Doctoral Thesis

Human leukocyte recruitment to the porcine endothelium implications for xenotransplantation

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Human Leukocyte Recruitment to the Porcine Endothelium: Implications for Xenotransplantation

A dissertation submitted to the
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presented by

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1. **SUMMARY**

The recent success with reversal of diabetes using porcine islets cells in a nonhuman primate model has demonstrated the great potential of xenotransplantation as an alternative to the limited supply of cells or organs in allotransplantation. For solid organs, progress has been slower, despite the availability of genetically altered pigs. For several reasons, the miniature swine was chosen as a suitable donor organ candidate for xenotransplantation. However, immunological barriers in pig-to primate xenotransplantation are not easily overcome. Porcine vascularised xenografts are hyperacutely rejected due to xenoreactive natural antibodies predominantly directed against the galactose-α(1,3)galactose (αGal) epitope expressed on most porcine cells, but absent on human cells. Nevertheless, one of the major advantages of pigs as donors is the possibility for genetic manipulation. The generation of αGal-KO pigs in 2002 represented a major progress toward the control of hyperacute vascular rejection, shifting the focus of immunological research to the next barriers: acute humoral and cellular rejection, and physiological incompatibilities such as the disparities in the coagulation system. One hallmark of acute cellular rejection is graft infiltration with different leukocyte subsets. Several *in vitro* studies characterized the cross-species receptor-ligand interactions mediating human leukocyte adhesion to porcine endothelial cells. However, little is known about the mechanism and receptor compatibilities involved in chemotaxis and transmigration from the vascular endothelium to the organ.

The potential for xenozoonosis represents a further major concern. Much attention has been given to donor-derived infections, neglecting host-derived infections with potential dire consequences for the graft. Reactivation of human cytomegalovirus (HCMV) is one of the most common infectious complications in transplantation medicine and has been associated with allograft rejection. Given the intense immunosuppression, HCMV reactivation can be expected in potential pig-to-human xenotransplantation trials. In preclinical pig-to-baboon xenotransplantation models, both porcine and baboon CMV reactivation have been observed leading to systemic coagulation abnormalities.

The intrinsic ability of the xenograft to resist the potential injuries mediated by graft infiltration and HCMV infection, and the following immune response is crucial for maintaining graft haemostasis. Many of the injuring factors involved in the rejection mechanism have a proinflammatory, cytokine-mediated component. Suppressor of cytokine signalling (SOCS) proteins were recently found as important regulators of cytokine signalling.
SOCS1 and SOCS3 are important negative feedback regulators of IFN-γ and IL-6, two cytokines implicated in graft injury.

The aims of this thesis were (i) to investigate the role of αGal, and the molecular mechanisms of interactions between human leukocyte subsets and αGal-negative porcine endothelial cells (PAEC-KO) during leukocyte recruitment to the endothelium, (ii) to analyze the influence of HCMV cross-species infection on PAEC-KO in terms of cell surface receptor expression and human leukocyte recruitment, and (iii) to establish a porcine-specific SOCS1 and SOCS3 detection system in order to quantify SOCS mRNA expression in allo- and xenotransplantation and after HCMV infection.

The results of this thesis demonstrate that there are no differences between PAEC expressing or lacking αGal in their ability to mediate human peripheral blood mononuclear cells (PBMC) chemotaxis and transendothelial migration (TEM), excluding a crucial role for αGal in leukocyte recruitment. In our in vitro model, which differentially measured adhesion and TEM, we documented a role for CD18 and CD99 receptors in human leukocyte TEM across PAEC-KO.

Cross-species infection of PAEC-KO by HCMV induced cytokine release, a modulation of cell surface receptor expression, and increased human leukocyte adhesion and chemotaxis. Finally, quantitative real time PCR conditions allowing to discriminate between the porcine and human SOCS1 and SOCS3 mRNA sequences and to quantify SOCS expression were established. With this tool we documented a strong increase in SOCS1 amplification in HCMV-infected PAEC-KO cultures.

Despite the significant progress achieved in the last years in xenotransplantation, the immunological, physiological, and coagulation barriers, and the concern for potential cross-species infections remain. Progress in genetical engineering will allow the production of αGal-KO pigs expressing protective transgenes for the graft, such as anti-coagulant genes and complement-regulatory proteins. Further efforts are currently directed towards the identification of additional targets to reduce cellular rejection. In this context, our results suggest that CD18 and CD99, or their respective porcine ligands, may constitute attractive targets for future therapeutical interventions aiming at specifically suppressing cellular infiltration. In addition, the results stress the importance to consider the consequences of HCMV cross-species infection of porcine xenografts in future clinical trials. Further studies
are needed in order to understand the mechanisms of infection and prevent graft damage. In this context, the role of SOCS proteins in the control of inflammation is of interest.

In conclusion, extensive efforts and investigations are required before solid organ xenotransplantation can become a clinical reality, and a solution to overcome the severe shortage of human organs.
2. RIASSUNTO

Il recente successo ottenuto con l’inversione del diabete usando cellule-β porcine in un modello primato non-umano, ha dimostrato l’enorme potenziale dello xenotrapianto come alternativa alla grave carenza di cellule e organi nell’allotrapianto. Nello xenotrapianto di organi solidi il progresso è stato più lento, malgrado la disponibilità di maiali alterati geneticamente.

Per varie ragioni il maiale è stato scelto come donatore appropriato di organi nello xenotrapianto. Tuttavia le barriere immunologiche nello xenotrapianto tra maiale-umano non sono facili da superare. Gli xenotrapianti porcini vascularizzati vengono rigettati in modo iperacuto a causa di anticorpi naturali xenoreattivi presenti nella circolazione sanguinea, diretti principalmente contro l’epitopo galattosio-α(1,3)galattosio (αGal), espresso sulla maggioranza delle cellule porcine ma assente su quelle umane. Ciò nonostante uno dei maggiori vantaggi del maiale come donatore è la possibilità della manipolazione genetica. La generazione di maiali non esprimenti αGal (αGal-KO) nel 2002 rappresentò il maggior progresso verso il controllo del rigetto vascolare iperacuto. Di conseguenza il centro della ricerca immunologica si è spostato verso le barriere successive: il rigetto acuto umorale e il rigetto cellulare e le incompatibilità fisiologiche come ad esempio quelle presenti nel sistema di coagulazione. Una caratteristica del rigetto acuto è l’infiltrazione del trapianto da parte di diverse sottoclassi di leucociti. Mentre le interazioni cross-specifiche (tra diverse specie) tra ricettore-ligando proemoventi l’adesione di leucociti umani alle cellule endoteliali porcine sono state caratterizzate in vitro, si conosce poco sui meccanismi e le compatibilità tra ricettori proemoventi chemiotassi e diapedesi dall’endotelio vascolare all’organo sottostante.

Il potenziale per xenozoonosi rappresenta un’ulteriore importante preoccupazione. È stata prestata molta attenzione alle infezioni derivate dal donatore, trascurando le infezioni derivate dal ricevente con possibili gravi conseguenze per il trapianto. La riattivazione del citomegalovirus umano (HCMV) è una delle complicazioni infettive più comuni nella medicina del trapianto ed è stata associata al rigetto. Data l’intensa immunosuppressione, ci si può aspettare che avvenga la riattivazione dell’HCMV in potenziali sperimentazioni di xenotrapianti tra maiale-umano. Nel modello preclinico di xenotrapianto tra maiale-babbuino, si è osservata la riattivazione di entrambi i CMV, porcino e babbuino, portando ad anomalie sistemiche nella coagulazione.
L’abilità intrinseca dello xenotrapianto nel resistere le potenziali lesioni provocate dall’infiltrazione del trapianto o dall’infezione con l’HCMV, e la successiva risposta immunitaria, è cruciale per mantenere l’omeostasi del trapianto. Diversi fattori danneggianti coinvolti nel meccanismo di rigetto hanno una componente pro-infiammatoria, mediata da citochine. Le proteine SOCS (suppressor of cytokine signalling) sono state scoperte recentemente come importanti regolatori della segnalazione delle citochine. SOCS1 e SOCS3 sono importanti regolatori retroattivi negativi di IFNγ e IL-6, due citochine implicate nel danneggiamento del trapianto.

Gli scopi di questa tesi erano (i) indagare il ruolo di αGal, e i meccanismi molecolari d’interazione tra le frazioni di leucociti umani e le cellule endoteliali porcine non-esprimenti αGal (PAEC-KO) durante il reclutamento dei leucociti verso l’endotelio, (2) analizzare l’influenza dell’infezione cross-specifica dell’HCMV nelle PAEC-KO in termini di espressione di ricettori superficiali cellulari e del reclutamento dei leucociti umani, e (3) stabilire un sistema di rilevamento specifico per le porcine SOCS1 e SOCS3, per poter quantificare l’espressione dell’mRNA delle SOCS negli allo- e xenotrapianti e dopo l’infezione con l’HCMV.

I risultati di questa tesi dimostrano che non ci sono differenze tra PAEC esprimenti o carenti αGal nella loro abilità nell’indurre chemotassi e diapedesi, escludendo quindi un ruolo cruciale di αGal nel reclutamento di leucociti umani. Nel nostro modello in vitro, separante adesione da diapedesi, abbiamo documentato un ruolo dei ricettori CD18 e CD99 nella diapedesi dei leucociti umani attraverso le PAEC-KO.

L’infezione cross-specifica delle PAEC-KO da parte dell’HCMV ha indotto la secrezione di citochine, la modulazione dell’espressione dei ricettori superficiali cellulari, e un aumento dell’adesione e della chemotassi.

Infine, abbiamo stabilito un sistema di PCR quantitativa che ci permette di discriminare tra le sequenze porcine e umane di SOCS1 e SOCS3 e di quantificare l’espressione del loro mRNA. Con questo strumento abbiamo documentato un forte aumento dell’amplificazione di SOCS1 nelle culture PAEC-KO infette dall’HCMV.

Malgrado l’importante progresso raggiunto negli ultimi anni nello xenotrapianto, rimangono barriere immunologiche, fisiologiche e della coagulazione, e la preoccupazione per potenziali infezioni cross-specifiche. Progressi nell’ingegneria genetica permetteranno la produzione di
maiali αGal-KO esprimenti transgeni protettivi per il trapianto, come ad esempio geni anti-coagulanti e proteine regolatrici del sistema di complemento. Ulteriori sforzi sono attualmente diretti all’identificazione di nuovi bersagli per ridurre il rigetto acuto. In questo contesto, i nostri risultati suggeriscono che CD18 e CD99, o i loro rispettivi ligandi porcini, potrebbero costituire attrattivi bersagli per futuri interventi terapeutici mirati alla soppressione specifica dell’infiltrazione cellulare. Inoltre i risultati sottolineano l’importanza del considerare le conseguenze dell’infezione cross-specifica dell’HCMV negli xenotraipi in future sperimentazioni cliniche. Sono richiesti ulteriori studi per comprendere il meccanismo d’infezione e prevenire il danneggiamento del trapianto. In questo contesto, è interessante il ruolo delle proteine SOCS nel controllo dell’inflammazione.

In conclusione, sono necessari ampi sforzi prima che lo xenotraipianto possa divenire una realtà clinica, e una soluzione per superare la critica carenza di organi umani.
3. INTRODUCTION

3.1 Xenotransplantation

Xenotransplantation is the transplantation of organs or tissues between members of different species. In the last years, it has gained importance as an alternative to overcome the severe organ shortage in clinical allotransplantation. Recently, encouraging results were obtained with reversal of diabetes using porcine islets cells in a nonhuman primate model [1,2]. In addition, encouraging preliminary data from an early-phase clinical trial were recently reported, where six patients were transplanted with encapsulated porcine islets [3]. Although no peer-reviewed reports of the data are available yet, the trial will expand for a second phase. Indeed, xenotransplantation is an ancestor of allotransplantation. The first transplantation attempts in the early 16th century were made without any knowledge of the immunological species barriers. The first documented xenotransplantation trial, in 1667, was a lamb-to-human blood transfusion. In the 19th century, testicle xenotransplantation from apes to man as “human revitalization transplantation” was the start for endocrinotherapy [4,5]. Advances in anastomotic techniques and the ability to restore organ vascularisation led to transplantation of whole organs. During the 1960s, with the introduction of modern pharmacological immunosuppression, a number of xenografts were transplanted from primates to human. The most successful chimpanzee-to-human kidney transplantation was performed in 1964 by Reemtsma et al [6]. The graft lasted for 9 months and represents the longest survival ever recorded for pre-clinical xenotransplantation. In 1967, Barnard realized the first human-to-human heart transplantation [7]. This success, coupled with improved immunosuppression and the widespread acceptance of deceased-donor organ donation shifted the interest to allotransplantation. During this period, great progress was achieved in terms of surgical techniques, immunosuppressive regimens, and pre-transplant diagnostic tools, all of which have contributed to increased patient and graft survival after allotransplantation. Allotransplantation of heart, lung, liver, kidney, and pancreas became fully established. However, this success also led to a shortage of human donor organs and the reconsideration of animal sources in the 1980s. The beginning of the new xenotransplantation era began in 1984, with the paediatric baboon heart transplant (and the introduction of cyclosporine immunosuppression) to the patient known as Baby Fae. Over the past 50 years, important progress was made in understanding the barriers to xenotransplantation and possible ways to overcome them. Today, the percentage of survival after allotransplantation at 1, 5, and 10
years is approximately 85%, 65%, and 50%, respectively, but the waiting lists for new organs are long, and many patients die without having the possibility of transplantation. In Switzerland, in 2008, despite 459 transplanted patients, there were still 942 patients on the waiting list and 62 patients died while waiting for a transplant.

Two types of xenotransplants are distinguished: transplantation of primarily vascularised organs, and transplantation of tissues or cells such as islets cells, bone marrow, neural cells, or hepatocytes. In the first case, the immediate exposure of donor vascular endothelium to the recipient’s circulation plays an important role in immune responses eventually inducing graft rejection. Although primates are immunologically very similar to humans, nonhuman primate species are either endangered or too small, and represent a risk for infectious disease transmission. The advantage of inbred miniature swines is their similarity to men in size, physiology, and anatomy. Furthermore, pigs represent a large source and offer the possibility to rear them in pathogen-free conditions. Finally, as pigs are currently sacrificed for food purpose, their use as organ donors will most likely generate less ethical concerns as compared with e.g. non-human primates [8,9]. The more distant genetic relationship to men can be both an advantage and a disadvantage. Despite the greater immunological barriers, major advantages of pigs as donors are the possibilities to alter their genetic background, and to use species-specific treatments such as antibodies which do not crossreact with the human host.

3.2 Rejection in xenotransplantation

3.2.1 Hyperacute vascular rejection

Experimental studies during the 1960s revealed that primarily vascularised xenografts between widely disparate species (i.e. discordant species) were immediately destructed. The responsible process, termed hyperacute rejection (HAR, Figure 1), is an irreversible event that results in graft loss within minutes to hours. Characteristic of this type of rejection are diffuse interstitial haemorrhage, edema and thrombi in capillaries and small vessels [5,10]. Because of the similarities to the rejection occurring in ABO-incompatible allografts, the mechanism was thought to be antibody-mediated. In 1984, Galili et al described preformed human natural antibodies (nAb) directed against Galα1,3Gal (αGal), called anti-αGal antibodies [11]. αGal is synthesized on the endothelial cell surface by the enzyme α1,3-galactosyltransferase that is functional in all mammals except for higher primates including humans, apes and Old World
monkeys [12]. As αGal is also expressed by many microorganisms present in the gastrointestinal tract, natural αGal antibodies are produced early in life [13]. During the 1990s, preformed nAb were found to initiate HAR by activating the classical pathway of the complement cascade [14], thus mediating complement deposition and endothelial cell activation and damage [15]. This further results in platelet activation and aggregation, coagulation and, in turn, disruption of vascular endothelial cell integrity with loss of endothelial functions. Additional studies revealed that the anti-αGal antibodies are the most important mediators of HAR [16-18] and that IgM is the predominant immunoglobulin isotype involved [19]. Strategies to prevent HAR, targeting αGal and the complement system, will be addressed below.

Figure 1: Hyperacute rejection. Binding of xenoreactive nAb to xenoantigenic epitopes (mainly αGal) on donor endothelial cells, leading to complement activation and graft rejection within minutes to hours. Adapted from Yang and Sykes, Nature Rev Immunol 2007 [20]

3.2.2 Acute humoral xenograft rejection
When HAR is prevented, other mechanism which eventually destroy the graft occur within days to weeks, in a process known as acute humoral xenograft rejection, AHXR (also called acute vascular rejection, AVR, or delayed xenograft rejection, DXR, Figure 2) [21,22]. This event is induced by an immune response, mediated first by IgM, then by IgG immunoglobulins against αGal and non-αGal epitopes [23]. Progressive deposition of Ig, complement, and fibrin is associated with activation, apoptosis, or necrosis of endothelial cells with consequent disruption of the endothelial layer. This results in vascular thrombosis
with blood extravasation, interstitial edema, and tissue injury [24]. Further on, xenografts are infiltrated with macrophages and neutrophils, and, to a lesser extent with T cells and NK cells [10,25]. Activation of cytokine genes, adhesion molecule expression, and changes from an anticoagulant to a procoagulant phenotype on the endothelial cell surface may all play a role in this process [8]. The activation of coagulation pathways and intravascular thrombosis can be further augmented by molecular incompatibilities in the regulators of the coagulation system [26]. The most important of these disparities is between porcine thrombomodulin (TBM) and human thrombin [26-28]. While porcine TBM is able to bind human thrombin, activation of the anticoagulant human protein C is diminished, resulting in enhanced thrombin and may favouring the initiation of clotting [26]. Data on the ability of porcine tissue factor pathway inhibitor (TFPI) to inhibit human factor Xa, and thus the activation of human prothrombin to thrombin, and tissue factor/activated factor VII complex are controversial. Some described molecular incompatibilities [29,30], while others did not [26]. Porcine von Willebrand Factor (vWF) has high affinity for human platelet receptors, probably leading to formation of platelet microthrombi and vascular injury [31]. Abnormalities in the regulation of human plasminogen activators by porcine plasminogen activator inhibitor type-1 (PAI-1) associated with vascular endothelial cells have also been described [32]. However, expression and cloning of PAI-1 has demonstrated that there are no obvious functional differences between human and porcine serpins [33,34]. Finally, upon xenogeneic endothelial cell activation, vascular injury may be exacerbated by the loss of vascular NTPDase/CD39, an important thromboregulatory factor that degrades the extracellular inflammatory mediators ATP and ADP into AMP, thereby inhibiting platelet activation and modulating vascular thrombosis [35].
Figure 2: Acute humoral xenograft rejection (AHXR). Binding of natural and elicited xenoreactive Ab to xenogeneic endothelial cells results in complement activation, antibody-dependent cell-mediated cytotoxicity, and endothelial cell activation. Histological features are thrombosis and vasoconstrictions. Adapted from Yang and Sykes, Nature Rev Immunol 2007 [20]

3.2.3 Cellular xenograft rejection
The presence of cellular infiltrates, associated with direct tissue damage in solid organ xenografts explanted from immunosuppressed primates, was defined as acute cellular xenograft rejection (Figure 3) [20]. Infiltrates may consist of T and B cells, some NK cells, macrophages and neutrophils. In 1996 an in vitro study revealed that human T cell responses against pig antigens, especially indirect responses, were stronger than those to alloantigens [36]. In a more recent study of pig-to-baboon xenotransplantation, where hearts from human-complement-regulatory-protein transgenic pigs were transplanted into recipients depleted from B cells and αGal antibodies, but not from T cells, confirmed the important role of T cells in xenograft rejection [25]. Moreover, additional studies indicated that T-cell suppressive treatments significantly prolonged the survival of porcine xenografts in non-human primates if nAb-mediated responses were suppressed [37-39]. Innate immune cells also contribute to graft rejection. NK cells are known to be regulated by a balance between activating and inhibitory signals. This balance is shifted towards activation in many xenogeneic interactions. According to the missing self-principle, an important factor accounting for this imbalance is the incompatibility between porcine class I receptor, SLA class I, and human NK inhibitory receptors. Indeed, porcine transgenic cells expressing human HLA molecules are partially protected from NK cell adhesion and cytotoxicity [40-43]. In contrast, porcine cells express ligands which are compatible with activating receptors
on human NK cells [44-47]. NK cell cytotoxicity can also be induced by antibody-dependent cell-mediated cytotoxicity (ADCC), mediated by NK cell recognition of Ab bound to target cells via Fc receptors [48]. Furthermore, NK cells release cytokines, such as IFNγ and TNFα, thus contributing to the induction of inflammation and to the activation of macrophages and endothelial cells [49]. Macrophages have been implicated in both cellular and solid-organ xenograft rejection in a number of different studies [20,50-52]. The incompatibility between SIRPα, an important macrophage inhibitory receptor, and its ligand (CD47) was shown to contribute to the phagocytosis of xenogeneic cells [53,54]. Finally, in vitro studies revealed a role for neutrophils in the activation of the xenogeneic endothelium [55,56].

