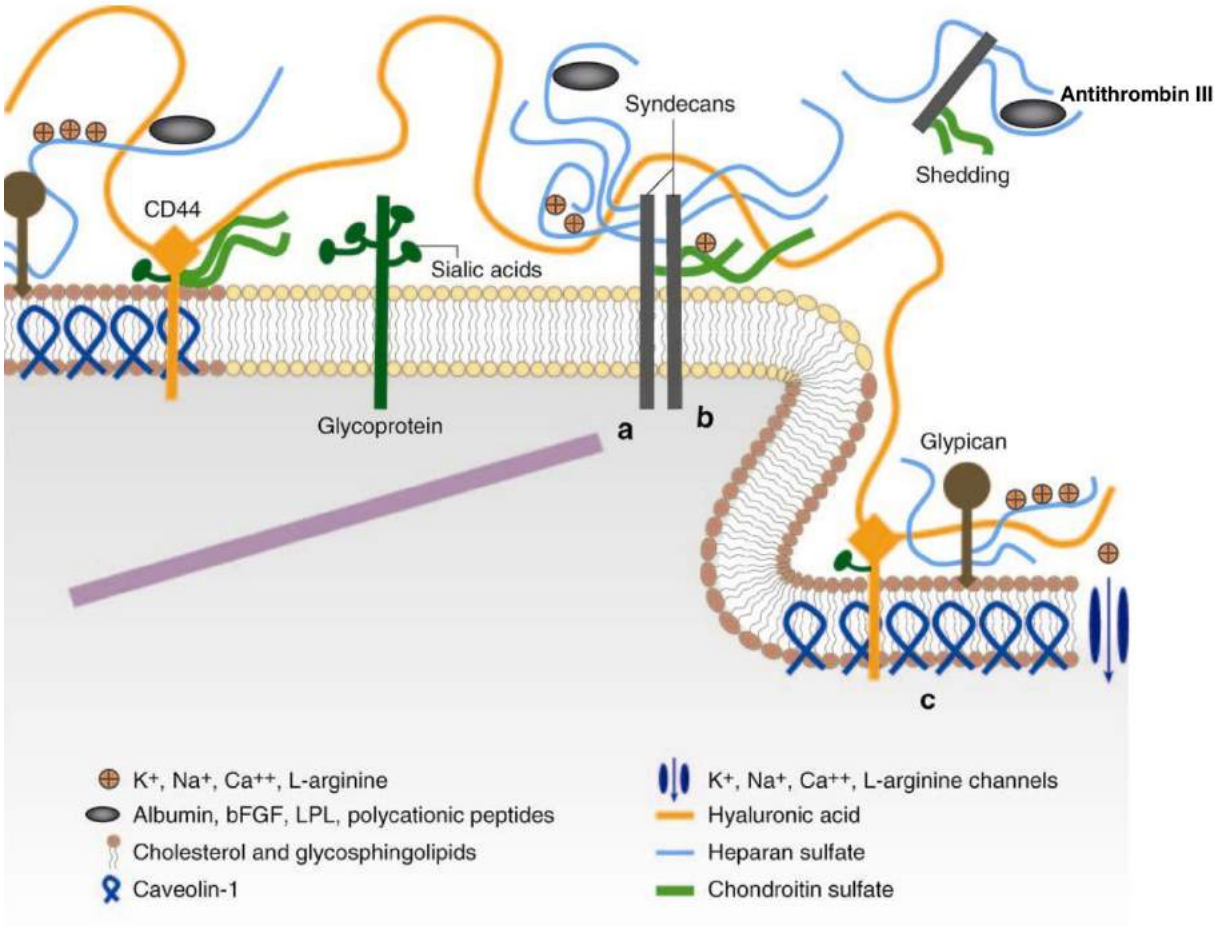


Figure 9: Illustration of glycoproteins and proteoglycans on the surface of endothelial cells (modified)[107].

2.8.2. Proteoglycans

Proteoglycans are the most important backbone molecules of the glycocalyx. They consist of a core protein with one or more glycosaminoglycan chains linked to it[91,104]. The core proteins of proteoglycans are diverse in regard to their size, number of attached glycosaminoglycan chains and connection to the cell membrane. Syndecans and glypicans are firmly connected to the cell membrane via a membrane-spanning domain (syndecan) or a glycosylphosphatidylinositol anchor (glypicans)[104]. The other proteoglycans, namely mimecan, perlecan and biglycan, are secreted after the assembly and their glycosaminoglycan chain modifications[105]. These soluble proteoglycans can reside in the glycocalyx or diffuse into the blood stream. The proteoglycans are promiscuous in terms of their binding of glycosaminoglycan chains. This means, that one core protein can include different types of glycosaminoglycans and this changes under different circumstances and stimuli[104]. Heparan sulfate, chondroitin sulfate, dermatan sulfate, keratan sulfate and hyaluronan are all glycosaminoglycan chains[106]. Glycosaminoglycans are linear polydisperse heteropolysaccharides with variable lengths that are modified by

sulfation and/or (de)acetylation to an extent[107]. The disaccharides contain an uronic acid and a hexosamine and the classification of the glycosaminoglycans heavily relies on the pattern of sulfation and the integration of the specific uronic acid or hexosamine[104]. The distribution of the different glycosaminoglycans is tissue specific and heparan sulfate proteoglycans dominate the vasculature with 50-90% of the total amount of proteoglycans present in the glycocalyx[108]. The expression rate of proteoglycans by endothelial cells is heavily impacted by various stimuli. Syndecans have tightly regulated expression patterns that are influenced by endothelial cell activation or stimulation with other chemokines. The second most abundant glycosaminoglycan expressed by endothelial cells is chondroitin sulfate/dermatan sulfate and the ratio between heparan sulfate and chondroitin sulfate in the vascular endothelium is typically 4:1[107,109].
Proteoglycans consisting of heparan sulfate and chondroitin sulfate/dermatan sulfate are synthesized in the endoplasmic reticulum and Golgi apparatus of the endothelial cell. A xylosyltransferase will transfer xylose from uracil-diphosphate xylose to specific serine residues after the ribosomal translation of the core protein[104]. After transportation to the cis-Golgi, galactosyltransferase type I and II will add two galactose-groups to the xylose, after which glucuronosyltransferase type I adds glucuronic acid, therefore completing the primary linker for glycosaminoglycan chains. The next step after building the primary linker determines the type of glycosaminoglycan that will be produced. Heparan sulfate is produced by adding alpha4-glucosamine to the construct[104]. After the building of the primary construct is finished, glucuronic acids and glucosamines are linked to the core protein and the growing glycosaminoglycan chain will undergo modifications including N- and O-sulfation after chain polymerization[102]. The chain modification is conducted in the cis- and the trans-Golgi, determining the final type and functionality of the proteoglycan.

2.8.3. Glycoproteins

Glycoproteins are connecting the glycocalyx to the endothelial cell membrane and are also viewed as backbone molecules. They are characterized by relatively small (2-15 sugar residues) and branched side chains[104]. Cell adhesion molecules belong to the family of glycoproteins that are crucial mediator of cell recruitment of the blood stream and in the cell signalling cascade. The cell adhesion molecules are grouped into three families, namely the selectin family, the integrin family and the immunoglobulin superfamily. The glycoproteins belonging to the selectin family contain a cytoplasmic tail, a transmembrane domain, an epidermal growth factor-like domain, several consensus repeats and a lectin domain. The lectin domain is important for the binding of carbohydrate groups on glycosylated proteins or lipids.
E- and P-selectins are the most prominent selectins in the vascular endothelium, both involved in leukocyte-endothelial cell interactions[107]. P-selectin is stored in the Weibel-Palade bodies of the endothelial cells and is constitutively produced. A rapid translocation of P-selectin to the cell surface is triggered by stimuli such as thrombin and histamine and leads to exocytosis of Weibel-Palade bodies. This P-selectin expression is rapidly internalized and redirected to lysosomal granules or the Golgi apparatus, where the P-selectin is stored in newly formed Weibel-Palade bodies[104]. Expression of E-selectin is mediated through de novo mRNA and protein synthesis and expressed on the cell surface and not stored in granules. Cytokines, such as interleukin-1, tumor necrosis factor-α and lipopolysaccharide stimulate endothelial cells and upregulate E-selectin expression.
The second family of cell adhesion molecules, the integrins, are heterodimeric molecules are composed of non-covalently bound alpha and beta subunits. The subunits contain a

cytoplasmic tail and a transmembrane domain and build an integral membrane protein together[104]. Endothelial cells, leukocytes and platelets express integrins.
The immunoglobulin superfamily of glycoproteins have a cytoplasmic tail, a transmembrane domain and variable amounts of immunoglobulin-like domains that overhang luminally[104]. Intercellular adhesion molecule 1 and 2 (ICAM-1 and -2), vascular cell adhesion molecule 1 (VCAM-1), and platelet/endothelial cell adhesion molecule 1 (PECAM-1), act as ligands for integrins on leukocytes and platelets and belong to the superfamily of immunoglobulin. These molecules are crucial mediators of leukocyte homing to the endothelium and diapedesis of the leukocytes. ICAM-1 and -2 and PECAM-1 are expressed with a baseline level, while VCAM-1 is only present after stimulation of endothelial cells by cytokines[107]. This stimulation also enhances ICAM-1 expression. The endothelial glycocalyx also consists of glycoproteins with a functionality in coagulation, fibrinolysis and haemostasis, for example the glycoprotein Ib-IX-V complex. This complex is expressed on endothelial cells and on platelets and consists of four glycoproteins: Ibα, Ibβ, IX and V that are all membrane-spanning polypeptides. The complex binds von Willebrand factor and is the main platelet vWf-receptor. The Ib-IX-V complex also binds P-selectin and facilitates the interaction of platelets with activated endothelial cells. Endothelial cells express all components of the Ib-IX-V complex which allows binding to the vWf substrate of the subendothelium and binding of Weibel-Palade body derived vWf that is secreted luminally by activated endothelial cells.

2.8.4. Functional importance of the endothelial glycocalyx

Due to its unique position between the blood stream and the endothelium, the endothelial glycocalyx is a crucial determinant of vascular permeability. It limits the access of certain molecules to the endothelial cell membrane which has been shown in a study using rat mesenteric arteries and fluorescently labelled dextran of various molecular weights[104]. Size and steric hindrance are not the only factors playing a role in glycocalyx dependent permeability. The electrostatic charges of the glycocalyx is impacted by the many glycosaminoglycan chains that are highly sulphated, leaving the glycocalyx as a net negative charged surface to the bloodstream that hinders certain permeating substances[104]. The glycocalyx impacts blood cell-vessel wall interactions by repulsing red blood cells from the endothelium. Upon breakdown of the glycocalyx a red blood cell exclusion zone parallel to the endothelium can be observed *in vivo*[104,107]. Proteins, such as albumin, in serum are charged negatively at pH7 preventing a transfer into the vascular wall through endothelial cells due to the negative charge between the proteins and the glycocalyx. The negative charge added by heparan sulfate in the basement membrane is viewed as a charge-dependent permeability barrier between the blood and the vessel wall[80]. Under physiological conditions, the mesh like structure of the glycocalyx covers the adhesion molecules, preventing pathological adhesiveness of the vascular wall only exposing them during inflammation to facilitate leukocyte rolling, adherence and diapedesis[105]. The endothelial glycocalyx is able to sense the strength of the shear stress of flowing blood and translates this stimuli throughout the cell to sites where this force is translated into biochemical responses, the so called mechanotransduction[109].
The endothelial glycocalyx regulates binding of cytokines the binding of cytokines to the cell surface receptors spread on the endothelial glycocalyx and therefore controls inflammatory responses of the vascular endothelium. Shedding of heparan sulfate from the glycocalyx leaves the glycocalyx more prone to activation by cytokines[104].
Endothelial cells are protected from shear stress and blood flow through the endothelial glycocalyx. Increased endothelial shear stress leads to the enhanced production of nitric oxide (NO) that then expands the vessels and results in reduction of stress. The glycocalyx

notably controls the anticoagulant and anti-inflammatory properties of the endothelium within as well as around the vessel lumen of several coagulation proteins: AT-III binds to heparan sulfate and its anti-coagulant activity is increased; Dermatan sulfate triggers heparin cofactor II, a thrombin-specific protease inhibitor; Factor VIIa and Xa are inhibited through binding of tissue factor pathway inhibitor to heparan sulfate[102].
Lastly, the glycocalyx binds an enzyme that scavenge oxygen radicals (superoxide dismutase) which leads to reduction of oxidative stress and helps to maintain nitric oxide bioavailability to prevent endothelial dysfunction[102,107].

2.9. Microfluidic experiments

Macroscopic cell culturing has been around for many years and was adapted and changed countless times to provide the best option for each different material that has been used in this context. A revolutionary new player was introduced with the culturing of cells in microfluidic systems. Microfluidic refers to the science and technology of a system that processes small (10^{-9} to 10^{-18} litres) amounts of fluids, using channels with a dimension of tens to hundreds of micrometres[110]. This approach allows for new and extraordinary experimental designs. Microscale cell culturing enables higher-throughput experimentation in drug testing, the study of complex biological processes and in analysing conditions for large-scale bioreactions[111]. A major advantage of designing microfluidic devices is the tailoring to the needs of the specific cell type[112]. One can add a chemical gradient or set up a continuous perfusion of the culture. All in all, it allows for a closer, more physiological experimental set up closer to the *in vivo* situation while using a tremendously lower amount of reagents. The microfluidic system allows, in accordance with the 3R principle, to reduce the number of animals that are used for experimental purposes[81].
The adaption from macroscopic culture environment to a microscopic small-scale culturing was a big step for cell culturing, since the majority of culturing protocols were designed for macroscopic systems. Extensive trials and revision of these protocols had to be conducted to adjust them for microscale culturing systems. Oxygen and CO_2 are essential for cell culturing and diffuse from the air inside of the incubator into the culture medium to provide support for cell growth, proliferation and medium buffering[112]. Controlling these levels in microscale cell culturing is essential since small changes have a bigger impact on the conditions due to the smaller cell-to-media ratio in this cell culture method. In macroscale cell culturing cell medium is added excessively while a regular exchange is necessary in microscale cell culturing to provide essential nutrients for the cells since the cell-to-medium ratio is so small.
Polydimethylsiloxane (PDMS) is the material of choice for microfluidic systems. PDMS belongs to the family of silicones, which are synthetic polymers with a repeating chain of Si-O molecules and various organic groups in the backbone. This silicone brings a lot of advantages: The material allows for the design of complex microfluidic systems; it is optically clear and therefore allows real-time, high-resolution optical imaging; it has a high flexibility and is cost-effective and easy to handle; it can be solidified through polymerization and cross-linking with a curing agent; it is biocompatible and non-toxic; it is permeable enough for gas, making it a great choice for on-chip cell culturing[113,114]. The PDMS has a high surface hydrophobicity that limits the affinity for mammalian cells to effectively adhere to the PDMS surface[115]. This limitation can be resolved by modifying the surface of the silicone with oxygen plasma treatment[116]. Oxygen plasma activates the synthetic surface of the PDMS, therefore reducing the hydrophobicity and switching to a hydrophilic surface. Adding APTES ((3-Aminopropyl)triethoxysilane) enhances the hydrophilicity and in combination with glutaraldehyde allowing to attach matrix proteins

to the chemically treated PDMS surface. The combination of APTES and glutaraldehyde resulted in covalent attachment of matrix proteins on the activated surface of the functional groups of PDMS[115,116]. A study tested the effects of fibronectin and collagen type I in combination or separately in terms of support for the attachment of endothelial cells and concluded, that the combination of both resulted in the best cell adherence[117].

