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# IN VITRO MODELS OF XENOGRAFT REJECTION

Studies on leukocyte-endothelial cell interactions

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To the memory of my grandfather, Johnny K. S. Cheng

## ORIGINAL PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-IV).

**I.** Porcine endothelium activated by anti-α-Gal antibody binding mediates increased human neutrophil adhesion under flow. **Ehrnfelt C.**, Serrander L. and Holgersson J. Transplantation, 2003, 76(7):1112-1119.

**II.** Aberrant expression of α-Gal on primary human endothelium does not confer susceptibility to NK cell cytotoxicity or increased NK cell adhesion. He Z.*, **Ehrnfelt C.***, Kumagai-Braesch M., Islam K. I. and Holgersson J. European Journal of Immunology 2004, 34(4):1185-1195.

**III.** Human monocytes adhere less to porcine than to human TNF-α-stimulated endothelium under conditions of physiological flow. **Ehrnfelt C.**, He Z. and Holgersson J. Manuscript.

**IV.** Adult porcine islets produce MCP-1 and recruit human monocytes *in vitro*. **Ehrnfelt C.**, Kumagai-Braesch M., Uzunel M. and Holgersson J. Xenotransplantation, 2004, 11(2):184-194.

* These authors contributed equally to the work.

## CONTENTS

## LIST OF ABBREVIATIONS

| | |
|---|---|
| α1,3GalT | α1,3-galactosyltransferase |
| α-Gal | Galα1,3Gal |
| Ab | Antibody |
| API | Adult porcine islet |
| AVR | Acute vascular rejection |
| EC | Endothelial cell |
| HAEC | Human aortic endothelial cell |
| HAR | Hyperacute rejection |
| HLA | Human leukocyte antigen |
| IBMIR | Instant blood-mediated inflammatory reaction |
| ICAM | Intercellular adhesion molecule |
| ICC | Islet-like cell-cluster |
| IDDM | Insulin-dependent diabetes mellitus |
| IFN | Inteferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| KRG | Krebs Ringer Glucose |
| LFA | Lymphocyte function associated antigen |
| m | Monoclonal |
| MCP | Monocyte chemoattractant protein |
| MHC | Major histocompatibility complex |
| NK | Natural killer |
| PAEC | Porcine aortic endothelial cell |
| PAF | Platelet activating factor |
| PBMC | Peripheral blood mononuclear cells |
| PBS | Phosphate buffered saline |
| PECAM | Platelet/endothelial cell adhesion molecule |
| PERV | Porcine endogenous retrovirus |
| RT-PCR | Reverse transcriptase-polymerase chain reaction |
| SLA | Swine leukocyte antigen |
| TNF | Tumor necrosis factor |
| VCAM | Vascular cell adhesion molecule |
| VLA | Very late antigen |
| XNA | Xenoreactive natural antibody |

# 1 ABSTRACT

Xenotransplantation is the transplantation of cells, tissues or organs between different species and is a potential alternative to allotransplantation because of the lack of human donors. The pig is believed to be a suitable donor-species to man for medical, technical, financial and ethical reasons. Porcine vascularized organs transplanted into humans are hyperacutely rejected (HAR) due to xenoreactive natural anti-pig Abs present in man. These Abs are mainly directed against the Galα1,3Gal (α-Gal) epitope expressed on most porcine cells, including the endothelium. Following their binding to the endothelium, the complement cascade is activated leading to graft destruction. If HAR is prevented, the vascularized grafts are generally lost in an acute vascular rejection (AVR). AVR is characterized by type II (*i.e.* transcriptional) endothelial cell (EC) activation, thrombosis with fibrin deposition and cellular infiltration dominated by monocytes/macrophages, natural killer (NK) cells and neutrophils. In contrast, cellular xenografts, *e.g.* isolated islets of Langerhans, are subjected to an instant blood-mediated inflammatory reaction (IBMIR) upon intra-portal transplantation and a subsequent cell-mediated rejection dependent on CD4+ T cells and macrophages.

The aims of the work outlined in this thesis were: (i) to investigate the interaction between human leukocyte subpopulations and porcine endothelium *in vitro*; and to evaluate the effect of the α-Gal epitope and human anti-α-Gal Abs in this interaction; (ii) to study the cytokine/chemokine profile of isolated adult porcine islets (APIs) and to examine if these factors could induce activation of human ECs and/or attract human leukocytes *in vitro*.

In the absence of xenoreactive natural anti-pig Abs and complement, human neutrophils and monocytes did not adhere more to porcine than to human non- and TNF-α-stimulated endothelium, as assessed under static and dynamic conditions. The adhesion of human neutrophils increased significantly after anti-α-Gal Ab-mediated activation of porcine endothelium. Porcine EC activation was demonstrated by an increase in E-selectin and VCAM-1 expression, and by the production of soluble factors such as IL-8 and PAF. These factors induced migration of human neutrophils. Aberrant expression of the α-Gal epitope on human endothelium using recombinant lentiviruses did not confer susceptibility to human NK cell-mediated cytotoxicity or increased NK cell adhesion. NK cells did not produce IFN-γ upon interaction with the α-Gal-expressing human endothelium. In addition, aberrantly expressed α-Gal epitopes did not increase the adhesion, under static or dynamic conditions, of human monocytes to human endothelium. Neither did it increase monocyte transendothelial migration. Recombinant lentiviruses proved to be effective vehicles for gene transfer into primary human ECs. Cultured APIs expressed mRNAs for MCP-1, IL-1β and TNF-α. Supernatants from cultured APIs induced migration of human monocytes, which could be blocked by an anti-MCP-1 Ab. Isolated islets and islets *in situ* produced MCP-1 as detected by immunohistochemistry. Recombinant porcine IL-1β and TNF-α induced the expression of E-selectin and VCAM-1 on human endothelium.

With regard to novel therapeutic strategies to disrupt the infiltration of human leukocytes into organ xenografts, our data suggest that efforts should be focused on adhesion receptor-ligand interactions rather than on carbohydrate remodeling of donor pigs. Induced Abs of α-Gal or non-α-Gal specificity may increase the adhesion and infiltration of, not only human neutrophils, but also of other human leukocyte subpopulations. Indeed, preventing the induced anti-porcine Ab response will be an important factor to prevent AVR. The work on isolated porcine islets highlighted the importance of pro-inflammatory and chemotactic factors produced by the graft itself, and that these factors may have profound effects on the rejection process *in vivo*.

# 2 INTRODUCTION

The immune system has evolved to protect us from hazardous agents, and especially microorganisms such as bacteria, viruses and fungi, which are present in the environment around us. The immune response is characterized by a prompt, non-antigen specific response known as the innate immune response, and a slower antigen-specific response known as the adaptive immune response, which takes several days to mount. The former serves as a first line of defense against pathogens, whereas the latter is specific for a particular pathogen. The adaptive immune response generates an immunological memory, which ensures a more rapid and effective response on a second encounter with the same pathogen. Another key feature of the immune system is its ability to distinguish between self and non-self, which is crucial in order to avoid autoimmunity. Although these characteristics of the immune system are essential for the survival of an individual, they cause many problems transplanting cells or organs from one individual to another.

Many patients have been cured from disease by transplantation, giving these individuals an improved quality of life and/or a longer life. Since the 1950s, when solid organ transplantation was first successfully performed, great advancements have been achieved in terms of surgical techniques, immunosuppressive regimens and pre-transplant diagnostic tools, all of which have contributed to increased patient and graft survival. However, a major limiting factor to allotransplantation (*i.e.* the transplantation between individuals within the same species) is the number of available human organ/tissue/cell donors. Today, the waiting list for new organs is long and many patients die while waiting. In Sweden every fifth patient waiting for a new kidney, liver or lung dies (*1*). This fact has led the research community to explore the possibility of using other species as organ/tissue/cell donors in human transplantation, so called xenotransplantation (from Greek *xenos,* foreign).

While there are many advantages with xenotransplantation as an alternative to allotransplantation, many problems and questions still exist with regard to ethical issues, possible microbial transmission between species and graft rejection. The work in this thesis has been focused on investigations of xenograft rejection, both of vascularized and non-vascularized xenografts, using different *in vitro* models with special emphasis on leukocyte-endothelial cell (EC) interactions.

## 2.1 XENOTRANSPLANTATION

As proposed by Calne, xenotransplantation can be divided into concordant (*e.g.* non-human primate-to-human and mouse-to-rat) and discordant (*e.g.* pig-to-man and rodent-to-man) transplantation (*2*). A discordant species combination is characterized by the presence, in the recipient species, of preformed antibodies (Abs) specific for the donor species. A vascularized organ transplanted across a discordant species barrier will be rapidly and vigorously rejected in a so called hyperacute rejection (HAR). In general, the greater the phylogenetical distance is between the donor and recipient species, the greater the immunological barrier becomes, and the more rapid and severe the rejection process is. So far, the pig has been suggested to be the most suitable organ/cell donor to man because of medical, technical, financial and ethical reasons. The anatomy of pig organs is similar to that of human organs, and it would be possible to breed pigs that have an appropriate organ size for any potential human recipient (infant or adult). The pig has been a domestic animal for thousands of years and is technically easy to breed in large numbers for clinical

transplantation purposes. Although many organ systems in pigs are physiologically similar to their human counterparts, *e.g.* the digestive and cardiovascular systems, incompatibilities do exist. The fact that organs from disparate species may not function adequately in a recipient from a different species constitutes a barrier towards successful xenotransplantation. For instance, cynomologus monkeys became anemic after porcine renal transplantation due to the failure of pig erythropoetin to support erythropoiesis in the recipient species (*3*).

Importantly, the risk of transmitting infectious agents, in particular porcine endogenous retroviruses (PERV) from pigs to humans, has to be carefully evaluated before xenotransplantation becomes a clinical reality. This is important in order to protect the xenograft recipient and the public from potential, yet unknown, infectious diseases. Endogenous retroviruses are integrated in the genome of all organisms and are harmless in their hosts. What will happen with these endogenous retroviruses post-xenotransplantation is not yet known. However, the infectious risk is considered less when transplanting a human being with a pig xenograft than with a non-human primate graft (*4, 5*). *In vitro* studies have shown that certain PERVs can infect human cells at low efficiency, and these PERVs were also shown to recombine with human viral elements (*6*). In addition, NOD/SCID mice became infected by PERV after pig-islet transplantation (*7, 8*). However, to date no humans exposed to porcine tissue have been reported to be infected by PERVs (*9-11*). Recently, in-bred mini-pigs that appear not to transmit retrovirus to human cells have been identified (*12*).

Another important issue to address is the ethical aspects of xenotransplantation. The most evident question is if humans are allowed to use animals for transplantation at all. Pigs have been bred for a long time to serve as a food supply for humans, and the part of the community that accept this fact would most likely also approve that pigs are used for treatment of human diseases. Concerns such as the risk-benefit ratio of xenotransplantation and religious perspectives on animal use have to be considered. Knowledge about the general opinion towards xenotransplantation is essential. Other important questions to reflect upon are: "How does the community regard a xenotransplanted patient?", "Who is entitled a human organ, from the limited supply, and who is not?" and "How much biological material from another species can a human receive and still be/feel human?".

### 2.1.1 VASCULARIZED ORGAN XENOTRANSPLANTATION

Today, transplantation can cure patients with irreversible cardiac disease, end-stage renal disease as well as acute and chronic liver failure. In 1905, Princeteau reported the first discordant clinical xenotransplantation (*13*). The patient survived 16 days after receiving kidney slices from a rabbit. Since then, several clinical xenotransplantations have been performed with hearts, kidneys and livers from donor animals such as pig, sheep and goat. However, none of the patients receiving a xenograft were cured from disease. Reemtsma performed one of the most successful clinical xenotransplantations in the 1960s (*14*). In one patient a chimpanzee kidney survived, with adequate function, for nine months. Due to the limited clinical experience of pig-to-man organ transplantation, it is not well known whether pig organs function adequately in the human body (*15*). Even though pig organs would prove not to function as well as human organs, they may be useful for short-term transplantation in patients waiting for a suitable human organ. Extensive studies on pig organ transplantation to non-human primates have been performed, and in this context, pig

hearts and kidneys have been shown to function well (*16, 17*). Xenotransplanted livers are believed to function less efficiently due to its inability to meet the metabolic demands of the recipient (*18*). The baboon is considered the most reliable non-human primate animal model, since they reject transplanted pig organs in a hyperacute fashion similar to man (*19*). Many *in vivo* studies on discordant transplantation have been carried out in rodents. For several reasons, these results should be interpreted with caution when extrapolated to clinical transplantation. For instance, the Galα1,3Gal (α-Gal) barrier (further discussed below) responsible for HAR in pig-to-man transplantation is not present in the pig-to-rodent species combination.

### 2.1.2 NON-VASCULARIZED CELLULAR XENOTRANSPLANTATION

In diseases where specific cells are degenerated or destroyed, such as in insulin-dependent diabetes mellitus (IDDM or type 1 diabetes) and Parkinson's disease, replacement of the damaged cells is a way to cure the disease. In addition, cellular transplantation is generally associated with less surgical complication, morbidity and mortality compared to solid organ transplantation.