Figure 3: Cellular rejection. The binding of NK cells to activating receptors on porcine cells, and the inability of porcine MHC class I molecules to interact with human NK cell inhibitory receptors makes porcine cells highly susceptible to NK cell-mediated lysis. Macrophages are activated by the interaction of a Gal and galectin-3. The incompatibility between porcine CD47 and the human inhibitory receptor SIRPα makes porcine cells susceptible to macrophage phagocytosis. Both NK cells and macrophages can be activated by binding of their Fc receptors to xenoreactive-Ab-coated porcine cells. The cytotoxicity of macrophages and NK cells can be significantly increased by cytokines released from activated T cells.


3.2.4 Strategies to prevent xenograft rejection
Several approaches have been considered in order to overcome xenograft rejection. One of the most important successes was the generation of the αGal KO pig in 2002. This was a major contribution to surmount the first immunological barrier represented by HAR. Thereafter, immunological research has been shifted to the next barriers, represented by acute humoral and cellular rejection and physiological incompatibilities, such as the disparities in the
coagulation system. The crossbreeding of αGal KO pigs with pigs transgenic for different human anticoagulant and/or complement regulatory proteins, and the induction of tolerance, will be one of the next attempts in order to significantly decrease immunological and cellular barriers. Recent progress and future perspectives to prevent xenograft rejection are discussed below.

**Hyperacute rejection** The first attempts to prevent HAR focused on the depletion of preformed αGal antibodies and the regulation of the complement system. The first strategies to deplete nAb against αGal included immunoadsorption, continuous infusion of soluble synthetic αGal sugars into the blood [57,58], and continuous intravenous infusion of bovine serum albumin conjugated to multiple αGal molecules [59]. Further approaches concentrated on the suppression of B cell activity in order to prevent antibody production. In an initial study, the level of αGal in transgenic pigs was reduced by the expression of a α1,2-fucosyltransferase [60]. The first approaches to interfere with the complement system included administration of cobra venom factor, soluble complement receptor-1, C1-inhibitor, intravenous Ig and anti-C5 antibody treatment. Despite the beneficial role of these treatments in the prevention of HAR in ex vivo and in vivo pig-to-primate models [61], systemic complement interference was costly and was associated with the risk of side-effects. The first “complement-transgenic pig” in 1992, was transgenic for human decay accelerating factor (hDAF), an important complement regulatory protein. With effective immunosuppressive therapy, there was a prolonged survival of organs from hDAF transgenic pigs but graft failure did still occur. Furthermore, many studies demonstrated that the presence of complement inhibitors was generally efficient in preventing HAR but not AHXR [23,62]. A major step forward was represented by the generation of αGal-KO pigs by the disruption of the αGal gene. Heterozygous αGal-KO pigs were generated by two separate groups in 2001 by homologous recombination and nuclear transfer [63,64]; double αGal-KO pigs were successfully bred one year later [65]. The introduction of αGal-KO pigs significantly prolonged xenograft survival: pig hearts survived for 2-6 months in baboons [38,66], and a shorter survival was reported for kidneys [37]. The absence of αGal expression, despite allowing longer survival with less immune modulation, did not completely solve the rejection problem. Even if HAR was prevented, graft failure eventually occurred following a development of thrombotic microangiopathy [67]. Thus, incompatibilities in the coagulation pathway and the prevention of AHXR, mediated partly by non-αGal epitopes, need further investigation.
**Acute humoral and cellular rejection** After the generation of αGal KO pigs, the focus of research shifted to acute xenograft rejection. As a consequence of the role of nAb against αGal in the onset of AHXR, HAR prevention also resulted in delayed AHXR. However, AHXR was not completely prevented, suggesting a role of non-αGal antibodies [68]. Interestingly, AHXR did not occur when T-cell responses were controlled [37,69], thus suggesting that it was possible to inhibit AHXR by effective T-cell suppression or tolerance. Although chronic nonspecific T-cell immunosuppressive therapy was able to prolong graft survival, the treatment was insufficient and toxic. Therefore, the induction of T-cell tolerance was preferred. Furthermore, as described above, identification of pig antigen targets for nAb, NK cells, or macrophages might allow additional genetic manipulation in order to prevent cellular infiltration and the onset of an immune response.

**T-cell tolerance** The induction of T-cell tolerance is essential for the success of clinical xenotransplantation. Several strategies have been taken into consideration, unfortunately with few promising results in large animal models [20]. These approaches include: costimulatory blockade, donor-specific transfusion (infusion of cells, e.g. splenocytes from the organ or tissue donor into the transplant recipient), vascularised thymic transplantation [37], and the induction of mixed haematopoietic chimerism (i.e. the coexistence between the recipient and xenogeneic donor haematopoietic cells).

**B-cell tolerance** Mixed xenogeneic chimerism has also been shown to promote the induction of B-cell tolerance. Other approaches include the induction of tolerance to the αGal epitope and tolerance of T-cell-independent B cells [20].

**Accommodation** Accommodation is referred to a situation in which a graft survives in the presence and deposition of antibodies directed against it without the need for continued anti-humoral rejection prophylaxis [20]. To date, accommodation has never been convincingly shown in discordant species combinations.

**Coagulation** An additional obstacle is represented by the coagulation incompatibilities between pig and man. As seen for xenografts from αGal-KO pigs, graft failure eventually occurred because of thrombotic microangiopathy [67]. Overexpression of anticoagulation genes (e.g. CD39, a key inhibitor of inflammation and thrombosis) or deletion of procoagulation genes, has been shown to prevent acute vascular rejection in rodent models [70-72]. Unfortunately, antithrombotic and anticoagulant agents had limited or no effect in the transplantation of organs from swine to non-human primates [73,74]. However, recently generated human CD39-transgenic pigs [69] may represent a step forward, eventually in combination with the αGal KO background, toward the improvement of xenograft survival.
3.3 Xenozoonosis

Zoonosis is derived from the Greek words zoon (animal) and nosos (disease) and indicates any infectious disease able to be transmitted between animals and humans. The potential transmission of pathogens between pig and human is one important concern in xenotransplantation. One of the major advantages of pig-to human xenotransplantation is the possibility to breed and maintain the donor pigs in specific-pathogen-free (SPF) conditions, under strict health surveillance and absence of known pathogens. In addition, the development of new sensitive and rapid tests allows the prompt screening of donor pigs for the presence of potential infectious agents. An alternative strategy is the early weaning of newborn pigs within the first 2 weeks after birth with subsequent isolation from other animals, that allows to successfully exclude porcine cytomegalovirus (PCMV) from donor animals [75,76]. Therefore, the potential transmission of donor pig pathogens along with organ transplantation may be reduced as compared to allotransplantation. Nevertheless, concerns have been raised with regard to the transfer and pathogenic potential of porcine endogenous retroviruses (PERV), which are integrated in the porcine genome and thus difficult to eliminate.

Conversely, infections of the graft by recipient pathogens, taken into consideration only recently, are more difficult to prevent. As discussed later in detail, reactivation of human cytomegalovirus (HCMV) is frequently occurring during allotransplantation and is a major concern. It is anticipated that reactivation of HCMV will occur during xenotransplantation, exposing the graft to an unknown risk.

3.3.1 Donor to recipient transmission: PERV

PERV are similar to human endogenous retroviruses (HERV), present in all human cells. Three classes of PERV have been found to be integrated in multiple copies in the genome of all porcine cells [77]. Human-tropic PERV are often recombinations of PERV-A and PERV-C [78-81]. Although there is no evidence that the virus can cause significant health problems in their hosts, concerns have been raised about the transfer of PERV to human recipients. Potentially, PERV may mutate or reassort with HERV to create new and more pathogenic mutants that would not only represent a risk for the xenograft recipient but also for general society. However, a number of studies involving xenotransplantation trials or exposure to porcine cells and tissues, did not prove any evidence for successful PERV infection in vivo [81-85]. Furthermore, PERV is susceptible to current antiviral agents, and antibodies against the highly conserved epitopes have been shown to neutralize PERV in vitro, suggesting that
there may be a basis for the generation of a PERV-vaccine [86]. In addition, pig lines have been described in which PERV seem incapable to infect human cells in vitro [87,88]; therefore the selection of such animals for breeding might minimize the risk of PERV transmission. Finally, genetic manipulation of the porcine genome via KO technology [86] or small interfering RNA [89-91], significantly reduced PERV expression and may provide an additional source for safer pig organs.

3.3.2 Recipient-derived infection: human Cytomegalovirus (HCMV)

Human cytomegalovirus (HCMV), also known as Herpes simplex virus 5 (HSV-5), is a member of the β-herpesvirus family, and has the characteristic to establish life-long latent infections [92,93]. HCMV is one of the most common pathogens: between 50-90% of the adult population are seropositive. Following infection, the virus is first persistently secreted in body fluids for months to years, then intermittently asymptptomatically reactivated and shed. In immunocompetent individuals, infection is usually subclinical or eventually manifests as a mononucleosis-like syndrome. This unnoticed virus infection or reactivation enables the virus to spread both horizontally and vertically. HCMV infection or reactivation is problematic in immunologically immature and immunocompromised individuals such as newborns, transplant patients, and HIV-infected individuals. Central nervous system (CNS) damage is a frequent feature of congenital infection [94] whereas in allotransplantation HCMV infection has been linked to graft rejection, an issue that will be further addressed below.

During natural infection, HCMV can replicate productively in a great variety of cells, including epithelial cells, endothelial cells, smooth muscle cells, mesenchymal cells, hepatocytes, granulocytes, and monocyte-derived macrophages. One reason for this broad tropism is the wide distribution of the poorly characterized HCMV receptors. The broad cell tropism is reflected in an increasing number of in vitro cell culture models. The cell type used for propagation of an isolate influences the cell tropism of the HCMV strain. It was shown that long-term propagation in fibroblasts selected for HCMV strains with low endothelial cell tropism, whereas long-term propagation in endothelial cells did not influence the broader cell tropism observed in recent clinical isolates [95]. Latent virus is mainly detected in cells of the bone marrow and in peripheral blood. Leukocytes and vascular endothelial cells contribute to the virus spread [95-99]. The virion of HCMV consist of an icosahedral nucleocapsid containing a 230 kbp, double-stranded linear DNA genome, surrounded by a tegument (proteinaceous layer) and enclosed by a lipid bilayer [92]. The envelope contains 6 virus-encoded glycoproteins (gB, gN, gO, gH, gM and gL) that play essential roles in virus entry
into host cells, cell-to-cell spread, and virion maturation [100]. HCMV infection begins with an initial low-affinity interaction to cellular HSPGs (heparan sulfate proteoglycans) mediated by the gM/gN complex and gB (Figure 4) [101-104]. Following a stable docking to integrin heterodimers and other unknown receptors, a trimeric gH/gL/gO complex and gB mediate the fusion of the virus envelope and cell membranes [105]. The role of EGFR as entry receptor is still controversial [103]. In a recent study, a critical role for the PDGFR-α receptor interaction with gB for HCMV entry into human and murine cells was demonstrated [106]. The interactions of glycoproteins with their receptors are enough to induce intracellular signal transduction pathways, leading to an alteration of cellular gene expression. After fusion, the nucleocapsid and tegument proteins enter into the host cytoplasm and are rapidly translocated into the nucleus.

The HCMV genome is the largest of all herpesviruses; it contains an arrangement of unique long (UL), unique short (US), and repeat regions, and encodes more than 200 proteins. During productive infection, these proteins are expressed in three overlapping phases, and are known as immediate early (IE or α), early (E or β), and late (L or γ) proteins. The transcription of IE proteins starts within 1 h post infection (p.i.) and includes transactivators and autostimulators of viral genes, as well as proteins that regulate the expression of a large number of host cells and thus potentially affect host cell response or host cell immune clearance mechanisms [93,107]. Expression of E genes starts within 4-24 h p.i., depends on IE genes, and is unaffected by inhibitors of DNA replication [107]. E genes encode mostly non-structural proteins, such as viral DNA replication factors, repair enzymes and proteins involved in immune evasion [108]. Finally, transcription of L proteins begins more than 24 h p.i. and requires prior viral DNA replication [107]. Their gene products have mainly structural roles and contribute to virus assembly, morphogenesis, and egress [108].

Unlike other herpesviruses, HCMV depends on the host cell metabolism for its DNA replication [109,110]. Thus, it stimulates cellular transcription and translation rather than shutting off the host macromolecular synthesis. However, in infected cells, cell cycle progression is arrested in order to prevent a competition for DNA precursors between the host DNA machinery and the virus [111,112]. After capsid formation and DNA packaging in the nucleus, nucleocapsids acquire a primary envelope by budding at the nuclear membrane. They then further mature through a de-envelopment/re-envelopment process in the cytoplasm, where they acquire their tegument. Finally, beginning at 72 h p.i., infectious virus is released via an exocytic-like pathway [108,113].
3.3.2.1 Host defence and HCMV escape mechanism

The induction of innate immune responses by HCMV does not require virus replication or cellular protein synthesis. Early events in HCMV infection affect hundreds of cellular genes; the most strongly induced are genes belonging to the inflammatory cytokine family and the interferon-stimulated genes [93,103]. The innate immune response serves to limit viral replication early during infection as well as to activate and promote adaptive immune responses. Cell-mediated immunity plays a major role in the control of viral infection. Studies performed in mice revealed that both T cells and NK cells are of primary importance for prevention of recurrence [114-116]. Experimental and clinical findings show a beneficial role of the humoral response; many HCMV proteins are recognized by the humoral immune system [92]. During primary infection, immunocompetent individuals produce anti-HCMV IgM Ab that persist for 3-4 months, followed a few weeks later by IgG Ab that persist for life. HCMV infection is kept under control by the immune system, however, total virus clearance is rarely achieved and HCMV can persist in a latent stage [117]. The ability of CMV to persist in the host is the result of (1) induction of a latent state, (2) exploitation of immunological privileged tissue for replication, and (3) expression of genes that interfere with the immune response [92,118,119]. Escape from CD8+ T cells is mediated by several mechanisms. By the expression of unique short (US) proteins, leading to e.g. HLA class I and class II degradation or internalization, HCMV can interfere with the HLA-restricted pathway of antigen
presentation, thus escaping the host T-lymphocyte-mediated antiviral immune response[120]. One strategy to escape from NK cell responses is the HCMV-gpUL40-mediated induction of HLA-E molecule expression on the host surface. HLA-E binding to NK cell inhibitory receptors then protects the infected cells from NK cell cytotoxicity [121]. Another way to avoid NK killing is to abrogate their activation. HCMV proteins gpUL16 and gpUL141 interfere with the binding and expression of NK activating receptor ligands for NKG2D and DNAM-1, respectively, thereby preventing the triggering of NK killing [122-126]. Further strategies to evade the immune response are represented by the interference of HCMV with chemokine-driven inflammation [127,128] and the inhibition of apoptosis [118,129].

3.3.2.2 HCMV in allograft- and xenograft rejection
HCMV infection of solid organ transplants (SOT) can result in both direct (CMV disease) and indirect effects. CMV disease is related to the presence of high rates of viral replication and includes fever, malaise and neutropenia, pneumonitis, gastrointestinal disease, hepatitis, retinitis and encephalitis. Conversely, indirect effects result from the presence of low rates of viral replication over prolonged periods of time and are caused by virus interaction with the host immune response [130,131]. The indirect effects have been associated with an increased risk of rejection and graft dysfunction, accelerated atherosclerosis, opportunistic infections, malignancies, post-transplant diabetes mellitus and Guillain-Barré syndrome. A correlation between HCMV infection and allograft rejection has been described for all types of SOT [132-134]: heart [135-138], lung [139,140], liver [141,142], and kidney [143,144]. A support for the role of HCMV infection in graft rejection is the observation that treatment of the recipients with ganciclovir delayed the development of allograft rejection. However, the relationship between HCMV infection and graft rejection appears to be bidirectional [143]. The direct effects accounting for allograft rejection are mediated by the result of immune clearance and the expression of pathogenic viral proteins. The indirect effects might be explained by the immunoactivation produced by the virus in the host that results in a recruitment of inflammatory cells and inflammatory effectors. Furthermore, CMV modifies different cellular functions; e.g. it induces cellular factors involved in angiogenesis and wound repair including adhesion molecules and growth factors and receptors [133].
It can be expected that in potential clinical xenotransplantation trials, HCMV reactivation will be observed in the light of the extensive immunosuppression necessary to prevent xenogeneic rejection. In the preclinical pig-to-baboon xenotransplantation model, both direct and indirect effects of porcine and baboon CMV reactivation have been observed. Baboon CMV was
reactivated in almost all recipients, resulting in disease and death in some of the baboons [145]. As in the clinical settings, prophylaxis with ganciclovir markedly reduced BCMV activation and prevented the disease.

HCMV has generally been described to be strictly species-specific. The species restriction usually results in a postpenetration failure in replication following IE gene expression and has been associated with induction of apoptosis [146]. However, cross-species productive infection of porcine endothelial cells by a clinical HCMV isolate has been demonstrated [147] and further characterized in our in our laboratory [148]. This study demonstrated that HCMV was able to productively infect porcine endothelial cells (pEC) of different anatomical background. The infection efficacy and the kinetics of cytopathogenicity depended on both HCMV tropism for human cells and on the anatomical origins of the pEC. Despite the restricted late viral protein expression in pEC, as compared to the human system, pEC supported a full replicative cycle. However, in contrast to the human setting, where HCMV efficiently suppresses apoptosis, HCMV-infected pEC showed increased apoptosis, probably in part reflecting the restriction of HCMV in pEC.