3. Hypothesis and Aim of the experiment

The species specificity of complement regulators is still controversial and highly discussed, but it was already shown numerous times that CD46 is able to function across species. We therefore hypothesized that human CD46 has a beneficial effect on the complement deposition in an allogeneic perfusion setting since both human and porcine CD46 are present and able to regulate complement activation. Furthermore, we hypothesized that we are able to replicate the results of the previously in our lab performed *ex vivo* study on myocardial infarction in pigs in an *in vitro* model. The *ex vivo* study showed that hCD46 is able to protect the endothelial cells and reduce the coagulation activation in inflammatory conditions such as ischemia reperfusion injury.

Our aim is to mimic the situation of an ABO incompatible allotransplantation by using wild type and transgenic (hCD46/HLA-E) porcine aortic endothelial cells (PAEC), human purified anti-Gal antibodies and porcine serum and a recirculating closed microfluidic system. We then aim to assess the role of hCD46 in modulating the allogeneic complement activation.

4. Materials and Methods

4.1. Cultivation of pig aortic endothelial cells

Wild type (WT) and hCD46/HLA-E transgenic (TG) pig aortic endothelial cells (PAEC) between passage two and five were stored at -150°C. The cells were cultured in a T-75 flask (TPP, Trasadingen, Switzerland and placed into a 37°C, 5% CO_2 incubator until confluence was reached. DMEM cell culture medium (Thermo Fisher Scienctific, Waltham, MA, USA) was used and supplemented with 10% heat-inactivated fetal bovine medium (FBS, Biochrom, Berlin, Germany), 100 IU/ml penicillin and 100µg/ml streptomycin (Thermo Fisher Scientific). The medium was changed every other day.

4.2. PDMS microchip fabrication

Polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, Wiesbaden, Germany) was mixed with a curing agent in a ratio of 10:1. This mixture was poured into a petri dish and a vacuum pump was utilized to remove air bubbles in the mixture. The mold needles

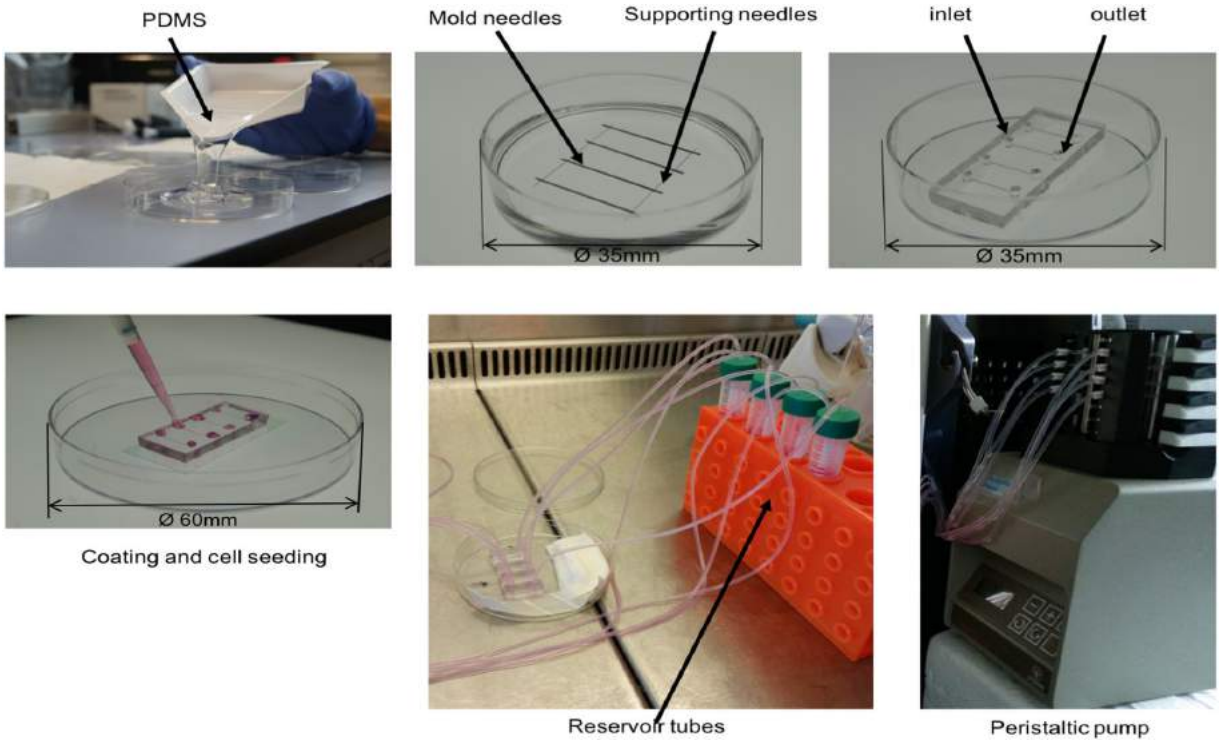


Figure 10: Schematic of microchannel fabrication and pump connection. Liquid PDMS is poured into a Petri dish. Support and mold needles are place into the liquid PDMS and the whole Petri dish is cured overnight at 60°C. Needles are removed, inlet and outlet holes are punched with a 2 mm biopsie puncher and the lateral holes are sealed with more PDMS. The microchip is bound to a glass slide after plasma oxygen treatment and coated with fibronectin and collagen I. Cells are seeded into the microchannels and a peristaltic pump is connected applying a shear stress of 15 dyn/cm^2 [81].

(B.Braun, Melsungen, Hessen, Germany) with a diameter of 550 µm were cleaned with isopropanol and dried on a tissue paper. Two support needles (Seirin, Hamburg, Germany) with a diameter of 120µm were placed vertically in the petri dish. Then, four

550µm mold needles were put on top, perpendicular to the previously placed support needles. The petri dish with the liquid PDMS and the needles were cured at 60°C in an oven over night. Once cured, the microchips were cut out of the petri dish and cut into single chips containing 4 microchannels. The mold needles were removed horizontally with tweezers and the support needles were cut away from both sides of each chip. An inlet and an outlet was punched with a 2mm biopsy puncher (kai Europe GmBH, Solingen, Germany) at a distance of 1 cm. The small hole, that was left from extracting the mold needles between the edge of the PDMS and the inlet and outlet, was sealed with liquid PDMS and the chip was again cured at 60°C overnight. The final microchip contained four microchannels with a diameter of 550 µm and a length of 1 cm.

4.3. Surface modification of PDMS chips

Before cells can be seeded into the microfluidic channels, the inner surface of the PDMS needs to be modified to allow covalent binding of extracellular matrix molecules[115]. The PDMS chip and standard glass slides were cleaned with water, soap water and isopropanol. The surface of the PDMS chip and the glass slide were activated in an oxygen plasma cleaner (Harrick Plasma, NY, USA) at 650 mTorr for 3 minutes.

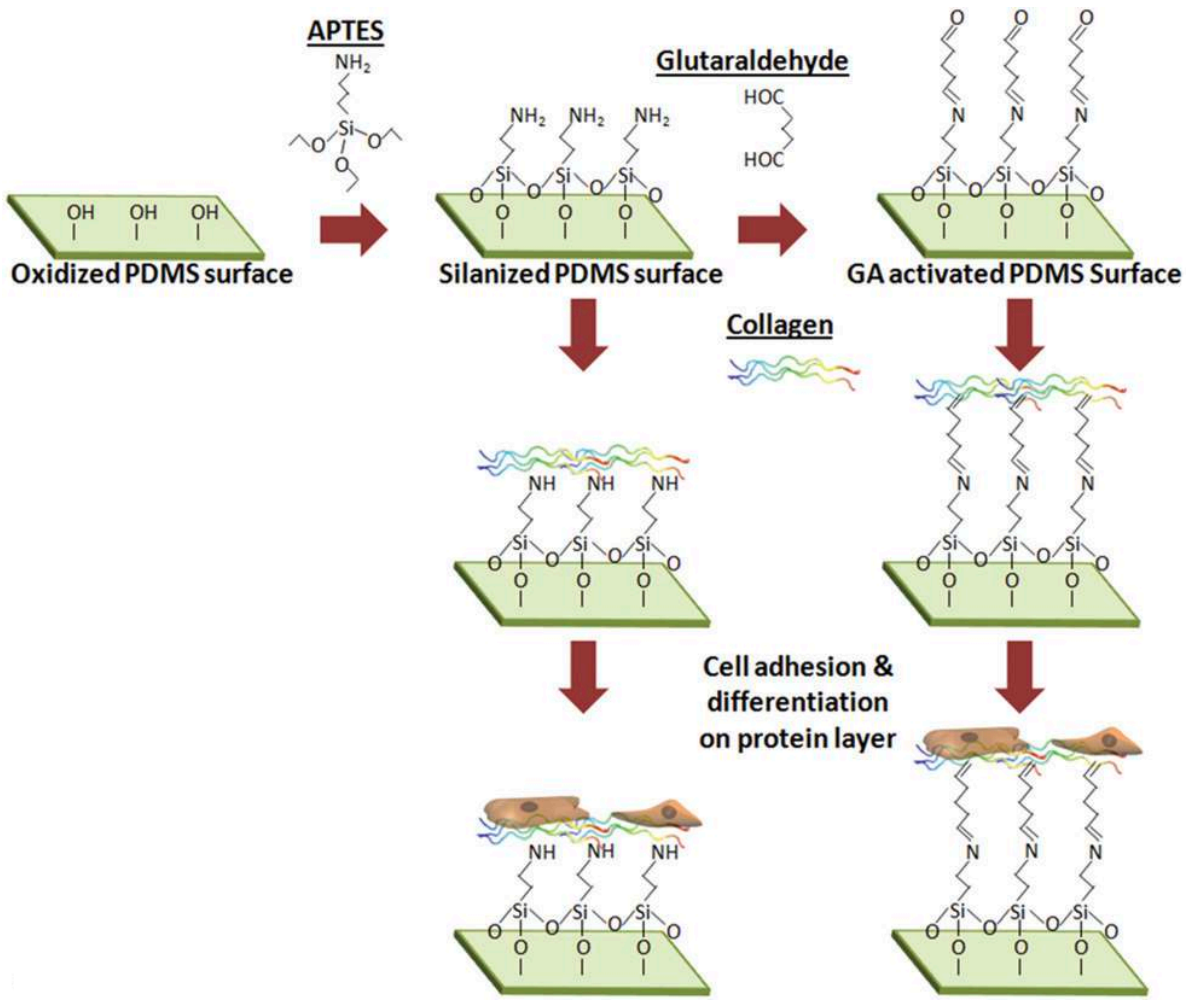


Figure 11: Schematic illustration of PDMS surface modification[115]: Activation through oxygen plasma treatment is followed by adding APTES to make the PDMS hydrophilic. Glutaraldehyde is used to allow the crosslinking of extracellular matrix proteins, namely fibronectin and collagen I. Cells are added and cultured on the protein layer generated on the PDMS surface.

During this treatment, the surface chemistry is changed by adding silanol (SiOH) groups as shown in figure 2. The microchips and the glass slide were bonded together immediately after the oxygen plasma cleaner. After bonding, the hydrophobic PDMS surface in the microchannels was silanized to make it hydrophilic by filling the channels with 5% 3-triethoxysilylpropylamine (APTES, Sigma-Aldrich, Buchs, Switzerland) and incubated for 20 minutes at room temperature. The channels were washed with ultrapure water and treated with 0.1% glutaraldehyde (Sigma-Aldrich) for 30 minutes. This provides a crosslinking substrate to immobilize extracellular matrix proteins. The microchannels were incubated with 50µg/ml human fibronectin (Millipore, Schaffhausen, Switzerland) in PBS for 1 hour in a 37°C incubator. After that, 100µg/ml bovine collagen I was diluted in 0.2 mol/l acetic acid (Gibco, Thermo Fisher Scientific) and added into the microchannels at room temperature for 1.5 hours. Cell culture medium containing 10% FBS and 100 IU/ml penicillin and 100µg/ml streptomycin was rinsed through the channels to block unspecific protein binding sites and to wash out any unbound collagen I before the cell loading.

4.4. Cell seeding and pump connection

Confluent culturing flasks of WT and TG PAEC were washed with PBS and incubated with 0.05% Trypsin-EDTA (Gibco, Thermo Fisher Scientific) at 37°C until all cells were detached. Cell culture medium was added to dilute and inactive the Trypsin. The cell suspension was centrifuged at 1200 rpm for 8 minutes. The supernatant was removed and the pellet was resuspended in 1 ml cell culture medium supplemented with 10% FBS, 100 IU/ml penicillin and 100µg/ml streptomycin and 4% dextran from Leuconostoc spp. (Mw ~ 70'000, Sigma-Aldrich) to increase the viscosity and promote cell adhesion. Cells were seeded into the microfluidic chip at a density of 1 x 10^6/ml. The whole chip was flipped upside down and placed in the incubator at 37°C/5% CO_2 for 10 minutes to promote the cell adhesion on the upper part of the microchannel. Afterward cell attachment was checked under the microscope and more cells were added. The unflipped device was placed back into the incubator for another 10 minutes. Cells were then cultured under static conditions with 2-3 cell culture medium changes at intervals of 2 hours, followed by an overnight incubation. A peristaltic pump – Minipuls 3 with 8 channels (Gilson, Villiers le bel, France) – was connected to the microfluidic channels via sterile silicon tubing with stoppers (Gilson, Villiers le bel) and extension silicon tubings (Gobatec, Bern, Switzerland) after reaching confluency. The extension tubings were autoclaved and extensively flushed with distilled water and PBS, followed by cell culture medium with 4% dextran. The silicone tubings with stoppers were washed with ethanol and ultrapure water and then treated with ozone gas in a CoolCLAVE Laboratory Bench Top Sterilizer (Genlantis, San Diego, CA, USA). A sterile 15 ml tube (Corning, Berlin, Germany) was used as a medium reservoir, containing 10 ml of culturing medium with 10% FBS, 100 IU/ml penicillin and 100µg/ml streptomycin, 4% dextran and 1 % bovine serum albumin (BSA) (Sigma Aldrich, St. Louis, MO, USA). A reservoir tube was connected to each microchannel and placed in the 37°C incubator together with the microfluidic device. The flow was set to 10 RPM, which corresponds to a shear stress of 15 dyn/cm^2. The medium in the reservoir tubes was changed every 24 hours and the cells were kept under flow for 48 hours before either a staining or a perfusion was performed.