#### 2.1.2.1 Diabetes mellitus and islets of Langerhans

DM, resulting in uncontrolled blood glucose levels, is one of the most common diseases worldwide and is estimated to affect 2.1% (124 million people) of the global population (*20*). Approximately 3.5 million individuals are diagnosed with IDDM, which is caused by a cell-mediated autoimmune destruction of insulin-producing β-cells in the islets of Langerhans (*20*). The only current treatment to maintain normoglycemia in these patients is daily administrations of exogenous insulin. However, this is not a cure for IDDM and does not prevent the late complications associated with the disease such as cardiovascular disease, renal failure, retinopathy and neuropathy. Today, the only cure for IDDM, thus achieving permanent normoglycemia, is to transplant whole pancreas or isolated islets of Langerhans. Until June 2003, more than 19,600 pancreatic allotransplantations had been performed worldwide, with a one-year survival rate of patients up to 95% and graft survival up to 84% (*21*). The results on patients transplanted with allogeneic isolated islets have not been very encouraging. Only 8% of transplanted patients remained insulin independent one year after transplantation (*22*). However, a breakthrough came in 2000 when seven IDDM patients were reported to be insulin independent after allo-islet transplantation using a modified transplantation protocol, including new immunosuppressive regimens without steroids, and increased tissue graft volume (*23*). The drawback of the new protocol is the need for at least two islet donors to reverse disease, and the lack of human cadaveric donors calls for alternative cell sources. There is limited experience in clinical xeno-islet transplantation. In the early 1990s, ten diabetic patients received porcine fetal islet-like cell-clusters (ICCs) at Huddinge University Hospital (*24*). Eight patients were transplanted through portal vein injections, and four of these patients excreted porcine C-peptide for 200-400 days post-transplantation. Two patients received their islets under the kidney capsule simultaneously with a renal allograft, and one of them showed intact porcine cells, surrounded by an infiltrate consisting of mononuclear and eosinophilic cells, in a biopsy taken three weeks after transplantation. Although transplanted ICCs showed signs of function and engraftment, none of the patients showed any decreased insulin dependence. All patients significantly

increased their titers of anti-pig Abs, which were mainly reactive against the α-Gal epitope (*25*).

#### 2.1.2.2 Parkinson's disease and dopaminergic neurons

Parkinson's disease is a disease caused by the degeneration of dopaminergic neurons of the nigro-striatal pathway resulting in rigidity, tremor and akinesia. The dopamine precursor, levodopa, and agonists of the dopamine-receptor are used as treatment, but after five to ten years the efficacy of these drugs decreases and side-effects appear. Immature neurons from human embryos and fetuses have been used to transplant Parkinson patients, and these cells were shown to survive the implantation procedure, exert neurological effects and, in a few patients, restore dopamine function for up to ten years (*26-29*). Until 2000, twelve patients had been transplanted with porcine fetal neural tissue and with modest clinical improvements post-transplantation (*30*). The brain of one xenotransplanted patient was autopsied and showed infiltration of T cells both inside and around the graft (*31*).

## 2.2 REJECTION OF XENOGRAFTS

The major hurdle preventing clinical xenotransplantation is the rapid rejection of xenografts. The type of vascularization, *i.e.* if the endothelium is of host or donor origin, determines the nature of the immune response elicited against a xenograft. In this section the rejection of vascularized, *i.e.* solid organs, and non-vascularized, *i.e.* tissue or cellular xenografts, will be discussed. An overview of the different types of immune responses triggered by different types of xenografts after transplantation is outlined in Fig. 1.

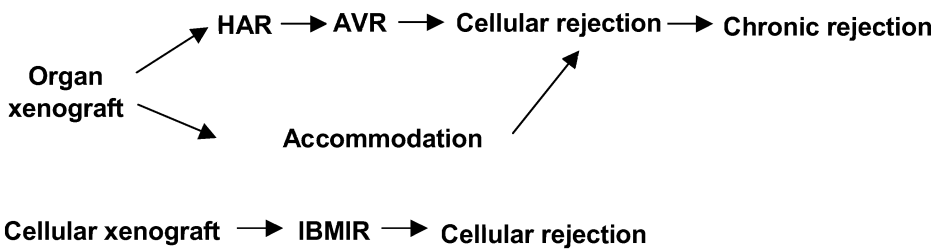


**Fig. 1.** Schematic figure of the different immunological responses triggered by different types of xenografts. AVR, acute vascular rejection; HAR, hyperacute rejection; IBMIR, instant blood-mediated inflammatory reaction.

### 2.2.1 HYPERACUTE REJECTION

In discordant species combinations, vascularized organ xenografts are subjected to engorgement and discolorisation with loss of function within minutes to hours post-transplantation. This type of rejection is called HAR and has for a long time been considered to be the primary barrier to xenotransplantation. The histology is marked by extensive intravascular thrombosis, edema and extravascular hemorrhage (Fig. 2) (*32*). Preformed xenoreactive natural antibodies (XNAs), mainly directed against the α-Gal

epitope, and complement have been found to be responsible for HAR of vascularized porcine xenografts transplanted into primates.

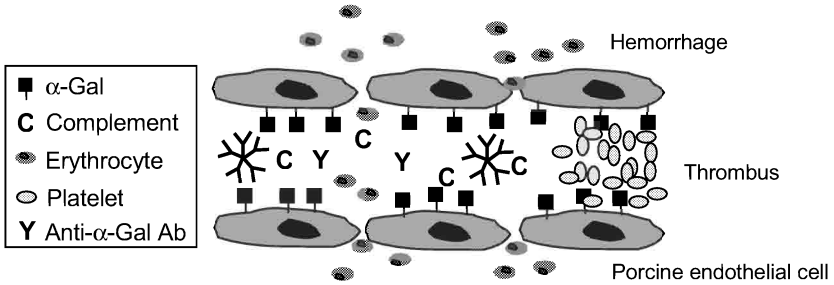


**Fig. 2.** Hyperacute rejection is initiated by the binding of anti-α-Gal Abs to α-Gal epitopes on porcine endothelium followed by complement activation. The histopathology of hyperacute rejection is characterized by intravascular thrombosis, hemorrhage and edema.

#### 2.2.1.1 The α-Gal epitope and anti-α-Gal antibodies

The initiating event of HAR is the binding of XNAs to the carbohydrate epitope Galα1,3Galβ1,4GlcNAc-R (α-Gal), on porcine endothelium. The α-Gal epitope is formed by the enzyme, α1,3-galactosyltransferase (α1,3GalT), which glycosylates N-acetyllactosamine on different glycoproteins, such as cell adhesion molecules, and glycolipids on many porcine cells including ECs (Fig. 3) (*32-34*). Using the lectin *Griffonia (Bandeiraea) simplicifolia* $IB_4$, known to bind α-Gal epitopes, it has been estimated that nucleated cells express $10^6$ to $3.5x10^7$ α-galactosyl epitopes most of which seem to have the structure Galα1,3Galβ1,4GlcNAc-R (*35*). The α1,3GalT is present in


**Fig. 3.** The biosynthesis of the α-Gal epitope is catalyzed by the glycosyltransferase, α1,3galactosyltransferase (α1,3GalT), which is present in porcine cells. UDP, uridine diphosphate.

New World monkeys and non-primate mammals, while absent in man, apes and Old World monkeys (*35*). Instead, man, apes and Old World monkeys produce Abs against α-Gal. The α1,3GalT gene in man and Old World monkeys contains a frame shift and nonsense mutations that result in gene inactivation (*36*). Most of the anti-α-Gal natural Abs are of immunoglobulin (Ig) M isotype, however, IgG and IgA are detected at lower levels (*37*). It has been estimated that anti-α-Gal IgG comprises 1% of total human serum IgG, whereas anti-α-Gal IgM comprises 4% of total IgM (*37, 38*). More than 80% of the total amount of XNAs in human serum is directed against the α-Gal epitope and approximately 1% of human circulating B cells produce anti-α-Gal Abs (*37, 39, 40*). Even though there are several isotypes among XNAs directed against the α-Gal epitope, only IgM is thought to initiate HAR (*41, 42*). This is probably due to its high binding avidity,

and the ability of IgM to efficiently trigger complement activation (*32*). The anti-α-Gal Abs is believed to arise as a result of the colonization of the gut by bacteria and of the exposure to viruses, protozoa or components in food carrying α-Gal related antigens (*43*). Although anti-α-Gal Abs pose problems for successful xenotransplantation, one may take advantage of their presence to clinically increase the immunogenicity of human autologous tumor vaccines that are modified to express the α-Gal epitope (*44, 45*).

#### 2.2.1.2 Complement

Ab-binding to porcine endothelium results in complement activation, which is the main mediator of HAR (*32*). The complement cascade is activated by the classical pathway, leading to the activation of C3 and C5, and finally the formation of C5-9 also called the membrane attack complex. The membrane attack complex will form pores in the plasma membrane of porcine endothelium and thereby cause their lysis. In the pig-to-man species combination, the complement is not activated via the alternative pathway, most likely because factor H, which prevents the binding of factor C3b with factor B, is active across this species barrier (*46*). In contrast, in some species combinations, such as guinea pig-to-rat, activation through the alternative pathway occurs (*47*).

#### 2.2.1.3 Endothelium

The endothelium is the major target in HAR and upon Ab-binding type I EC activation, *i.e.* non-transcriptional activation, occurs (*48*). This activation is quick and leads to EC retraction, expression of P-selectin (CD62P) and von Willebrand factor, secretion of platelet activating factor (PAF) and loss of its anti-thrombotic phenotype (*48, 49*). These EC changes result in edema, hemorrhage and intravascular thrombosis. The observed EC-retraction may be caused by rapid redistribution of platelet/endothelial cell adhesion molecule (PECAM-1/CD31) and VE-cadherin away from cell junctions as a result of α-Gal cross-linking (*50*).

#### 2.2.1.4 Prevention of HAR

Treating the xenograft recipient with immunosuppressive drugs alone does not prevent HAR in the pig-to-non-human primate animal model. However, combining immunosuppressive drugs with some of the following strategies can effectively avert HAR. First, HAR can be prevented by removal or neutralization of XNAs by plasmapheresis or by immunoabsorption using columns with a solid phase carrying α-Gal oligosaccharides (*51-54*). Second, HAR can be avoided by inactivation or depletion of complement, for example by treating the xenograft recipient with soluble complement receptor 1 (*55*). Cobra venom factor, which activates C3b and thereby deplete complement, and monoclonal (m) Abs directed against various complement factors have also been shown to be effective (*56*). Complement regulatory proteins, such as membrane cofactor protein (MCP/CD46), decay-accelerating factor (DAF/CD55) and CD59, are not fully active across the pig-to-man species barrier (*57, 58*). The human counterparts of the complement regulatory proteins have been successfully expressed on the endothelium in transgenic pigs, and have proven to be effective in preventing HAR (*59-62*). A third way to eliminate HAR is by reducing the α-Gal epitope expression in donor pigs. An initial study reduced the level of α-Gal in transgenic mice and pigs by the expression of an α1,2fucosyltransferase (*63*). ECs from such mice were protected from the binding of

XNAs and from complement-mediated lysis. In 2002, two studies were published reporting the production of cloned pigs in which one allele of the α1,3GalT had been eliminated (*64, 65*). However, more importantly, in 2003 the generation of α1,3GalT deficient pigs was reported and, hopefully, HAR is now eliminated for good in clinical xenotransplantation (*66*).

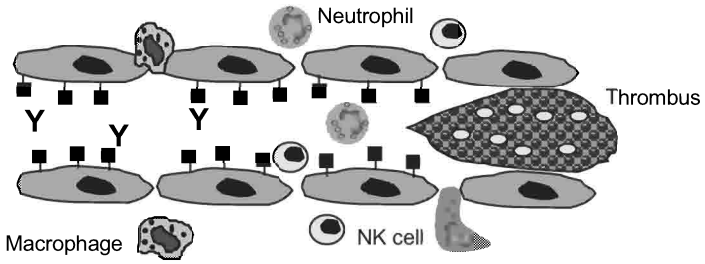


**Fig. 4.** Acute vascular rejection is thought to be triggered by anti-porcine Abs inducing a type II (*i.e.* transcriptional) EC activation resulting in infiltration of human leukocytes (monocytes/macrophages, NK cells and neutrophils) into the graft. The histopathology of acute vascular rejection is characterized by EC swelling, diffuse thrombosis with fibrin deposition, and infiltration of innate immune cells.

### 2.2.2 ACUTE VASCULAR REJECTION

Although HAR can be prevented using any of the above mentioned strategies, discordant organ xenografts are usually lost in an acute vascular rejection (AVR), also called delayed xenograft rejection, occurring hours to days post-transplantation. Today when HAR can be averted, AVR is viewed as the major obstacle to clinical xenotransplantation. Leventhal et al. were the first to identify AVR in a guinea-pig-to rat transplantation model (*67*). AVR is histopathologically characterized by EC swelling, focal ischemia, a diffuse microvascular thrombosis with fibrin deposition and a cellular infiltrate, dominated by monocytes/macrophages, natural killer (NK) cells and neutrophils (Fig. 4) (*48, 67, 68*). A key factor in the pathogenesis of AVR is the so called type II EC activation, *i.e.* transcriptional activation, recognized by increased transcription of genes encoding cell adhesion molecules [E-selectin (CD62E), vascular cell adhesion molecule-1 (VCAM-1/CD106) and intercellular adhesion molecule-1 (ICAM-1/CD54)], cytokines/chemokines [interleukin (IL)-1, IL-6, IL-8 and monocyte chemoattractant protein (MCP-1)] and pro-thrombotic molecules (tissue factor) (*48, 69*). These EC changes will promote leukocyte recruitment, platelet aggregation and loss of thromboregulation.