3.3.2.3 Role of HCMV in alterations of endothelial cell phenotype

One of the indirect effects of HCMV infection is the alteration of adhesion molecule expression on endothelial cells. Localized at the interface between the allo- or xenograft and the host immune system, the endothelium plays an important role during rejection [149,150]. Several lines of evidence indicate that HCMV infection enhances human leukocyte recruitment to the endothelium during acute cellular rejection. Infection of human cells with HCMV induced the production of the chemokines IL-8 (CXCL8) and GROα (CXCL1) [98] and cytokines, such as IL-1β and IL-6 and IFNγ [151,152]. Moreover, CMV infection resulted in alterations of adhesion-molecule expression, including upregulation of Lewisx (CD15) and sialyl-Lewisx (CD15s) [153], platelet-endothelial cell adhesion molecule I (PECAM-I or CD31) [99] intracellular adhesion molecule I (ICAM-1) [99,151,154-156], vascular adhesion protein-I (VAP-1) [157], and α6-integrin (CD49f) and downregulation of α2 and α5-integrins (CD49b and CD49e, respectively) [158]. Some studies also showed an upregulation of E-selectin (CD62E) and vascular cell adhesion molecule-I (VCAM-1, CD106) [99,151,159,160] upon CMV infection whereas others did not [154,156]. The different results may in part be explained by the cell source, as shown by Sindre et al [161]. Finally, HCMV infection of endothelial cells induced disruption of actin stress fiber
formation and decreased formation of lateral junction proteins, thus increasing endothelial cell permeability [99].

The release of chemokines and the increased adhesion receptor expression was linked to enhanced leukocyte recruitment. Increased neutrophil chemotaxis and leukocyte adhesion have been described [98,159,162]. In addition, the migration of T cell subsets, polymorphonuclear neutrophils (PMN), and monocytes across the endothelium was enhanced upon HCMV-infection [99,163,164]. Moreover, PMN and monocytes were infected by HCMV during transmigration across infected endothelium and contributed to virus dissemination [98,99].

An important aim of this thesis was to elucidate the phenotypical consequences of HCMV crossinfection of porcine endothelial cells on cell-surface receptor expression and the recruitment of human leukocyte.

3.3.2.4 HCMV prophylaxis and treatment

Strategies to prevent HCMV infection in risk groups can be planned in accordance to the virus transmission routes. In the case of solid organ transplant recipients, the risk of primary infection could be reduced by matching seronegative donors and recipients, although this is difficult due to the scarcity of donor organs. Prior to the availability of ganciclovir, CMV disease was often fatal. Prophylaxis with antiviral agents introduced in the late 1970, and pre-emptive therapy have made CMV-associated mortality rare [165-167].

The development of improved diagnostic techniques and the availability of oral antiviral agents has opened a debate over the importance of preventing the disease. There is significant controversy in the transplant literature regarding timing and type of therapy, i.e. pre-emptive therapy versus prophylaxis [168-173]. In prophylaxis, the antiviral drug is administered before active HCMV infection is detected in order to prevent its occurrence. On the other hand, pre-emptive treatment consists in withholding antiviral drugs until they would be maximally effective to reduce the incidence of HCMV disease. Pre-emptive therapy has the advantage of minimizing patient exposure to costly anti-HCMV drugs. Although it has been effective in preventing the disease in most cases, low-level and asymptomatic viral replication is not impeded, exposing patients to the potential indirect effects [131]. Prophylaxis suppresses viraemia for the period of its application in the initial months after transplantation. One of its advantages is that it reduces both direct and indirect effects of HCMV infection, and may decrease patient morbidity and mortality [168,174,175]. However, with prophylaxis the patient is more exposed to the drug with the consequence of side effects and a possible
increase in viral resistance. A further problem of therapeutic drugs is their association with neutropenia. The best available drugs currently used in prophylaxis are ganciclovir and its prodrug valganciclovir. The answer to whether prophylaxis is a better strategy than preemptive therapy should be confirmed by further studies.

The development of new agents for the treatment of CMV disease is a highly attractive area of pharmaceutical research. In this context, xenotransplantation could represent an advantage given the possibility to genetically modify the pig organ in order to render it more resistant to HCMV infection, thus reducing the need of antiviral therapy.

3.3.2.5 HCMV vaccines

An effective HCMV vaccine would be beneficial in decreasing the need of antiviral drugs and their side-effects. Most importantly, it would be a solution for congenital HCMV infection, since no drug has been approved for use in the treatment of congenital HCMV. Many efforts have been made over 30 years in order to develop HCMV vaccines, however with little success. The main problems are: (1) the host immune correlates of protective immunity are not yet clear, (2) the viral proteins that should be included in the vaccine are uncertain, (3) clinical trials have largely focused on immunocompromised patients, a population that may not be relevant in the case of congenital infections, (4) the ultimate target population for vaccination remains unclear, and finally, (5) there has been insufficient education about the problem of HCMV infection [176]. A variety of strategies have been employed in the development of vaccines that can be divided into live, attenuated vaccines, and subunit vaccines. Since the immunocompromised individuals represent a target population for vaccination, it would be preferable to use a virus-free vaccine. Vaccines under clinical trials have targeted HCMV structural proteins, such as gB and pp65, because of their strong induction of humoral and cellular responses, respectively [177-179]. Recently a gB vaccine evaluated in phase 2 clinical trials showed potential results in prevention of maternal and congenital CMV infection [180]. Nonstructural proteins, such as IE1, an important target of the CD8+ T cell response, have also been used. A trivalent DNA vaccine targeting gB, pp65, and IE1, was evaluated in a phase I trial [181], and another trivalent vaccine was evaluated using an alphavirus-like replicon system [182]. Other formulations of recombinant gB or pp65 vaccines evaluated in clinical trials are the combinations with adjuvants, the use of vector vaccine expression system based on canarypox vectors (ALVAC), and DNA vaccination. Novel vaccine approaches include dense bodies vaccines, peptide-based
vaccines, the exploitation of viral genomes cloned in E.Coli as BACs (bacterial artificial chromosomes), and the search for new HCMV protein targets.

3.4 Cellular recruitment to the porcine endothelium

As previously described, during acute vascular rejection or after HCMV infection, leukocytes are recruited to the endothelium and participate in the process of graft rejection. The recruitment of human leukocytes to the porcine endothelium can be divided in 3 major steps: chemotaxis, adhesion and transmigration (Figure 5). An important prerequisite for a successful interaction between human leukocytes and the porcine endothelial cells (pEC) is the compatibility between human and porcine receptors.

3.4.1 Chemotaxis

The initial step in the recruitment of human leukocytes to pEC is mediated by the release of chemotactic factors from pEC that trigger leukocyte chemotaxis toward the site of inflammation. Certain chemokines released from pEC have been shown to act over the species barrier and recruit human leukocytes from the circulation by binding to chemokine receptors on their surface [183]. Monocytes and neutrophils infiltrate a xenograft within minutes and subsequently amplify NK and T cell recruitment to the graft [184]. Porcine monocyte chemoattractant protein (MCP-1) released from porcine islet cells [185] and RANTES [186] induce strong monocyte recruitment. Endothelial cell activation induces the release of IL-8 and platelet activating factor (PAF), known to promote chemotaxis of human neutrophils (PMN). Whereas PAF released from pEC resulted in significant PMN chemotaxis, the effect of porcine IL-8 on human neutrophils is still unclear [187,188].

3.4.2 Adhesion

Human leukocyte rolling and adhesion on pEC relies mainly on interactions between human selectins and integrins and their porcine ligands [183]. For neutrophils, adherence is mediated by binding of L-selectin (CD62L) to undefined ligands on pEC, PSGL-1 (CD162) interactions with porcine CD62E and CD62P, and by β2-integrins (CD18) which bind porcine ICAM-1 and -2 (CD54 and CD102) [183,189]. Adhesion under low shear stress of human monocytes, NK cells, and T cells on pEC strongly depends on binding of the β1-integrin VLA-4 (CD49d/CD29) to porcine VCAM-1 (CD106), but is also mediated by interactions between
CD62L and β2-integrins and their respective porcine ligands [190-192]. In intraspecies in vivo models, the transition from rolling to arrest and firm adhesion has been shown to depend on leukocyte activation mediated by EC-bound chemokines [193]. However, this process has still to be investigated in the human-porcine xenogeneic setting.

### 3.4.3 Transmigration

Human intraspecies transmigration is a multistep process. After adhesion, monocytes undergo locomotion, in which they move from a site of firm adhesion to the nearest junction to begin diapedesis. For monocytes, this step is mainly mediated by the interactions between β2-integrins and endothelial ICAM-1 and ICAM-2 [194]. Transendothelial migration (TEM) relies on homotypic CD31 and CD99 binding [195-197] and homotypic and heterotypic junctional adhesion molecules (JAM) binding [198]. Less is known about the steps involved in the migration of human leukocytes across pEC. The homotypic CD31 binding was shown to be incompatible across the porcine-human species barrier [199]. Nevertheless, other junctional interactions may still be functional. Hauzenberger et al. demonstrated that the porcine endothelium was able to support TEM of human leukocytes [200]. Blocking porcine CD106 inhibited TEM of both human monocytes and NK cells, whereas masking of human CD18 reduced only TEM of monocytes. The first aim of this thesis was to analyze the role of human cell surface receptors in TEM across pEC in a model separating adhesion from TEM.

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**Figure 5: Recruitment of human leukocytes to porcine endothelial cells**

Human leukocyte to porcine EC can be divided by chemotaxis, rolling, firm adhesion and transmigration. Illustrated are also the receptor interactions involved at each step.

Adapted from Schneider and Seebach, Curr Opin Organ Transplant 2008 [183].
3.5 SOCS and the control of inflammation

The intrinsic ability of the graft to counteract injuring mechanisms following transplantation and to repair damages may be crucial for ultimately maintaining graft function. Many of the injuring factors have a pro-inflammatory cytokine-mediated component, the extent and duration of which may determine short-term graft damage as well as long-term outcome [201]. Control of inflammatory responses represents an attractive approach to prevent both innate and adaptive immune mechanisms. Beside TNF-α and other pro-inflammatory cytokines, IFN-γ is detected very early in the graft in experimental transplantation models [202-204]. The effects of IFN-γ in transplantation are still controversial. Another cytokine that has been implicated in graft injury and rejection is IL-6, which was markedly increased in the serum and the graft after allogeneic, syngeneic and alymphoid transplantation in mice [203].

In the 1990s, a family of inhibitory proteins was identified and termed “suppressors of cytokine signalling (SOCS) proteins” [205-208] and were discovered as important negative regulators of the cytokine-JAK/STAT pathway. Further studies revealed important roles of SOCS proteins in immunological and pathological processes (Figure 6) [208,209]. The SOCS family comprises 8 members, each consisting of (1) a central SH2 domain, responsible for the binding to target proteins, (2) a variable amino-terminal domain, and (3) a conserved carboxy-terminal domain known as SOCS-box, involved in the recruitment of the ubiquitin-transferase system, responsible for proteosomal degradation.

The last part of this thesis focused on SOCS1 and SOCS3 due to their involvement in the regulation of inflammatory mediators associated with transplantation, IFN-γ and IL-6. Both SOCS1 and SOCS3 are inhibitors of JAK tyrosine kinase via their kinase inhibitory region (KIR) in their N-domain. SOCS1 and SOCS3 were found to be induced by TLR stimulation by LPS and CpG-DNA in macrophages and DCs [210-212]. TLRs play a crucial role in inflammation, septic shock, and in innate and adaptive immunity. TLR are also involved in the rejection process: in mice lacking the universal TLR adaptor protein MyD88, rejection in a skin allograft model was reduced, probably by reducing expression of IFN-γ [213].

SOCS1-KO mice are normal at birth but die within 3 weeks by acute SOCS1-/- disease, characterized by severe lymphopenia, activation of peripheral T cells, fatty degeneration and necrosis of the liver, and macrophage infiltration of major organs. This is mainly due to uncontrolled inflammation mediated through IFN-γ [206,207,214,215]. SOCS1 has been shown to be essential for the T cell development in the thymus, for the activation of peripheral CD8+ T cells, and for the Th1-Th2 cell balance. Furthermore, SOCS1 has been
implicated in the development of T_{H}17 cells, a subset of cells with important roles in inflammation and autoimmune diseases [208]. T and NKT have been reported to play an essential function in the SOCS1-/- disease, but not alone. Other possible candidates are antigen presenting cells, such as DC and macrophages. SOCS1 has been implicated in hyperresponsiveness to cytokines such as IFN-γ after the exposure of macrophages to LPS. SOCS1 is involved in DC development, maturation and activation. SOCS1-deficient dendritic cells (DC) are hyperresponsive to IFN-γ and IL-4 [216]. In mice on a SOCS1 knock-out background, but with restored SOCS1 expression in B and T cells, DC abnormally accumulated in the thymus and spleen inducing aberrant expansion of B cells and autoreactive antibody production. Furthermore, SOCS1-deficient DC activated not only B cells but also allogeneic T cells [217]. In addition to the role of SOCS in the IFN-γ response, lipopolysaccharide (LPS), IL-12, IL-6, IL-2 and TNF-α are also negatively regulated by SOCS1 [218-223]. Both LPS and TNF-α expression in the graft have been associated with graft failure. As SOCS1 is an inhibitor of both type I and type II IFN signalling, it plays a crucial role in the balance of the beneficial antiviral and detrimental inflammatory effects of IFN signalling [208]. SOCS1-deficient cells and KO mice were shown to be resistant to viral infections but also to non-viral infectious agents, especially intracellular parasites [224]. SOCS3 has an essential role in placental development. SOCS3-KO mice die during the embryonic stage [225,226]. Recent studies revealed SOCS3 as a key regulator of diet-induced leptin and insulin resistance [227]. Furthermore, SOCS3 is an important negative regulator of IL-6 and G-CSF [228,229]. SOCS3 has been shown to be a crucial regulator of the divergent functions of IL-6, and IL-10. These are important cytokines: the first has a pro-inflammatory function and the second is an immunoregulatory cytokine with anti-inflammatory roles. Whereas SOCS3 is induced by both IL-6 and IL10 in the presence of LPS, it selectively inhibits IL-6 signalling via the binding to gp130 [230]. As IL-6 inhibits LPS signalling, the net effect in this model is an increase in inflammation. Consistent with this observation, mice with SOCS3-deficient macrophages and neutrophils are resistant to LPS-induced shock [230]. On the other hand, SOCS3 has a role in preventing IFN-γ-like responses in cells stimulated by IL-6 [228]. Thus, the effect of SOCS3 appears to be dependent on the experimental conditions. In a more recent study, SOCS3 was upregulated in intestinal epithelial cells after stimulation with IL-28A or IL-29. These two interleukins are potent mediators of antiviral activity, and increased IL-28A levels were shown after infection with CMV. It raises the question whether SOCS proteins may play a role in controlling inflammation mediated by infection [231]. Further studies revealed that SOCS3-transduced DCs were highly effective
inducers of T\(_{H2}\)-cell differentiation [208]. On the other hand, SOCS3-deficient DCs were activators of effector CD4\(^+\) T cells but induced FOXP3\(^+\) regulatory T cells. Therefore, the expression of SOCS3 by DCs might have a crucial role in the balance between effector T\(_{H2}\) cells and regulatory T cells. In addition, in T cells SOCS3 has been reported to negatively regulate the induction of T\(_{H3}\), an inducible regulatory T cell subset, and to negatively regulate T\(_{H17}\) cells differentiation.

![Figure 6: SOCS1 and SOCS3 involvement in the negative regulation innate and adaptive immune responses. Adapted from Dimitriou, Immunol Rev 2008 [232].](image)

In summary, SOCS1 and SOCS3 are important regulators of cytokine activity, primarily of IFN-\(\gamma\) and IL-6, by acting as negative feedback inhibitors, and mostly, their effect is anti-inflammatory. Both have direct effects on B and T cell regulation \textit{in vitro} and \textit{in vivo} and might therefore alter the adaptive immune response in transplantation. So far, little is known about the role of SOCS proteins in experimental and clinical transplantation. There are few studies about SOCS proteins and islet transplantation. Using mice with islet cell-specific expression of SOCS3 and SOCS3-transduced rat islet cells, cytokine-mediated destruction
was inhibited and allograft rejection was delayed [233]. In another model, overexpression of SOCS1 in islet grafts protected them from apoptotic loss and prolonged graft survival [234]. In contrast, in spontaneous diabetic mice, SOCS1 expression in islets delayed islet allograft rejection but could not circumvent islet autoimmune destruction [235]. In a rat islet cell transplantation model, mRNA expression of SOCS1 and SOCS3 was increased as early as one day after transplantation [236]. Finally, there are some studies focusing the involvement of SOCS3 in the development of regulatory T cells, reported to be involved in the achievement of transplantation tolerance [237-239].

Recently, the porcine SOCS3 gene was cloned by Zhao et al showing a 93% nucleotide and 96% amino acid identity, compared with human SOCS3 [240]. So far, porcine SOCS1 is found only as Expressed Sequence transcript (EST) in The Institute for Genomic research database. However, since murine SOCS1 is highly identical on the nucleotide level to human SOCS1, it is also likely that the porcine gene can be easily identified. There are only few studies addressing porcine SOCS as part of a more general interest in the immune response to various stimuli. SOCS1 expression was increased in response to porcine reproductive and respiratory syndrome virus (PRRSV) vaccination [241] and in response to Salmonella enterica serovars Choleraesuis and Typhimurium [242]. SOCS3 mRNA expression in porcine PBMC was increased by butyrate [243]. In another study it was downregulated by Pasteurella multocida toxin (PMT) as a mechanism to maintain activation of STATs [244]. It was also suggested that SOCS3 gene expression levels in adipose and muscle tissues, and its relationship to obesity (ob) and insulin-like growth factor I (IGF-I), which are involved in adipose metabolism or muscle development, might contribute to the different fat deposition and muscle development ability between obese and lean pigs [245]. The purpose of this work was to establish quantitative PCR settings in order to discriminate between the human and porcine SOCS1 and SOCS3 sequences and, thus, allowing the detection and quantification of SOCS mRNA expression in allo- and xenotransplantation settings or after HCMV infection.
4. AIM OF THE STUDY

Xenograft rejection occurs in two important phases: HAR, mediated by the binding of xenoreactive nAb and the activation of the complement system, followed by AHXR and cellular rejection, the latter characterized by graft infiltration with different leukocyte subpopulations. Human leukocyte recruitment to xenografts depends on chemotaxis, adhesion, and transmigration. Whereas the cross-species receptor-ligand interactions mediating human leukocyte adhesion to porcine endothelial cells have been well studied *in vitro*, little is known about the mechanism and receptor compatibilities mediating chemotaxis and transmigration. Besides immunological and physiological barriers, the potential for xenozoonosis represents a further major concern. Reactivation of HCMV is one of the most common infectious complications in transplantation medicine and has been associated with allograft rejection for all types of solid organ grafts. Many of the injuring factors involved in the rejection mechanism have a proinflammatory, cytokine-mediated component. SOCS proteins are important regulators of cytokine signalling.

The following aims were outlined in order to investigate the mechanism of cellular recruitment during xenograft rejection and cross-species infection and to develop possible strategies to overcome graft rejection.

**Aim #1:** Investigate the role of αGal in mediating xenogeneic chemotaxis and transendothelial migration (TEM) on specific leukocyte subsets. Investigate the molecular mechanisms of interactions between human leukocyte subsets and αGal-negative porcine endothelial cells (EC) by testing the influence of receptor blocking in a model separating adhesion and TEM.

**Aim #2:** Investigate the influence of human cytomegalovirus (HCMV) cross-species infection on αGal-negative porcine EC in terms of cell surface receptor expression and recruitment of human leukocytes by chemotaxis and adhesion.