4.5. Perfusion reagents

The microchannels were perfused with TNF-α, human anti gal antibodies and wt pig serum. Pig serum was drawn from experimental animals into polypropylene tubes containing glass beads (S-Monovette, Sarstedt, Germany) and allowed to clot for 30

minutes at room temperature. The clot was removed by centrifugation for 10 minutes at 20000 x g in a cooled centrifuge at 4°C and the supernatant was collected and stored at -80°C.
Anti-Gal antibodies were isolated from NHS through depletion by repeated passage through a chromatography column (Bio-Rad Laboratories, Hercules, CA). The columns were filled with synthetic Galα1-3Gal oligosaccharides in the form of flexible, hydrophilic polyacrylamide conjugates that were covalently coupled to Fast-Flow Sepharose (Lectinity Corp., Russia). The anti-Gal depleted serum (aNHS) was then stored on ice until it was frozen in aliquots at -80°C. The columns were extensively rinsed with PBS and 0.1M glycine-HCl pH 2.3 was used to elute the bound anti-Gal antibodies into tubes that contained $NaHCO_3$ to bring the pH to neutrality. The anti-Gal aliquots were then frozen at -80°C. Recombinant human TNF-α derived from E. coli (R&D Systems, Minneapolis, USA) was diluted to a concentration of 20μg/ml in pure DMEM without any supplements.

4.6. Perfusion of microfluidic channels

The perfusion of the microchannels was done after 48 hours of shear stress on the WT and TG PAEC. There were X groups: Group 1: TNF-α + anti-Gal antibodies + pig serum, Group 2: TNF-α + anti-Gal antibodies + heat inactivated pig serum, Group 3: TNF-α + normal human serum, Group 4: TNF-α + pig serum. All the perfusion reagents were diluted in pure cell culturing medium without any supplements. Anti-Gal antibodies were diluted 1:10 and perfused for 30 minutes. TNF-α was diluted to a concentration of 20μg/ml and perfused for 4 hours. Pig serum was diluted 1:5 and perfused for two hours. The microfluidic system was washed in between every perfusion step with pure cell culture medium without any supplements.

4.7. Immunofluorescence staining

Immunofluorescence staining was performed to assess the complement activation as well as to check for transgene expression, characterization of the endothelial cells and to measure other markers of endothelial cell activation, complement deposition and the coagulation cascade. Cells in the microfluidic channels were washed with PBS at the end of the experiment, then fixed with 4% paraformaldehyde for 10 minutes that was heated up to 60°C for 10 minutes to avoid any debris. Channels were washed with PBS after fixation and then blocked with PBS-3% BSA for 30 minutes at room temperature on a shaker. Permeabilization was done with 0.5% TritonX in PBS for 10 minutes on a shaker if needed. Incubation with primary antibodies was done at room temperature for 1 hour followed by a 1 hour incubation of the secondary antibodies and DAPI. Antibodies were diluted in PBS-1% BSA or PBS-1%BSA-0.05% Tween20 for intracellular antigens. Microchannels were washed in between the antibody solutions to rinse of any unbound antibodies. After staining, the microchannels were washed with PBS and stored at 4°C and covered with aluminium foil and parafilm to prevent bleaching and evaporation.
Here is a list of the different antibodies used, grouped in primary, secondary and directly labelled antibodies:

Primary Antibodies	Dilution	Company
Sheep anti human Tissue Factor	1:200	Affinity Biologicals
Mouse anti porcine E-Selectin	1:100	Sigma
Rabbit anti human FGL-2	1:100	Aviva Systems Biology
Rat anti pig CD31	1:100	R&D Systems
Ve-Cadherin mouse monoclonal IgG	1:100	Santa Cruz Biotechnology
Mouse anti human CD46	1:100	Hycult Biotech

Mouse anti human HLA-E	1:100	Biolegend
Mouse anti porcine CD46	1:200	Bio-Rad

Secondary Antibodies	**Dilution**	**Company**
Goat anti mouse 546 Alexa Fluor	1:500	Invitrogen
Goat anti mouse Alexa Fluor 488	1:500	Invitrogen
Goat anti rabbit Alexa Fluor 633	1:500	Invitrogen
Donkey anti sheep Alexa Fluor 488	1:500	Invitrogen
Goat anti rat IgG	1:500	Jackson Immuno Research

Directly labelled antibodies	**Dilution**	**Company**
4',6-diamidino-2-phenylindole (DAPI)	1:1000	Boehringer Mannheim GmbH
Directly labelled rabbit anti human C3b/c FITC	1:500	Dako
Directly labelled rabbit anti human C4b/c FITC	1:200	Dako
Lectin from Bandeiraea simplicifolia FITC conjugated	1:200	Sigma Aldrich

A confocal laser-scanning microscope (LSM 710, Zeiss) was used at 10x magnification to acquire the pictures. Image J (National Institutes of Health) was used for image analysis.

4.8. Statistical analysis

All data are presented as mean ± standard deviation (SD). The statistical analysis was performed with GraphPad Prism 8 software (GraphPad, San Diego, CA, USA) using the one-way analysis of variance (ANOVA). P values < 0.05 were considered statistically significant.

5. Results

5.1. Wild type and transgenic pig aortic endothelial cell characterization

Wild type and transgenic pig aortic endothelial cells ranging from passage 2 to 5 were used for all the conducted experiments. Cells were cultured in a fibronectin coated glass chamber slide and grown until confluency before fixation and immunofluorescence staining. Cells were characterized by their expression of typical endothelial cell markers such as VE-Cadherin, CD31 and vWF. Figure 12a and c show wild type pig aortic endothelial cells expressing VE-Cadherin and CD31; Figure 12b and figure 12d show hCD46/HLA-E transgenic pig aortic endothelial cells expressing VE-Cadherin and CD31. The two cell types expressed the endothelial cell markers and we were able to define them as endothelial cells.

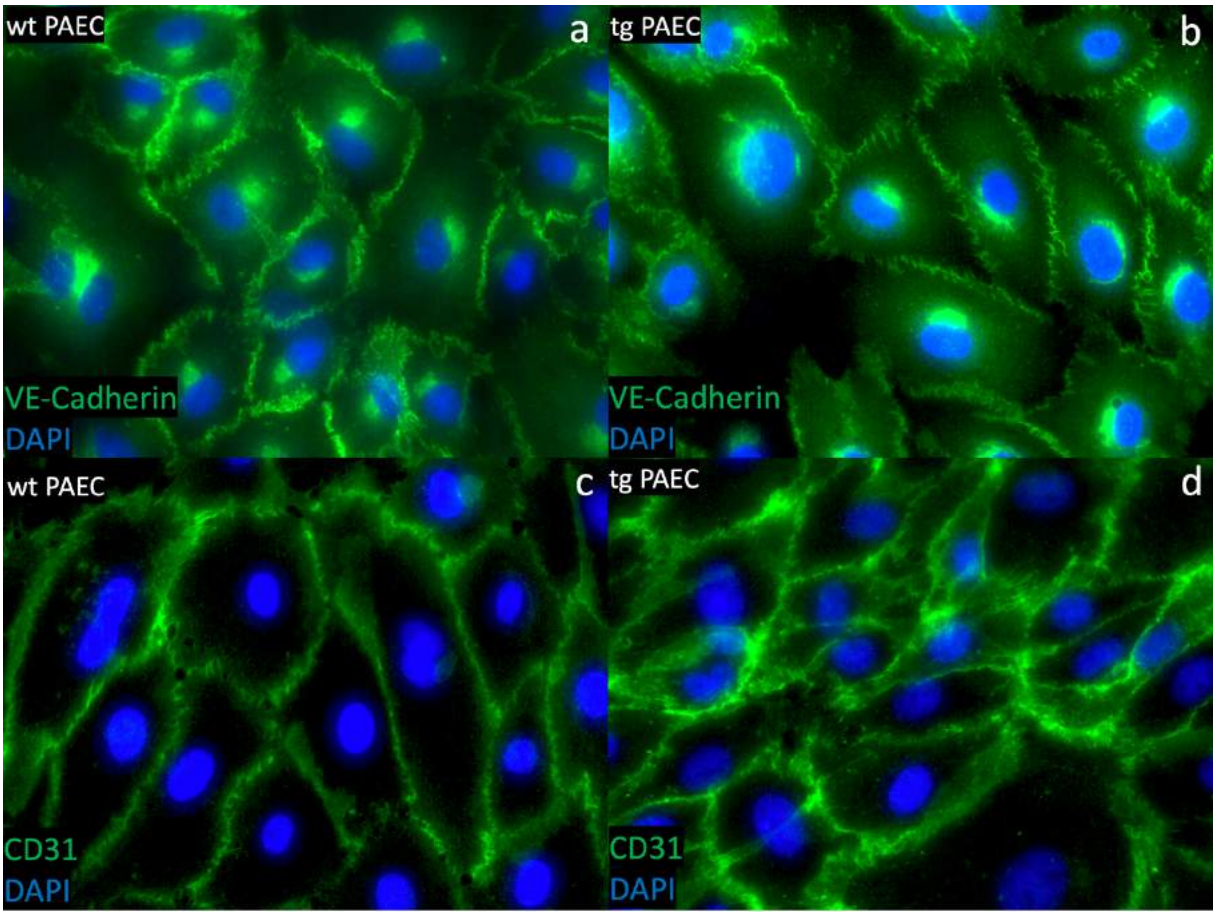


Figure 12: Characterization of wild type and transgenic pig aortic endothelial cells. a) wt PAEC with a staining for DAPI and VE-Cadherin; **b)** tg PAEC stained for DAPI and VE-Cadherin; **c)** wt PAEC with a staining for DAPI and CD31; **d)** tg PAEC stained for DAPI and CD31; The cells were cultured under static condition with daily medium exchange. Pictures were acquired with a fluorescence microscope (Leika DMI 4000b)

5.2. Transgene expression on transgenic pig aortic endothelial cells under flow exposure

Before we started experimenting with different conditions we had to check for the expression of the transgene in the transgenic cell line. The cells should have human CD46 (a complement regulator protein) and HLA-E expression (better known as MHC class I antigen E and plays an important role in recognition by NK cells). We exposed wild type and transgenic cells to a shear stress of 15 dyn/cm^2 for two days and fixed the cells and did the immunofluorescence staining.

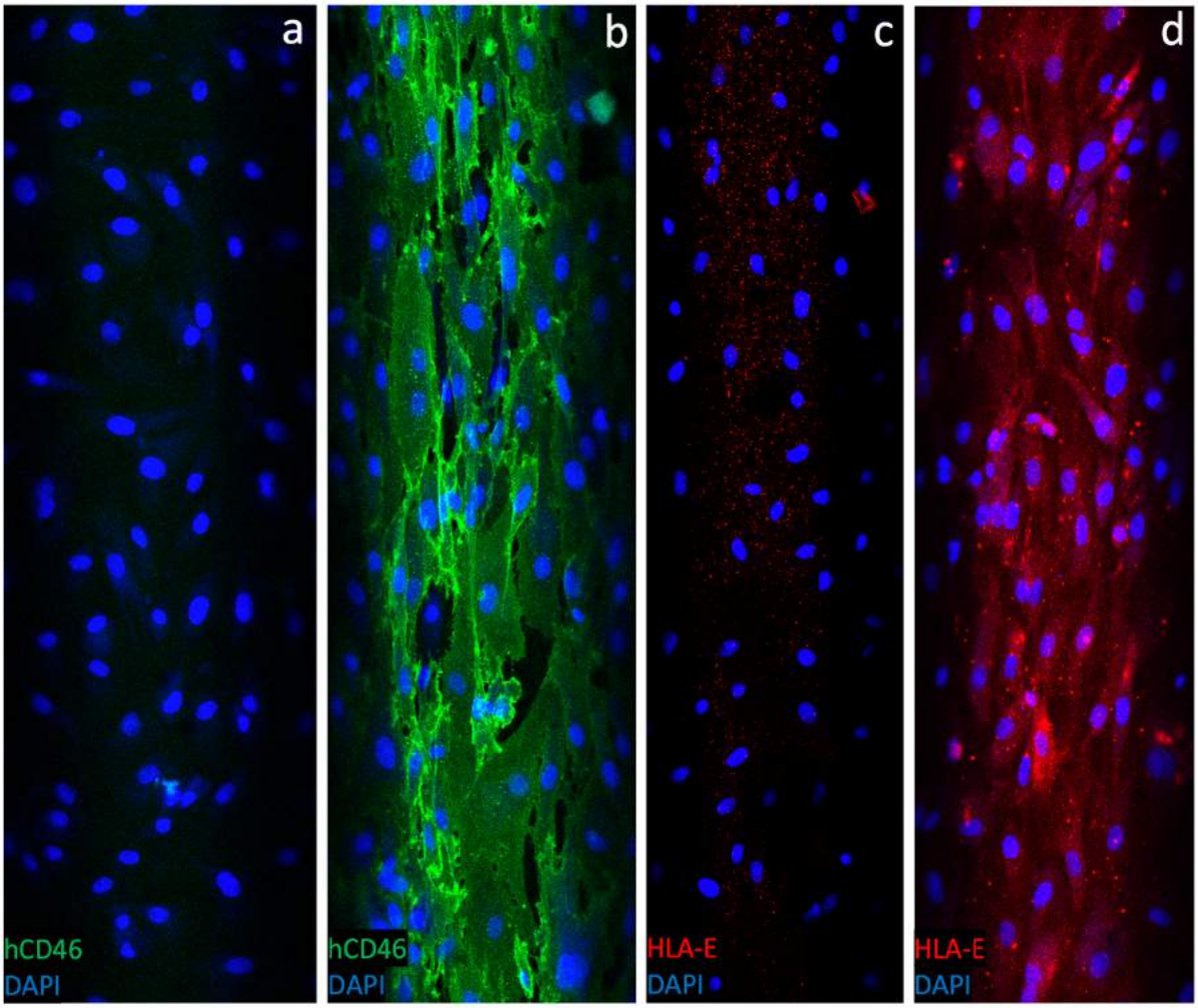


Figure 13: Expression of transgenes under flow conditions in both wild type and transgenic pig aortic endothelial cells. a) wt PAEC stained for hCD46 and DAPI; **b)** tg PAEC stained for hCD46 and DAPI; **c)** wt PAEC stained for HLA-E and DAPI; **d)** tg PAEC stained for HLA-E and DAPI; Cells were kept under flow with a shear stress of 15 dyn/cm^2 for two days with daily medium exchange.