The triggering events leading to AVR are still controversial and different mechanisms that may initiate AVR are discussed below.

#### 2.2.2.1 Antibodies and acute vascular rejection

Complement-independent type II EC activation by preformed or induced xenoreactive Abs is believed to be important for the initiation of AVR and there are several findings supporting this hypothesis. For instance, in the earlier mentioned guinea pig-to-rat model of heart xenotransplantation, AVR developed in complement-depleted recipients where the anti-donor Abs remained (*67*). Further, baboons transplanted with transgenic pig hearts that expressed human DAF and CD59, in order to inhibit complement activation and

HAR, and whose circulating Abs were depleted, did not develop AVR (*70*). Recently, it was demonstrated that in baboons, whose anti-α-Gal Abs were eliminated by extracorporeal perfusion, DAF/CD59-transgenic pig organs were not rejected by AVR (*71*). Thus, these data indicate that anti-α-Gal Abs may mediate AVR. In addition, when the α-Gal Abs returned, porcine organs continued to function and appeared accommodated (discussed below).

*In vitro*, α-Gal epitope-mediated activation of porcine aortic ECs (PAECs) has been reported to induce both type I and type II EC activation (*72, 73*). Furthermore, affinity-purified human IgM Abs, containing XNAs, induced an increased transcription of IL-8 and plasminogen activator inhibitor, but did not increase the transcription of E-selectin and VCAM-1 in PAECs (*74*). In addition, porcine endothelium exposed to human serum upregulated E-selectin expression; this effect was associated with the presence of IgG3 and IgM anti-α-Gal Abs in the serum (*75*). Our work described in paper I showed that affinity purified anti-α-Gal Abs induced an upregulation of E-selectin and VCAM-1 on PAECs, and subsequently increased adhesion of neutrophils. Further, PAECs treated with human serum, as a source of XNAs and complement, supported human leukocyte adhesion under flow, possibly through the activation of NF-κB (*76*). Finally, long exposure (18-24 hrs) of porcine endothelium to human serum or anti-α-Gal mAbs has been shown to induce EC-apoptosis (*77, 78*).

There may also be other pathways leading to the initiation of AVR. Some studies have demonstrated that low levels of systemic complement activation following Ab deposition may be sufficient to induce type II EC activation and cause AVR (*79*). Thus, the induction of AVR may be mediated by a complement-dependent mechanism(s). The binding of Abs to EC antigens may mediate binding to Fc receptors on different effector cells, such as NK cells (*80, 81*). Also, the binding of xenoreactive Abs to shed or soluble endothelial antigens, may lead to the formation of immune complexes that can induce inflammatory or pro-thrombotic reactions seen in AVR.

#### 2.2.2.2 Molecular incompatibilities as a cause of AVR

Molecular incompatibilities between adhesion receptor pairs, hormones, cytokines, chemokines and their receptors, as well as other immune receptors and their ligands, are likely to be more severe following xenografting than allografting. Such incompatibilities may contribute to AVR. For example, porcine endothelial complement regulatory proteins are not efficient in inhibiting the actions of human complement. Moreover, the porcine tissue factor pathway inhibitor is not effective in inhibiting human factor Xa, rendering the porcine endothelium pro-thrombotic (*82*). Some incompatibilities may, however, act to reduce the strength of xenograft rejection. For instance, the human pro-inflammatory cytokines, IL-1β and interferon (IFN)-γ, are not able to activate porcine endothelium (*83*). Yet, some investigators do not believe that these molecular differences would initiate AVR, but rather, that they may contribute to the severity of the rejection process once it is set off by another triggering event (*70*).

#### 2.2.2.3 Cellular infiltration during AVR

The cellular infiltrate seen in xenografts during AVR suggests an important role for the innate immune system in the course of this rejection. The current model for leukocyte extravasation into inflamed tissues can be divided into four phases: capture, rolling, firm adhesion and transendothelial migration (Fig. 5) (*84*). Under shear stress, each step is a

prerequisite for the following step to occur. Many receptor-ligand pairs are involved in each step, and only a brief description of the most important molecules involved in each step will be presented here. The selectins, L- (CD62L), E- and P-selectin, mediate capture and rolling of leukocytes. L-selectin is expressed on leukocytes, whereas E- and P-selectin are found on the endothelium. The selectins bind to the carbohydrate sialyl-Lewis$^X$, and sulfated modifications thereof, present on different proteins. The firm adhesion is carried

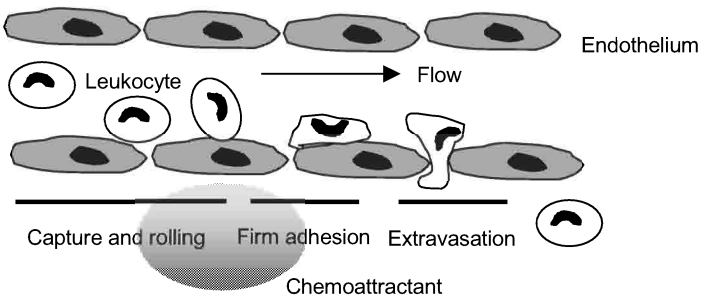


**Fig. 5.** Leukocyte transendothelial migration is initiated by the capture and rolling of leukocytes. The rolling phase is mediated by the selectins, E-, P-, and L-selectin. E- and P-selectin are expressed on endothelium, whereas L-selectin is expressed on leukocytes. The selectins bind to the tetrasaccharide sialyl-Le$^x$. In the next step, leukocytes firmly adhere to endothelium; a step carried out by the integrins LFA-1, Mac-1 and VLA-4 on leukocytes and the Ig superfamily members ICAM-1-3 and VCAM-1 on endothelium. During rolling and firm adhesion leukocytes are close to the endothelium and can be activated by chemokines secreted by the endothelium or by cells residing in the underlying tissue. The migration of leukocytes across endothelium is carried out by PECAM-1, CD99 and Junctional adhesion molecules.

out by $\beta_1$ [Lymphocyte function associated antigen-1 (LFA-1; CD11a/CD18) and Mac-1 (CD11b/CD18)] and $\beta_2$ [Very late antigen-4 (VLA-4; CD49d/CD29)] integrins. These integrins are expressed on the cell surface of leukocytes. The endothelial counter receptor for LFA-1 and Mac-1 is ICAM-1, ICAM-2 (CD102) and ICAM-3 (CD50), and VLA-4 binds to VCAM-1 on the endothelium. These cell adhesion molecules belong to the Ig superfamily. The fourth step, extravasation, is mediated by another Ig superfamily member, PECAM-1. PECAM-1 may bind in a homo- or heterophilic fashion and its expression is especially abundant in areas of cell-cell contact (*85, 86*). Recently, other molecules involved in transendothelial migration have been identified, such as CD99 and Junctional adhesion molecules A-C (*87, 88*). In addition, chemoattractants, such as chemokines, produced by the ECs or by the underlying tissue, are also important in the recruitment of leukocytes (*84*). These are important in activating and directing the leukocytes to a site of inflammation or infection. The chemokines also act to activate the integrin adhesiveness. The two major families of chemokines are the CXC and CC chemokines. The CXC chemokines [IL-8, GRO-$\alpha$, -$\beta$, -$\gamma$ and Epithelial derived neutrophil attractant (ENA)-78] tend to act on neutrophils, whereas the CC chemokines [MCP-1-4, Regulated on activation normal T cell expressed and secreted (RANTES) and Macrophage inflammatory protein (MIP)-1$\alpha$, -1$\beta$] act on monocytes, lymphocyte subpopulations and eosinophils. Chemokines bind to seven transmembrane spanning, G protein-coupled receptors on leukocytes. The initial, selectin-mediated, steps, capturing and rolling, will bring the leukocyte in close proximity to the endothelium, which exposes the leukocyte to chemokines for a longer period of time.

A prerequisite for human leukocytes to infiltrate porcine xenografts is that compatibility exists between cell adhesion molecules and chemokines with their respective ligands or receptors, or that other molecules may act as substitutes. The compatibility between human cell adhesion molecules and their porcine counter-receptors is yet incompletely characterized, but most ligand-receptor pairs investigated so far seem to be compatible (Table 1) (*89*). In general, the adhesion process of leukocytes to endothelium appear to be conserved across the human-to-pig species barrier (*90*). Furthermore, it has been claimed in the literature that a direct recognition of porcine endothelium by human leukocytes lacking antigen-specific receptors can occur, and that the α-Gal epitope may be involved in this recognition. In the next sections, the involvement of human neutrophils, NK cells, and monocytes in AVR, and their recognition of porcine endothelium will be discussed.

| | | Compatibility | | |
|---|---|---|---|---|
| Adhesion molecule | Major ligands | Porcine adhesion molecule-human ligand | Human adhesion molecule-porcine ligand | Anti-porcine (or cross-reactive anti-human) mAb available |
| CD18 | See CD11a/18 | Yes | Yes | Yes |
| CD11a/18 | CD50, 54, 102 | Yes | Yes | Yes |
| CD29 | See CD49 | Yes | Yes | Yes |
| CD31 | CD31, others | ND | No | No |
| CD34 | CD62L, others | ND | No | No |
| CD44 | ECM proteins, HA | Yes | Yes | Yes |
| CD49d/29 | CD106, fibronectin | Yes | Yes | Yes |
| CD49e/29 | Fibronectin | No | ND | Yes |
| CD49f/29 | Laminin | Yes | ND | Yes |
| CD54 | CD11a/18 | Yes | Yes | Yes |
| CD62E, P, L | Sialyl Lewis X, CD34 | Yes | Yes | Yes |
| CD106 | CD49d/29 | Yes | Yes | Yes |

**Table. 1.** Compatibility of porcine and human adhesion-receptor pairs. Abbreviations: ECM, extracellular matrix; HA, hyaluronic acid; ND, not determined. Modified from Simon et al. (*89*).

#### 2.2.2.4 Neutrophils

Neutrophils are the most abundant leukocyte circulating in the body, representing 50-70% of the total number of leukocytes, and are implicated in both HAR and AVR of xenografts (*67, 91*). The exact role of neutrophils in xenograft rejection has not yet been established. However, it is thought that these cells play a role in the later phase of the rejection process (*92*). It has been shown that human complement can upregulate P-selectin on PAECs, which increased the number of adhering neutrophils (*93*). In addition, Vercelotti et al. showed that the human complement factor iC3b deposited on PAECs induced an increased adhesion of human neutrophils through Mac-1 on the neutrophils (*94*). Also, blocking Mac-1 and complement decreased neutrophil infiltration and prolonged graft survival (*95, 96*). Human DAF-expressing PAECs have been claimed to support neutrophil adhesion less efficiently (*97*). Further, neutrophils have been shown to adhere

better to porcine than to human endothelium, and to activate the former independently of complement and XNAs in static and flow adhesion assays (*98, 99*). Under static conditions, the binding was blocked by addition of Abs to ICAM-1 on porcine endothelium or to its ligands, LFA-1 and Mac-1, on the neutrophils. However, under conditions of flow, neutrophil adhesion to PAECs was independent of ICAM-1, LFA-1, Mac-1 and the α-Gal epitope (*99*). On the other hand, our study presented in paper I demonstrates that human neutrophils were not able to distinguish between allo- and xenogeneic endothelium, neither under static conditions nor under physiological flow. Similar results were obtained by Robinson et al. and Rollins et al. (*90, 100*). Also, transendothelial migration of human neutrophils across porcine endothelium is not different from the migration through human endothelium (*101*).

#### 2.2.2.5 NK cells

NK cells make up approximately 15% of circulating lymphocytes in human blood. Phenotypically, they are characterized as CD56+ (Neural cell adhesion molecule) CD16+ (Fcγ receptor III) CD3- cells. It has been demonstrated in xenogeneic human-to-rat and human-to-pig *ex vivo* and pig-to-non-human primate *in vivo* models of AVR that NK cells infiltrate rejecting organs abundantly (*102-104*). This phenomenon has also been shown in concordant small animal models such as the guinea pig-to-rat model (*67, 105, 106*). Further supporting the involvement of NK cells in AVR is the observation that NK cells have the ability to lyse porcine endothelium. The lytic ability was increased by the presence of human serum as a source of anti-pig Abs (*54, 107*) and was enhanced by exogenous administration of IL-2 (*108*). In our laboratory, we have not been able to detect lysis of porcine endothelium by fresh, unstimulated human NK cells, however, upon stimulation with IL-2, a lytic effect is induced but does not exceed the lytic effect observed against human ECs. Thus, the ability of human NK cells to lyse porcine endothelium is a subject of controversy.