**Aim #3:** Establish a porcine-specific SOCS1 and SOCS3 detection system in order to discriminate between the porcine and human SOCS sequences, and detect and quantify SOCS mRNA expression in allo- and xenotransplantation and after HCMV infection, and investigate the consequences and morphological or functional changes related to SOCS protein regulation.
The experimental work is based on an *in vitro* model using porcine aortic endothelial cells (PAEC) devoid of αGal, the most important epitope recognized by nAb in the induction of HAR. PAEC have been adopted as a standard model to investigate leukocyte adhesion and TEM *in vitro*. The material and methods used in this thesis are described in detail in each publication or chapter.
5. RESULTS

5.1 PART I

Human Leukocyte Transmigration Across Galα(1,3)Gal-Negative Porcine Endothelium is Regulated by Human CD18 and CD99

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Abbreviations used in this paper: EC, endothelial cells; FM, fully migrated; FM÷TA, fully migrated out of total adhered; Gal, Galα(1,3)Gal; HAEC, human aortic endothelial cells; MC, membrane compartment; PAEC, porcine aortic endothelial cells; PAEC-KO, PAEC derived from α1,3GT null pigs; PAEC-WT, PAEC derived from Gal-expressing pigs; PBMC, peripheral blood mononuclear cells; pEC, porcine endothelial cells; PMN, polymorphonuclear neutrophils; sDMEM, supplemented DMEM; TA, total adhered; TEM, transendothelial migration
5.1.1 Abstract

Background. In pig-to-human xenotransplantation cross-species receptor interactions mediate cellular infiltration and rejection of porcine grafts. However, the mechanisms responsible for recruitment of human leukocyte subsets across porcine endothelial cells (EC) remain largely unknown. Here we investigated the role of CD99, CD18, and Galα(1,3)Gal (Gal) in this process.

Methods. Adhesion and transmigration of human peripheral blood mononuclear cell (PBMC) subsets on Gal⁻/⁻ and Gal⁺/+ porcine EC and on human EC was analyzed using a two-compartment system separated by a permeable membrane. The mechanisms of human PBMC recruitment to pEC was investigated by blocking cell surface receptors and by differentially measuring adhesion and transendothelial migration (TEM).

Results. Blocking of CD18, but not CD99, decreased human PBMC adhesion on pEC, whereas blocking of either CD18 or CD99 strongly reduced the subsequent human PBMC TEM across pEC. The inhibitory effect of CD99 blockade was slightly stronger across pEC as compared to human EC. A critical role for Gal in TEM of human monocytes, B, NK, NK/T, and T cells was excluded by evaluating TEM across pEC derived from Gal⁻/⁻ and Gal⁺/+ pigs.

Conclusions. CD99 and CD18, but not Gal, play a critical role in human monocyte and lymphocyte TEM across pEC, and their respective porcine ligands may serve as targets to specifically inhibit human leukocyte recruitment in pig-to-human xenotransplantation.
5.1.2 Introduction

The generation of pigs lacking Galα1,3Gal (Gal) [63,65] largely prevents hyperacute rejection, the first major obstacle in discordant pig-to-primate xenotransplantation. Hyperacute rejection is characterized by binding of human xenoreactive natural antibodies to Gal followed by activation of complement and coagulation factors [246]. The initial preclinical studies using Gal-negative pigs as donors for baboon recipients revealed prolonged survival of renal and heart xenografts [37,38], shifting the focus of xenotransplantation research to the subsequent immunological barriers, including acute humoral xenograft rejection and cell-mediated rejection. The latter is characterized by graft infiltration by leukocytes including polymorphonuclear neutrophils (PMN), monocytes/macrophages, NK cells, and T cells, together with endothelial cell activation, thrombus formation, and focal ischemia [20,183,247].

The recruitment of human leukocytes from the vasculature to porcine xenografts depends on cross-species receptor-ligand interactions mediating capture, adhesion, and transendothelial migration (TEM) [183,187,188,248]. Monocytes and PMN infiltrate xenografts within minutes to hours and subsequently stimulate and attract NK and T cells to the graft [184]. Aortic porcine endothelial cells (pEC) have been adopted as a standard model to investigate leukocyte adhesion and TEM in vitro [189-191,200,249-253]. Whilst the compatibility between receptors mediating human leukocyte adhesion on pEC has been addressed in a number of studies [189,190,249], little is known about the mechanisms of xenogeneic chemotaxis and TEM. Previous studies showed that platelet-activating factor and proteinaceous chemokines released by pEC synergistically induce human PMN chemotaxis [187,188], and that porcine islet-derived MCP-1 attracts human monocytes [185]. Human monocytes and NK cells induce the expression of IL-8, MCP-1, PAI-1, CD62E, and CD106 in pEC [254-257], and release endogenous chemokines such as IL-12 and IFN-γ [258,259]. Adhesion of human leukocytes to pEC is mediated by cross-species interactions between human CD18 (β2-integrins), CD29 (β1-integrins), CD62L, and CD162 and their respective porcine ligands [189,191,260,261]. These adhesive interactions facilitate human leukocyte capture and adhesion from free flow and subsequent TEM, as described in an allogeneic model where CD18 binding to CD54 (ICAM-1) and CD102 (ICAM-2) mediated leukocyte locomotion to the junctions [194]. Conversely, homotypic CD31 binding [195,262] is incompatible across the porcine-human species barrier [199]. Nevertheless, other junctional
interactions described in intraspecies settings may still be functional in the porcine-human setting. These include homotypic CD99 [196,197] and junctional adhesion molecule (JAM) binding [198], as well as heterotypic interactions between β1- or β2-integrins and JAM molecules [198], and possibly β3-integrins and CD31 [263,264]. Hauzenberger et al found no significant differences in the capacity of human EC and pEC to support TEM of human PMN and PBMC [200], although preferential PMN migration across pEC was reported subsequently [251]. The migration of human monocytes and NK cells across TNF-α-stimulated pEC was inhibited by masking porcine CD106 [200]. However, whether or not this reflected an active role of CD106 in the actual transmigration, rather than an inhibition of the preceding adhesion, is unclear. Finally, CD18 blockade reduced migration of monocytes across pEC, but for NK cells it only inhibited migration across human, but not porcine, endothelium [200].

Oligosaccharide ligands, including Gal, may constitute potential binding sites on pEC for human leukocytes [49,184,265-267]. Recent studies suggested that monocyte binding to pEC mediated by interactions between Galectin-3 and Gal leads to conformational changes in β2-integrins, associated with a higher affinity and increased adhesion [252,253]. However, other studies did not support these observations since blocking of Gal on pEC had no impact on PMN or NK cell adhesion [256,268]. Furthermore, adhesion of freshly isolated total PBMC, PMN, and purified NK cells was similar on primary EC derived from inbred pigs expressing or lacking Gal [269]. Finally, similar levels of human NK cell adhesion to untreated and Gal-transduced human aortic EC were reported [270]. Therefore, the question of whether Gal mediates adhesion of human leukocytes to pEC remains controversial.

In this study, we addressed the role of the human cell surface receptors CD18, CD31, CD49d, and CD99 in TEM of leukocyte subsets across pEC in blocking experiments and by differentially measuring adhesion and TEM. Furthermore, we investigated the role of Gal in mediating xenogeneic chemotaxis and TEM on specific leukocyte subsets, including human monocytes, B cells, NK cells, T cells, and NK/T cells.
5.1.3 Material and Methods

Cells

Primary aortic endothelial cells, PAEC-WT and PAEC-KO (Fig. 1A), were isolated from normal and α1,3GT null pigs [271], and cultured in sDMEM-10% (DMEM supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, non-essential amino acids, 20 mM HEPES (all Invitrogen, Basel, Switzerland), and 10% FCS (PAA Laboratories, Lucerne, Switzerland), as described earlier [269]. Cell-free supernatants from PAEC-WT and PAEC-KO were collected after 48 h culture in the presence of 100 U/ml human TNFα (PeproTech EC, London, UK) in sDMEM with 1% FCS only (sDMEM-1%). Human aortic endothelial cells (HAEC, Lonza, Basel, Switzerland) were cultured in EGM-2 medium (Lonza). The isolation of human PBMC and NK cells was performed as previously described [48,188]. Monocytes were isolated from PBMC using the Monocyte Isolation Kit II (Miltenyi-Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instructions.

Antibodies and Flow Cytometry

The following mAb were used for phenotyping and blocking studies: anti-human CD3 (UCHT1), CD14 (MφP9), CD19 (B43), CD45 (HI30), CD69 (FN50), and Galectin-3 (B2C10) (all BD-Pharmingen, San Diego, CA), CD18 (7E4) and CD49d (HP2/1) (both from Beckman Coulter, Fullerton, CA), CD31 (hec7, Endogen, Rockford, IL), CD99 (hec2, kindly provided by W. Muller, Cornell University, NY [196]), and CD62L (FMC46, Sigma, Buchs, Switzerland). Surface expression of Galectin on PAEC was analyzed by anti-Gal (M86, Alexis Corporation, Lausen, Switzerland) and FITC-labelled isolectin B4 from Bandeiraea simplicifolia (BS-IB4, Sigma). Stainings with purified mAb were followed by secondary staining with goat-anti-mouse Ig-FITC (Boehringer-Mannheim, Rotkreuz, Switzerland). Isotype-matched mAb (Sigma) were used as controls and dead cells were excluded by propidium iodide gating. Cells were analyzed on a FACScan or FACSCanto (Becton-Dickinson, Basel, Switzerland).

Dynamic Adhesion

Adhesion of human monocytes on PAEC was analyzed under shear stress as previously described [191]. Briefly, PAEC were grown to confluency on an area of approximately 3 cm². The resulting monolayers were washed and 10⁶ monocytes preincubated for 30 min with 10
\(\mu g/ml\) anti-human CD49d, CD31, or Galectin-3, or isotype-matched control mAb in 100 \(\mu l\) sDMEM-1% were added. The dishes were then rotated at 64 rpm, 37\(^\circ\)C, for 10 min and washed. Adherent monocytes were counted by light microscopy on four fields of 0.16 \(mm^2\) per dish at 6 mm from the center, corresponding to a shear stress of 0.7 dynes/cm\(^2\).

**Transmigration**

To study TEM, transparent cell culture inserts (3 \(\mu m\) pores, BD-Falcon) were coated with fibronectin (Fisher Scientific, Wohlen, Switzerland) and seeded with PAEC-WT, PAEC-KO, or HAEC at 5\(x\)10\(^4\) cells per insert. After reaching confluency the inserts were washed and transferred to wells containing 700 \(\mu l\) sDMEM-1% or supernatant from TNF\(\alpha\)-stimulated PAEC-WT or PAEC-KO. PAEC integrity on the inserts was confirmed by microscopy (Fig. 1B) and absence of medium permeability (data not shown). Inserts with intact continuous cell layers and absence of medium permeability were chosen for the experiments. To the upper compartment 10\(^6\) human PBMC were added in 100 \(\mu l\) sDMEM-1%. In blocking studies PBMC were preincubated for 30 min with 10 \(\mu g/ml\) anti-human CD18, CD49d, CD31, CD99, or isotype-matched control mAb at a concentration of 10\(^7\) cells/ml in sDMEM-1%. The cells were then added in 100 \(\mu l\) (10\(^6\) cells/insert) to the upper compartments without washing. Successful preincubation and stability of mAb binding throughout the assay were confirmed by staining preincubated PBMC before seeding and after TEM with secondary goat-anti-mouse Ig-FITC and flow cytometry (Fig. 1C).

After incubation in a humidified incubator (37\(^\circ\)C, 5% \(CO_2\)) for 4 or 20 h cells in the lower compartment were collected as the 'fully migrated' (FM) cell fraction (Fig. 1D). In blocking TEM assays the inserts were transferred to empty plates and non-adherent cells in the upper compartment were removed by gently washing twice with 500 \(\mu l\) HANKS (Kantonsapotheke, Zurich, Switzerland). Inserts were then incubated with 50 \(\mu l\) trypsin (0.25%, Invitrogen) and detached cells were collected by resuspending the upper compartment twice with 500 \(\mu l\) sDMEM-10% and flushing the lower side of the insert with 500 \(\mu l\) sDMEM-10%. The detached cells were defined as the 'membrane compartment' (MC) cell fraction, representing adherent cells and cells at different stages of TEM but still attached to the insert. The FM and MC fractions were quantified by counting the number of cells in 60 \(\mu l\) on a FACScan or FACSCanto, and then multiplying the acquired cell count with the total FM or MC volume in \(\mu l\) divided by 60. Individual PBMC subsets (T cells, NK cells, NK/T cells, monocytes, and B
Results – Part I

cells) were enumerated by relating cell counts with percentage of CD3⁺CD56⁻, CD3⁺CD56⁺, CD3⁺CD56⁻, CD14⁺, and CD19⁻ cells, respectively, and excluding EC based on size, granularity, and negative CD45 staining.

FM ratio was calculated as (# FM cells ÷ # cells seeded) x100 (Fig. 1D). The total amount of cells in the FM and MC fraction was defined as 'total adherent' (TA), representing the number of cells which at a given time point were either adherent or further on in the process of TEM. TA ratio was calculated as (# TA cells ÷ # cells seeded) x100. To separate between the influence of receptor blocking on TEM and adhesion, the FM out of TA (FM÷TA) ratio was calculated as (# FM cells ÷ # TA cells) x100. In blocking assays PBMC interdonor adhesion and TEM variability was adjusted for by normalizing FM÷TA ratios for each intra-assay isotype control to 100%, and then adjusting FM÷TA ratios in the presence of test mAb accordingly.

Statistical Analysis
Statistical analysis was performed by unpaired student T testing to analyze differences in TEM assays. P values < 0.05 (*) were considered to be statistically significant. NS depicts non-significance.
5.1.4 Results

No Difference in Human PBMC Adhesion and Transendothelial Migration Across PAEC-WT and PAEC-KO

TEM is facilitated by leukocyte capture and adhesion, mediating leukocyte locomotion to the junctions. We previously reported that adhesion of human PBMC and PMN on PAEC is independent of Gal [269]. Phenotypic analysis using a panel of mAb including CD31, CD54, CD62E, CD62P, CD86, CD102, and CD106 did not reveal any differences between PAEC-WT and PAEC-KO apart from Gal expression (Fig. 1A and data not shown), consistent with our previous observations [269,272]. Nevertheless, since another study reported that human monocyte adhesion on pEC was inhibited by blocking Galectin-3 [253], we investigated adhesion under shear stress of monocytes preincubated with anti-Galectin-3, isotype-matched control, or adhesion inhibiting or non-inhibiting binding controls (anti-CD49d and anti-CD31, respectively) to PAEC-WT and PAEC-KO (Fig. 2A). The absolute mean numbers per counting field of adherent monocytes treated with isotype control mAb were 159±11 and 165±12 on PAEC-WT and PAEC-KO, respectively, confirming that monocyte adhesion was unaffected by the presence of Gal on pEC. Whereas adhesion on both EC types was strongly reduced by anti-CD49d, treatment with anti-Galectin-3 or anti-CD31 mAb did not decrease adhesion (Fig. 2A).

The influence of Gal expression on TEM was tested by adding human PBMC to PAEC-WT or PAEC-KO on permeable inserts (Fig. 1B). To the lower compartments supernatants from human TNF-α-stimulated PAEC-WT and PAEC-KO, respectively, were added to establish a chemotactic gradient. These supernatants induced similar chemotactic activity in human PBMC as tested in chemotaxis assays using empty inserts (Supplementary Fig. S1). In 4 h TEM assays no statistically significant differences between FM ratios across PAEC-WT and PAEC-KO were detected for any of the tested cell populations (Fig. 2B). The FM ratios of total PBMC across PAEC-WT and PAEC-KO were 1.4% and 2.0%, respectively, which increased to 6.7% and 7.0%, by the addition of supernatant from stimulated PAEC to the lower compartment. FM ratios for the monocyte fraction reached 18.6% and 19.1% across PAEC-WT and PAEC-KO, respectively, whereas the corresponding ratios for the lymphocyte fraction were only 1.8% and 2.3%. After 20 h, with supernatant from stimulated PAEC in the lower compartment, the FM ratios for the total PBMC population were 13.9% and 14.9% across PAEC-WT and PAEC-KO, respectively, allowing TEM analysis also for the individual
Results – Part I

lymphocyte subsets (Fig. 2B). The FM ratios were 45.4% and 51.1% for monocytes, 4.9% and 2.7% for B cells, 7.9% and 7.6% for NK cells, 14.5% and 14.4% for NK/T cells, and 5.5% and 5.7% for T cells, across PAEC-WT and PAEC-KO, respectively. In addition, no significant differences in FM ratios across PAEC-WT and PAEC-KO were observed for IL-2 activated NK cells and T cells (data not shown). Because TEM was similar across PAEC-KO and PAEC-WT, PAEC-KO were chosen for the subsequent experiments based on their higher relevance for xenotransplantation studies using α1,3GT null pigs.

FIGURE 1. Transendothelial migration assay. (A) PAEC-WT (open solid histograms) and PAEC-KO (grey histograms) used in the TEM assays were analyzed by flow cytometry for cell surface expression of Gal using the lectin BS-IB4 or the Gal-specific mAb M86. Open hatched histograms depict fluorescence of the respective negative control. (B) Representative phase contrast pictures of PAEC-WT and PAEC-KO grown on permeable inserts. (C) Human PBMC were preincubated for 30 min with purified blocking mAb before addition to PAEC on permeable inserts and coculture for 4 or 20 h. To verify mAb binding throughout the TEM assay, PBMC from the prestaining and from the lower compartment after full migration were incubated with secondary FITC-conjugated Ab and analyzed by flow cytometry. Shown are stainings (open hatched histogram, isotype control; open solid histogram, CD31; filled histogram, CD99) on monocyte-gated cells before addition to the inserts (prestaining) and from the lower compartment at 20 h (fully migrated) in a representative experiment. (D) Schematic overview of the TEM assay set up. Indicated are the different compartments and the calculation of the TA, FM, and FM÷TA ratios used in this study.
FIGURE 2. Adhesion and transmigration across PAEC is not influenced by Gal. (A) Purified human monocytes were analyzed on PAEC-WT and PAEC-KO under shear stress. Shown is mean percent (±SEM) of adhered monocytes in the presence of blocking mAb against human CD31, CD49d, and Galectin-3 as compared to adhesion in the presence of isotype-matched control mAb (normalized to 100%), as calculated from 4 experiments. (B) Total PBMC were added to PAEC-WT and PAEC-KO grown on permeable inserts. Lower compartments contained supernatants from the corresponding human TNF-α-stimulated PAEC. After 4 and 20 h fully migrated (FM) cells in the lower compartments were enumerated and analyzed for cell lineage marker expression. Bars represent mean percentage (±SEM) FM cells out of seeded for total PBMC, monocytes, and total lymphocytes (4 h) or individual lymphocyte subsets (20 h), as calculated from 6 experiments.
Transition from Adhesion to Transendothelial Migration

To estimate the transition from adhesion to TEM for different PBMC subsets, FM out of TA (FM÷TA) ratios were calculated after determining PBMC numbers in both membrane and lower compartments (Fig. 1D), using cell lineage gating as depicted in Fig. 3A-C. At 4 h after seeding of PBMC on PAEC-KO inserts placed over supernatant from TNF-α-stimulated PAEC-KO, TA ratios were 32% for monocytes, but only 3% for lymphocytes (Fig. 3D). Moreover, the FM÷TA ratio was 65% for the monocyte fraction, but only 8% for lymphocytes (Fig. 3F). The small numbers of TA lymphocytes at 4 h did not allow an appropriate analysis of individual lymphocyte subsets. However, after 20 h the TA ratios for lymphocytes ranged from 3% for T cells to 9% for B and NK cells, and 11% for NK/T cells (Fig. 3G). Here, the FM÷TA ratios varied extensively among the different subsets: 15% for B, 18% for NK, 37% for T, and 54% for NK/T cells (Fig. 4I). Nevertheless, at the same time the most prominent TA, FM, and FM÷TA ratios were seen for monocytes, reaching 55%, 48% and 86%, respectively (Fig. 3G-I).