The wild type microfluidic channels worked as a control, since we do not expect to see any expression of these human genes in 'normal' wild type pig aortic endothelial cells. We observed low levels of background staining in figure 13c for the HLA-E staining on wild type endothelial cells and no background in figure 13a for the hCD46 staining on wild type endothelial cells. The staining for hCD46 on transgenic cells (figure 13b) looks specific and the expression is ubiquitously in the channel and on all cells. The complement regulator hCD46 is expressed on the cell surface and we can clearly observe a concentrated staining on the cell membrane of our endothelial cells. The HLA-E staining

in figure 13d is distinctive compared to the control in figure 13c. We were able to observe a surface staining on the transgenic endothelial cells, even though it is tremendously weaker than the hCD46 staining. We observed a perinuclear intracellular staining and a scattered dot-like staining of HLA-E on the transgenic pig aortic endothelial cells.

5.3. Human CD46 and porcine CD46 expression on wild type and transgenic pig aortic endothelial cells under static conditions

Human CD46 and porcine CD46 staining was tested under different culturing conditions starting with static conditions by culturing cells in fibronectin coated glass chamber slides until confluency.

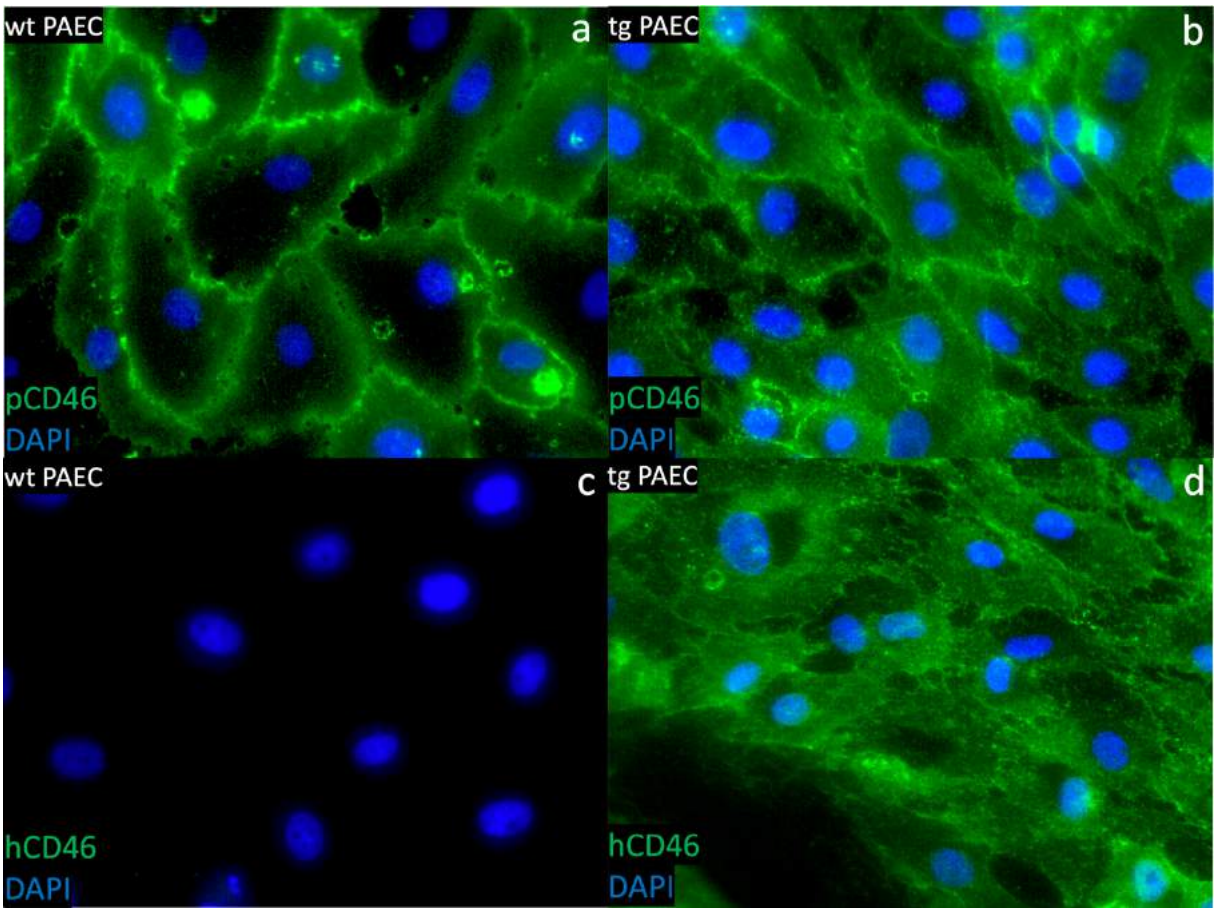


Figure 14: Chamberslide staining of wt and tg pig aortic endothelial cells for the expression of human CD46 and porcine CD46. a) Staining of porcine CD46 and DAPI on wt PAEC; **b)** Staining of porcine CD46 and DAPI on tg PAEC; **c)** Staining of human CD46 and DAPI on wt PAEC; **d)** Staining of human CD46 and DAPI on tg PAEC; The cells were cultured under static conditions with daily medium exchange and pictures were taken with a fluorescence microscope.

The transgene was only expressed in transgenic cells and evenly distributed with a clear concentration on the cell surface (visible in figure 14d). The corresponding wild type staining in figure 14c showed no background staining and no expression in wild type cells. Porcine CD46 is present in both cell types, wild type and transgenic, visible in figure 14a for wt and figure 14b for transgenic cells.

5.4. Human CD46 and porcine CD46 expression on wild type and transgenic pig aortic endothelial cells under flow conditions

The previously performed experiment to check porcine and human CD46 expression was repeated under different culture conditions. Cells were cultured under flow at 15 dyn/cm^2 for two consecutive days with daily medium exchange. After that, the microchannels were stained for porcine and human CD46 expression. The results regarding the expression of

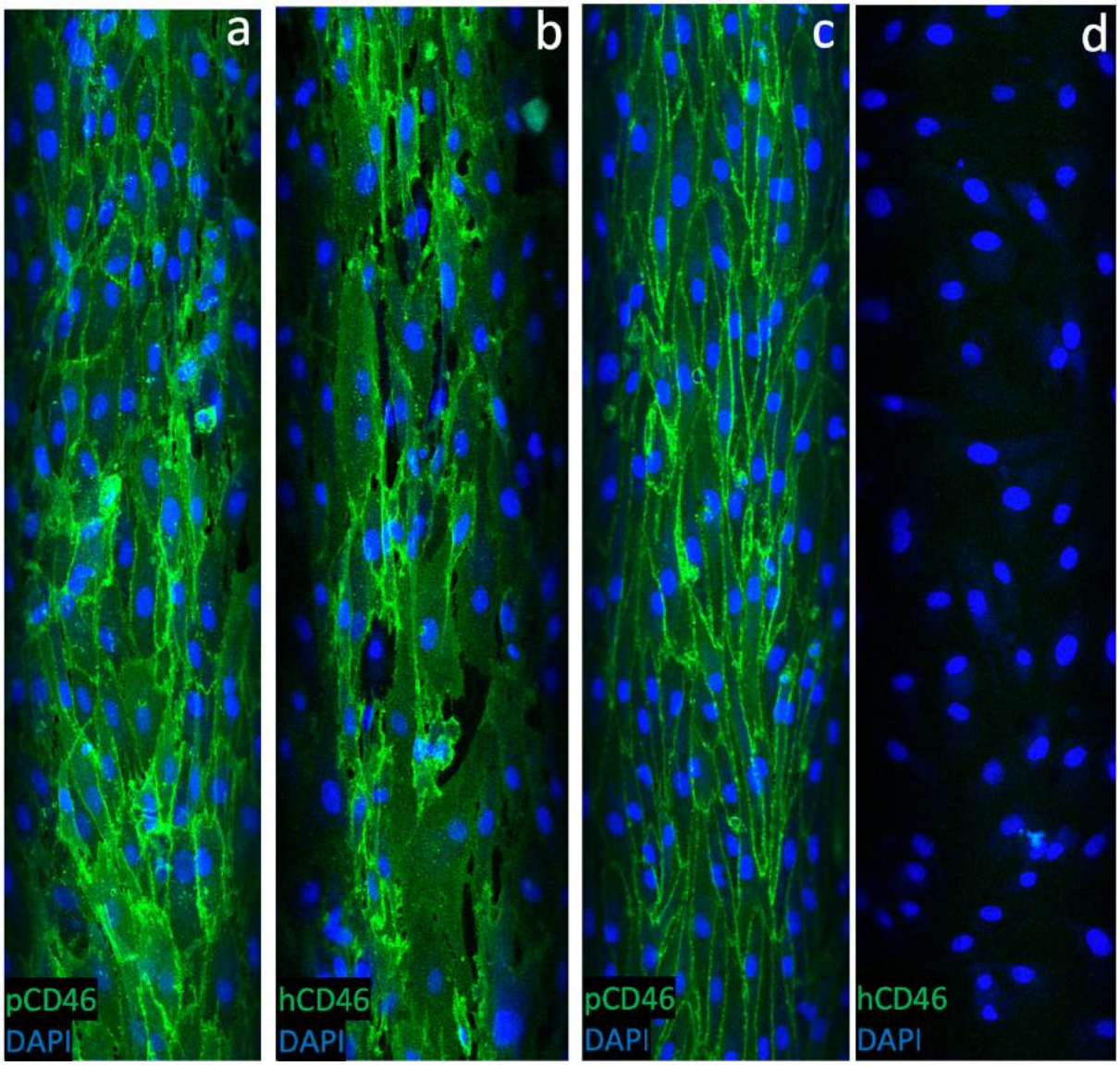


Figure 15:Expression of porcine and human CD46 on wildtype and transgenic PAEC in a microchip under flow. a) tg PAEC stained for porcine CD46; **b)** tg PAEC stained for human CD46; **c)** wt PAEC stained for porcine CD46; **d)** wt PAEC stained for human CD46; cells were kept under a flow of 10 RPM (15 dyn/cm^2) for two days with daily medium exchange.

porcine and human CD46 are identical to the staining under static conditions in 5.3.. The human CD46 was only expressed in the transgenic cell line in figure 15b and was completely absent in the wild type cells in figure 15d. The porcine CD46 was expressed in both the wild type cells (figure 15c) and the transgenic cells (figure 15a).

5.5. Transgenic endothelial cells are less activated after perfusion with allogenic pig serum

Transgenic and wildtype porcine endothelial cells were compared in terms of their levels of cellular activation. Both cell types were previously kept under flow for two days at 10

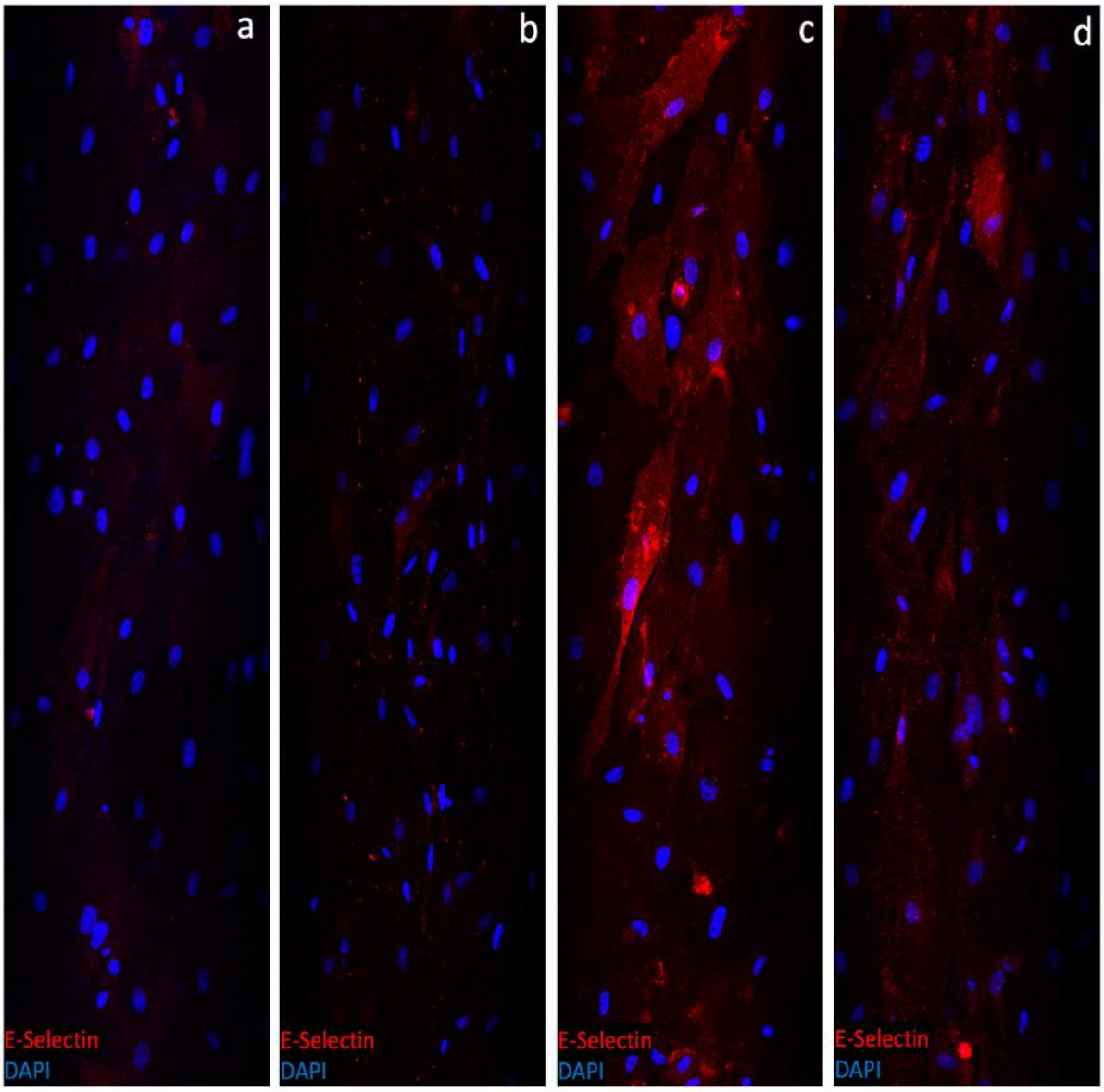


Figure 16: E-Selectin expression on wild type and transgenic PAEC after 2 hours of 20% allogeneic pig serum perfusion. a) wt PAEC control, perfused only with medium and stained for E-Selectin and DAPI; **b)** tg PAEC control, perfused only with medium and stained for E-Selectin and DAPI; **c)** wt PAEC perfused with 20% allogeneic serum for two hours and stained for E-Selectin and DAPI; **d)** tg PAEC perfused with 20% allogeneic serum for two hours and stained for E-Selectin and DAPI; Cells were kept under flow for two days before the perfusion with allogeneic serum.