##### *2.2.2.5.1 Human NK cell cytotoxic mechanisms against porcine targets*

The damage caused by NK cells in the xenogeneic graft may occur by at least two different mechanisms. First, NK cells may mediate cellular lysis in the presence of anti-graft IgG Abs, which will bind to Fcγ receptor III on NK cells; a mechanism called Ab-dependent cellular cytotoxicity. Second, NK cells may bind directly to the target EC and cause damage by the release of lytic components. The cytolytic mechanism is believed to be dependent on the perforin/granzyme B pathway in the human-to-pig species combination (*109*). It has been observed that, upon adhesion of human NK cells to porcine endothelium, gaps between ECs are formed and the endothelium becomes pro-thrombotic by induction of tissue factor activity (*110, 111*). Further, NK cells are able to activate porcine endothelium to express E-selectin and secrete IL-8 in a cell-to-cell contact-dependent manner (*112*). The EC activation was enhanced in the presence of human IgG Abs.

##### *2.2.2.5.2 Human NK cells and porcine endothelium*

Human NK cells have been claimed to adhere more to porcine than to human endothelium, and to activate the former under both static and dynamic conditions (*112, 113*). NK cells also become activated under these circumstances (*113*). Investigations in

which adhesion molecules and their respective ligands were masked have revealed that the interaction between VLA-4 and VCAM-1 is crucial both in the rolling and firm adhesion phase of human NK cell binding to porcine endothelium (*114*). In addition, blocking CD2 on human NK cells reduces static adhesion to porcine EC (*115*). In transendothelial migration assays, the masking of porcine VCAM-1 was effective in blocking the migration of human NK cells across porcine EC (*101*). These blocking studies do not fully prevent the adhesion of NK cells, suggesting that also other molecules or carbohydrate epitopes may be involved in the recognition of porcine endothelium by human NK cells. The first group to suggest that there could be a direct cellular recognition of, amongst other epitopes, the α-Gal determinant was Inverardi et al. (*116*). Human NK cells were shown to bind COS-7 cells expressing the porcine α1,3 GalT and thus α-Gal, but did not bind to non-transfected COS-7 (*116*). In addition, certain carbohydrates abrogated the binding efficiency of both NK cells and XNAs to porcine endothelium. Transfection of porcine endothelium with different glycosyltransferases, including the α1,2-fucosyltransferase competing for the same substrate as α1,3GalT and thus reducing the α-Gal expression, led to a reduced sensitivity to direct NK cell-mediated cytolysis (*108, 117*). However, other studies claim the interaction of human NK cells and porcine endothelium to be independent of α-Gal (*113*). In addition, in paper II we show that aberrant expression of the α-Gal epitope by human endothelium does not confer susceptibility to human NK cell cytotoxicity, increased NK cell adhesion under static and dynamic conditions, or activation of NK cells as measured by their secretion of IFN-γ.

##### *2.2.2.5.3 Human NK cells and SLA molecules*

NK cell cytotoxicity is negatively regulated by the recognition of self major histocompatibility complex (MHC) class I molecules, called the "missing-self" hypothesis (*118*). Receptors responsible for the inhibitory signals are killer inhibitory receptors and CD94/NKG2 receptors that belong to the Ig superfamily and C-type lectins, respectively (*119*). The recognition of swine leukocyte antigen (SLA) class I on porcine endothelium by human NK cell-inhibitory receptors may not be fully functional across the species barrier. In line with this hypothesis, it was demonstrated that sequences in MHC class I molecules recognized by human NK cell-inhibitory receptors were not conserved in SLA class I molecules (*120*). These data may provide an explanation for the susceptibility of porcine EC to human NK cells observed by some authors. On the other hand, upregulation of SLA class I by tumor necrosis factor (TNF)-α was shown to reduce the lytic activity of human NK cells (*115*). Introducing human leukocyte antigen (HLA) class I molecules in porcine endothelium may be a way to protect from NK cell lysis. Indeed, transient expression of HLA-Cw and HLA-G in porcine endothelium resulted in partial protection from human NK cells (*121-123*). In addition, HLA-G expression on porcine endothelium has been suggested to inhibit the migration and rolling adhesion of human NK cells (*124, 125*).

#### 2.2.2.6 Monocytes/Macrophages

Monocytes constitute approximately 2-10% of the total number of leukocytes in human blood. They constantly circulate between blood and tissues and can differentiate into antigen-presenting macrophages while in tissues. *In vivo* models of AVR have shown that monocytes are involved in this rejection process and the infiltration by monocytes/macrophages and their cytokine production have been shown to occur in the

absence of complement, XNAs and T cells (*67, 68, 104-106, 126*). It has also been demonstrated that activated macrophages can act as direct effectors in the rejection of both cellular and organ xenografts (*127, 128*). Further, macrophages were required for T cell infiltration to occur (*128*). Depletion of macrophages resulted in a prolonged graft survival in a guinea pig-to-C6-deficient rat heart xenograft model (*129*). In static adhesion experiments, human monocytes adhered more to unstimulated porcine than to human endothelium, and this binding was dependent on the interaction of VLA-4 and VCAM-1 (*130, 131*). In contrast, we could not detect any difference in monocyte adherence to non- or TNF-α-stimulated porcine or human endothelium under static conditions, as investigated in paper III. Upon human monocyte adherence to porcine endothelium, the endothelium displayed a type II EC activation, which suggests an important role for monocytes in the pathogenesis of AVR (*132*). The α-Gal epitope has been claimed to be involved in static adhesion of monocytes to porcine endothelium (*131, 133, 134*). In paper III, however, human endothelium aberrantly expressing the α-Gal epitope did not support increased monocyte adhesion, as compared to non-α-Gal-expressing human endothelium. In addition, under physiological flow, human monocytes adhered significantly less to TNF-α-activated PAECs as compared with human endothelium. Finally, porcine endothelium support transendothelial migration of human monocytes equal to human endothelium as shown in paper III and elsewhere (*101*).

#### 2.2.2.7 Avoiding acute vascular rejection

Since Abs seem to play a central role in the initiation of AVR, much attention have been given to develop strategies to control the anti-porcine humoral immune response. Combining splenectomy with immunosuppressive drugs, such as cyclophosphamide and leflunomide, has proven effective in some studies (*135, 136*). However, to accomplish complete and sustained Ab depletion has proven to be very difficult. Another approach to inhibit AVR is to target the transcription factor, NF-κB, which is activated during EC type II activation (see below). Preserving the anti-thrombotic properties of ECs, *e.g.* by stable expression of thrombomodulin or sustained activity of ADPase, may also prevent AVR (*137, 138*). Finally, to inhibit leukocyte infiltration by specifically target cell adhesion molecules and their respective ligands may protect the graft from AVR.

### 2.2.3 ACCOMMODATION

This phenomenon was originally described in ABO-incompatible kidney allografts by Alexandre et al., and is a state of continuing graft function in spite of the presence of anti-graft Abs and complement (*139*). Accommodation has been observed in animal models of xenotransplantation. In a rhesus monkey treated with plasmapheresis and immunosuppression, a pig heart transplant survived eight days without signs of rejection in the presence of XNAs and complement (*140*). It has also been demonstrated that an accommodated graft is resistant to the levels of Abs and complement that is sufficient to induce HAR of a naïve graft (*141*). Accommodation is believed to be accomplished by up-regulation of so called "protective genes", including the stress-responsive gene hemeoxygenase-1 and the anti-apoptotic genes A20, bcl-2 and bcl-$x_L$, by the graft endothelium (*142*). These genes, when over-expressed in endothelium, have been shown to impede the activity of NF-κB and thereby suppress the expression of pro-inflammatory genes associated with EC activation (*143, 144*). However, it is not yet fully understood what factors would contribute to a pro-inflammatory response and which factors would

induce a protective response in ECs. In addition, there is evidence that a Th2 dependent immune environment can promote accommodation of xenografts (*143*).

### 2.2.4 ACUTE CELLULAR REJECTION AND CHRONIC REJECTION

Due to the strength of both HAR and AVR, it has been difficult to study the T lymphocyte-mediated acute cellular and the chronic rejections *in vivo*. Initially, it was thought that the acute cellular response to xenografts would be weaker than that against allografts due to molecular incompatibilities between T lymphocyte co-stimulatory molecules and their ligands (*145, 146*). However, it was shown that the T lymphocyte-mediated response against xenogeneic cells was even stronger than the alloresponse, and is considered to be too vigorous to be controlled by conventional immunosuppression. Human T cells can recognize porcine antigens by both direct and indirect antigen-presenting pathways (*147*). Porcine endothelium has been shown to induce a proliferative human T cell response (*148, 149*). The power of the T cell response might be explained by the fact that porcine endothelium, unlike human endothelium, constitutively expresses the important costimulatory molecule CD86 that bind to CD28 on the human T cell (*150*). Perhaps induction of T cell unresponsiveness or tolerance would be a way to overcome acute cellular rejection.

It is not clear to what extent chronic rejection occurs in vascularized xenografts. It could be speculated that induced anti-donor Abs, responsible for causing chronic rejection in certain experimental allograft models, also can have the ability to give rise to chronic rejection in xenografts (*151*).

## 2.3 NON-VASCULARIZED XENOGRAFTS

The rejection mechanism of islet xenografts is different from organ xenografts due to the absence of a vascular network upon transplantation. During the isolation of pancreatic islets, they lose their capillary bed and require a period of revascularization post-transplantation (*152*). *In vivo* experiments have shown that hamster pancreatic islet isografts have a very high blood perfusion already one week post-transplantation (*153*) and that the endothelium of revascularized xenografts is of host origin (*154*). Thus, transplanted islets are protected from HAR and AVR, but are instead subjected to an immediate blood-mediated inflammatory reaction (IBMIR) upon intra-portal transplantation and a subsequent cell-dependent rejection.

### 2.3.1 THE INSTANT BLOOD-MEDIATED INFLAMMATORY REACTION

Intra-portal injection is the preferred route for administrating islets in clinical islet transplantation and, consequently, the islets will be directly exposed to human blood. The interaction between human blood and human or porcine islets have been studied *in vitro*; studies that revealed that both human and porcine islets are subjected to IBMIR. IBMIR is characterized by platelet and complement activation, initiation of coagulation and islet damage (*155, 156*). The islets are entrapped in fibrin clots, which are infiltrated by CD11b+ leukocytes (monocytes and neutrophils). Compared to human islets, porcine islets were more severely damaged as indicated by insulin “dumping” and loss of islet integrity. An explanation for this may be that complement was deposited on porcine islets but not on human islets. However, no binding of human Ig was observed on porcine islets indicating complement activation through the alternative pathway. Recently, it was

demonstrated that human endocrine cells produce tissue factor, which could explain the coagulation detected in patients receiving human islets (*157*). Whether porcine islets produce tissue factor remains to be investigated. Clotting and islet damage was also demonstrated upon intra-portal injection of adult porcine islets (APIs) into pigs (allogeneic) and cynomologus monkeys (xenogeneic) (*155, 156*). These data suggest that IBMIR may be the cause of the observed early islet loss and subsequent poor engraftment following transplantation. Yet, administration of soluble complement receptor 1 and heparin before islet injection inhibited complement activation and coagulation, and reduced insulin "dumping" (*155, 156*). Furthermore, fetal porcine ICCs express the α-Gal epitope and are susceptible to complement-mediated lysis triggered by the binding of anti-α-Gal Abs. In contrast, adult porcine islets seem to lack this epitope and are subjected to lysis via Ab-independent complement activation (*158*). A means to reduce the susceptibility of porcine islets to human serum may be to express human complement regulatory proteins such as DAF or CD59 on the islets (*159*).

### 2.3.2 CELLULAR REJECTION

Islet xenografts escaping the immediate destruction by IBMIR will be rejected in an acute cellular xenograft rejection. This type of rejection has been extensively studied in pig-to-rodent models, where islets are transplanted under the kidney capsule. In general, the rejection of islets in rodents is completed by day 10. The rejection is specific because only xenogeneic islets are rejected after transplantation of a mixture of xenogeneic and syngeneic islets (*160*). CD4+ T cells are one of the major players in islet xenograft rejection in rodents. Administration of an anti-CD4+ T cell Ab was shown to prolong porcine islet graft survival in mice (*161*). Furthermore, hyperglycemic RAG-deficient mice that received *in vitro*-stimulated human PBMCs and which were subsequently transplanted with porcine fetal ICCs, rejected these through an acute cellular rejection in which CD4+ T cells were shown to be sufficient to induce rejection (*162*). In a more clinically relevant model of islet xenograft rejection in which porcine islets were transplanted to cynomologus monkeys, the CD8+ T cells were the major graft-infiltrating cell (*163*).