As an indication of leukocyte activation, the majority of PBMC lost cell surface expression of CD62L after adhesion on PAEC-KO, and remained negative for CD62L after full migration (Fig. 4A). In contrast, only a minority (4-8%) of the lymphocyte population expressed the activation marker CD69 on the cell surface after full migration (Fig. 4B).
FIGURE 3. Analysis of adhesion and transmigration of human PBMC across porcine endothelium. Human PBMC were added to PAEC-KO on permeable inserts and cultured for 4 (D-F) and 20 (A-C and G-I) h in the presence of supernatants from stimulated PAEC-KO in the lower compartment. (A-C) Representative flow cytometry analysis of PBMC subpopulations among trypsinized cells from the MC fraction. (A) Viable PBMC were gated based on CD45 expression, granularity, size, and viability (lack of propidium iodide (PI) staining. The leukocyte population was divided into small (R3) and large (R4) size fractions. Gated cells were analyzed for expression of the lineage markers CD3, CD56, CD14, and CD19 to determine the proportions of monocytes, T, NK/T, NK, and B cells. Due to high autofluorescence background in the monocyte fraction after adhesion and TEM, the R3 (B) and R4 (C) regions were analyzed separately. (D-I) Fully transmigrated (FM) cells were collected from the lower compartment, and the MC fraction was harvested by trypsinization. Cells of each fraction were counted and analyzed for lineage marker expression as shown in A-C. Bars show mean percent (±SEM) total adhered (TA) out of seeded (D and G), FM out of seeded (E and H), and FM out of TA (F and I) at 4 (D-F) and 20 (G-I) h post seeding (p.s.) of total PBMC, as calculated from 4 experiments.
Blocking of CD99 or CD18 on PBMC Reduces Transendothelial Migration Across PAEC

The analysis of FM±TA ratios for PBMC in the presence of blocking mAb allowed estimating the role of various cell surface receptors in TEM in a manner distinct from prior adhesion steps. Only mAb specific for human receptors were used, since mAb against a putative porcine ligand for human CD99 are not available. In agreement with previous studies [189, 191], blocking of CD18 and/or CD49d on human PBMC significantly reduced adhesion (Supplementary Table S1). In contrast, at 4 h CD99 blockade decreased the FM±TA ratios for monocytes by 72% and lymphocytes by 90% (Fig. 5A-C), without having a significant effect on the TA ratio (Supplementary Table S1). Blocking of CD18, but not CD49d, also reduced the FM±TA ratios, particularly in the lymphocyte fraction (Fig. 5A-C).

At 20 h the higher number of transmigrated lymphocytes allowed the appropriate analysis of individual lymphocyte subpopulations (Fig. 5D-I). Blocking of CD18 reduced the FM±TA ratios for all lymphocyte subsets by approximately 50%, whereas masking of CD49d decreased the FM±TA ratios for NK, NK/T, and T cells, but not for B cells (Fig. 5F-I). An accumulative blocking effect by simultaneous masking of CD18 and CD49d was only
observed for NK/T cells and B cells (Fig. 5F,H). CD99 blockade revealed a significant reduction of the FM+TA ratios for all lymphocyte subsets, ranging from 44% for T cells up to 76% for NK cells (Fig. 5F-I). At 20 h the reducing effect of CD99 mAb on the monocyte FM+TA ratio was much smaller as compared to 4 h (Fig. 5E), suggesting the ability of monocytes to utilize alternative receptor interactions for xenogeneic TEM between 4 and 20 h after seeding. Human CD31 blocking did not influence TA, FM, or FM+TA ratios at 4 or 20 h for any PBMC subset (data not shown). Finally, simultaneous blocking of CD18, CD49d, and CD99 did not further decrease TEM as compared with the maximum reductions achieved by masking either CD18+CD49d simultaneously or CD99 alone (data not shown).

**FIGURE 5. Blocking of CD99 or CD18 on human PBMC reduces TEM across porcine EC.** Total PBMC were preincubated with blocking antibodies to CD18, CD49d, and CD99, alone or in combination, and added to PAEC-KO on permeable inserts and allowed to migrate for 4 (A-C) or 20 (D-I) h towards lower compartments containing supernatant from stimulated PAEC-KO. Fully transmigrated (FM) cells were collected from the lower compartment, and the MC fraction was harvested by trypsinization. Cells of each fraction were counted and analyzed for lineage marker expression as described in Figures 2B and 3. To compare separate experiments, the FM+TA ratio of each internal isotype control was normalized to 100%, and the FM+TA ratios for PBMC incubated with test mAb were adjusted accordingly. The bars represent the mean (±SEM) FM+TA ratios adjusted to internal controls of 4 experiments for total PBMC (A), monocytes (B) and lymphocytes (C) at 4 h, and of 6 experiments for total PBMC (D), monocytes (E), and the indicated lymphocyte subpopulations (F-I) at 20 h.

**CD99 Blockade Also Reduces Human Lymphocyte Transmigration Across Human EC**

As shown previously, homotypic CD99 interactions mediate human allogeneic TEM of monocytes [196] and neutrophils [197], whereas the impact of CD99 on lymphocyte TEM has not been reported to date. Therefore we investigated whether CD99 mediated allogeneic TEM...
across HAEC similar to that observed in the xenogeneic setting described in Fig. 5 and Supplementary Table S1. Blocking mAb against human CD31 was used as a positive blocking control since CD31 is known to mediate allogeneic TEM of both lymphocytes and monocytes [273]. After 4 h the percentage of total adhered cells in the allogeneic setting reached 5.0%, 18.2%, and 1.3% (TA ratios) for total PBMC, monocytes, and lymphocytes, respectively, i.e. lower than in the xenogeneic setting (10%, 32%, and 4%, Fig. 3D). In contrast, compared to the xenogeneic setting, the mean FM÷TA ratios were slightly but not significantly higher in the allogeneic setting, reaching 60.1%, 72.5%, and 9.8% for the total PBMC, monocyte, and lymphocyte populations, respectively. At 4 h CD99 blockade significantly reduced the FM÷TA ratios for both monocytes and lymphocytes (Fig. 6A-C), although the blocking effect was slightly lower than in the xenogeneic setting (Fig. 5A-C). In agreement with the xenogeneic setting, CD99 blockade significantly reduced the FM÷TA ratios for NK, NK/T, and T cells by roughly 50% after 20h, whereas the CD99 blocking effect on the monocyte population again was much smaller than after 4 h (Fig. 6D-I). In contrast, CD99 blocking did not influence FM÷TA ratios for B cells. Masking of either CD31 or CD99 revealed similar reductions of the FM÷TA ratios except for lymphocytes at 4 h and B cells at 20 h.

FIGURE 6. Blocking of CD99 or CD31 on human PBMC reduces TEM across human EC. Total PBMC were preincubated with blocking mAb to human CD31 or CD99, added to HAEC on permeable inserts and allowed to migrate for 4 (A-C) or 20 (D-I) h towards lower compartments containing supernatant from stimulated HAEC. Cells of each fraction were counted and analyzed for lineage marker expression as described in Figures 2B and 3. To compare separate experiments, the FM÷TA ratio of each internal isotype control was normalized to 100%, and the FM÷TA ratios for PBMC incubated with test mAb were adjusted accordingly. The bars represent the mean (±SEM) FM÷TA ratios adjusted to internal controls of 4 experiments for total PBMC (A), monocytes (B) and lymphocytes (C) at 4 h, and of 4 experiments for total PBMC (D), monocytes (E), and the indicated lymphocyte subpopulations (F-I) at 20 h.
5.1.5 Discussion
The role of Gal in leukocyte recruitment in models of pig-to-human xenotransplantation is a matter of controversy. In this study, a crucial role of Gal in TEM was excluded by comparing the capacity of PAEC-WT and PAEC-KO to mediate human PBMC chemotaxis, adhesion, and TEM. A recently published pig-to-human whole blood model including xenoreactive antibodies and the same pEC batches as in the present study demonstrated a role of Gal in pEC activation leading to cytokine release and CD62E upregulation [259]. In contrast, in the absence of xenoreactive antibodies a number of studies observed no influence by Gal on human leukocyte adhesion [256,268-270]. However, others reported that monocyte adhesion on PAEC depended on Gal-mediated activation [252], and was reduced by blocking Galectin-3 despite very low cell surface expression [253]. It was suggested that Gal was not directly mediating adhesion, but was inducing a CD18 high affinity state on monocytes [252]. In contrast to the latter studies, we analyzed the role of Gal in the different steps of leukocyte recruitment by directly comparing PAEC-WT and PAEC-KO. The negligible role of Gal in adhesion and TEM may in part be explained by the prominent and uniform expression of classical adhesion molecules on PAEC-WT and PAEC-KO ([269] and data not shown), overruling a putative contributing effect of Galectin-3-mediated monocyte activation. In fact, we found no significant difference in monocyte adhesion on PAEC-WT and PAEC-KO in the presence of anti-Galectin-3 mAb.

We demonstrate a novel role for CD99 in regulating the transmigration of human leukocytes through porcine endothelium. For monocytes, the inhibition by anti-CD99 was most pronounced at 4 h post seeding but almost reversed at 20 h, indicating the capability of monocytes to use alternative receptors. Previous studies showed that homotypic CD99 ligation mediates TEM of human monocytes and PMN across human EC, but not the preceding adhesion [196,197]. The porcine ligand(s) of human CD99 has still to be identified; the anti-human CD99 mAb used in the present study did not stain porcine cells excluding an effect of the mAb on pEC. Our results also revealed a role for CD99 in TEM of human lymphocytes across human EC, confirming the importance of CD99 in both allogeneic and xenogeneic leukocyte trafficking. Nevertheless, organ specific inhibition of TEM across a potential xenograft without affecting the human immune responses in host tissues relies on the identification of, and generation of species-specific antibodies against, the porcine ligand for human CD99.
The role of CD18-integrins in human leukocyte adhesion on pEC has been described previously [189,191]. However, to our knowledge this is the first study to show a specific role of CD18 in the subsequent xenogeneic TEM, by a model separating adhesion from TEM. It may be hypothesized that CD18 binding to porcine ICAM mediates leukocyte locomotion to the junctions, or that CD18 interacts with porcine JAM molecules, as shown in human allogeneic models [194,274]. In a previous study, TEM of human monocytes and NK cells across pEC was strongly inhibited by masking porcine CD106, whereas human CD18 blocking only reduced TEM of monocytes [200]. However, since that study did not separate between adhesion and TEM, the question remained whether CD18 and CD106 had active roles in TEM, or if the reduced TEM rather resulted from an inhibition of the preceding adhesion. In the present study, CD49d blocking alone resulted in a minute reduction of monocyte TEM, but a more pronounced inhibition of NK, NK/T, and T cell TEM. Whether this reduction was due to inhibition of the transition from adhesion to TEM, or of CD49d interactions with junctional ligands, needs further investigation. Finally, in agreement with the reported incompatibility between human and porcine CD31 [199], blocking of CD31 did not influence TEM of human PBMC.

We studied the mechanisms of TEM across PAEC by analyzing the effect of mAb blockade on the transmigration of individual PBMC subsets subsequent to adhesion. TEM of individual PBMC subsets was analyzed after seeding total PBMC, allowing cellular interactions between different leukocyte cell types during the assay. However, we did not investigate at which locations between the apical surface and the abluminal side of the endothelium that TEM was inhibited as done in other protocols [275]. Certainly, PBMC may have remained attached to the abluminal side of the membrane compartment after TEM, and therefore not been recorded as transmigrated. Nevertheless, the validity of our method for the study of TEM mechanisms is demonstrated by the significant fractions of adhered PBMC subsets which eventually fully migrated to the lower compartment, reaching up to 86% for monocytes.

In conclusion, Gal is probably not a dominant direct mediator of human leukocyte recruitment to pEC. Instead, CD99 and CD18 receptors and their respective porcine ligands have regulatory roles in xenogeneic TEM and may constitute attractive targets for future therapeutical interventions aimed at specifically suppressing cellular xenograft infiltration.

Acknowledgements

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### 5.1.6 Supplemental material

**Supplementary Table S1**: Influence of adhesion receptor blockade on adhesion and TEM.

<table>
<thead>
<tr>
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<tr>
<td></td>
<td>B cells</td>
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<tr>
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<sup>1</sup>The TA (total adhered), FM (fully migrated), and FM÷TA (fully migrated out of total adhered) ratios of each internal isotype control were normalized to 100%, and the corresponding ratios for PBMC incubated with indicated test mAb were adjusted accordingly. Data represent mean values ± SEM. Significant differences (p<0.05) to the internal isotype controls are depicted by *. 

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**Chemotaxis of human PBMC** (A) or activated purified NK cells (B) towards supernatants from human TNF-α-stimulated PAEC-WT (WT sup) or PAEC-Gal-KO (KO sup), or medium control was analyzed using empty permeable inserts in 4 h assays. Shown is percent fully migrated (FM) out of seeded for total PBMC, lymphocytes, monocytes (A), and activated NK cells (B), after adding total PBMC (A) or purified activated NK cells (B), respectively. Unspecific chemotactic effect of human TNF-α was investigated by comparing 4 h chemotaxis of human PBMC or activated purified NK cells towards medium containing 100 U/ml human TNF-α or not (C). Bars represent mean percentage (±SEM) as calculated from 4 (A) and 3 (B and C) independent experiments.
5.2 PART II
Human CMV Infection of Porcine Endothelial Cells Increases Adhesion Receptor Expression and Human Leukocyte Recruitment

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Keywords: xenotransplantation, cytomegalovirus, endothelial cells, adhesion, leukocyte recruitment

Abbreviations used in this paper: αGal, Galα(1,3)Gal; dpi, day(s) post infection; HCMV, human cytomegalovirus; hEC, human EC; IE antigen, immediate early antigen; MFI, geometric mean fluorescence intensity; MFIR, mean fluorescence intensity ratio; PAEC-KO, primary porcine aortic endothelial cells derived from α1,3GT null pigs; PCMV, porcine cytomegalovirus; pEC, porcine endothelial cells; SN, cell-free supernatant; SLA-I, porcine MHC class I / swine leukocyte antigen class I
5.2.1 Abstract

Background. Potential xenozoonosis is a concern for the clinical application of xenotransplantation. Human cytomegalovirus (HCMV) is one of the most important pathogens in allotransplantation, but the consequences of HCMV cross-species infection of porcine xenografts are unknown. Therefore, we investigated the effects of HCMV infection of porcine endothelial cells (pEC) on cell surface molecule expression and human leukocyte recruitment.

Methods. Infection of pEC inoculated with untreated, UV-inactivated, or heparin-treated HCMV at an MOI of 1 was analyzed by immediate early (IE) antigen expression. Cell surface receptor expression was studied by flow cytometry on pEC bulk cultures and differentially on IE-positive and -negative pEC. Adhesion of human PBMC was tested on pEC monolayers. pEC supernatants were analyzed for cytokine content, chemotactic activity, and stimulatory effect on resting secondary pEC cultures.

Results. At day 2 post infection IE staining was evident in 10-20% of HCMV-infected cells. Cell-surface expression of E-selectin and VCAM-1 was upregulated in both IE-negative and positive fractions of HCMV-infected pEC. In contrast, porcine MHC class I expression was upregulated in IE-negative, but reduced in IE-positive cells. The receptor alterations in the IE-negative fraction were mediated by pEC-derived soluble factors. The increased adhesion receptor expression was paralleled by enhanced human leukocyte chemotaxis and adhesion to infected pEC cultures. Pretreatment of HCMV with heparin, but not UV-inactivation, prevented adhesion-receptor modulation, and reversed the increased adhesion and chemotaxis.

Conclusions. Following pig-to-human solid organ transplantation HCMV may infect and activate the porcine endothelium, rendering the xenograft more susceptible to human leukocyte recruitment and rejection.
5.2.2 Introduction

The clinical success of allotransplantation has led to a shortage of available human organs. Considerable progresses in preclinical research suggest that xenotransplantation has the potential to relieve this shortage. The generation of genetically engineered pigs devoid of Galα(1,3)Gal (αGal) [63,65,246] has overcome hyperacute rejection. Thus, current efforts focus on finding solutions to minimize acute humoral and cellular xenograft rejection. Another concern is the potential risk for human recipients to be exposed to porcine pathogens (xenozoonosis). Indeed, a preclinical pig-to-baboon xenotransplantation model showed porcine CMV (PCMV) reactivation in the graft [145]. However, the majority of known pathogens leading to xenozoonoses can be successfully eliminated by the use of stringent protocols in the rearing of pig donors [82]. In contrast, host derived infection of the xenograft remains a largely unexplored potential problem. Reactivation of baboon CMV was observed in a pig-to-primate model in several tissues and contributed to the death of one animal [145].

Human cytomegalovirus (HCMV) is one of the most important pathogens following allotransplantation [276] and has been correlated to allograft rejection for all types of solid organ transplants [132,135,136,139]. The direct effects of CMV infection (CMV disease) can be prevented by prophylactic or preemptive therapy. However, there are important indirect CMV effects that may contribute to long term graft dysfunction and graft rejection [131]. Consequently, intense systemic immunosuppression is usually required to prevent rejection. The even stronger immunosuppressive regimens required for xenografts (5) would most likely lead to HCMV reactivation in potential pig-to-human xenotransplantation clinical trials. Until a few years ago HCMV had been considered as a strictly host-specific pathogen, when the possibility of cross-species infectivity of HCMV to porcine EC (pEC) was raised by Degre et al [147]. However, this study left the question of the immunological consequences of cross-species HCMV infection of pEC open.

Localized at the interface between the graft and the host immune system, the endothelium of vascularized grafts plays a crucial role during rejection [149]. While under physiological conditions it maintains an anti-inflammatory and anti-coagulant environment, HCMV infection might contribute to graft rejection by disrupting this delicate balance. Infection of human EC (hEC) with HCMV induces the production of chemokines, IL-8 (CXCL8) and GROα [98], and cytokines, IL-1β, IL-6 and IFNγ, respectively [151,152]. Moreover, CMV infection results in alterations of adhesion-molecule expression [99,151,153-158], including controversial data on upregulation [99,151,159,160] and downregulation [154-156] of E-
selectin (CD62E) and VCAM-1 (CD106). During acute rejection, human leukocytes are recruited to the graft and subsequently amplify the immune and inflammatory response. The HCMV-induced release of chemokines and the increased adhesion receptor expression is linked to enhanced leukocyte recruitment, including increased neutrophil chemotaxis and leukocyte adhesion [98,159,162]. In addition, the migration of T-cell subsets, neutrophils, and monocytes across EC is enhanced upon HCMV infection [99,163,164]. Moreover, neutrophils and monocytes are infected during transmigration across the infected endothelium and thus contribute to virus dissemination [98,99]. HCMV also modulates MHC molecule expression by the action of unique short proteins that interfere with MHC maturation and translocation to the cell surface [120]. Studies on MHC class I regulation are controversial, showing both an upregulation [152,277-279] and a downregulation [120,280] upon HCMV infection.