RPM (15dyn/cm^2) with daily medium exchange. After that, the microchannels were flushed with pure DMEM cell culture medium without any supplements, followed by a 2 hour perfusion with 20% allogeneic pig serum. In figure 16a are wild type PAEC and 16b are transgenic PAEC after a medium only perfusion. This control experiment was done to

measure the baseline activation of the endothelial cells in our system. The baseline activation was very minor for both cell types. This is showed in figure 16a for wild type and 16b for transgenic cells and presented as a graph in figure 17. A significant difference in the levels of activation was measurable between the wild type and transgenic cells that were perfused with allogeneic serum (visible in figure 17). This big difference is already visible by looking at figure 16c and 16d. Overall, the baseline activation of transgenic cells compared to wild type cells was already lower. There was a significant increase in the level of activation for the medium perfused transgenic cells vs. the serum perfused transgenic cell line. This increase was not significant for the wild type cell line when comparing the medium perfused vs. the allogeneic serum perfused cells. We compared the % increase of activation to minimize the already higher baseline activation of the wild type cells. The wild type cells activation level after serum perfusion was 6.99 % increased where as the transgenic cell activation after serum perfusion was 29.08 % increased.

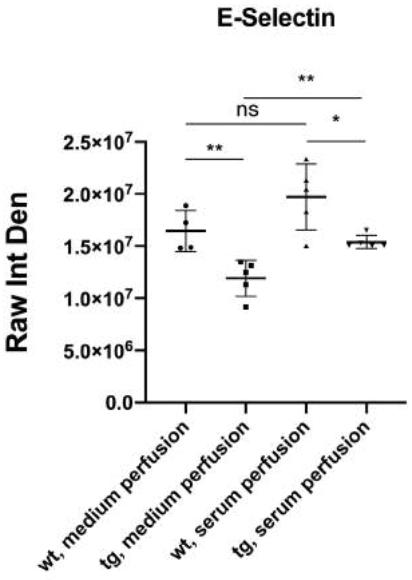


Figure 17: Statistical analysis of the microchannels from figure 7. Each dot represents a picture on a channel. 4-5 pictures were taken for each channel and one channel per group was analysed for this experiment.

5.6. Endothelial cell activation and coagulation markers on transgenic and wild type cells after allogenic serum perfusion

The E-Selectin staining was repeated and the coagulation markers FGL-2 and tissue factor (TF) were stained additionally. The cells were in an overall healthy state and looked fine after the 20% allogeneic serum perfusion. The staining for E-Selectin showed similar results as previously described in 5.5. Wild type (figure 18c) and transgenic (figure 18d) cells were stained for E-Selectin. The wild type cells in figure 18c showed a stronger

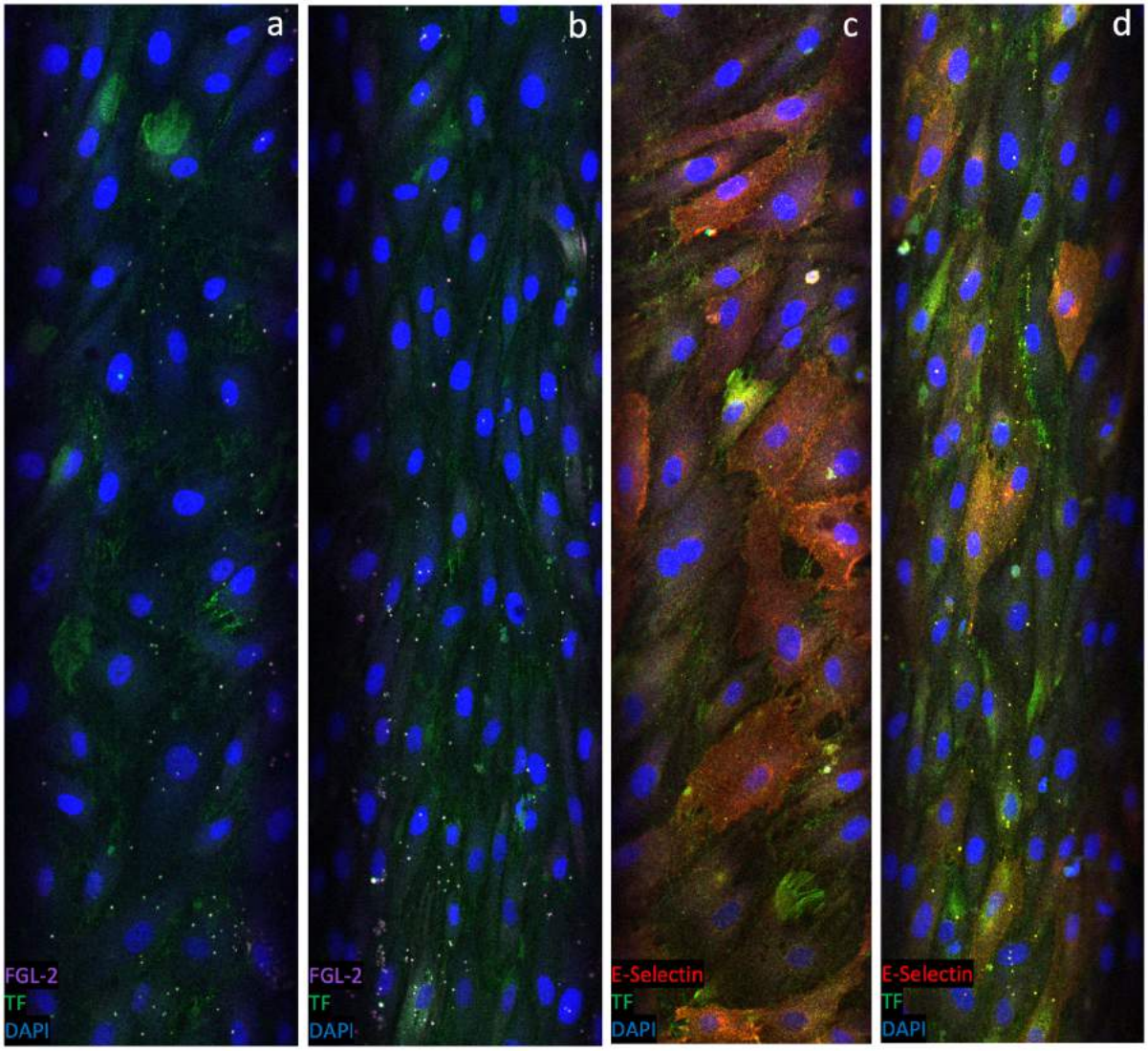


Figure 18: Expression of cell activation markers and coagulation markers on wild type and transgenic endothelial cells after two hours of 20% allogeneic serum perfusion. a) Expression of FGL-2 and TF on wt cells after two hours of allogeneic serum perfusion; **b)** Expression of FGL-2 and TF on tg cells after two hours of allogeneic serum perfusion; **c)** Expression of E-Selectin and TF on wt cells after two hours of allogeneic serum perfusion; **d)** Expression of E-selectin and TF on tg cells after two hours of allogeneic serum perfusion; the cells looked healthy in all microchannels and the perfusion was done after two days of 10 RPM (15 dyn/cm^2) flow exposure.

E-Selectin expression, compared to the transgenic cells in figure 18d. The tissue factor staining in figure 18a and 18b appears to be very dot-like and it is hard to say whether it is really specific or not. The same staining looks completely different in figure 18c and 18d. It has a perinuclear characteristic and looks less random and spread. It is not possible to say whether one cell type has a higher or lower tissue factor expression with this

staining. The FGL-2 staining in figure 18a and 18b did not work at all and we can not observe staining.

5.7. Complement deposition and endothelial cell alignment of wt and transgenic pig aortic endothelial cells after allogeneic serum perfusion

Both wild type and transgenic cells were perfused with 20% allogeneic serum perfusion for two hours. These cells were previously cultured under flow for two days at 10 RPM (15dyn/cm^2) before the perfusion with serum. Both C3b/c and C4b/c were stained and CD31 was stained to see the elongation and overall health of the endothelial cells. The staining for CD31 is strong and ubiquitously distributed in the microchannels in figure 19a and 19b.

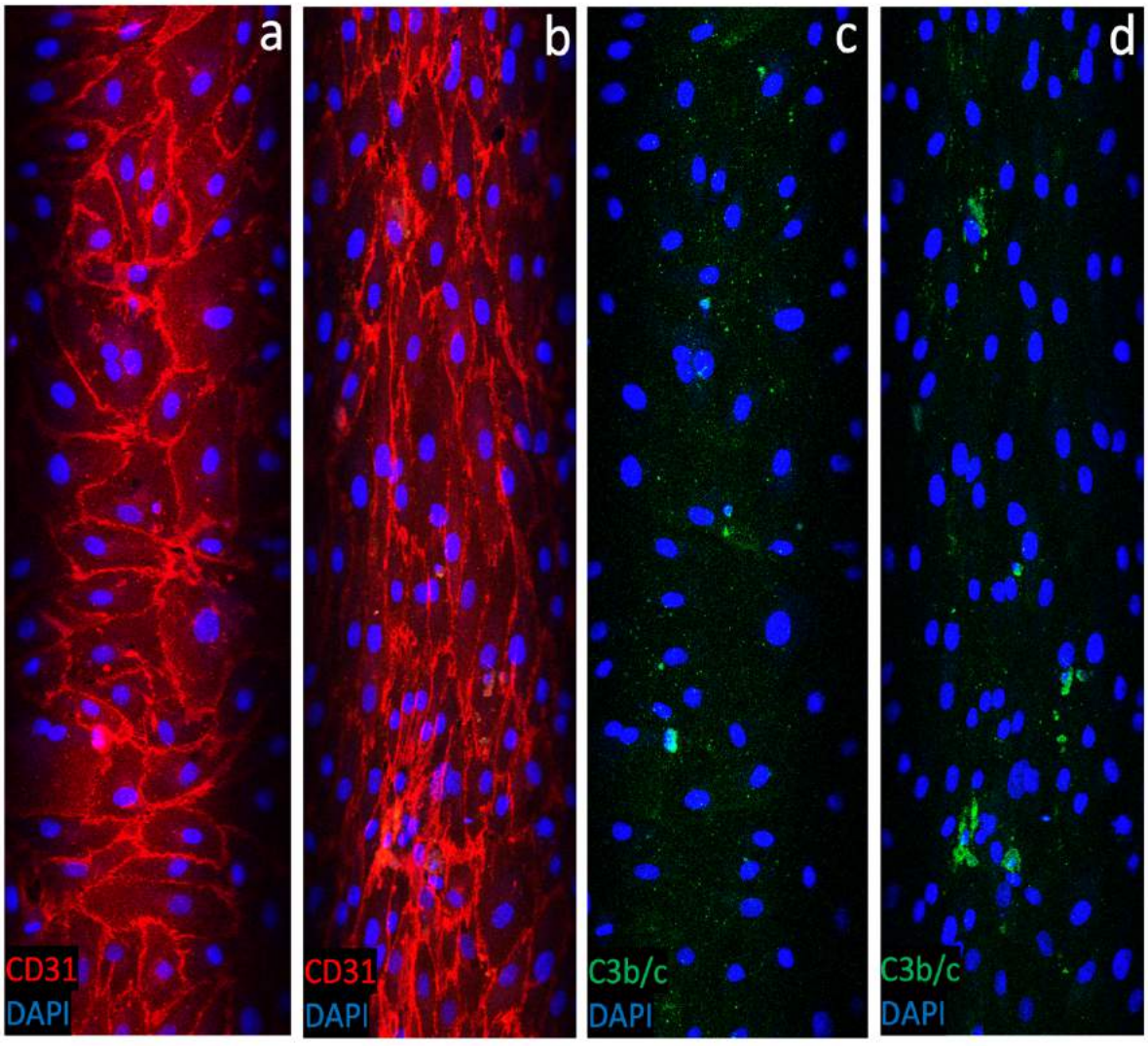


Figure 19: Wild type and transgenic PAEC stained for CD31 and C3b/c after 2 hours of 20% allogeneic serum perfusion. a) wt PAEC stained for CD31 and DAPI; **b)** tg PAEC stained for CD31 and DAPI; **c)**wt PAEC stained for C3b/c and DAPI ;**d)** tg PAEC stained for C3b/c and DAPI; the cells were two days under flow at 10 rpm (15dyn/cm^2) and were then perfused with 20 % allogeneic serum for two hours. The cells looked healthy overall after the perfusion.

The microchannels are still covered with a monolayer of endothelial cells after the serum perfusion and the overall health of the cells looks perfectly fine. The transgenic cells in figure 19b show a better elongation and alignment compared to the wild type cells in

figure 19a. In terms of complement deposition, there is barely any C3b/c staining visible in the wild type cell line in figure 19c except for single apoptotic cells. The transgenic cells show more C3b/c staining in figure 19d but the staining does not look very specific since it is not really cell associated and looks very random.