Macrophages heavily infiltrate rejecting porcine islet xenografts and CD4+ macrophages have been shown to be the main graft-infiltrating cell in fetal porcine ICCs transplanted to normoglycemic rats (*164*). In fact, upon histological analysis the pattern of infiltrating cells is similar to that of a delayed type hypersensitivity reaction; macrophages are in the center close to the collapsing endocrine cells, whereas T cells are found in the periphery surrounding the rejecting tissue. Further, it has been shown that a Th1 immune response precedes a Th2 response, which fits well with the fact that the delayed type hypersensitivity reaction is a prototype of a Th1 response and that eosinophil-infiltration is observed later during the rejection process (*164, 165*). Macrophages activated by CD4+ T cells are believed to be direct effectors of cellular xenograft rejection and specific depletion of macrophages delays cellular infiltration and rejection of APIs in mice (*127, 166*). In addition, in a pig-to-mouse model, it was recently demonstrated that macrophages activated by CD4+ T cells were capable of both recognition and rejection of pancreatic islet xenografts (*167*). How the macrophages find the xenoislet graft in the first place is not yet established. However, the MCP-1/CC-receptor 2 pathway has been reported to be of major importance for islet xenograft rejection (*168*). It is also possible that MCP-1 produced by the islet xenograft itself may contribute to macrophage infiltration as

demonstrated in paper IV. Moreover, islet xenograft destruction is dependent on an MHC-specific interaction between T cells and macrophages, as shown in a pig-to-mouse model (*169*).

In the pig-to-rodent islet transplantation model it has been shown that B cells and Abs (*170, 171*), IL-4 (*172*), IL-5 and eosinophils (*173*), IFN-γ (*174*), Fc receptors (*170*), NK cells (*175*) and perforin (*176*) are each alone not essential for xenograft rejection.

# 3 AIMS OF THE PRESENT STUDY

The general aims of this thesis were to investigate the interaction between human leukocyte subpopulations and porcine endothelium *in vitro* - an interaction that could be important in the initiation of AVR *in vivo*, and to study the cytokine/chemokine profile of isolated APIs *in vitro*, which could contribute to cell-mediated rejection *in vivo*.

**The specific aims for each paper or manuscript were:**

**Paper I.** In this paper we investigated the adhesion under flow of naïve human neutrophils to porcine endothelium stimulated with human anti-α-Gal Abs. The ability of human neutrophils to adhere to human and porcine endothelium under static and flow conditions was also evaluated.

**Paper II.** Recombinant lentiviruses encoding a porcine α1,3GalT was used to obtain α-Gal expressing primary human endothelium in order to evaluate the importance of this epitope for NK cell adhesion under static and dynamic conditions, and for susceptibility to NK cell-mediated cytotoxicity.

**Paper III.** Human monocyte adhesion to, and their migration across, human and porcine endothelium were examined. The role of the α-Gal epitope in these processes was evaluated using α-Gal expressing human endothelium.

**Paper IV.** The aim of this study was to investigate if isolated APIs themselves produce and/or secrete chemokines (MCP-1) and/or inflammatory cytokines (IL-1β, IL-6 and TNF-α). The ability of these factors to induce activation of human ECs and/or attract human monocytes was analyzed.

# 4 METHODOLOGICAL CONSIDERATIONS

The materials and methods used in this thesis are described in detail in each paper or manuscript. In this section some considerations will be given concerning some of the methods used.

## 4.1 *IN VITRO* MODELS

The experimental work in this thesis is based on different *in vitro* models aimed at mimicking the *in vivo* situation of xenograft rejection. *In vitro* models have a great potential for the study of specific, isolated interactions that are integrated parts in a more complex biological process such as xenograft rejection. *In vitro* models are also a means to circumvent the use of animals. In the context of studying different cellular interactions in xenograft rejection, one can use the most clinically relevant species combination, *i.e.* pig-to-human, without the ethical concerns that such an *in vivo* model would raise. However, *in vitro* data have to be interpreted with caution since the *in vivo* situation is much more complex, and *in vitro* data are thus not always directly applicable in the clinical situation.

## 4.2 PRIMARY AORTIC ENDOTHELIAL CELLS

HAECs (Clonetics, Walkersville, MD; Cascade Biologics, Portland, OR) of passage <15 were cultured in gelatin-coated cell culture flasks using EBM-2 medium or Medium 200 supplemented according to the instructions of the suppliers. The cells were cultured at 37°C in 5% $CO_2$, and the culture medium was changed every 48-72 hrs.

PAECs were isolated as previously described (*101*). Briefly, the abdominal aorta was obtained from outbred pigs (Swedish landrace) at a local slaughterhouse. After clamping one end of the aorta, the vessel was filled with 2% (w/v) collagenase A (Boehringer Mannheim, Mannheim, Germany) in phosphate buffered saline (PBS) and clamped in the other end. The vessel was immersed in PBS at 37°C for 5-10 min and cut open. The released ECs were collected in PBS containing penicillin and streptomycin. The ECs were washed twice in PBS and seeded into a gelatin-coated 24-well plate. The cells were cultured in supplemented EBM-2 medium and areas of cells exhibiting the EC-characteristic cobblestone morphology were handpicked with pipette and expanded. The cells were phenotyped by flow cytometry using mAbs specific for E-selectin, PECAM-1 and VCAM-1. The cells were also assessed for reactivity with the anti-fibroblast and anti-human smooth muscle actin mAbs. The PAECs were cultured as described for the HAECs.

The ECs used in this thesis were primary aortic endothelium of human and porcine origin, respectively. It is well known that EC from different vascular beds vary both phenotypically (*177, 178*) and functionally (*179*). Further, it is likely that the endothelium will undergo changes when cultured *in vitro* (*180*). Probably, the most relevant type of endothelium to be used in these studies is ECs of microvascular origin. However, we chose aortic endothelium due to its accessibility and the relative ease by which it can be kept in culture. If endothelium from different species is to be compared it is essential that the ECs originate from the same anatomical site. Primary endothelium is likely to be superior to EC lines since the cells may undergo changes upon transformation. It is also of importance how EC activation is defined; in our studies activation was defined as an increase in surface expression of E-selectin and VCAM-1.

## 4.3 ISOLATION OF HUMAN LEUKOCYTE SUBPOPULATIONS

Human PBMCs were obtained from peripheral blood of healthy volunteers by Ficoll-Hyperpaque gradient centrifugation. Monocytes and NK cells were isolated using a Monocyte Negative Isolation kit and Dynabeads (CD3, CD14 and CD19), respectively, according to the instructions of the manufacturer (Dynal, Skøyen, Norway). To avoid activation and phagocytosis (monocytes), the cells were kept on ice until further analysis. Neutrophils were isolated according to Bøyum (*181*). In short, 4% dextran was added to peripheral blood (1:2 dilution), which was allowed to sediment for 40 min at room temperature. The top layer was washed in PBS and separated by Ficoll gradient centrifugation for 15 min at 410 g. The pellet was resuspended in PBS and erythrocytes were lysed using distilled water. The neutrophils were used within 4 hrs. Since neutrophils are easily activated, cold solutions, polypropylene plastics and gentle handling were applied.

## 4.4 PURIFICATION OF HUMAN ANTI-α-GAL ANTIBODIES

Human anti-α-Gal Abs was purified from pooled human AB serum using α-Gal-conjugated agarose beads (Calbiochem, La Jolla, CA). Briefly, 4 ml of slurry (2 ml of packed beads) were poured into a column of 10 mm diameter and washed with PBS. Two hundred ml of serum was applied at 0.5 ml/min, the column was extensively washed with PBS and bound Abs were eluted with 0.1 M glycine/HCl (pH 2.5) at 1 ml/min. Four ml fractions were collected in tubes containing 0.4 ml of neutralizing buffer (1.5 M Tris/HCl, pH 8.8). The absorption at 280 nm was read spectrophotometrically, and tubes containing protein were pooled, dialyzed against 1% PBS, lyophilised, and resuspended in distilled water. The protein concentration was determined using the BCA Protein Assay kit (Pierce, Rockford, IL).

## 4.5 ADHESION ASSAYS

*Static adhesion.* One million of human leukocytes were added to confluent monolayers of HAECs and PAECs, respectively, and incubated for 5 (neutrophils) or 15 (NK cells and monocytes) min at 37°C (Fig. 6A). Non-adhered cells were carefully removed by rinsing three times with medium. The number of adhered cells was counted in an inverted phase contrast microscope at a magnification of 100x.

*Flow adhesion.* A flow chamber (Glycotech, Rockville, MD) was mounted and held in place by vacuum on plastic culture dishes with confluent monolayers of ECs (Fig. 6B). Human leukocyte subpopulations ($5x10^5$ cells/ml) were perfused through the chamber at 1 Dyne/$cm^2$ at 37°C or at room temperature. One Dyne/$cm^2$ corresponds to the shear stress in post-capillary venules, which is the site where the interactions studied are likely to occur. The cells were observed in an inverted phase contrast microscope at a magnification of 100x. The number of adhered cells was counted after 5, 10, 15 or 20 min of leukocyte perfusion.

Leukocyte-EC adhesion assays are aimed at mimicking the *in vivo* adhesion of leukocytes to the endothelium lining the blood vessels. Leukocyte adhesion and arrest in the flow assay is dependent on both rolling and firm adhesion, whereas adhesion under static conditions is mainly dependent on adhesion receptor pairs responsible for firm adhesion. For the adhesion assays the ECs were cultured in petri dishes, which were

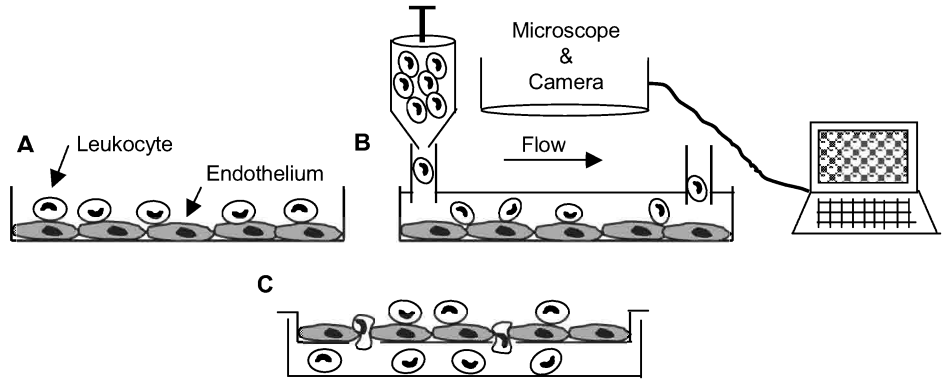


**Fig. 6.** Schematic figure of the static adhesion (A), flow adhesion (B) and transendothelial migration (C) assays. In the static adhesion assay (A), leukocytes ($1x10^6$ cells) were added to confluent monolayers of endothelium and allowed to adhere for 5 or 15 min. The non-adherent cells were carefully removed by gentle washing, and adhered cells were counted. In the flow adhesion assay (B), a flow chamber was mounted and held in place by vacuum on plastic culture dishes with confluent monolayers of ECs. Leukocytes ($5x10^5$ cells/ml) were perfused at 1 Dyne/$cm^2$ for 5-20 min and adhered cells were counted. The cells were monitored using a microscope coupled to a CCD camera and a computer. The transendothelial migration assay performed in paper III is illustrated in (C). Monocytes ($1x10^6$ cells) were added to the upper compartment and allowed to migrate for 2 hrs and the migrated cells were counted using a Bürker chamber. The migration assays performed in paper I and IV are based on the same principle, although in these assays the endothelium was absent and the cells migrated across filters.

carefully monitored by phase contrast microscopy prior to the assay to make sure that the endothelium was confluent. The temperature at which the adhesion assay is performed is an important factor because adhesion molecules and their respective ligands may display varying affinities at different temperatures. The static and flow adhesion experiments included in this thesis were all performed at 37°C, except the flow adhesion assays including lentivirus-transduced cells which were performed at room temperature for technical reasons. The concentration of leukocytes that are perfused or allowed to statically adhere to ECs and the perfusion or incubation time are important factors that will influence the results. The concentration of leukocytes was optimized to obtain a number of adhering cells that was reasonably easy to count, and the time of perfusion or static adhesion was adjusted to how fast the different leukocyte subpopulations adhered.

## 4.6 MIGRATION ASSAYS

*The Transwell® system.* Transwell filters (pore size of 3 μm) were used to measure migration of human neutrophils towards supernatants from PAECs stimulated with anti-α-Gal Abs. The filters were immersed in 1% BSA/PBS at low pressure and room temperature for 20 min. Freshly isolated neutrophils were labeled with 10 μM Calcein AM in Krebs Ringer Glucose (KRG) for 20 min at 37°C, washed three times in KRG, resuspended in 1 mM $Ca^{2+}$-KRG and kept on ice protected from light. Labeled neutrophils ($2x10^6$ cells) were added to the upper compartment, and the different supernatants were added to the lower compartment. Recombinant human IL-8 and medium alone served as controls. After 60 min at 37°C, the upper compartments were removed and Triton X-100

was added to the migrated cells in the lower compartment to release Calcein from cells. The fluorescence in the lower compartment was a measure on how many cells that had migrated.

*Transendothelial migration.* ECs were cultured on gelatin-coated Transwell inserts (8 μm pore size) for at least five days to reach confluence (Fig. 6C) (*101*). Where indicated, the endothelium was stimulated with TNF-α for 5 hrs before the assay. Recombinant human MCP-1 was added to the lower compartment as a chemotactic agent. Freshly isolated monocytes ($1x10^6$ cells) were added to the upper compartment and allowed to migrate for 2 hrs at 37°C. The cell suspensions in the lower compartments were concentrated by centrifugation; the cells were fixed in 1% paraformaldehyde and counted using a Bürker chamber.