We hypothesized that HCMV infection of pEC may result in alterations of pEC phenotype that may trigger human immune responses. Therefore, we studied cell surface expression of adhesion receptors and porcine MHC class I (SLA-I) on HCMV-infected pEC derived from Galα1,3-Gal deficient pigs, and investigated the consequences of infection on the recruitment of human leukocyte in terms of adhesion and chemotaxis.
5.2.3 Material and Methods

Cells
Primary aortic endothelial cells (PAEC-KO) were isolated from α1,3GT null pigs [271], following standard procedures [48]. PAEC-KO (passages 13-19) were cultured in DMEM supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, non-essential amino acids (1x), 20 mM HEPES (all Invitrogen, Basel, Switzerland), and 10% FCS (LabForce, Nunningen, Switzerland). Human PBMC were isolated by Ficoll gradient as previously described [48].

HCMV
TB40/E [281] was kindly provided by Dr. Christian Sinzger (University of Tübingen, Germany) and AD169 was obtained from ATCC (American Type Culture Collection, Rockville, MD). Both virus strains were propagated on human fibroblasts (MRC5) and purified by ultracentrifugation over a 15% sucrose cushion (50mM Tris-HCl, 12mM KCl, 5mM Na2EDTA) at 20,000 rpm for 90 min at 4°C using a SS-34 rotor (Beckman Coulter, Fullerton, CA).

Infection of Porcine Endothelial Cells
PAEC-KO were grown in 6-well plates (Becton-Dickinson, Basel, Switzerland) for phenotypic and adhesion assays, or in shell vials (IG Instrumenten-Gesellschaft, Switzerland) for immunocytoprotectionstaining, after precoating with bovine fibronectin (Invitrogen). When 70% confluency was established, PAEC-KO were incubated with either normal medium (mock) or with TB40/E or AD169 (MOI 1). As control, HCMV was either UV-inactivated (5 min, 30 cm distance from a 30W, 230V, 50 Hz UV lamp (Osram) [282]) and/or preincubated for 1 h with 2-20 μg/ml heparin (Sigma-Aldrich, Buchs, Switzerland) [101,283,284] before addition to PAEC-KO. After 90 min incubation (37°C, 5% CO2), the virus was removed, cells were washed twice with Hanks’ balanced salt solution (Kantonsapotheke, Zurich, Switzerland) and further incubated with fresh medium for 1 to 2 days. UV inactivation was confirmed by IE staining and PCR. MOI of 1 was chosen to be possibly close to physiological conditions while still allowing the documentation of HCMV-mediated effects in vitro.
Incubation of PAEC-KO With Supernatants from HCMV-Infected Cultures

To study the effects of soluble factors secreted by infected PAEC-KO, cell-free supernatants (SN) from HCMV-infected cells collected at 1 or 2 day post infection (dpi) were preincubated for 1 h with heparin to block any potential residual virus, and added to fresh PAEC-KO cultures. As a positive control for EC stimulation, cells were incubated with 100 U/ml human TNFα (PeproTech, Rocky Hill, NJ). The phenotype of the secondary cultures was analyzed after 24 h incubation.

Detection of HCMV Infection

At 1 or 2 dpi, HCMV-infected PAEC-KO monolayers were washed with PBS, fixed in cold acetone-ethanol mix (1:2) for 10 min, and air-dried. The monolayers were then covered with 50 μl of a 1:50 dilution of mouse mAb directed against HCMV immediate early antigens (IE) (E13, Argene, Varhiles, France) and incubated for 30 min at 37°C. Thereafter monolayers were washed with PBS, and incubated at 37°C for 30 min with 1:50 dilution of FITC-conjugated goat anti-mouse-IgG Fab’2 (Boehringer-Mannheim, Rotkreuz, Switzerland) and Evans blue (Argene). After staining, each coverslip was rinsed, mounted in 1 drop of fluokeep (Argene), and analyzed using a Nikon fluorescence microscope.

Adhesion Assay

Adhesion of freshly isolated human PBMC on PAEC-KO was analyzed in static assays as previously described [191]. At 2 dpi, monolayers were washed with Hanks, overlaid with 5x10^6 freshly isolated PBMC in 800 μl and incubated for 15 min in a humified incubator. Thereafter non-adherent cells were removed and the monolayers were washed twice with Hanks. To collect adherent cells, cells were trypsinized (Invitrogen) and fixed in 4% formalin for 15 min at room temperature. Adherent cells were quantified on a FACSCanto (Becton-Dickinson, Basel, Switzerland). Individual PBMC subsets were enumerated by relating cell counts with percentage CD3⁻CD56⁻ (T), CD3⁻CD56⁺ (NK), CD14⁺ (monocytes), and CD19⁺ (B) cells. Contaminating PAEC-KO were excluded by size, granularity, and CD45-negative staining. Adhesion ratios were calculated by dividing cell-subset numbers with the originally seeded populations.

Chemotaxis Assay

SN harvested at 2 dpi were added in 900 μl to the lower compartment of 24-well companion plates (Becton-Dickinson) as described [285]. Empty cell-culture inserts (3 μm pores, Becton-
Dickinson) were placed into the wells and 10^6 freshly isolated human PBMC were rapidly added in 300 µl culture medium to the upper compartment. After 4 h incubation (37°C, 5% CO₂), migrated cells were collected from the lower compartment and quantified on a FACSCanto. Migration ratios were calculated by dividing total cell subset numbers with the originally seeded populations. As a positive control SN from PAEC-KO stimulated for 48 h with 100 U/ml human TNFα was used.

**Cell Staining and Flow Cytometry**

For cell surface single stainings PAEC-KO were fixed and incubated with anti-CD31/PECAM-1 (LCl-4, Serotech, Oxford, UK), anti-CD106/VCAM-1 (10.2C7, kindly provided by D. Haskard, Hammersmith Hospital, London, UK), anti-CD62E/E-selectin (1.2B6, Serotec, Kidlington-Oxford, UK), and anti-SLA-I (74-11-10, VMRD, Pullman, WA). MOPC21 (IgG1, BD-Pharmingen, San Diego, CA) and MOPC141 (IgG2b, Sigma) were used as isotype-matched controls. For combined cell surface and IE stainings, cells were first stained with mAb to cell surface receptors, fixed and permeabilized following the BD Cytofix/Cytoperm protocol (Becton-Dickinson), and then incubated with alexa-fluor conjugated IE1 mAb (8B1.2, Chemicon International, Temecula, CA). Secondary PE- or APC-conjugated goat anti-mouse Ig (Bohringer-Mannheim, and Becton-Dickinson, respectively) were used for detection of unconjugated mAb. Phenotype and purity of PBMC was analyzed using anti-CD3-FITC (UCHT1), anti-CD14-FITC (MφP9), anti-CD19-PE (B43), anti-CD45-APC (HI30), and anti-CD56-PE (B159) mAb (all BD-Pharmingen). Cells were analyzed on a FACSCanto. To compare the levels of surface expression the mean fluorescence intensity ratios (MFIR) were calculated by dividing the geometric mean fluorescence intensity (MFI) of the test mAb with the MFI of the control mAb.

**Cytokine Quantification**

Cell-free SN from HCMV-infected PAEC-KO, cultured in DMEM supplemented with 1% FCS, were collected at 1 and 2 dpi and tested for cytokine content, including porcine IL-1β, IFNγ, TNFα, IL-6 and IL-8, following the SearchLight protocol (Pierce, Rockford, IL). Briefly, 50 µl of each sample was added in duplicates in precoated 96-well plates. After 3 h incubation at room temperature, the plates were washed and incubated for 30 min with biotinylated antibodies, then washed and incubated for 30 min with streptavidin-HRP. After washing, SUPERSIGNAL substrate was added, and luminescence was immediately detected.
on a Kodak Image system. Data were analyzed with the Array-Vision Software (GE-Healthcare).

Statistical Analysis
Statistical analysis was performed by column statistics test (GraphPad Prism) to analyze the differences in receptor expression, adhesion and chemotaxis. P values < 0.05 (*) were considered statistically significant.
5.2.4 Results

HCMV Infection of PAEC-KO Modulates Cell Surface Receptor Expression

In line with a previous study [147], infection of PAEC-KO with HCMV strains TB40/E and AD169 was successful, resulting in 10-20% of infected cells (Fig. 1A and data not shown). Heparin is known to inhibit virus entry into human cells by competitive binding to heparan sulfate receptors on HCMV [101,283,284]. In agreement, titration studies revealed a 70% reduction of IE staining upon preincubation of TB40/E with 2 µg/ml heparin (Supplemental Fig. 1), whereas complete blocking was reached with 20 µg/ml (Fig. 1B). In contrast, no blocking effect was observed when PAEC-KO were preincubated with heparin (Fig. 1C). At 1 dpi the infected cultures showed no clear morphological changes whereas significant alterations in cell surface receptor expression were observed. As compared with mock-infected culture, CD31 cell surface intensity was decreased by 8%, while CD62E, CD106, and SLA-I were increased by 44%, 58% and 24%, respectively, in TB40/E-infected cultures (data not shown). At 2 dpi, clear morphological changes, as manifested by increased size and granularity, and partial cell detachment, were observed in cultures inoculated with HCMV or with UV-inactivated virus. These cultures also showed significant changes in receptor expression (Fig. 1D-G), indicating that HCMV entry, but not replication, is necessary to mediate these effects. At 2 dpi, baseline receptor expression in mock culture, as indicated by MFIR, was 2.69 for CD31, 1.08 for CD62E, 15.92 for CD106 and 13.86 for SLA-I. In average, incubation of PAEC-KO with TB40/E or UV-inactivated virus induced a 37% increased expression intensity of CD62E, a 60% increase of CD106, a 20% decrease of CD31, and a 42% increase of SLA-I (Fig. 1H-K). In contrast, no significant changes in receptor expression were observed after incubation with heparin-pretreated HCMV. The same patterns were observed in cells infected with AD169 (Fig. 1F,G). To confirm that heparin was solely blocking virus entry and was interfering neither with detection antibody binding, nor with cytokine receptor ligation on endothelial cells, PAEC-KO were cultured with human TNFα [269], in the presence of heparin during culture and/or antibody incubation. TNFα stimulation resulted in increased adhesion receptor expression that was unaffected by both heparin preincubation and coincubation, or by incubating PAEC-KO with heparin immediately before the addition of detection antibodies (Supplemental Fig. 2). Thus, an interfering role of heparin in cytokine stimulation or detecting antibody binding was ruled out, justifying heparin as an appropriate negative control in our further experiments.
Results – Part II

FIGURE 1. Immediate early (IE) antigen expression and modulation of receptor expression in PAEC-KO is blocked by heparin. (A-C) Immediate early antigen staining of PAEC-KO infected with TB40/E (MOI 1) at 2 dpi (A). Either the virus (B) or PAEC-KO (C) were preincubated with 20 μg/ml heparin. Shown is one representative experiment out of 6. (D-K) PAEC-KO were incubated with HCMV, UV-inactivated or heparin-treated HCMV (MOI 1) and combinations thereof, and analyzed for cell surface receptor expression at 2 dpi. Expression of CD62E (D, F) and CD106 (E, G) on PAEC-KO cultured in control medium (mock, filled solid histograms), or inoculated with HCMV (open solid histograms), heparin-treated (open dotted histograms), or UV-inactivated (open dashed histograms) TB40/E (D, E) or AD169 (F, G). (H-K) Relative expression intensity of CD62E (H), CD106 (I), CD31 (J) and SLA-I (K) on treated PAEC-KO as related to mock culture (normalized to 100%). The bars depict mean (±SD) percent of control culture expression of the indicated markers on PAEC-KO as calculated from 10 independent experiments. * = p<0.05.

Receptor Expression Modulation is Observed in Both IE-Positive and IE-Negative Fractions

In cytoimmunological stainings, only 10-20 % of PAEC-KO were positive for IE at 1 and 2 dpi in cultures infected with TB40/E at an MOI 1. However, an overall shift in the cell surface receptor expression pattern was observed (Fig. 1D-G). Therefore, to differentially investigate expression intensities in IE-positive and negative fractions, we performed double stainings of
IE versus CD62E, CD106, CD31 or SLA-I. As in cytoimmunological stainings, a minor fraction of PAEC-KO was IE-positive in HCMV-inoculated cultures, whereas PAEC-KO incubated with UV-treated TB40/E were IE-negative (Fig. 2A,B). Differential gating on IE-positive or negative PAEC-KO revealed a uniform change in CD62E, CD106, and CD31 expression intensities in both populations (Fig. 2C-E). In contrast, SLA-I expression was slightly decreased in IE-positive, but increased in IE-negative PAEC-KO (Fig. 2F).

**FIGURE 2.** Receptor modulation is observed both in the IE positive and negative fractions of HCMV-infected cultures. PAEC-KO cells were incubated with TB40/E (MOI 1) (A) or UV-inactivated TB40/E (B). At 1 dpi PAEC-KO were analyzed for the expression of IE antigens. Cell surface expression of CD62E (C), CD106 (D), CD31 (E), and SLA-I (F) was separately analyzed on the IE positive and negative gated fraction. The histograms represent an overlay of mock-infected PAEC-KO (filled grey histograms), and IE-negative (open dashed histograms) and IE-positive cells (open solid histograms) in TB40/E-infected PAEC-KO cultures. The isotype control is depicted by the light grey histograms. Shown is one representative experiment out of 5.

Receptor Expression Changes in the IE-Negative Fraction are Mediated by Soluble Factors
In order to investigate the bystander effect inducing receptor modulation on IE-negative cells in HCMV-infected cultures, cell-free SN from PAEC-KO (primary cultures) inoculated with TB40E (TB40/E-SN) or UV-inactivated (TB40/E-UV-SN) and/or heparin-treated TB40/E, were added to untreated secondary cultures. To avoid infection of secondary cultures with potentially remaining HCMV, SN were preincubated with heparin before addition to PAEC-KO. After 24 h incubation with TB40/E-SN or TB40/E-UV-SN collected at 2 dpi (2 dpi SN), we observed changes in receptor expression similar to those seen in primary cultures: CD62E was increased by 43% and 28%, CD106 by 95% and 92%, and SLA-I by 63% and 54%, respectively (Fig. 3). Similar trends were observed with AD169 SN (data not shown). SN from primary cultures incubated with heparin-pretreated HCMV did not induce receptor expression modulation. These data indicate that phenotypical changes were mediated by both
HCMV infection and by soluble factors released from HCMV-infected cells acting on bystander cells and possibly also on infected cells in an autocrine manner.

**FIGURE 3.** Receptor upregulation is mediated by soluble factors released by infected cells. Secondary PAEC-KO cultures were incubated with 2 dpi SN from primary cultures inoculated with TB40/E, UV-inactivated or heparin-treated TB40/E (MOI 1). SN were preincubated with heparin to block residual virus. As a positive control, cells were stimulated with 100 U/ml human TNFα. After 24 h incubation, the expression of cell surface receptors was analyzed by flow cytometry. Shown is relative expression of CD62E (A), CD106 (B), CD31 (C) and SLA-I (D) on SN-incubated PAEC-KO as related to mock control culture (normalized to 100%). Bars represent mean (±SD) percent of control culture expression of the indicated markers on PAEC-KO as calculated from 5 independent experiments. * = p<0.05.

**Increased Receptor Expression Promotes Human Leukocyte Adhesion to PAEC-KO**

Considering the important role of CD62E and CD106 in human leukocyte adhesion to pEC [183], we investigated the functional consequences of receptor upregulation on PBMC adhesion to PAEC-KO inoculated with HCMV (Fig. 4A-E) or incubated with SN from HCMV-treated cultures (Fig. 4F-L). To adjust for interdonor variability in baseline adhesion, the adhesion to mock-infected PAEC-KO, or to secondary cultures incubated with SN from mock-infected primary cultures, was normalized to 100% for each donor, and the adhesion under the experimental conditions were adjusted accordingly. At 2 dpi, the adhesion ratios on mock-infected PAEC-KO ranged from 7% for T cells, to 12% for B cells, 21% for NK cells and 31% for monocytes. The overall PBMC adhesion increased by 158% and 73% on PAEC-KO incubated with TB40/E or UV-inactivated virus, respectively (Fig. 4A). For the different subpopulations the increase was 85% and 43% for monocytes, 192% and 90% for B cells, 125% and 60% for NK cells, and 158% and 73% for T cells, respectively, (Fig. 4B-E). A similar pattern was seen on cells incubated 24 h with 2 dpi SN: the overall PBMC adhesion increased by 71% and 52% on PAEC-KO treated with TB40/E-SN or TB40/E-UV-SN,
respectively (Fig. 4F). The corresponding increase for the different subpopulations was 45% and 28% for monocytes, 128% and 91% for B cells, 71% and 60% for NK cells, and 102% and 81% for T cells, respectively, (Fig. 4G-L). No increased adhesion was observed on PAEC-KO incubated with heparin-pretreated HCMV or with SN from primary cultures incubated with heparin-pretreated virus.

**FIGURE 4. Increased adhesion receptor expression promotes human leukocyte adhesion to PAEC-KO.** Adhesion of freshly isolated human PBMC on PAEC-KO was analyzed in static assays. At 2 dpi (A-E) or after 24 h incubation with 2 dpi SN (F-L), PAEC-KO monolayers were overlaid with freshly isolated PBMC. Adherent leukocytes were quantified on a FACScanto. Individual PBMC subsets were enumerated by relating cell counts with percentage CD3⁺CD56⁻, CD3⁻CD56⁺, CD14⁺, and CD19⁺ cells, respectively, and excluding contaminating PAEC based on size, granularity, and CD45 negative staining. The adhesion ratios were calculated by dividing cell subset numbers by the number of originally seeded cells. Bars represent mean (±SD) percent of adherent cells, as related to cells adhered to PAEC-KO incubated with mock or mock-SN (normalized to 100%) for total PBMC (A, F), monocytes (B, G), and lymphocyte subset (C-E, H-L). * = p<0.05. The mean is calculated from 6 (A-E) and 4 (F-L) independent experiments.
HCMV-Infected PAEC-KO Induce Chemotaxis in Human PBMC

As previously reported, HCMV infection of hEC induces the production of chemokines [98]. Here we tested the chemotactic activity of SN from HCMV-treated PAEC-KO on human leukocytes. After 4 h incubation, the baseline chemotaxis toward control medium was 5% for total PBMC, 1% for lymphocytes and 26% for monocytes. However, incubation with 2 dpi TB40/E-SN or TB40/E-UV-SN increased the chemotaxis by 59% and 27% for total PBMC, 60% and 31% for monocytes, and 35% and 16% for lymphocytes, respectively (Fig. 5 and Supplemental Fig. 3). Similar results were obtained with AD169 (data not shown).

FIGURE 5. Soluble factors released from HCMV-infected PAEC-KO induce chemotaxis in human PBMC. SN from HCMV-infected or control treated PAEC-KO, or from PAEC-KO cells stimulated with TNFα, was added in the lower compartment of 24-well companion plates. Freshly isolated total human PBMC were added to permeable inserts and allowed to migrate for 4 h. Cells were counted on a FACScanto. Bars represent mean (±SD) percent chemotaxis adjusted to the mock control of 6 independent experiments for total PBMC (A), monocytes (B) and lymphocytes (C). * = p<0.05.