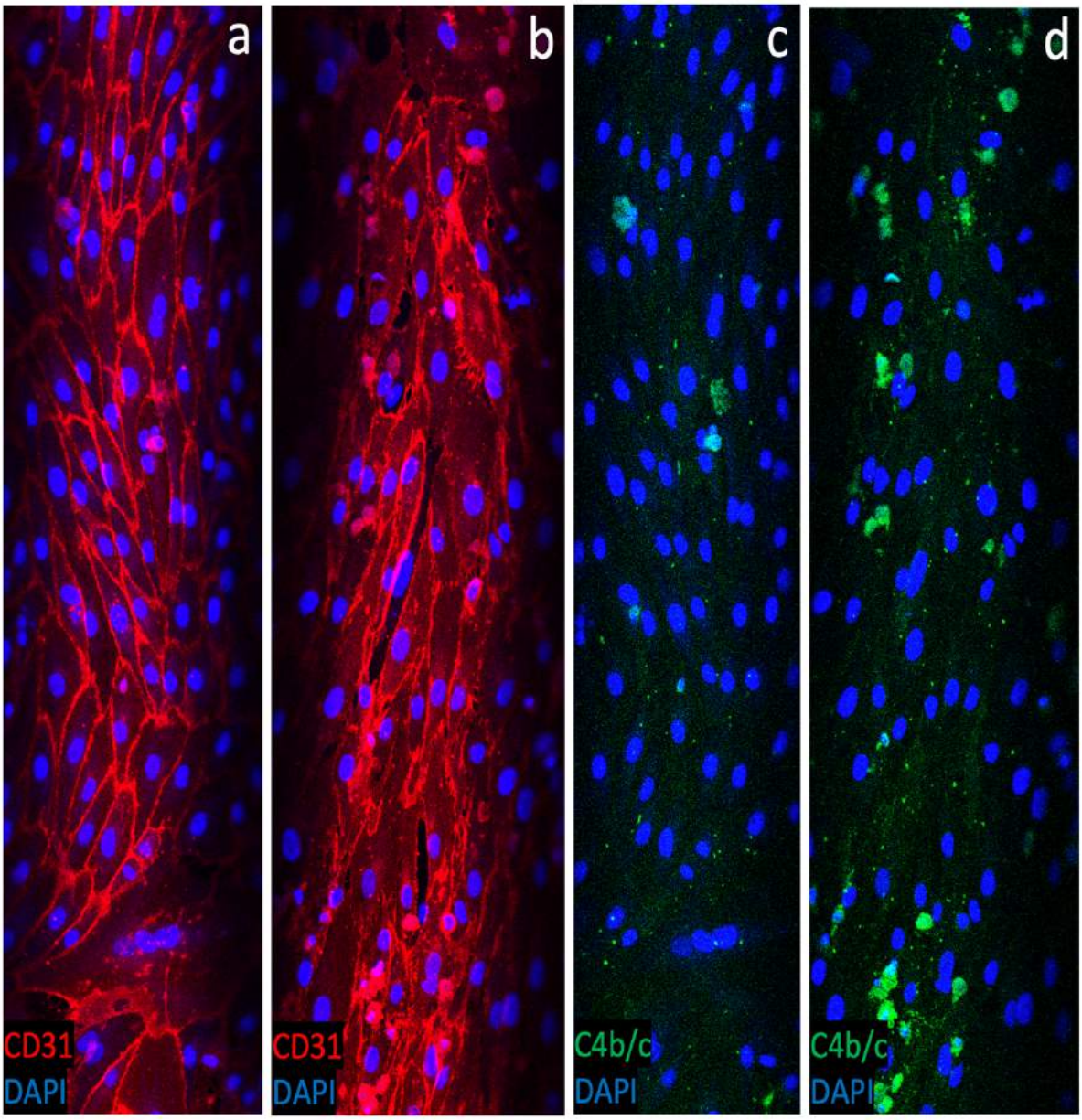


Figure 20: Wild type and transgenic PAEC stained for CD31 and C4b/c after 2 hours of 20% allogeneic serum perfusion. a) wt PAEC stained for CD31 and DAPI; **b)** tg PAEC stained for CD31 and DAPI; **c)**wt PAEC stained for C4b/c and DAPI ;**d)** tg PAEC stained for C4b/c and DAPI; the cells were two days under flow at 10 rpm (15dyn/cm^2) and were then perfused with 20 % allogeneic serum for two hours. The cells looked healthy overall after the perfusion.

The CD31 staining in figure 20a and 20b is again evenly distributed and a monolayer of endothelial cells is still present after the perfusion. The elongation of the wild type cells (figure 20a) is better than in figure 19a and is more comparable to the transgenic cells in figure 20b. Overall the cells look very healthy after the perfusion. The C4b/c staining on the wild type cells in figure 20c is again very minor and comparable to the previously

observed C3b/c staining. The staining in figure 20d for the transgenic cells looks very fragmented and scattered.

5.8. Checking for the alpha gal antigen on wild type and transgenic pig aortic endothelial cells

We planned on using human anti gal antibodies to enhance complement deposition in our experiments. The transgenic cell line that we use did not have a α-gal KO and anti-gal antibodies should therefore bind to the alpha Gal antigen. We stained for this binding site in both the wild type and transgenic cells with a Lectin antibody. This antibody is able to bind to the alpha Gal antigen and can therefore be used to visualize this antigen. The transgenic cells (figure 21a) and the wild type cells (figure 21b) both clearly expressed this antigen epitope on the surface of the endothelial cells.

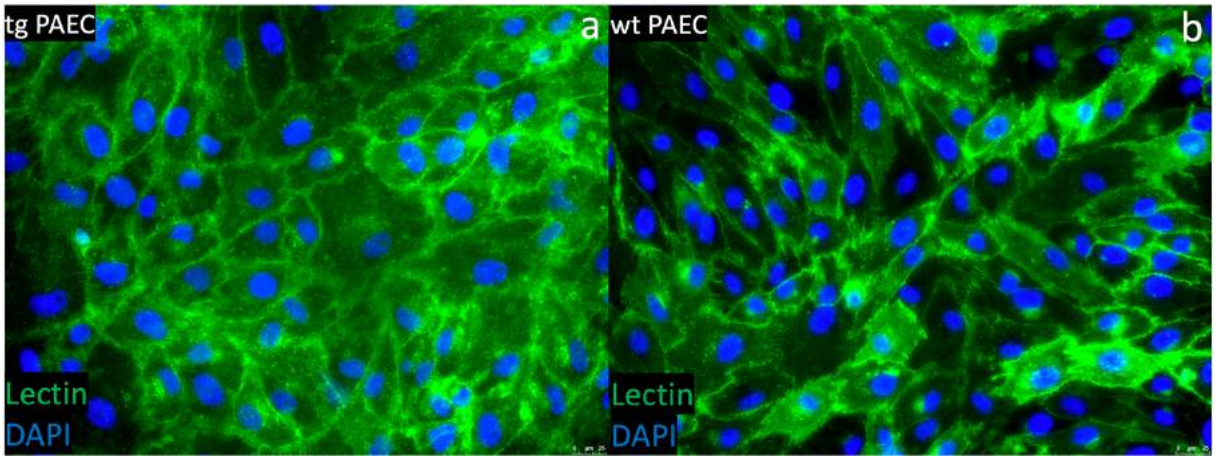


Figure 21: wild type and transgenic cells under static conditions stained for the alpha Gal antigen and DAPI. a) transgenic PAEC stained for the alpha Gal antigen and DAPI; **b)** wild type PAEC stained for the alpha Gal antigen and DAPI; the cells were cultured in a fibronectin coated glass slide chamberslide until confluency and then stained.

5.9. Complement deposition is lowered on transgenic pig endothelial cells compared to wild type pig endothelial cells when pig cells are pre-perfused with human anti-Gal

A pre-perfusion with human anti-gal antibody before the actual allogeneic serum perfusion was performed. The overall health of the cells after the double perfusion was good and no cell loss was observed. The C3b/c staining on the wild type cells in figure 23a was strongly expressed. The transgenic cells in figure 23b showed a massively lower C3b/c deposition when compared to the wild type cells. The wild type cells in figure 23c showed a high C4b/c deposition on almost all cells visible in the picture. The C4b/c staining on the transgenic cells in figure 23d was comparable to the C3b/c staining of this cell type. There was almost no visible staining for the transgenic cells. Compared to kind of signal we saw in 5.7 we now had a cell associated complement deposition.
Furthermore, statistical analysis of these data shown in figure 23 showed a significant difference for both stained complement markers.

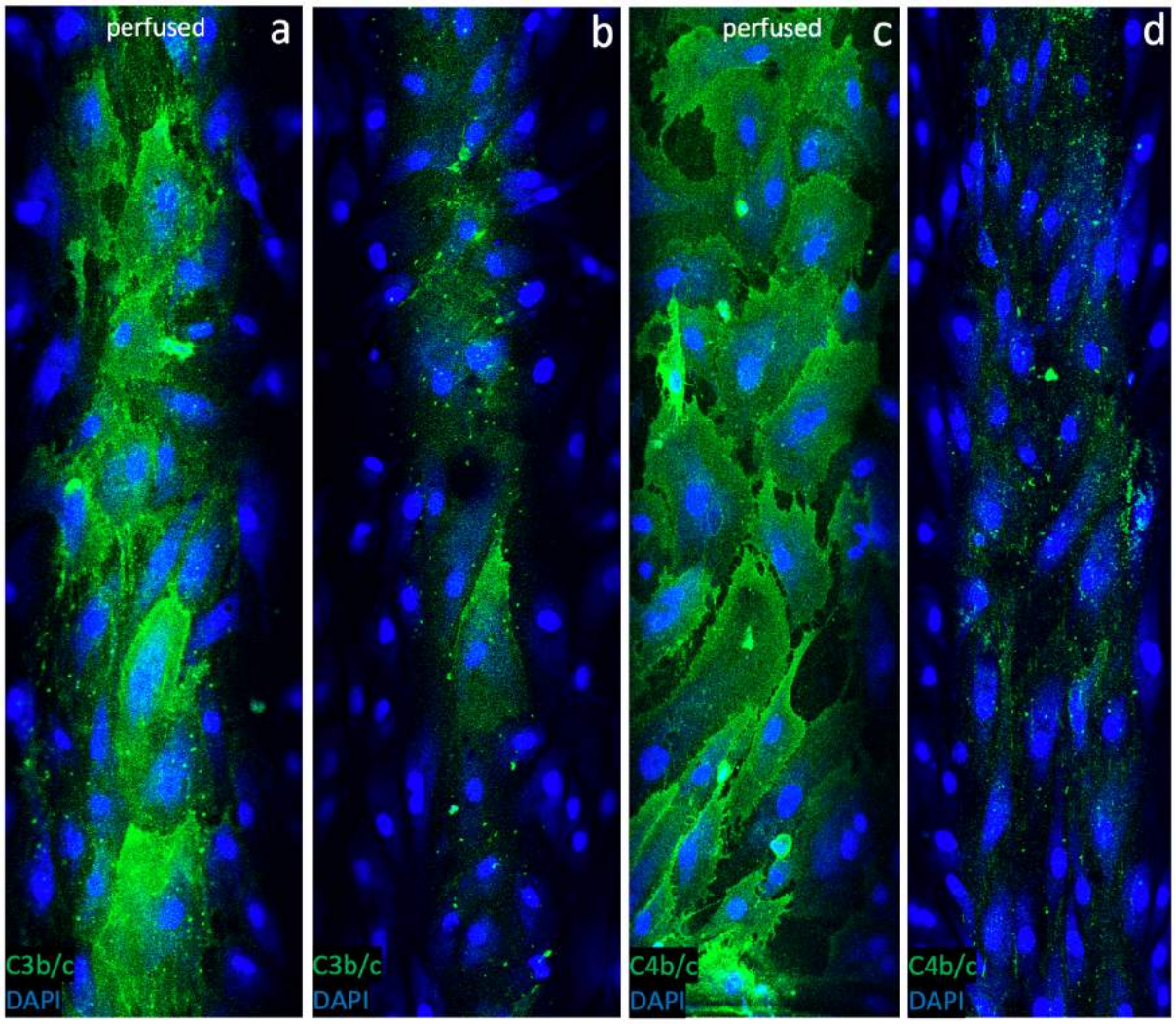


Figure 21: Complement deposition on wild type and transgenic PAEC after 30 minutes of human anti-gal antibody pre-perfusion and 2 hours of 20% allogeneic serum. a) wt PAEC stained for C3b/c and DAPI; **b)** tg PAEC stained for C3b/c and DAPI; **c)** wt PAEC stained for C4b/c and DAPI; **d)** tg PAEC stained for C4b/c and DAPI; Cells were cultured two days under flow at 10 RPM (15 dyn/cm^2) with daily medium exchange. The cells looked healthy after the perfusion with a slight cell loss in the wild type channels.

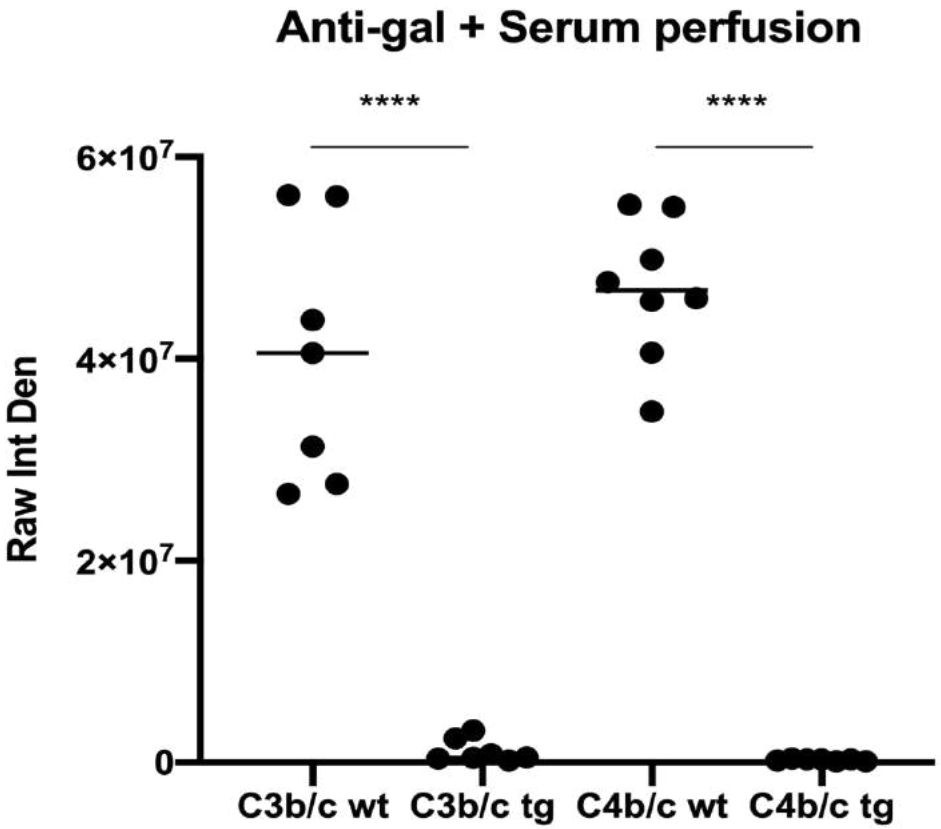


Figure 23: Statistical analysis of C3b/c and C4b/c deposition on both wild type and transgenic pig aortic endothelial cells after 30 minutes of human anti-gal antibody pre-perfusion and 2 hours of 20% allogeneic serum perfusion. Each dot represents a picture on a channel. At least 5 pictures were taken for each channel and one channel per group was analysed for this experiment.