*Boyden chamber assay.* Supernatants from cultured APIs were loaded in the lower chamber of a microchemotaxis chamber. Isolated human monocytes ($5x10^5$ cells/ml) were added to the upper chamber. A filter (pore size of 3 μm; polyvinylpyrrolidone-free) coated with gelatin was used to separate the lower and upper chambers. Medium and recombinant human MCP-1 were used as controls. After 2 hrs of incubation at 37°C, the filter was removed, fixed in methanol and stained with Giemsa (1:10 dilution in $dH_2O$). The number of migrated cells was counted in a light microscope.

The migration assays are intended to simulate the migration of leukocytes from the blood stream across the endothelium and into the underlying tissues. Migration occurs as a consequence of firm adhesion of leukocytes to ECs. In paper I and IV, adhesion assays across coated filters were performed to test the chemotactic effect of different supernatants, whereas in paper III the ability of monocytes to migrate across different EC-types were evaluated. We found that it was important for the leukocytes to bind to a matrix in order to firmly adhere and migrate in the absence of ECs. In the absence of ECs, a filter pore size of 3 μm was used to ensure that the leukocytes had to actively migrate to transverse the filter. Prior to the transendothelial migration assays, ECs were split and allowed to reach confluence for five days according to Hauzenberger et al. (*101*). However, a more accurate way to test the cells for confluence would probably be to use electric resistance to measure the barrier function of the endothelium. For the same reasons as mentioned above, the concentration of the leukocytes and the time of migration are important parameters also in the migration assays. Relatively short migration times were used since monocytes and neutrophils migrate quickly across the endothelium *in vivo*. Furthermore, long incubation times may lead to cell activation from intercellular cross-talk, which may influence migration in a way that would not be seen *in vivo* because of the dynamic conditions.

## 4.7 LENTIVIRUS TRANSDUCTION

A significant concern surrounding the use of viral vectors for gene transfer into ECs is the risk of EC activation. This is especially a problem if the ECs are to be used in functional assays, such as adhesion or migration assays. Previous studies have shown that rat ECs become activated with increased expression of MHC class I and CD59 following adenovirus infection (*182*). By the use of EC activation markers (E-selectin, VCAM-1 and MHC class II) in flow cytometry, primary HAECs were not found to be activated upon lentivirus transduction. Furthermore, transduced ECs were as responsive to TNF-α and IFN-γ stimulation as non-transduced ECs.

## 4.8 REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

In paper IV, cultured APIs were investigated for the presence of IL-1β, IL-6, TNF-α and MCP-1 transcripts by reverse transcriptase-polymerase chain reaction (RT-PCR). These cytokines/chemokine were chosen for this study based on the fact that they are well-known inflammatory cytokines, and that MCP-1 is an important chemoattractant for monocytes. However, it is not at all certain that these proteins are the most important ones in the context that they were studied. Ideally, an initial cytokine/chemokine array would have been suitable to assess a broad number of candidate genes. Importantly, the presence of mRNA does not imply that the corresponding protein is translated. Furthermore, the obtained RT-PCR results were not quantitative. However, by relating the mRNA expression levels of each cytokine/chemokine tested to the housekeeping gene mRNA expression levels, an approximate quantitative estimate of each message was obtained. If this experiment had been performed today, real time RT-PCR would have been chosen since it is a highly sensitive and quantitative method. Control reactions were run with genomic pig DNA to exclude that the obtained RT-PCR products were generated from genomic DNA sequences.

## 4.9 SDS-PAGE AND WESTERN BLOTTING

SDS-PAGE and Western-blotting were performed to investigate the staining pattern of α1,3GalT-transduced and non-transduced EC-lysates. Purified human anti-α-Gal Abs were considered superior to the *Griffonia simplicifolia* $IB_4$ lectin for detecting α-Gal-bearing proteins, as the lectin also binds α-Gal epitopes that are not α1,3-linked (*183*). Since Western blotting is a very sensitive method, it is important to include negative and positive controls in each gel. For instance, if the membrane is over exposed, a high background will be obtained in which unspecific bands may appear as if they were actually true bands.

# 5 RESULTS AND DISCUSSION

## 5.1 HUMAN LEUKOCYTE-PORCINE ENDOTHELIAL CELL INTERACTIONS-A ROLE FOR THE α-GAL EPITOPE?

As discussed in the introduction to this thesis, it is extensively debated in the literature whether human leukocyte subpopulations have the ability to distinguish between allo- and xenogeneic endothelium. In addition, the role of the α-Gal epitope in the interaction of human leukocytes with porcine endothelium is not yet established. In paper I-III, we sought to investigate these issues further.

As shown in paper I and III, human naïve neutrophils and monocytes did not adhere, under either static or dynamic conditions, more to resting or TNF-α-stimulated PAECs than HAECs in the absence of XNAs and complement. As expected, TNF-α-stimulation augmented the number of adhering leukocytes. In fact, monocytes adhered significantly less to TNF-α-stimulated PAECs than to HAECs under conditions of flow (Fig. 7), although stimulated PAECs readily increased their expression of E-selectin and VCAM-1 indicating an activated phenotype. Similar results with neutrophils were observed after stimulating PAECs with TNF-α for 30 min. The less effective binding of human monocytes may be explained by an impaired rolling on porcine endothelium. In man, both the selectins (E-, P- and L-selectin) and the VLA-4 integrin can mediate rolling of monocytes (*84, 184*). The selectins have been suggested to support rolling of human leukocytes to porcine endothelium, however, the ability of VLA-4 to mediate rolling of human monocytes on porcine endothelial VCAM-1 has not yet been investigated even though this receptor pair is compatible (*90, 185*).

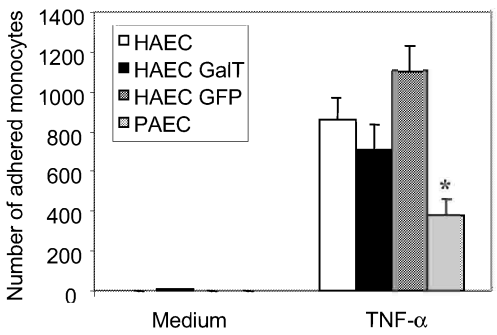


**Fig. 7.** Adhesion of human monocytes to PAECs, GalT-, GFP- and non-transduced HAECs under conditions of physiological flow. ECs were treated with medium or TNF-α (20 ng/ml) for 6 hrs before the flow adhesion assay. Human monocytes ($5x10^5$ cells/ml) were perfused over confluent monolayers of ECs at 1 Dyne/$cm^2$ for 15 min. The number of adhered cells was counted in ten visual fields for every condition at a magnification of 100x. The results are expressed as mean ± SEM from five experiments. $p$=0.03 vs. adhesion to TNF-α-stimulated non-transduced HAECs (*).

In paper II and III, high frequencies (70-95%) of α-Gal-positive HAECs were obtained upon lentiviral transduction, which allowed us to evaluate the effect of this epitope on human monocyte and NK cell adhesion, and on NK cell cytotoxicity, in assays

in which primary ECs with otherwise identical genetic background were used as controls. No difference was detected between non-α-Gal and α-Gal expressing HAECs in terms of their susceptibility to NK cell-mediated lysis or ability to stimulate IFN-γ production by NK cells. Further, no difference in the ability of TNF-α-stimulated or non-stimulated, transduced or non-transduced HAECs to support NK cell or monocyte adhesion under static and dynamic conditions could be observed.

There are several potential explanations for the contradictory results present in the literature with regard to the adhesion of human leukocyte populations to porcine endothelium; (i) the conflicting data may be due to differences in experimental set up including the use of different shear forces, temperatures, and incubation or perfusion times; (ii) to compare the ability of ECs from different species and from different anatomical sites to support adhesion may be irrelevant since the endothelium is known to vary phenotypically between different vascular beds (*130, 177, 178*); (iii) to use COS cells as a model system to elucidate the role of the α-Gal epitope in the interaction between human leukocytes and porcine endothelium may not be relevant because their repertoire of α-Gal structures may be different from that of ECs, and they lack EC-specific cell adhesion molecules (*133, 134*); (iv) in addition to the expression of the α-Gal epitope on porcine endothelium, there are ample phenotypic differences between human and porcine endothelium. For instance, the MHC class I molecules on porcine cells have been suggested to be incompatible with killer inhibitor receptors expressed on human NK cells (*120*); (v) α-Gal binding lectins or IgG $F(ab')_2$ may sterically block α-Gal substituted molecules that are involved in the conjugation of the NK cell to the target even though the α-Gal epitope *per se* is not involved, or they may activate porcine ECs which may affect their susceptibility to NK cell-mediated lysis (*34, 72*); (vi) reducing the α-Gal epitope density on porcine endothelium by over-expression of an α1,2fucosyltransferase competing for the same precursor as the α1,3GalT, or by treating ECs with an α-galactosidase cleaving terminal α-Gal, may have additional profound effects on the glycosylation phenotype of these cells. In the case of α1,2fucosyltransferase over-expression, the degree of sialylation decreases making the cell surface less negatively charged, which in itself will decrease the repulsive forces between two opposing cells and thereby increase adhesion (*186-188*).

In a transendothelial migration experiment performed in paper III, porcine and α-Gal- or non-α-Gal-expressing human endothelium supported monocyte transendothelial migration equally well. As previously demonstrated by our lab, porcine ECs supported migration of different human leukocyte subpopulations, including monocytes, as well as human endothelium did (*101*). Even though porcine and human endothelium support monocyte migration equally well, the process may be different on a molecular level. The migration across TNF-α-activated endothelium in the presence of MCP-1 was decreased for all EC-groups as compared with the addition of MCP-1 alone. A possible explanation may be that TNF-α-activated endothelium supports monocyte EC adherence to a degree preventing migration.

Furthermore, we have shown by flow cytometric analysis that unstimulated human neutrophils were unable to bind polyacrylamide-conjugates or mucins carrying multiple α-Gal epitopes (unpublished observation). This supports our conclusion, at least in terms of the α-Gal epitope, that human naïve neutrophils do not recognize porcine endothelium differently from human endothelium.

Taken together, our data strongly argue against a differentiated recognition of human and porcine endothelium by human neutrophils and monocytes. The α-Gal epitope

does not seem to be involved in the interaction between human monocytes or NK cells and porcine endothelium, neither during adhesion nor by mediating increased NK cell cytotoxicity. Neutrophil adhesion was not tested on α-Gal-expressing HAECs, but it is conceivable that the α-Gal epitope is not involved in their interaction with PAECs, because their adhesion to PAECs was not increased compared to HAECs. As no α-Gal epitope-binding lectin or receptor on leukocytes has been described, and until then, data indicating a direct cellular recognition of α-Gal epitopes based on carbohydrate-, lectin- or Ab-blocking experiments should be interpreted with caution.

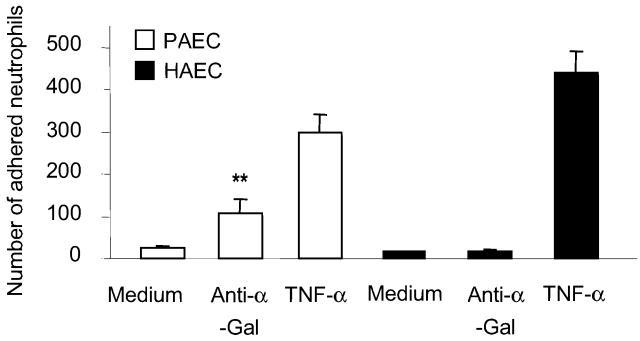


**Fig. 8.** Human neutrophils were perfused over confluent monolayers of HAECs and PAECs in a flow adhesion assay. ECs were stimulated with medium alone, TNF-α (500 U/ml) for 6 hrs or with anti-α-Gal Abs (100 μg/ml) for 30 min followed by gentle washing, and further incubation in medium alone for 6 hrs. The number of adhered cells was counted in ten set visual fields at 100x magnification after 15 min of neutrophil perfusion. The neutrophils were perfused at 1 Dyne/cm$^2$ and at a concentration of 5x10$^5$ cells/ml. Results are expressed as mean ± SEM from five experiments for each condition. $p<0.01$ as compared to PAECs treated with medium (**).

## 5.2 THE ROLE OF ANTI-α-GAL ANTIBODIES IN NEUTROPHIL ADHESION

In paper I, the role of human anti-α-Gal Abs in the adhesion of human neutrophils to porcine and human endothelium was investigated. In a flow adhesion assay, a significant increase in adhesion of human neutrophils to PAECs, but not to HAECs, was detected 6 hrs after anti-α-Gal Ab-binding (Fig. 8). Flow cytometric analysis of PAECs 30 min, 4 hrs and 6 hrs after stimulation with anti-α-Gal Abs revealed an increased expression with time of the cell adhesion molecules E-selectin and VCAM-1. Previously, α-Gal epitope-mediated activation of PAECs has been reported to induce both type I and type II endothelial activation (*72-74, 76*). Even though we could not detect an increased expression of ICAM-1 on PAECs at any time after anti-α-Gal Ab-stimulation, PAECs used in our assay expressed ICAM-1. ICAM-1 may be involved in mediating firm adhesion of human neutrophils to PAECs, and the constitutive level of expression is likely to be sufficient for that. However, Sheikh et al. demonstrated that ICAM-1 and its ligands were not involved in the adhesion of human neutrophils to PAECs under conditions of flow (*99*). Although speculative, human neutrophils may use other adhesion molecules and ligands, *e.g.* the $\beta_1$-integrins, to firmly adhere to xenogeneic endothelium. However, this remains to be investigated. Nevertheless, the activation of PAECs caused by anti-α-Gal Ab-binding or TNF-α-stimulation seemed essential for arrest and firm adhesion of human neutrophils to occur since adhesion was not detected on non-stimulated PAECs.