HCMV-Infected PAEC-KO Secrete IL-6 and IL-8

To determine possible soluble factors responsible for EC activation and chemotaxis induction, cell-free SN from HCMV- or control-infected PAEC-KO were analyzed for different porcine cytokines, including IL-1β, TNFα, IFNγ, IL-6 and IL-8. Of these, IL-6 secretion was strongly
induced and IL-8 secretion moderately increased in 2 dpi TB40/E-SN or TB40/E-UV-SN (Table 1). Pretreatment of the virus with heparin prevented the enhanced cytokine release.

**TABLE 1.** Production of IL-6 and IL-8 in HCMV-infected cultures.a

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*aSN from PAEC-KO cultures inoculated with TB40/E, UV-inactivated or heparin-treated TB40/E (MOI 1) at 2 dpi was tested in a sandwich ELISA for the presence of different porcine cytokines. The values represent the mean concentrations (pg/ml) as calculated from 6 (IL-6) or 4 (IL-8) independent experiments. * = p<0.05.

**5.2.5 Discussion**

For clinical xenotransplantation, both transmission of graft-derived infection and reactivation of latent infection in the host are of concern. Improvement in pig rearing protocols has reduced the transmission risk of a number of pathogens including PCMV, but reactivation of host-derived HCMV can be expected. In this study we analyzed phenotypical and immunological aspects of HCMV infection of pEC, focusing on adhesion receptor expression and human leukocyte recruitment. HCMV infection of PAEC-KO modulated cell surface receptor expression in the bulk pEC population, including a significant upregulation of CD62E, CD106, and SLA-I, and a minor downregulation of CD31, in line with previous studies of HCMV-infected hEC [151,159,277-279]. Inhibition of viral replication by UV-inactivation of HCMV [282] did not prevent the upregulation of CD62E and CD106 on PAEC-KO, as previously reported for hEC [151,159]. In contrast, blocking viral entry by heparin [101,283,284] abrogated receptor regulation. Taken together, the receptor modulation depends on virus entry and/or attachment to the cell, but not on viral replication and IE gene expression. In agreement, HCMV-mediated effects in human cells, such as the induction of antiviral genes [102,103,286], did not require gene expression but depended on virus-to-cell contact and/or entry. In HCMV-incubated PAEC-KO cultures an overall change in receptor expression was observed, despite the small percentage of IE-positive cells. CD31, CD62E, and CD106 expression was similarly modulated in both the IE-positive and IE-negative subpopulations, in concordance with a study by Dengler et al. on HCMV-infected hEC [151].
In contrast, while SLA-I surface expression was increased in the larger IE-negative fraction, and thereby also in the overall population, it was downregulated in the smaller IE-positive fraction. This explains the observation that overall MHC expression was decreased in human fibroblasts infected with HCMV at high MOI, resulting in a high percent of infected cells, whereas MHC expression was slightly increased at lower MOI [152]. Moreover, in human fibroblasts MHC-I was downregulated upon infection with HCMV, but upregulated when incubated with UV-inactivated virus [286]. Taken together, the controversial observations reported for MHC-I expression in human cells probably result from differences in the percentage of infected cells. Thus, it may be hypothesized that an early entry event activates the infected cells, resulting in altered expression of CD62E, CD106 and SLA-I in infected PAEC-KO, and secretion of soluble factors promoting a receptor upregulation on uninfected bystander cells. Indeed, incubation of secondary cultures with supernatants from HCMV-infected primary cultures, induced a receptor upregulation similar to that seen in primary cultures. While CD62E and CD106 expression remained upregulated after viral gene expression, SLA-I receptor expression was abrogated in the IE-positive fraction. In human cells, HCMV proteins expressed at different stages of infection, are responsible for MHC-I modulation by interfering with MHC maturation and translocation to the cell surface [120]. This exemplifies an early HCMV entry-event that does not require virus gene expression itself, but is later counteracted by viral gene expression [103].

CD62E and CD106 are important mediators of human leukocyte adhesion to pEC [183,189,190]. Consequently, HCMV-induced upregulation of these adhesion receptors was paralleled by a strong increase in human PBMC adhesion to PAEC-KO. A similar pattern was observed for secondary PAEC-KO incubated with SN from HCMV-infected cultures. Furthermore, SN from HCMV-infected cultures induced chemotaxis of human leukocytes. In agreement, previous studies reported enhanced neutrophil chemotaxis and leukocyte adhesion to HCMV-infected hEC [98,159,162]. In HCMV-incubated PAEC-KO, a minor downregulation was seen for CD31, a junctional molecule promoting human leukocyte transmigration [262]. However, homotypic CD31 binding is incompatible across the porcine-human species barrier [199]. Nevertheless, a minor decrease of CD31 expression may contribute to intracellular gap formation, thus facilitating increased leukocyte infiltration.

Dengler et al. reported that adhesion receptor upregulation on hEC was mediated by secretion of IL-1β [151]. We did not detect significant levels of porcine IL-1β in HCMV-SN, neither other cytokines such as TNFα and IFNγ. However, porcine IL-6 and IL-8 were present as possible mediators for the observed bystander effects on uninfected PAEC-KO and for the
Results – Part II

Induction of leukocyte chemotaxis. IL-6 has both pro-inflammatory and anti-inflammatory features, and plays a critical role in human leukocyte migration, activation and apoptosis [287,288]. Whether or not the increased chemotaxis observed with SN from HCMV-infected cells was partly mediated by a crossreactive action of porcine IL-6 on human monocytes needs further investigation, using recombinant porcine IL-6 and blocking antibodies. In contrast, a role for IL-6 in the receptor modulation on noninfected bystander cells is less likely, since at least hEC can release IL-6 but lack an IL-6 receptor [289,290]. However, secondary effects of IL-6 secretion in vivo may be mediated by soluble IL-6 receptor/IL-6 complexes, shed from neutrophils and binding to gp130 on hEC [291]. This process results in release of chemotactic factors and upregulation of adhesion receptors on EC, thus promoting leukocyte recruitment [287,288]. In accordance with human studies [98], we found porcine IL-8 in the SN of HCMV-incubated PAEC-KO. However, although porcine IL-8 binds human leukocytes, its potential effect on human leukocyte chemotaxis is a matter of debate [187,188]. Together with previous studies, our data stress the importance of soluble factors released from infected cells that contribute to an amplification of the immune and inflammatory response [151,152,292,293]. Furthermore, HCMV induced apoptosis in HCMV-infected pEC cultures (Millard, unpublished data). Apoptosis of pEC would probably result in direct graft damage, but may also contribute, together with the release of proinflammatory and chemotactic cytokines, to an amplification of the inflammatory and immune responses.

In conclusion, HCMV cross-species infection modulates SLA-I and adhesion receptor expression on pEC, and induces the secretion of mediators promoting the recruitment and activation of human leukocytes, which are important contributors of graft rejection. Therefore, further studies are needed to understand the mechanisms of infection and to find strategies aimed at rendering pEC resistant to HCMV infection, before xenotransplantation can become a clinical reality.
5.2.6 Supplemental material

**Supplemental Figure 1:**

**Heparin titration.** To determine the concentration required for complete blocking of virus entry, TB40/E was preincubated with increasing concentration of heparin ranging from 2 mg/ml to 20 mg/ml, before being added to PAEC-KO. To verify that heparin was binding only on the virus side, PAEC KO were preincubated with 20 mg/ml heparin before the addition of the virus. The values represent the mean percent of infected cells, as counted from 6 different fields in one representative experiment.

**Supplemental Figure 2:**

**TNFα** is neither interfering with cell surface receptor ligation, nor with detection antibody binding to PAEC-KO. PAEC-KO were cultured with 100 U/ml human TNFa, in the presence of heparin (20 mg/ml) during culture and/or antibody incubation. TNFα stimulation resulted in an increased adhesion receptor expression on PAEC-KO (solid histogram) that was unaffected by both heparin preincubation (open dashed line) or coincubation (open dotted histogram), and by incubating the cells with heparin immediately before the addition of detection antibodies (open dashed-dotted histogram).
Supplemental Figure 3:

**Chemotaxis.** Soluble factors released from HCMV-infected PAEC-KO induce chemotaxis in human PBMC. SN from TB40/E-infected or control treated PAEC-KO, was added in the lower compartment of 24-well companion plates. Freshly isolated total human PBMC were added to permeable inserts and allowed to migrate for 4 h. Shown is one representative experiment out of 6.
5.3 PART III
Detection of SOCS1 and SOCS3 expression in porcine endothelial cells

5.3.1 Abstract

*Introduction:* Transplanted grafts are exposed to a variety of challenges, such as rejection and infection. Graft damage is partly caused by the strong pro-inflammatory mediators IFN-γ and IL-6. We hypothesized that negative regulation of inflammation is crucial for haemostasis of the graft. Suppressors of cytokine signaling proteins (SOCS) 1 and 3 are two important regulators of the inflammatory mediators IFN-γ and IL-6 associated with injury during transplantation.

*Material and Methods:* Different primers and probes were evaluated for SOCS species specificity and quantification and were then used to measure SOCS mRNA expression in porcine endothelial cells devoid of αGal (PAEC-KO) after HCMV infection and after coculture with human PBMC.

*Results:* The high nucleotide homology between porcine and human SOCS posed a great challenge for specificity. By choosing primer and probes in regions of discrepancy between the porcine and human sequences we were able to specifically detect porcine SOCS mRNA. SOCS1, but not SOCS3 expression, was upregulated in HCMV infected PAEC-KO. In contrast, coculture with human PBMC did not significantly alter SOCS1 and SOCS3 mRNA expression levels in PAEC-KO.

*Conclusion:* This preliminary report shows that SOCS proteins are functional in porcine endothelial cells and leukocytes. SOCS message expression is, at least in part, correlated to infection and endothelial activation. Control of inflammatory responses might represent an attractive approach to reduce graft damage.
5.3.2 Introduction

SOCS proteins are of interest in the context of allo- and xenotransplantation because of their role in the control of inflammatory responses. Pro-inflammatory cytokines, in particular IFN-\(\gamma\) [202-204] and IL-6 [203] are detected in the graft in experimental transplantation models, and may be involved in graft rejection. In a negative feedback loop, SOCS1 and SOCS3 are inhibitors of IFN-\(\gamma\) and IL-6.

Little is known about the role of SOCS proteins in experimental transplantation. A partially protective effect of SOCS1 and SOCS3 in delaying islet allograft rejection was described [233-235], and another study showed increased mRNA expression of SOCS1 and SOCS3 after islet transplantation [236]. Other studies focused on the involvement of SOCS3 in the development of regulatory T cells, reported to be important for the achievement of transplantation tolerance [237,238].

The porcine SOCS3 gene was recently cloned showing a 93% nucleotide and 96% amino acid identity, compared with human SOCS3 [240]. So far, porcine SOCS1 is found only as Expressed Sequence Transcript (EST). A comparison of the EST sequence with the human open reading frame (ORF) reveals a high homology sequence also for SOCS1. The interest for porcine SOCS is limited to few studies in the context of a more general interest in the immune response to various stimuli [241-244].

The aim of this final part of the thesis was to develop a specific real time PCR in order to specifically quantify porcine SOCS mRNA expression in various experimental settings.
5.3.3 Material and Methods

Cells

Primary aortic endothelial cells (PAEC-KO) were isolated from α1,3GT null pigs [271], following standard procedures [48]. PAEC-KO (passages 13-19) were cultured in DMEM supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, non-essential amino acids (1x), 20 mM HEPES (all Invitrogen, Basel, Switzerland), and 10% FCS (LabForce, Nunningen, Switzerland). Human PBMC were isolated by Ficoll gradient as previously described [48]. Human aortic endothelial cells (HAEC, Lonza, Basel, Switzerland) were cultured in EGM-2 medium (Lonza). Human and porcine PBMC were isolated by Ficoll gradient as previously described [48,188]. For LPS stimulation, 5x10^6 PBMC or confluent EC
were seeded in 1 ml medium in 6-well plates (Becton Dickinson, Basel, Switzerland) and incubated with 100 ng/ml LPS (Sigma-Aldrich, Buchs, Switzerland) for 5h.

**HCMV**

TB40/E [281] was kindly provided by Dr. Christian Sinzger (University of Tübingen, Germany) and AD169 was obtained from ATCC (American Type Culture Collection, Rockville, MD). Both virus strains were propagated on human fibroblasts (MRC5) and purified by ultracentrifugation over a 15% sucrose cushion (50mM Tris-HCl, 12mM KCl, 5mM Na2EDTA) at 20,000 rpm for 90 min at 4°C using a SS-34 rotor (Beckman Coulter, Fullerton, CA).

**Infection of Porcine Endothelial Cells**

PAEC-KO were grown in 6-well plates (Becton-Dickinson) after precoating with bovine fibronectin (Invitrogen). When 70% confluency was established, PAEC-KO were incubated with either normal medium (mock) or with TB40/E or AD169 (MOI 1). As control, HCMV was either UV-inactivated (5 min, 30 cm distance from a 30W, 230V, 50 Hz UV lamp (Osram) [282]) and/or preincubated for 1 h with 20 μg/ml heparin (Sigma-Aldrich, Buchs, Switzerland) [101,283,284] before addition to PAEC-KO. After 90 min incubation (37°C, 5% CO2), the virus was removed, cells were washed twice with Hanks’ balanced salt solution (Kantonsapotheke, Zurich, Switzerland) and further incubated with fresh medium for 1 to 2 days. UV inactivation was confirmed by immediate early (IE) antigen staining and PCR.

**RNA isolation and reverse transcription**

Infected or stimulated cells were washed once with Hanks buffer, and collected by trypsinization (Invitrogen). Cells were then pelleted, washed again and RNA was isolated following the QIAGEN RNAeasy mini kit protocol (QIAGEN, Düsseldorf, Germany). To eliminate possible genomic DNA contamination, the samples were treated with the RNase-free DNase I (QIAGEN). For SOCS protein detection and quantification, RNA was reverse transcribed following the Superscript III (Invitrogen) protocol, using either specific primers (microsynth, Balgach, Switzerland) or OligodT primers (Promega, Madison, WI, USA). The cDNA was then amplified by PCR and loaded on 2% agarose gel or quantified by TaqMan PCR amplification either as single reaction or in multiplex (HotStarTaq Master Mix Kit, QIAGEN; iQ Multiplex powermix, BioRad, Hercules, CA, USA, respectively)
Primers and Probes

porcine SOCS1 standard:
fw primer: 5’CGTGGGGCACCTTCCTG 3’
rv primer: 5’CCGCCACGTAGTGCTCCA 3’
amplicon:
CGTGGGGCACCTTCCTGGTGCGCGACAGCCGCCAGCGGAACGTCTTCTTCTGCCCCTC
AGTGTGAAGATGCTTCTCGGCGCCCCACAAAGCATCCGGGTGCATTCCAGGGCGGCCGC
GCTTCCACCTGGGACGCGGCCAGCGGAGAGGCTTGACTGCTTCTGAGCTGCTGGA
GCACTACGTGCGG

porcine SOCS3 standard:
fw primer: 5’GAGAAGATCCCTCTCTGGTTG 3’
rv primer: 5’AAAGTGGGGCCATGTACTGG 3’
amplicon:
GAGAAGATCCCTCTGGTTGAGCCGGCCCCTCCTCCTCAACGTGGCCACTCTCC
AGCAtCTCTGTGAAAGACGATTCACGGCCACCTGAACTCTATAGAAGATCAG
CCAGCTGCCTGGGCTTGGACTCCTATGAGAAAGTCACCAGCTG

porcine SOCS1 taqman:
fw primer: 5’GGAACTGCTTCTTTCCGCCCCTC 3’
rv primer: 5’GAACGCGGCAGGCTGG 3’
probe: 5’FAM-TGGGGGCGGAAGCATBHQ1 3’
amplicon:
GGAACTGCTTCTTTCCGCCCCTCAGTGTCAGATGGCTGCTTGCCGCCCACAAAGCAT
CCGCCGTGCACCTCCAGGCGCGGCTTCC

porcine SOCS3 taqman:
fw primer: 5’CCACGCTGCCACTCTCCCGCATCTCTGTCGAAAACAGTCAACGCGCCACCT
GCCGTCACCTCCAGGCCCGGCCCCTC

porcine GAPDH:
fw primer: 5’ACATGGGCTCCAAGGAGTAA 3’
rv primer: 5’GATCCAGTGGGCTGGTACT3’
probe: 5’HEX-CCCAACCAACACCGAAGAGC-CBHQ1 3’

porcine HPRT:
fw primer: 5’GTGATAGATCCATTCCTATGACTGTAAGA3’
rv primer: 5’TGGAGATACATCTCCACCAATTACTT3’
probe: 5’Cy5-ATCGCCCGTTGCACTGCTACTAGCT-CBHQ2 3’

Quantitative PCR
Reactions were performed in a real-time thermocycler (i-cycler; Biorad): 30 min at 50°C; 15
min at 95°C followed by 60 cycles of 5s at 95°C and 1.20 min at either 60°C (single reaction),
or 58 °C (multiplex) monitoring fluorescence at 60°C. Calibration was performed by use of the i-cycler software. Under these conditions, target DNA were detected with a linear dynamic range from $10^1$ to $10^7$ copies. For quantification of SOCS mRNA expression the threshold cycle, or Ct, values of PCR amplification were used in the delta-delta Ct method.

### 5.3.4 Results

SOCS1 and SOCS3 are detected in human and porcine endothelial cells and PBMC

To detect SOCS1 and SOCS3 expression in human (HAEC) and porcine (PAEC) endothelial cells (EC) or PBMC, total RNA was isolated from untreated or LPS stimulated cells and reverse transcribed using previously published primers [241,243,294,295] and new designed primers. The resulting cDNA was amplified by PCR, and analyzed by gel electrophoresis. SOCS expression could be detected in both human and porcine EC and PBMC, and was slightly enhanced after LPS stimulation (Figure 2). By testing different primers we noticed that not all primers were species specific, but some amplified both the human and the porcine SOCS sequences (data not shown). This was in line with the high similarity between the two sequences.

![Image](71x755)

**Figure 2: SOCS1 and 3 expression in human and porcine EC and PBMC.**

Clear amplification for human SOCS1, and for human SOCS3 but with some background. Porcine SOCS1 and SOCS3 amplification, with some little background for porcine SOCS1 in PAEC cells but not in PBMC. po SOCS1 primer NEW was not specific. Every second band is from LPS-stimulated cells.
*Porcine-SOCS specific quantitative PCR*

In order to specifically detect porcine SOCS1 and SOCS3 we designed and tested different primers and probes. To discriminate between the porcine and human sequences, much attention was given in choosing a mismatch at the 3’ end for primers and 5’ end for probes, important for the binding to the target sequence.

The annealing temperature was carefully chosen. As shown in Figure 3 for SOCS1, by decreasing the temperature from 60 °C to 58 °C there was an enhancement of SOCS1 amplification without an increase of primer dimers in the negative control.

**Figure 3: Melt peak chart of porcine SOCS1 amplification at 58 and 60 °C.**

Solid line: PAEC-KO, 60 °C; triangles: PAEC-KO, 58 °C; crosses: neg control 58°C; squares: neg control, 60 °C

The combination of primers and probe resulted specific amplification of porcine SOCS. The primes and probes chosen and used for further experiments are highlighted in bold characters in Figure 1.