5.10. Checking the complement deposition on wt and transgenic pig aortic endothelial cells after endothelial cell activation

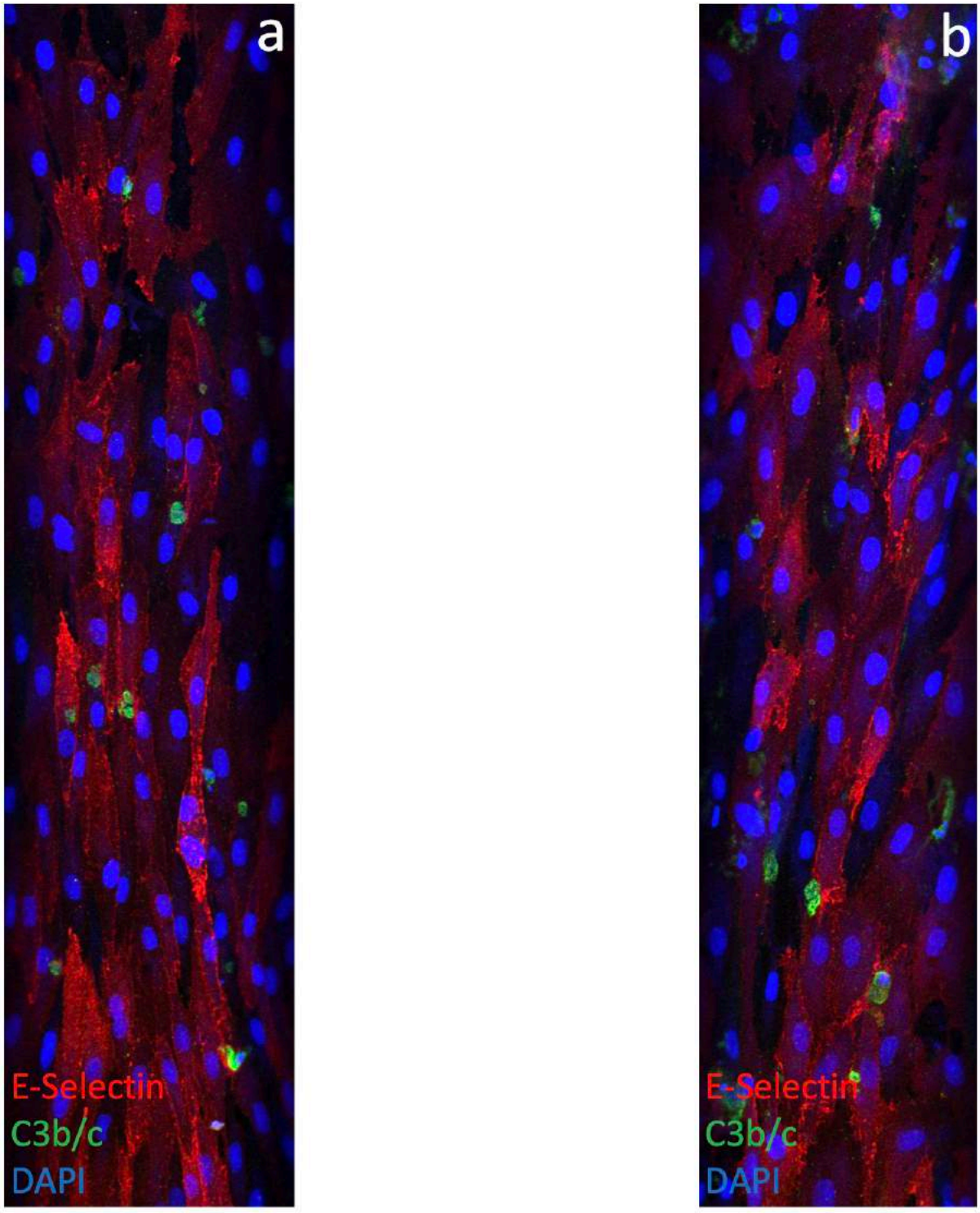


Figure 24: Staining for complement deposition and endothelial cell activation after perfusion with TNF-α and 20 % allogeneic serum. a)wild type PAEC stained for E-Selectin and C3b/c ; **b)** transgenic PAEC stained for E-Selectin and C3b/c; Cells were cultured for two days under flow at 10 RPM (15 dyn/cm^2) with daily medium exchange. Perfusion with 20μg/ml TNF-α for 4 hours was followed by a 2 hour 20% allogeneic serum perfusion.

Wild type and transgenic PAEC were perfused with 20 μg/ml TNF-α for 4 hours to check for differences in endothelial cell activation as well as complement deposition.
The cells were perfused with 20% allogeneic serum for two hours right after the TNF-α perfusion. Overall, the cells look still very healthy with almost no cell detachment even after the long exposure to TNF-α. The staining for E-Selectin shows high endothelial cell activation for both wild type (figure 24a) and transgenic (figure 24b) endothelial cells. There is no real difference observable in the levels of activation between the two different cell types. The C3b/c staining for both cell types looked similar to the staining we observed earlier in the first trials of complement stainings. There are single apoptotic cells visible in both cell types with the respective complement staining around it but other than that, the staining is not cell associated and appears random and not specific. It is nowhere comparable to the clear staining we obtained in the pre-perfusion experiment.

5.11. Assessing complement deposition on wild type and transgenic pig aortic endothelial cells after endothelial cell activation and perfusion with anti-gal and allogeneic serum

The experiment was divided into four different groups: TNF-α, anti-gal antibodies and allogeneic serum perfusion; TNF-α and allogeneic pig serum perfusion; TNF-α and normal human serum perfusion; TNF-α, anti-gal antibodies and heat inactivated allogeneic serum perfusion.

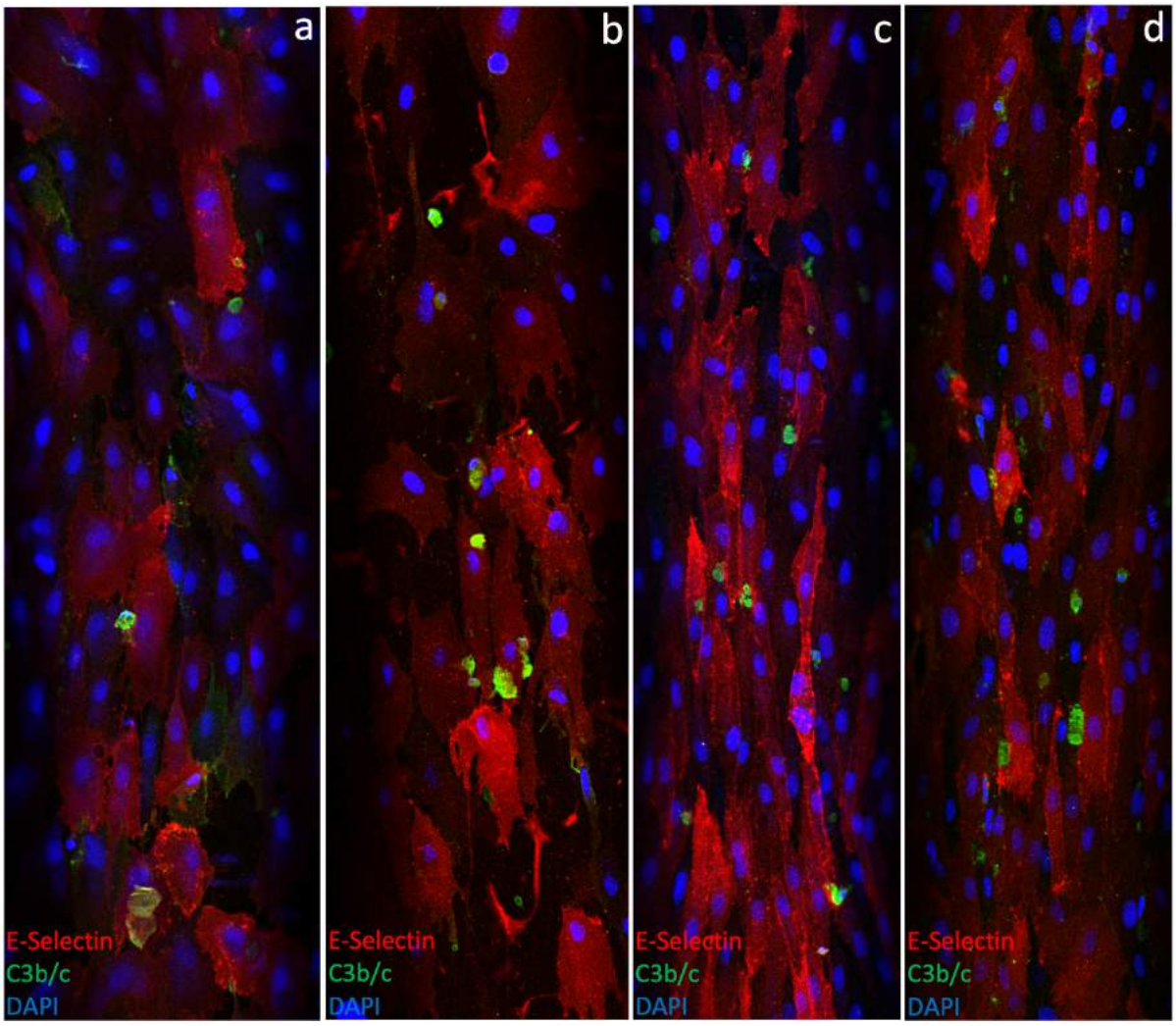


Figure 25: Staining for E-Selectin and C3b/c on wild type and transgenic PAEC after perfusion with TNF-α, anti-gal antibodies and 20% allogeneic pig serum or TNF-α and 20% allogeneic pig serum. **a)** wt PAEC perfused with TNF-α, anti-gal antibodies and allogeneic pig serum and stained for E-Selectin and C3b/c; **b)** tg PAEC perfused with TNF-α, anti-gal antibodies and allogeneic pig serum and stained for E-Selectin and C3b/c; **c)** wt PAEC perfused with TNF-α and allogeneic pig serum and stained for E-Selectin and C3b/c; **d)** tg PAEC perfused with TNF-α and allogeneic pig serum and stained for E-Selectin and C3b/c; Cells were previously cultured under flow at 10 RPM (15 dyn/cm²) for two days with daily medium exchange.

TNF-α was perfused for 4 hours, anti-gal antibodies for 30 minutes and the serum was perfused for 2 hours and diluted to 20% for allogeneic serum and 10% for normal human serum.
The perfusion with normal human serum worked as a positive control and the perfusion with the heat-inactivated pig serum was the negative control.

Overall the cells were healthy, and monolayers were still present after the perfusions except for the normal human serum perfusion in figure 26a and 26b and the transgenic cells that were perfused with TNF-α, anti-gal antibodies and allogeneic pig serum in figure 25b. In figure 25a and 25b, the E-Selectin staining is strongly expressed, and the complement staining is very concentrated but again not very cell associated. We can observe some cell loss in figure 25b and the cells are slightly more activated compared to the respective wild type channel in figure 25a. In figure 25c and 25d the staining looks identical to the staining from the first TNF-α experiment.

The E-Selectin staining is very strongly present, and the complement staining is barely present and very concentrated and not really cell associated.

The E-Selectin staining is no longer visible in figure 26a and 26b. A lot of cells detached and there is no longer a monolayer of endothelial cells visible. The wild type channel in figure 26a has lost more cells compared to the transgenic channel in figure 26b. The C3b/c staining is heavily expressed in both channels and is present all over the cell.