Some binding through Fcγ receptors cannot be excluded, although considered less important because we did not detect any increased binding 30 min after anti-α-Gal Ab-binding. In this study, only preformed anti-α-Gal Abs were used, and it is possible that induced, affinity-matured anti-porcine Abs, directed against the α-Gal epitope or non-α-Gal epitopes, may have a more profound effect on the endothelium and, consequently, on leukocyte-mediated effector functions following transplantation.

## 5.3 MCP-1 IS PRODUCED AND SECRETED BY ADULT PORCINE ISLETS *IN VITRO*

In paper IV, APIs cultured for 1, 4, 8 and 11 days post-isolation expressed mRNA for the chemokine MCP-1 at all time points. Culture supernatants from APIs induced migration of human monocytes, which was significantly blocked by an anti-MCP-1 Ab. Further, immunohistochemistry revealed MCP-1 in the cytoplasm of α- and β-cells in isolated islets and in islets *in situ* (Fig. 9). MCP-1 produced and secreted by cells in the islets may

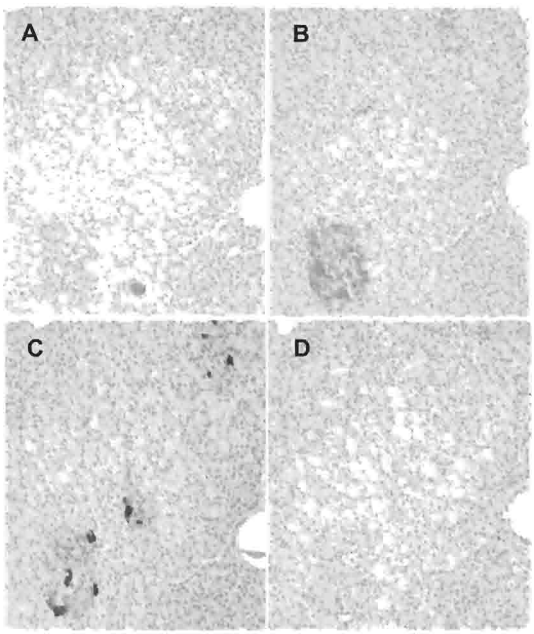


**Fig. 9.** Immunohistochemical staining of consecutive sections (5 μm) of porcine pancreas. Cells reactive with anti-insulin (B) and glucagon (C) mAbs, respectively, within islets were positive also for MCP-1 (A). Anti-CD45 staining is shown in (D), and no infiltrating leukocytes were detected within the islets, which suggests that these cells were not the major contributor of MCP-1 production. Sections were counterstained with hematoxylin. Magnification 200x.

contribute to the rejection of islets in many ways. First, MCP-1 may induce the recruitment of CD11b+ leukocytes directly from the blood into the clot-embedded islets undergoing IBMIR shortly after intraportal injection. This could result in islet damage as well as inducing the presentation of xenoantigens by macrophages. MCP-1 has also been reported to induce expression of tissue factor in human monocytes, which could be detrimental to the transplanted islets (*189*). Second, MCP-1 may also be important during the islet rejection phase after revascularization by recruiting monocytes of recipient-origin. Infiltrating monocytes may, in turn, produce chemokines, which will recruit leukocytes that can initiate the rejection process. Our results also indicated that MCP-1 is expressed during non-inflammatory conditions in islets *in situ*, suggesting other functions of MCP-1

than as a chemoattractant. For instance, MCP-1 has been shown to promote angiogenesis, which could be an important property of MCP-1 within islets in order to ensure a high degree of vascularization (*190*). Further, endothelial progenitor cells have been suggested to derive from the monocytic cell lineage (*191*). It may be speculated that MCP-1 is produced by islets to recruit "endothelial progenitor cells" which, after differentiating into mature ECs, may support pancreatic endocrine cell-differentiation (*192*). Since it appeared as if not all islets produced MCP-1 in our tissue sections, it is possible that only islets in certain developmental or renewal stages express this chemokine.

## 5.4 COMPATIBILITY OF CHEMOKINES/CYTOKINES ACROSS THE PIG-TO-MAN SPECIES BARRIER

Knowledge about how chemokines and cytokines affect cells from another species is important in xenotransplantation. Human TNF-α is well known to activate porcine endothelium, whereas human IFN-γ is species restricted (*83*). However, little is known about the possible effects of porcine chemokines/cytokines on human immune or ECs.

To investigate if anti-α-Gal Ab-stimulated PAECs produced any factors triggering neutrophil motility, we collected supernatants 6 hrs post-stimulation and assessed them in a migration assay. As demonstrated in paper I, supernatants from Ab-stimulated PAECs induced migration of human neutrophils, which was partially blocked by anti-porcine IL-8 Abs or an antagonist to PAF (Fig. 10). The supernatants were found to contain 4.6±0.1 ng/ml of porcine IL-8, a concentration inducing a limited migratory response of neutrophils as revealed by a dose-response analysis with recombinant human and porcine IL-8. Thus, it appears as if human neutrophils are able to respond to porcine IL-8. In accordance with our data, a previous study has shown that PAECs synthesize PAF after stimulation by anti-α-Gal Abs (*193*). The presence of factors stimulating migration in the supernatants of anti-α-Gal Ab-stimulated PAECs are in line with our finding that stimulation of PAECs with anti-α-Gal Abs induced an activated EC phenotype. Finally, it is plausible that anti-α-Gal Ab-mediated activation of PAECs induce the production and secretion of other factors, known or yet to be described, that may have a chemoattractant effect on neutrophils and other leukocytes.

In paper IV, we found that supernatants from cultured APIs induced migration of human monocytes. The migration could be blocked by an anti-MCP-1 Ab. These data indicate that porcine MCP-1 is active across the pig-to-human species barrier. The collected APIs also contained mRNA for IL-1β and TNF-α. These cytokines could influence the rejection process by activating the ECs revascularizing the islets and thereby support the recruitment of leukocytes into the graft. Unexpectedly, IL-1β and TNF-α were not detected on the protein level by ELISAs. The apparent absence of IL-1β and TNF-α in API supernatants was also supported by the inability of these to activate HAECs following a six-hour incubation. Also, co-culturing APIs and HAECs failed to activate the ECs. It is possible that the cytokines, for which we detected the mRNA, remained stored in intracellular compartments, were bound to specific receptors present on cells within the islets preventing their accumulation in the culture supernatants, or were not translated into proteins at all. However, we found that both recombinant porcine IL-1β and TNF-α were able to activate human ECs inducing E-selectin and VCAM-1 expression on the cell surface.

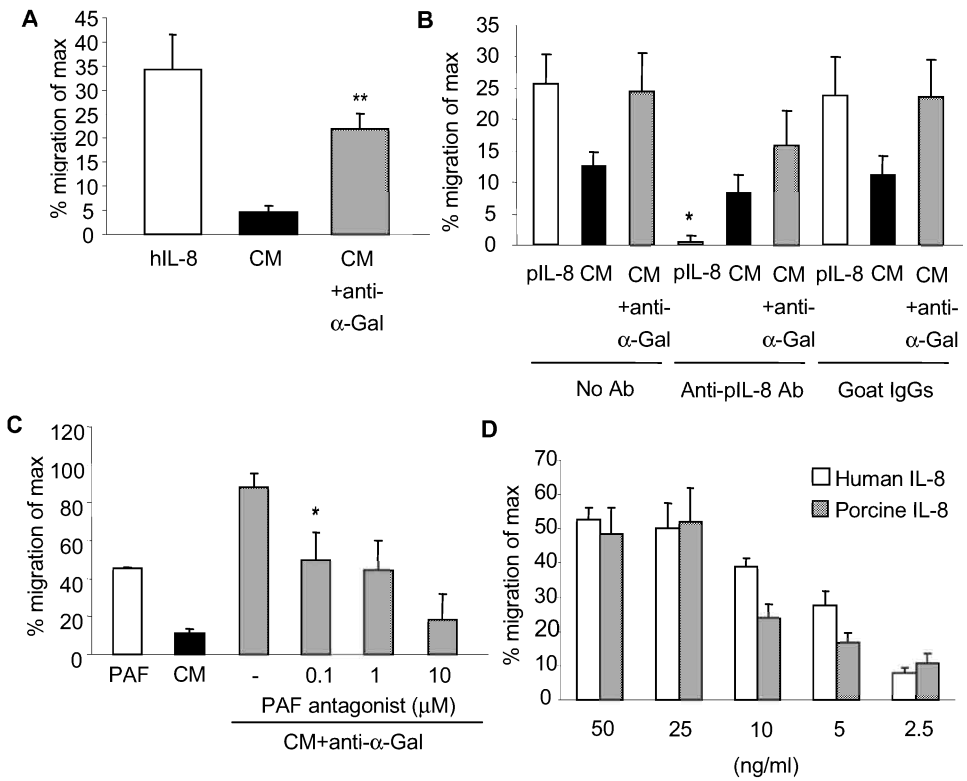


**Fig. 10.** The effect of supernatants collected from PAECs on the migration of human naïve neutrophils. PAECs were stimulated with medium alone or anti-α-Gal Abs (100 μg/ml) for 30 min and left in medium alone for 6 hrs prior to the assay. In (A), recombinant human IL-8 (50 ng/ml) was used as positive control. $p<0.01$ versus migration to medium (**). Anti-porcine IL-8 Ab (10 μg/ml) was added in order to block migration (B). Recombinant porcine IL-8 (50 ng/ml) was used as positive control and goat IgGs (10 μg/ml) were used as control Abs. $p<0.05$ versus migration to recombinant porcine IL-8 without blocking Ab (*). In (C) an antagonist to PAF was added to the neutrophils at the following final concentrations: 0.1, 1.0 and 10 μM. PAF served as a positive control at 0.1 μM. $p<0.05$ (*) as compared to migration without antagonist added. Dose-response analysis of human and porcine IL-8 at 2.5, 5, 10, 25 and 50 ng/ml (D). Results are expressed as mean ± SEM from at least three experiments for each condition. CM, conditioned medium from ECs cultured in medium alone; CM + anti-α-Gal, CM from ECs stimulated with anti-α-Gal Abs; h, human; p, porcine. The value for maximal migration was obtained by measuring the fluorescence of cells directly added to the lower well of the Transwell®.

## 5.5 LENTIVIRAL TRANSDUCED ENDOTHELIUM AS A TOOL TO STUDY LEUKOCYTE-ENDOTHELIAL CELL INTERACTIONS

The *in vitro* characterization of EC-gene function and its effect on EC-leukocyte interactions has so far been difficult to study for two main reasons. First, proper cell lines displaying the phenotypic and functional properties of primary ECs are lacking. Second, a high gene expression in primary ECs has been hard to obtain due to low transfection efficiencies. In paper II, we used green fluorescent protein as a reporter, and could show

that the frequency of lentiviral transduced HAECs increased until day 5 post-transduction. These data suggest that lentiviruses are efficient gene carriers in primary human ECs. Another crucial factor to consider is that the ECs must not be activated as a consequence of viral infection, which is particularly important if the ECs are to be used in functional assays. Lentiviral transduction did not result in activation of HAECs as detected with EC activation markers (E-selectin, VCAM-1 and MHC class II) by flow cytometry (Fig. 11). Further, transduced HAECs responded as expected to TNF-α and IFN-γ stimulation.

To ascertain the importance of the α-Gal epitope for NK cell adhesion, it is important that the density of the α-Gal epitope on α-Gal-expressing HAECs is similar to the density normally found on PAECs. Using human anti-α-Gal Abs in flow cytometry, overlapping fluorescence intensities was obtained of α-Gal-expressing HAECs and PAECs, suggesting equal α-Gal densities. Two populations with distinct α-Gal epitope expression were identified among the α-Gal epitope expressing HAECs. This result may be explained by a regulatory event not related to the expression of the transgene but acting at an epigenetic level, for example, carbohydrate precursor chain or activated sugar-donor availability. As analysed by using anti-α-Gal Abs in Western blot experiments, PAECs and α-Gal-expressing HAECs exhibited a very similar staining pattern under reducing-conditions with one major protein component of 155 kDa stained. This finding indicates that proteins carrying precursor chains for the α1,3GalT may be phylogenetically conserved and that the glycosylation of particular proteins is tightly regulated.