**Figure 4: SOCS amplification in specific for porcine cells.** SOCS1 (left panel) and SOCS3 (right panel) amplification in PAEC (triangles) or HAEC (crosses) using porcine-specific primers and taqman probes. The negative control is depicted as solid line.
**Results – Part III**

*SOCS1 mRNA expression is upregulated in HCMV-infected cultures*

HCMV infection of solid organ transplants is known to induce both direct and indirect effects contributing to graft loss. The indirect effects might be explained by the immunoactivation produced by the virus in the host resulting in recruitment of inflammatory cells and inflammatory effectors. Because of the role of SOCS protein in the regulation of important mediators involved in graft rejection, we decided to compare SOCS1 and SOCS3 mRNA expression between mock and HCMV-infected PAEC-KO at 1-2 dpi. As a control the virus was either preincubated with heparin, known to block virus attachment/entry and/or with UV, to prevent viral gene expression. SOCS mRNA expression was detected in a multiplex real time PCR, with the parallel amplification of two housekeeping genes, porcine GAPDH and HPRT. Changes in SOCS mRNA expression were calculated using the delta-delta Ct method. There was a clear increase of SOCS1 (Figure 5), but not SOCS3 (not shown) mRNA expression in TB40/E-infected PAEC-KO compared to the level of the housekeeping genes which did not show a significant variation between the different samples. The SOCS1 upregulation was very strong but showed large variations between separate experiments, ranging from a 50-fold to a 100-fold change. SOCS1 upregulation was prevented by blocking virus attachment/entry (heparin), but not by preventing viral gene expression (UV). Similar results were obtained with HCMV strain AD169 (data not shown).

**Figure 5: Increased SOCS3 expression in cells incubated with HCMV +/- UV pretreatment.**

SOCS1 (left panel), GAPDH (upper right panel) and HPRT (lower right panel) amplification for medium +/- heparin (solid plain line), TB40/E (triangles), TB40/E + heparin (crosses), TB40/E-UV (diamonds) or TB40/E-UV + heparin (squares). The negative control is in grey. The figures are representative for one experiment out of four.
Establishment of SOCS standard curves

For a more accurate quantification of SOCS mRNA expression, we created a standard by PCR amplification and gel purification of longer SOCS1 and SOCS3 fragments. The primers used are listed in the Material and Methods section.

Figure 6: porcine SOCS1 and SOCS3 standard curves.

Porcine SOCS1 (left panel) and SOCS3 (right panel) standard curves, using the standards obtained with primers listed in Material and Methods. The figure shows 100-fold dilutions, starting from 5x10^7 copies/μl.

SOCS1 and SOCS3 mRNA expression is not affected by coculture of porcine endothelial cells with human PBMC

To analyze if the interaction with human leukocytes affects SOCS1 and SOCS3 mRNA expression, PAEC-KO were cocultured with human PBMC. However, after 5h coincubation no significant changes in SOCS mRNA expression were detected in PAEC-KO (data not shown).

5.3.5 Discussion

In this study we established real time PCR settings allowing us to discriminate between the porcine and human SOCS1 and SOCS3 sequences. This tool enables us to measure mRNA expression in different conditions such as in xenotransplantation settings, when both human and porcine cells are present.

SOCS1 is an inhibitor of both type I (IFNα and IFNβ) and type II IFN (IFNγ) signaling [208,296]. Whereas type I IFN are well known for their antiviral activity, IFNγ plays a major role as a powerful immunomodulatory cytokine, being involved in the activation of the adaptive and innate immune responses and in the upregulation of MHC on antigen presenting cells. The proinflammatory features of IFNγ may also be involved in graft rejection. Not surprisingly, many viruses develop defence mechanisms in order to abrogate IFN production.
by either interfering with IFN-activating pathways [297] or by the induction of inhibitory pathways. SOCS1 and SOCS3 are upregulated by different viruses in human models. SOCS3 upregulation by herpes simplex virus infection contributed to the inhibition of the interferon signalling pathway thus leading to efficient viral replication [298,299]. SOCS3 was upregulated by HCV [300], and hepatic SOCS3 expression correlated to non-response to HCV therapy [301]. Both SOCS1 and SOC3 were upregulated upon Influenza A virus challenge and negatively regulated the host innate immune response [302]. SOCS1 upregulation may also be responsible for the immunological defects observed after HIV-infection [303].

HCMV was shown to interfere with the interferon pathway by downregulation of Jak-1 and interferon regulatory factor 9 (IRF9) [304,305]. A recent publication showed that the HCMV-encoded homolog of the immunosuppressive cytokine IL-10 did not only suppress the transcription of proinflammatory cytokines through direct inhibition of NF-κB but also enhanced the transcription of the negative immune regulator SOCS3 [306].

We observed increased SOCS1 mRNA expression in HCMV-infected PAEC-KO cultures. As observed in part II of this thesis, for the regulation of adhesion molecules, SOCS1 upregulation was dependent on an early attachment and/or entry event but not on viral gene expression. In agreement with the above mentioned studies, SOCS1 upregulation may contribute to viral escape mechanisms by inhibiting the antiviral and inflammatory responses. In contrast, SOCS3 expression was unaffected in HCMV-infected cells. However, there may have been an effect which was not detected in our system because of the time point chosen for mRNA detection [307]. If the upregulation is dependent on an early HCMV attachment/entry event, the mRNA levels might be upregulated early but already decreased again at 1-2 dpi. Further kinetic experiments analyzing earlier timepoints are required before a regulation of SOCS3 by HCMV can be excluded. The same also accounts for SOCS1 and SOCS3 expression in PAEC-KO cocultured with human PBMC. An additional question is whether the observed upregulation of SOCS1 is specific for the directly infected cells or if it occurs also in uninfected bystander cells in the same culture, mediated by the action of soluble factors secreted from infected cells, as shown for the modulation of cell surface adhesion receptors in part II of this thesis. This question could be answered by comparing SOCS1 mRNA expression in cells infected at increasing MOI and analyze if the expression is changing with increasing percentage of infected cells. The variation reported for SOCS1 upregulation in the different experiments can be controlled by the introduction of a standard for the quantification, thus allowing a more precise comparison.
Finally, it would be of great interest to also investigate SOCS modulation in human cells infected with HCMV.
6. GENERAL DISCUSSION

The shortage of human organs is a major challenge in allotransplantation. The number of patients in need of a transplant keeps increasing, while the quantity of available donor organs does not. In Switzerland, only 459 patients could be transplanted in 2008; 942 non-transplanted patients were left on the waiting list, and, unfortunately, 62 persons died while waiting for an organ transplant. The urgency for transplantation may depend on the affected organ. In case of heart, liver or lung dysfunction there are few alternative procedures to keep the patient alive, and heart transplantation requires brain-dead donor with a beating heart. In contrast, kidney transplants can be obtained also from living donors but the organ supply is still insufficient for the high demand. Potential solutions to overcome the shortage of human organs are (1) artificial organs and mechanical devices, (2) stem-cell-derived organ and tissue regeneration, and (3) xenotransplantation [20]. The first option is currently used as a temporary solution to support the circulation as a bridge to transplantation or recovery, but has not shown the potential to supersede transplantation as a long-term curative therapy in the near future. Stem cell transplantation may delay the need for transplantation but at present cannot replace entire organs. Attempts to grow organs hold great promise for the future, but progress in this field towards clinical organ replacement will require many years of research. Xenotransplantation might provide the most imminent solution to overcome the lack of available organs. Recently, the successful reversal of diabetes using porcine islet cells in a nonhuman primate model [1,2], and the encouraging preliminary data from an early-phase safety clinical trial where six patients with type I diabetes were transplanted with encapsulated neonatal porcine islets [3], have rekindled enthusiasm and enhanced the possibility of clinical trials of pig-to-human xenotransplantation.

For several reasons primates have been replaced by miniature swine as potential donors for clinical xenotransplantation [8]. However, immunological barriers are not easily overcome in pig-to-primate xenotransplantation. Since the 1990s, with the identification of the crucial role of preformed natural antibodies against the αGal epitope and of the complement system in HAR [5,308], great efforts where undertaken to overcome this first rejection step. The generation of pigs transgenic for complement regulatory proteins represented an important step forward, but the major achievement toward control of HAR occurred in 2002, when αGal KO pigs were successfully bred [63,65]. Recent studies showed a reduced impact of humoral graft rejection when grafts from αGal KO pigs were transplanted into non-human primates. The survival of porcine xenografts in baboons increased significantly to 2-6 months for
hearts, and up to 83 days for kidney. However, graft failure eventually occurred in all transplants where thrombotic microangiopathy was the main histological finding [309]. After the success obtained in preventing HAR, the focus of immunological research shifted to the next immunological barriers: AHXR and cellular rejection, the latter characterized by graft infiltration with different leukocyte subpopulation [52,247,310], and physiological incompatibilities such as the disparities in the coagulation system [26].

The first aim of this thesis was to elucidate the role of αGal in, and receptor interactions responsible for, human leukocyte recruitment to the porcine endothelium. The role of αGal as a binding receptor for human leukocytes is controversial. While some studies showed that αGal-binding via Galectin-3 promoted monocyte adhesion [252,311], others reported that blocking αGal on porcine EC did not significantly reduce total PBMC, PMN or NK adhesion [256,268-270]. In accordance with these findings, our study ruled out a significant role of αGal in human leukocyte recruitment to porcine aortic endothelial cells (PAEC), by directly comparing the ability of PAEC expressing or lacking αGal (PAEC-KO) to mediate chemotaxis, adhesion, and TEM. Furthermore, we showed an important role for CD18 and CD99 receptors in xenogeneic TEM. In line with studies on allogeneic human leukocyte TEM across human EC [196,197], we showed that CD99 on human leukocytes is involved in TEM across, but not adhesion on, porcine EC. The role of CD18 integrins is well described in human leukocyte adhesion to porcine EC [189,191], however, in this study we showed a specific role of CD18 also in the subsequent TEM. Further studies are needed to clarify if CD18 blocking is inhibiting locomotion to the junctions or the actual TEM. This will allow to specifically block the interaction, i.e. with mAb against ICAM-1 and ICAM-2, or against JAM molecules, reported to have an important function in human allogeneic models, respectively [194,274]. Our findings and further studies elucidating the receptor interactions mediating the recruitment of human leukocytes to the xenograft endothelium, may contribute to the discovery of novel targets for future therapeutical interventions aiming at specifically prevent xenograft infiltration.

Besides immunological and physiological barriers, xenozoonosis raises important concerns. The risk of pig-to-human virus transmission can be reduced by breeding and maintaining the pigs in specific-pathogen-free (SPF) conditions, and by screening of donor pigs. Nevertheless, major concerns have been raised with regard to the transfer of porcine endogenous retroviruses (PERV). However, a number of different studies did not provide any evidence for
successful PERV infection \textit{in vivo} [81-84]. In contrast, infections of the graft by recipient pathogens are more challenging to prevent and have been taken into consideration only recently.

The \textbf{second aim} of this thesis focused on the effects of human cytomegalovirus (HCMV) infection on PAEC-KO in terms of cell surface receptor expression and human leukocyte recruitment. HCMV is an important pathogen in allotransplantation whose reactivation in immunosuppressed transplant recipients has been linked to graft rejection. The results obtained show that cross-species infection of PAEC-KO with HCMV induced an alteration of cell surface SLA-I, an increase of adhesion receptor expression, and secretion of the pro-inflammatory cytokines IL-6 and IL-8. PAEC activation by HCMV was shown to be independent of gene expression but dependent on virus attachment and/or entry. HCMV-infected cells released soluble factors acting on bystander cells and possibly also on infected cells in an autocrine manner, responsible for the modulation of receptor expression. The increase in adhesion receptor expression was paralleled by increased human leukocyte recruitment as observed by enhanced chemotaxis toward, and adhesion to HCMV-infected cultures. An additional study in our group demonstrated productive HCMV cross-species infection of pEC from different anatomical origins [148]. In contrast to the human setting, where HCMV efficiently suppresses programmed cell death, apoptosis in HCMV-infected pEC was increased.

Taken together, HCMV reactivation during the extensive immunosuppression required for xenotransplantation would probably lead to direct graft damage and may contribute to an amplification of the inflammatory and immune response via the induction of porcine endothelial activation, thus leading to xenograft rejection. Therefore, HCMV cross-species infection of the xenograft needs to be carefully prevented in order to protect the graft from rejection. Currently, reactivation of HCMV is prevented by pharmacological intervention. While successful in the majority of cases, associated risks such as side effects and the arising of viral resistance are potential problems. The development of new therapeutical agents and of effective HCMV vaccines is a very attractive area of pharmaceutical and vaccine research. Xenotransplantation could offer an advantage compared to allotransplantation, giving the possibility to genetically modify the pig organ in order to render it less susceptible to HCMV infection and thus reducing the need for antiviral therapy.

The intrinsic ability of the graft to counteract injuring mechanisms following transplantation and to repair damages may be crucial for ultimately maintaining graft function. Control of
inflammatory responses represents an attractive approach to prevent both innate and adaptive immune mechanisms. In the late 1990s, a family of inhibitory proteins was identified and termed “suppressors of cytokine signalling (SOCS) proteins” [205,208]. The third aim of this study focused on porcine SOCS1 and SOCS3, due to their involvement in the regulation of inflammatory mediators associated with transplantation, e.g. IFN-γ and IL-6 [202-204]. Given the high similarities between the human and porcine SOCS sequences, it was challenging to establish a detection tool able to discriminate between the two species. By testing different primers we were able to define porcine-specific sequence targets allowing to specifically quantify porcine SOCS1 and SOCS3 mRNA expression. This tool allowed us to document an increase of SOCS1 expression in HCMV-infected cultures. SOCS1 and/or SOCS3 are upregulated by different viruses and contribute to the viral evasion strategies [298-300,302,303]. The regulation of SOCS3 in HCMV cultures needs further investigation by analyzing different timepoints after infection.

This tool will allow to analyze mRNA expression under different conditions, such as in allo- and xenotransplantation models or after administration of immunosuppressants. Furthermore, the consequences and morphological or functional changes related to SOCS protein regulation can be investigated.

To conclude, much progress has been made in the field of xenotransplantation in the last years by characterizing and overcoming important immunological barriers. Graft survival has been prolonged from minutes or hours, as it was 20 years ago, to weeks or months, thus increasing the possibility of clinical trials. The generation of αGal KO pigs provided an opportunity to identify new barriers and, thus, new potential targets. In line with that, the blocking of receptors mediating leukocyte recruitment to the xenograft could contribute to protecting the graft from cellular infiltration and prevent rejection. One big advantage of xenotransplantation is the possibility to specifically block receptors on the porcine xenograft thus avoiding the potential side effects associated with unspecific blocking in allotransplantation. Promising results were obtained with a combination of anti-CD4 with anti-LFA-1 and anti-ICAM-1 [312] or anti-VLA-4 and anti-LFA-1 [313] in promoting long-term survival of neonatal porcine islets xenograft in mice and in prolonging graft survival in murine allo- and xenotransplantation models [314-316]. Current studies aim at the generation of a transgenic pig on the αGal KO background, expressing additional protective genes such as coagulation inhibitors or human complement regulators. Additional approaches in order to reduce xenograft rejection focus on the induction of T- and B-cell tolerance, inhibition of NK cell
responses, and the promotion of accommodation. To prevent the risk associated with xenozoonosis, in addition to the control of PERV expression in porcine xenografts, HCMV infection in the recipient needs to be carefully taken into consideration. A potential approach could focus on the development of strategies aimed at rendering the graft more resistant to infection. Therefore cross-species infection of porcine grafts needs to be further characterized in order to identify novel potential targets.

Extensive efforts and investigations are required before xenotransplantation can become a clinical reality and a solution to overcome the severe shortage of human organs.
7. APPENDIX

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7.2 List of Abbreviations

\(\alpha_{\text{Gal}}\) Gal\(\alpha(1,3)\text{Gal}\)

\(\text{Ab}\) antibody

\(\text{AHXR}\) acute humoral xenograft rejection

\(\text{dpi}\) day(s) post infection

\(\text{EC}\) endothelial cells

\(\text{HAEC}\) human aortic endothelial cells

\(\text{HAR}\) hyperacute vascular rejection

\(\text{HCMV}\) human cytomegalovirus

\(\text{hEC}\) human EC

\(\text{HSPGs}\) heparan sulfate proteoglycans

\(\text{IE antigen}\) immediate early antigen

\(\text{MFI}\) geometric mean fluorescence intensity

\(\text{MFIR}\) mean fluorescence intensity ratio

\(\text{nAb}\) natural antibody

\(\text{NK}\) natural killer cell

\(\text{PAEC}\) porcine aortic endothelial cells

\(\text{PAEC-KO}\) PAEC derived from \(\alpha_{1,3}\text{GT null pigs}\)

\(\text{PAEC-WT}\) PAEC derived from Gal-expressing pigs

\(\text{PBMC}\) peripheral blood mononuclear cells

\(\text{PCMV}\) porcine cytomegalovirus

\(\text{pEC}\) porcine endothelial cells

\(\text{PERV}\) porcine endogenous retrovirus

\(\text{PMN}\)

\(\text{SLA-I}\) porcine MHC class I / swine leukocyte antigen class I

\(\text{SN}\) cell-free supernatant

\(\text{SOCS}\) suppressor of cytokine signaling

\(\text{TEM}\) transendothelial migration

\(\text{VCAM-1}\) vascular cell adhesion molecule 1 (CD106)
7.3 Curriculum vitae

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Appendix

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Abstracts:

Ghielmetti M., Millard A-L., Häberli L., Bossart W., Seebach JD, Schneider KJ., Müller NJ. Human cytomegalovirus infection of porcine endothelial cells induces increased adhesion receptor expression and human leukocyte adhesion. Annual Congress of the SSAI/SSPT, April 17-18, 2008, Fribourg, Switzerland


Ghielmetti M., Millard A-L., Häberli L., Bossart W., Seebach JD, Schneider KJ., Müller NJ. Human cytomegalovirus infection of porcine endothelial cells induces increased adhesion receptor expression and human leukocyte adhesion. Day of Clinical Research, March 27., 2008, University Hospital Zuerich, Switzerland

Ghielmetti M., Millard A-L., Häberli L., Bossart W., Seebach JD, Schneider KJ., Müller NJ. Human cytomegalovirus infection of porcine endothelial cells induces increased adhesion receptor expression and human leukocyte adhesion. Wolfsberg meeting, March 17-19, 2008, Wolfsberg, Switzerland


Schneider MKJ, Ghielmetti M, Rhyner DM, Seebach JD. Xenogeneic leukocyte transmigration partly depends on interactions between human CD99 and beta2-integrins with porcine endothelium. Annual Congress of the SSAI/SSPT, April 19-20, 2007, Basel, Switzerland

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Ghielmetti M, Rhyner DM, Seebach JD, Schneider MKJ. Recruitment of human leukocytes to porcine endothelium expressing or lacking Gal-alpha(1,3)Gal. 4th International Conference on Innate Immunity, June 4-9, 2006, Corfu, Greece.


Schneider MKJ, Rhyner DM, Antsiferova MA, Ghielmetti M, Seebach JD. Recruitment of human leukocytes to porcine endothelial cells expressing or lacking Gal-alpha(1,3)Gal. 5th Day of clinical research, University Hospital Zurich, March 23-24, 2006
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