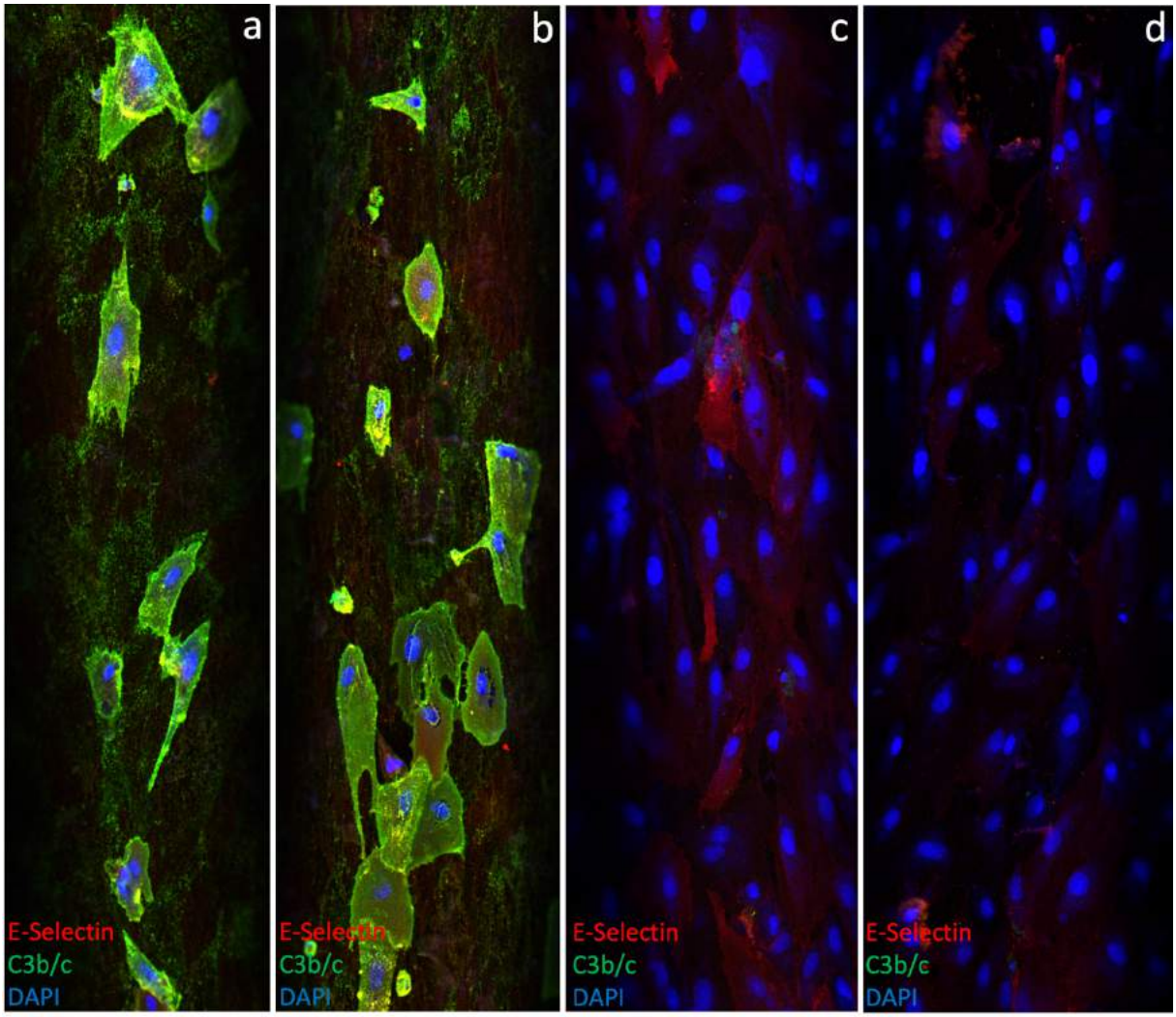


Figure 26: Staining for E-Selectin and C3b/c on wild type and transgenic PAEC after perfusion with TNF-α, anti-gal antibodies and 20% heat inactivated allogeneic pig serum or TNF-α and 10% normal human serum (NHS). **a)** wt PAEC perfused with TNF-α and NHS and stained for E-Selectin and C3b/c; **b)** tg PAEC perfused with TNF-α and NHS and stained for E-Selectin and C3b/c; **c)** wt PAEC perfused with TNF-α, anti-gal antibodies and heat inactivated allogeneic pig serum and stained for E-Selectin and C3b/c; **d)** tg PAEC perfused with TNF-α, anti-gal antibodies and heat inactivated allogeneic pig serum and stained for E-Selectin and C3b/c; Cells were previously cultured under flow at 10 RPM (15 dyn/cm^2) for two days with daily medium exchange.

This staining is comparable to the staining observed in experiment 5.9.. The cells in figure 26c and 26d are still forming a monolayer of endothelial cells. The E-Selectin staining in these two channels is weak and a slightly higher staining is observable in the wild type cells. There is overall no C3b/c staining visible in figure 26c and 26d.

6. Discussion

It was essential to first characterize the cells thoroughly to evaluate the purity and to ensure that the transgenes and the typical endothelial cell markers are expressed ubiquitously. We were able to say, that the wild type pig aortic endothelial cells and the transgenic (hCD46 / HLA-E) pig aortic endothelial cells clearly expressed endothelial cell markers such as CD31 and VE-Cadherin after visualizing and analysing the immunofluorescence stainings that we performed.
Furthermore, we stained for the transgenes in the transgenic cells to confirm the presence and to check the expression levels of said transgenes. The results from the hCD46 and HLA-E staining on a microchip showed that both transgenes were evenly expressed in the transgenic cell line under physiological flow conditions. The hCD46 staining showed no background in the wt channel staining, highlighting the specificity of the antibody to its target. The hCD46 expression was clearly localized on the cell surface of the transgenic endothelial cells while the HLA-E staining was more scattered and present on the cell surface. We did not take any 3D pictures of the transgene expression and are thus unable to say whether the staining is also present within the cell or only on the cell surface. It was previously shown, that HLA-E staining in human endothelial cells was localized on the outer surface of endothelial cells as well as intracellularly in a perinuclear distribution[118]. The surface staining and the perinuclear staining was rather weak and we had a high background in the HLA-E staining on the transgenic cells compared to the hCD46 staining. The high background is also visible in the HLA-E staining of the wt channel. Overall, we had a lower specificity of the HLA-E staining, suggesting that we should have permeabilized the cells and used a different antibody that has less background staining. Since we were only interested in the presence of the staining and not the level of the staining we continued using this antibody.

We then continued characterization with a porcine and human CD46 expression staining on glass chamber slides and under flow conditions in a microchip. The results of the static chamber slide staining showed again a hCD46 staining all over the surface of the transgenic cells, confirming the previously obtained 'under flow' results. We did not measure any staining of hCD46 on the wild type cells, underlining the specificity of the antibody for human CD46. The porcine CD46 staining was similar for both cell types and just like with the human CD46, the expression was mainly concentrated on the cell surface. This staining pattern is identical in our microchip under more physiological conditions. The pCD46 is expressed universally over the cell surface of both cell types and on the other hand the hCD46 is only expressed in transgenic cells. We were again not able to measure any signal of hCD46 staining for the wild type cells, confirming again the specificity of the antibody in a different experimental setting.

As mentioned in the hypothesis and aim of the study, we were interested in the function of the introduced transgene in the transgenic porcine aortic endothelial cells with a special emphasis on the hCD46 gene. We started checking for the beneficial activity of hCD46 in terms of endothelial cell activation by perfusing both wild type and transgenic PAEC with allogeneic pig serum and stained for E-Selectin. E-Selectin is an important marker of endothelial cell activation. The E-Selectin staining on the perfused transgenic cells was less strong compared to the perfused wild type cells and when statistically analysed resulted in a significant difference. However, also the baseline expression of E-selectin on transgenic cells was already lower than the one observed on wild type PAEC. Because cell culture conditions *per se* cause stress for the cells (reference), we conclude

that the additional hCD46 expression of the transgenic PAEC leads to the expression of a more resting, healthy endothelial cell phenotype *in vitro* as compared to the wild type cells. Overall, we could prove that the transgene expression is beneficial at keeping the endothelial cells in a more quiescent state after allogeneic serum perfusion.

We then switched our focus to coagulation markers and wanted to see if the transgene has also a beneficial effect in terms of the regulation of coagulation. We perfused wild type and transgenic cells again with allogeneic serum and stained for E-Selectin, Tissue factor and FGL-2. The previously seen trend of transgenic cells being better in regulating the cell activation than wild type cells was observed again. The transgenic cells were barely activated and the wild type cells were full on activated throughout the whole channel. The Tissue factor and FGL-2 staining did not work out well. The Tissue factor staining was all dot-like and did not look specific while the FGL-2 staining was not present at all. The main issue with this staining is, that we used allogeneic serum as a perfusate. The majority of coagulation proteins are not in the serum but in the plasma. We cannot expect to see the same signal as with a plasma or even whole-blood perfusion when we perfuse our system with serum. We were not able to perfuse with plasma or whole blood because it is not possible to use these with our system as of right now due to clotting problems within the tubings. Furthermore, we would have to have damage to the endothelial cell layer to expose tissue factor on the surface of the endothelial cells. As long as the endothelial cells in our microchips are healthy and quiescent, we do not expect to see any possible tissue factor binding.

We measured complement binding on the two endothelial cell types also by perfusing the microchips with allogeneic pig serum and stained for C3b/c and C4b/c. Deposition of these two complement components is regulated by the membrane cofactor protein CD46. We expected to see major differences between the two cell lines, since the transgenic cell line has also the human CD46 as a regulator expressed. The complement staining in this experimental set up is for both the C3b/c and C4b/c has a quite high background staining that could be explained due to the FITC labelled antibody that we used which tends to have a high background, especially with an overall weak staining like we had in our setting. We did not expect to see a high staining since we used pig serum on pig cells. The staining is not really cell associated and looks random. We are still not certain what exactly activated the complement since the source of complement would have been the allogeneic pig serum and that would not lead to a high complement deposition. We can only speculate and say that low flow, activation of the coagulation or something up to this point unknown to us could have potentially led to this complement deposition.

We wanted to do a pre-perfusion with human anti-Gal antibodies, that would then bind to the carbohydrate epitope galactose-α1,3-galactorse (Gal) and would then simulate the immunological reaction of a hyperacute rejection, thus inducing strong complement deposition[7,40]. This would mean, that the source of complement proteins would still be the allogeneic pig serum, whereas the human anti gal antibodies would hopefully enhance the complement staining due to their nature of binding to the Gal epitope.

We first had to confirm the presence of the alpha-Gal carbohydrate epitope on the surface of our endothelial cells. We performed an immunofluorescence staining in fibronectin coated glass chamber slides on both wild type and transgenic pig aortic endothelial cells and stained for the anti-Gal antigen with the help of a Lectin antibody that binds to it. This

antigen was present ubiquitously on both cell lines and was concentrated on the cell surface of the endothelial cells.

After confirming the presence of this antigen, we then isolated human anti-Gal antibodies from serum in our lab and pre-perfused the microchannels for 30 minutes and followed that with the regular two hour perfusion with allogeneic pig serum. We stained again for C3b/c and C4b/c as complement markers. The resulted staining was more intense and clearer and very cell associated. There was a striking difference between both the C3b/c and C4b/c staining of the wild type and the transgenic cells. The wild type cells had a very clear C3b/c and C4b/c staining that was all over the endothelial cell in the microchannels, whereas the transgenic cells had only minimal staining on single cells for the C3b/c staining and no real staining for the C4b/c. The statistical analysis underlined the result that was visible by eye. There was a highly significant difference between the C3b/c and C4b/c deposition on the wild type and transgenic endothelial cells, clearly showing that the transgenic cells were strikingly better at regulating the complement deposition. This was a huge difference compared to the previously obtained complement staining and the human anti-Gal antibodies enhanced the staining immensely. The source of complement proteins was still the allogeneic serum and the human anti gal antibodies worked as a kick-starter for the complement deposition. This result was according to our hypothesis that the hCD46 was beneficial for the complement regulation in the allogeneic setting.

We then repeated the same experiment several times and had issues with the health of the cells and the specificity of the staining, thus replicating the results we obtained was difficult. We tried to find a more reliable experimental set up that would be easier to replicate. The previously mentioned issues led to adapting our experimental set up and restarting with measuring the endothelial cell activation but this time we perfused with TNF-α to enhance the level of cell activation, followed by the allogeneic serum perfusion and additionally stained for C3b/c deposition. We chose four hours of TNF-α perfusion to a previous study, that showed peak E-Selectin expression of endothelial cells after four hours of TNF-α perfusion[119]. The wild type and transgenic cells were clearly activated after the TNF-α perfusion and the overall health of the cells was good. We could not see a difference between the activation level of wild type and transgenic cells after the TNF-α stimulation. This can be due to the perfusion with TNF-α that leads to maximal expression of E-Selectin at four hours perfusion time, thus resulting in no difference since the cells will be fully activated at this time point regardless of their ability to regulate cell activation. The complement staining was now again similar to the first trials that we performed where we had a scattered non cell-based staining that did not look specific at all. We expected to see a lower complement deposition, since we did not have the anti-gal antibodies that boost the complement deposition. We performed this TNF-α perfusion to see if cell-activation alone with the addition of allogeneic pig serum perfusion as complement protein source was enough to trigger complement deposition, but we were not successful.

7. Conclusion

Our microfluidic system is a recirculating closed *in vitro* system that allows for the usage of a higher amount of allogeneic serum thus resembling the *in vivo* situation where an indefinite amount of complement is circulating. Standard flatbed culture *in vitro* systems are limited by the volumes of allogeneic serum and cells are cultured under static conditions in these culturing methods. The microfluidic system allows for culturing of cells under a pulsatile flow and exposes the cells to shear stress. All these previously mentioned perks resemble the *in vivo* situation more closely compared to standard culture systems. We were able to observe allogeneic complement deposition in an *in vitro* system after perfusing the cells with human anti-gal antibodies and allogeneic pig serum. We observed a tremendous difference in the ability of wild type pig cells and pig cells transgenic for human CD46 and HLA-E to regulate the complement deposition after the previously mentioned perfusion. The transgenic cells were noticeably better at regulating the complement deposition, thus brining us to the conclusion that human CD46 participates in the cleavage of porcine complement proteins. This study shows the beneficial effect of the overexpression of human CD46 in preventing the complement activation in an allogeneic perfusion situation.
Our results suggest that human CD46 is able to cleave porcine C3b and C4b, confirming earlier data obtained in acute myocardial infarction experiments with hCD46 transgenic pigs performed by our group. Furthermore, we are able to show the beneficial effect of overexpressing human CD46 in preventing the complement activation in an allogeneic perfusion situation.

8. Outlook

We will have to conduct further experiments and try to replicate our results to obtain a sufficient n-number and perform valid statistical analysis. This will allow us to show the discrepancy in the ability of wild type and transgenic PAEC in regulating the complement deposition. We have to keep in mind that the transgenic cells have the gene for porcine CD46 and human CD46. We will have to perform more experiments and analyse if the combination of porcine and human CD46 is better in an allogeneic perfusion setting in regard to complement deposition. We then have to test transgenic PAEC with pCD46 knockout and only the human CD46 gene and compare them to the cells with porcine and human CD46 as well as the wild type. After these experiments we will be able to say if the porcine CD46 in combination with the human CD46 or the human CD46 alone is better to regulate the complement deposition in an allogeneic perfusion setting.

9. References

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11. Declaration of consent

Declaration of consent

on the basis of Article 30 of the RSL Phil.-nat. 18

Name/First Name:	Luther Fabian
Registration Number:	14-204-184
Study program:	Master of Science in Molecular Life Sciences with special qualification in Microbiology/Immunology

Bachelor ☐ Master ☒ Dissertation ☐

Title of thesis:	Assessment of the protective role of human CD46 on allogeneic complement activation in vitro
Supervisor:	Prof. Dr. R. Rieben Dr. phil. nat. R. Sfriso Dr. phil. nat. N. Sorvillo

I declare herewith that this thesis is my own work and that I have not used any sources other than those stated. I have indicated the adoption of quotations as well as thoughts taken from other authors as such in the thesis. I am aware that the Senate pursuant to Article 36 paragraph 1 litera r of the University Act of 5 September, 1996 is authorized to revoke the title awarded on the basis of this thesis.
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