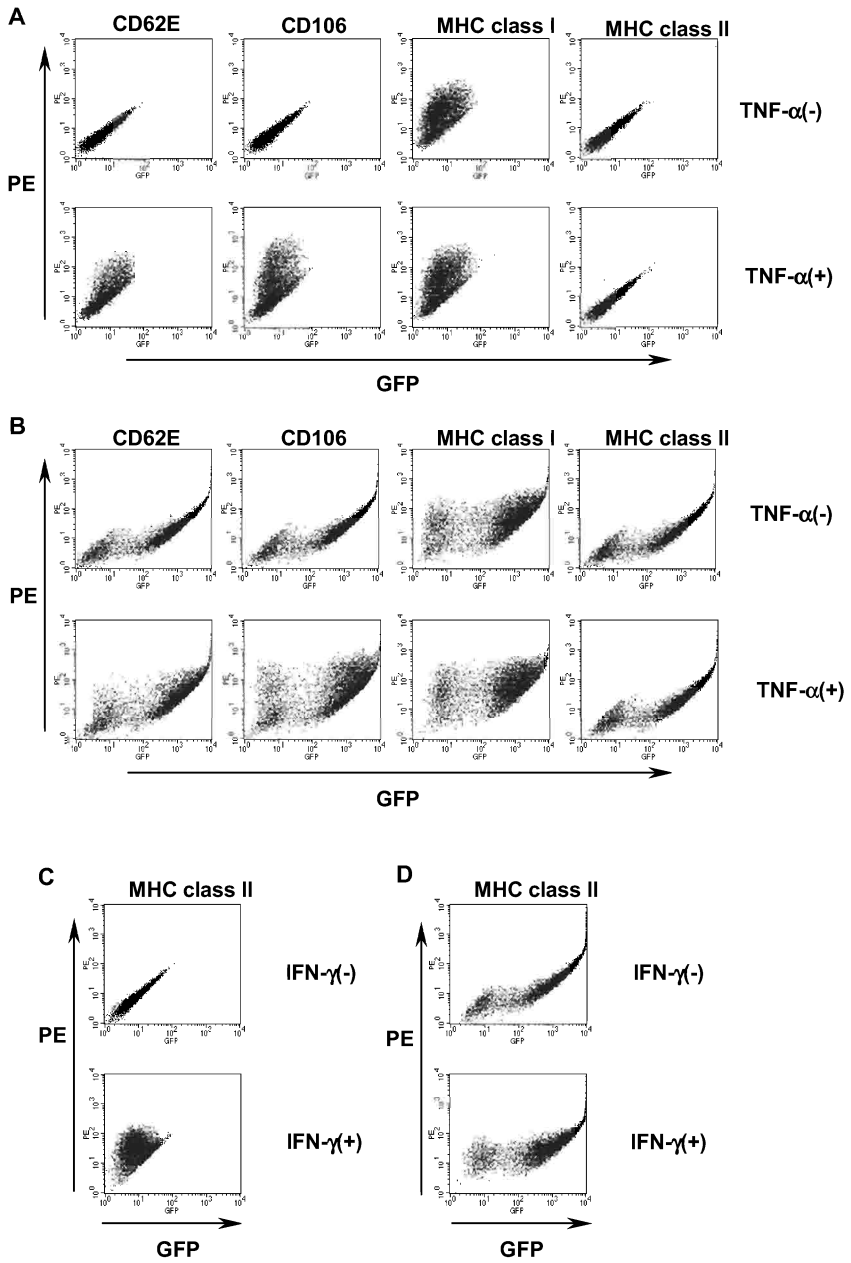


**Fig. 11.** Phenotypic analyses of green fluorescent protein (GFP)-transduced and non-transduced primary HAECs. On day 5, non-transduced (A, C) and transduced (B, D) primary HAECs were cultured with or without TNF-α or IFN-γ. Following staining with PE-conjugated mAbs, the expression of CD62E (E-selectin), CD106 (VCAM-1), MHC class I and II was assessed (y-axis) on GFP-expressing (B, D) and non-GFP-expressing (A, C) cells (x-axis) by flow cytometry.

# 6 CONCLUSIONS

- The adhesion of human neutrophils increased significantly after anti-α-Gal Ab-mediated activation of porcine endothelium. Porcine EC activation was demonstrated by an increase in E-selectin and VCAM-1 expression, and by the production of soluble factors such as IL-8 and PAF. These factors induced migration of human neutrophils.

- Aberrant expression of the α-Gal epitope on human endothelium did not confer susceptibility to human NK cell-mediated cytotoxicity or increased NK cell adhesion. NK cells did not produce IFN-γ upon interaction with the α-Gal-expressing human endothelium.

- Recombinant lentiviruses proved to be effective vehicles for gene transfer into primary human ECs.

- Aberrantly expressed α-Gal epitopes did not increase the adhesion, under static or dynamic conditions, of human monocytes to human endothelium. Neither did it increase monocyte transendothelial migration.

- There was no difference in the adhesion of human neutrophils and monocytes to allo- and xenogeneic endothelium under static and dynamic conditions.

- Cultured APIs expressed mRNAs for MCP-1, IL-1β and TNF-α. Supernatants from cultured APIs induced migration of human monocytes, which could be blocked by an anti-MCP-1 Ab. Isolated islets and islets *in situ* produced MCP-1 as detected by immunohistochemistry. Recombinant porcine IL-1β and TNF-α induced the expression of E-selectin and VCAM-1 on human endothelium.

With regard to novel therapeutic strategies aiming at disrupting the infiltration of human leukocytes into organ xenografts, our results from paper I-III suggest that efforts should be focused on adhesion receptor-ligand interactions rather than on carbohydrate remodeling of donor pigs. Induced Abs of α-Gal or non-α-Gal specificity may increase the adhesion and infiltration of, not only human neutrophils as suggested in paper I, but also of other human leukocyte subpopulations. Indeed, preventing the induced anti-porcine Ab response will be an important factor to prevent AVR.

The work in paper IV highlighted the importance of pro-inflammatory and chemotactic factors produced by the graft itself, which can have profound effects on the rejection process *in vivo*.

# 7 FUTURE PROJECTS

- In the context of porcine EC-activation by human anti-α-Gal Abs, it would be interesting to determine which α-Gal carrying proteins that mediate EC-activation and to identify the signaling pathway(s) downstream of such proteins. If the players of such an activation pathway were known, it would be possible to silence signaling molecule(s) of importance by the siRNA technology. This could potentially serve as a means of future therapeutic intervention in AVR by inhibiting the Ab-mediated EC-activation.

- The studies of manuscript III will be taken further to compare human monocyte activation markers acquired after transendothelial migration across human and porcine endothelium. Monocytes/macrophages are abundant in organ xenografts undergoing AVR, and it is possible that the interaction of monocytes with porcine ECs during transendothelial migration is different from their interaction with allogeneic ECs. Such differences may affect the differentiation of monocytes to macrophages and, thus, their ability to present graft-derived antigens. Also of interest is to test whether human monocytes have the ability to bind soluble α-Gal. This can be tested with our polyacrylamide-conjugates or mucins carrying multiple α-Gal epitopes using flow cytometry. Furthermore, it will be of interest to investigate whether phagocytosis by human macrophages of particles coated with the α-Gal epitope is different from their phagocytosis of particles devoid of this epitope. This will have implications on our understanding of how α-Gal antigens influence antigen presentation.

- The finding in paper IV, that porcine APIs produce MCP-1 is very interesting, and it would be significant to study the role of MCP-1 *in vivo* in the course of IBMIR and islet rejection after revascularization. Using an anti-MCP-1 Ab in a rodent model of islet xenotransplantation or transplanting a wild-type recipient with xenogeneic islets from an MCP-1 knock-out donor would perhaps shed some light on the importance of graft-produced MCP-1 in xenogeneic islet rejection *in vivo*. Our data also indicate that some islets *in situ* produce MCP-1, under non-inflammatory conditions, suggesting that MCP-1 may play a role in normal islet physiology. Future investigations should be aimed at studying the role of MCP-1 in, for instance, islet vascularization, islet morphology and physiology. The use of MCP-1 or CC-receptor 2 knock-out mice may be a relevant model. Such mice have been reported to develop normally and to have normal numbers of circulating leukocytes, however, these studies were not focused on pancreatic or islet development and physiology (*194, 195*).

# 8 POPULÄRVETENSKAPLIG SAMMANFATTNING

Immunsystemet har uppstått för att skydda organismen mot angrepp från yttre faktorer, t.ex. bakterier, virus och svampar. En nyckelfunktion hos immunförsvaret är dess förmåga att skilja på kroppseget och icke kroppseget, vilket är centralt för att undvika angrepp från immunförsvaret på kroppens egna vävnader. I de fall som denna särskiljande funktion inte fungerar talar man om s.k. autoimmunitet, vilken anses ligga bakom flera sjukdomar t.ex. typ 1 diabetes. Immunförsvarets egenskaper är helt livsnödvändiga men skapar samtidigt en rad problem vid transplantation av celler, vävnader eller organ mellan olika individer.

Flera sjukdomar kan idag behandlas m.h.a. transplantation. Emellertid råder idag brist på mänskliga cell- eller organdonatorer varför man studerar möjligheten att transplantera vävnad från andra djurarter till människor genom s.k. xenotransplantation. Grisen anses vara en lämplig organdonator av såväl medicinska, etiska som ekonomiska skäl. Dessutom anses risken för att ett gristransplantat ska smitta den mänskliga mottagaren med virus som liten. Dock måste många immunologiska hinder överbryggas innan xenotransplantation som behandlingsmetod kan bli verklighet.

Kort efter en transplantation av ett grisorgan till en människa avstöts den transplanterade vävnaden i en s.k. hyperakut avstötning. Detta beror framför allt på att grisen på de flesta av sina celler uttrycker en typ av kolhydrat, som kallas α-Gal, mot vilken människokroppen har naturligt förekommande antikroppar. Den hyperakuta avstötningen kan förhindras antingen genom att minska uttrycket av kolhydraten på grisens celler eller genom att avlägsna de naturliga antikropparna, eller genom att inaktivera det s.k. komplementet (ett av blodets försvar). Även om man lyckats avstyra den hyperakuta avstötningen kommer emellertid transplantatet att stötas bort i ett senare skede i en s.k. akut vaskulär avstötning. Denna involverar en aktivering av endotelceller (klär blodkärlens insida) och en infiltration i transplantatet av olika vita blodkroppar (t.ex. neutrofiler, monocyter och NK celler). Cellulära xenotransplantat [t.ex. Langerhanska öar (innehåller insulinproducerande celler) som isolerats från sitt värdorgan bukspottskörteln] avstöts dock genom en direkt cellmedierad process.

I arbete I-III i denna doktorsavhandling har vi studerat interaktionen mellan mänskliga vita blodkroppar och endotelceller från gris med fokus på den akuta vaskulära avstötningen. I motsats till tidigare studier fann vi att mänskliga vita blodkroppar inte binder till grisendotelceller i större utsträckning än till mänskligt endotel, varken under statiska eller dynamiska förhållanden och att kolhydraten α-Gal inte förefaller vara inblandad i denna interaktion. Däremot upptäckte vi i arbete I att mänskliga naturliga antikroppar mot α-Gal aktiverar grisens endotel och att detta leder till en ökad adhesion av neutrofiler under dynamiska förhållanden. Dessutom utsöndrade det aktiverade grisendotelet ämnen som orsakar vandring av neutrofiler. Våra resultat tyder på att man i utvecklandet av behandlingsmetoder för att undvika cellulär infiltration vid xenotransplantation bör fokusera på adhesionsreceptorer och deras ligander istället för att förändra kolhydratuttrycket på donatorgrisarnas celler. Det är troligt att inducerade antikroppar riktade mot α-Gal eller andra målmolekyler på grisens celler kan öka adhesionen och infiltrationen även av andra vita blodkroppar än neutrofiler i ett transplanterat grisorgan. Att förhindra uppkomsten av inducerade antikroppar mot gris är förmodligen en mycket viktig faktor för att förhindra den akuta vaskulära avstötningen.

I arbete II och III använde vi ett lentivirus för att få mänskliga endotelceller att uttrycka α-Gal. Lentivirus visade sig vara ett effektivt verktyg för att överföra de aktuella grisgenerna till mänskliga endotelceller.

I arbete IV, studerade vi om isolerade Langerhanska öar från gris producerar ämnen som skulle kunna få mottagarens vita blodkroppar att påskynda avstötningen vid en transplantation. Vi fann att sådana Langerhanska öar producerade och utsöndrade ett ämne som kallas MCP-1. MCP-1 orsakar migration av monocyter, vilka är viktiga celler vid avstötningsprocessen av bukspottkörtelceller från gris. I migratonsstudier visade sig MCP-1 från griscellerna även vara verksamt på mänskliga monocyter. Vi fann också att vissa Langerhanska öar i grisens bukspottskörtel normalt producerade och utsöndrade MCP-1. Detta fynd antyder möjligen att MCP-1 även spelar en roll i utvecklingen och funktionen hos Langerhanska öar. Resultaten från arbete IV understryker vikten av att känna till vilka ämnen som ett xenotransplantat producerar och hur dessa påverkar avstötningsprocessen.

Förhoppningsvis har dessa studier bidragit till en ökad kännedom om hur mänskliga vita blodkroppar interagerar med endotelceller från gris och hur de attraheras till cellulära xenogena transplantat. Dessa kunskaper är av vikt för att man i framtiden ska kunna förhindra den cellulära avstötningsprocessen av xenogena cell- och organtransplantat.

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# 11 PAPERS