Doctoral Thesis

Xenotransplantation: prevention of human innate immune responses

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Xenotransplantation:
Prevention of Human Innate Immune Responses

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presented by

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1. Summary

Xenotransplantation is the transplantation of cells, tissues, and organs between members of different species. It has the potential to overcome the increasing gap between available and required donor organs in transplantation medicine. For several reasons, the pig is viewed as a suitable organ donor candidate for xenotransplantation. However, porcine vascularized organs transplanted into humans are hyperacutely rejected due to xenoreactive natural anti-porcine antibodies (NAb) present in the recipient’s blood circulation. These NAb are predominantly directed against galactose-α(1,3)galactose (Gal), an epitope expressed on most porcine cells, including the endothelium. As revealed from in vitro studies and pig-to-primate animal transplantation models, NAb binding to porcine endothelial cells (pEC) induces endothelial cell activation and a complement-driven hyperacute xenograft rejection (HAR). Furthermore, anti-Gal NAb mediate lysis of pEC by both complement and human natural killer (NK) cells. The latter recognize NAb bound to pEC by their Fcγ receptor III, leading to antibody-dependent cell-mediated cytotoxicity (ADCC). It has been postulated that the recent generation of Gal-deficient pigs will largely overcome the barrier of HAR. The focus of research has therefore shifted to other rejection mechanisms such as cellular immunity including NK-cell cytotoxicity. Human NK cells lyse porcine cells and are a potent source of activating cytokines such as interferon γ, and may therefore play an important role in the cell-mediated rejection of pig-to-human xenografts as shown in several in vivo models. Lysis is probably a consequence of the failure of human major histocompatibility complex (MHC)-specific inhibitory NK receptors to interact with porcine MHC class 1 molecules and the recognition of hitherto unknown ligands by activating NK receptors.

The aims of this thesis were: (1) to examine the characteristics of human anti-porcine NAb with special focus on the binding of non-Gal anti-porcine NAb to Gal-deficient pEC; (2) to evaluate whether the lack of Gal protects pEC from NAb/complement-induced lysis, NAb-dependent ADCC, direct xenogeneic NK cytotoxicity, and adhesion of human leukocyte subpopulations; and (3) to analyze whether the expression of the MHC class I molecule HLA-E on the surface of porcine cells protects against direct xenogeneic NK cytotoxicity.

The results of this thesis demonstrate that the absence of Gal on pEC leads to a substantial reduction of NAb binding, complement lysis, and ADCC in vitro and, thus, seems to correlate with the notion that hyperacute NAb-mediated xenograft rejection in pig-to-nonhuman
primate xenotransplantation is overcome. Nevertheless, the present data also indicate that non-Gal anti-porcine NAb represent a potential immunologic hurdle for clinical xenotransplantation, at least for a subgroup of prospective human recipients. Furthermore, the lack of Gal did not resolve adhesion of human leukocyte subsets and direct xenogeneic NK cytotoxicity, indicating that Gal is not a dominant target molecule for human NK cells. Finally, the expression of HLA-E on porcine cells partially protected them from lysis mediated by polyclonal NK populations. However, the capability of different porcine cell types to express HLA-E on the cell surface and, consequently, the protective potential differed considerably depending on the nature and availability of the peptides that form a trimeric complex together with HLA-E and β2-microglobulin.

With regard to novel therapeutic strategies our data suggest that future research efforts should be focused on the identification of non-Gal epitopes recognized by human xenoreactive NAb and of the porcine ligands for activating NK receptors. Although the data of this thesis are based on in vitro experiments, they reveal important molecular mechanisms which potentially also play a role in vivo. This thesis has focused on particular aspects of xenotransplant rejection and proposed new avenues of research that might contribute to overcome the immunological hurdles. Other aspects such as the potential risk of xenozoonosis, immunological incompatibility, and chronic rejection mechanisms also need to be examined carefully. Only the combined efforts of many researchers in the field of xenotransplantation will make xenotransplantation a clinical reality, and hopefully will alleviate the current shortage of organs in human transplantation medicine.
2. Zusammenfassung


Die Ziele dieser Dissertation waren: (1) die Analyse der Eigenschaften von humanen antiporzinen NAb, im Speziellen die Bindung von nicht-Gal-spezifischen NAb an Gal-negative pEC; (2) die Untersuchung der Auswirkungen fehlender Gal Expression auf folgende Vorgänge: Antikörper-abhängige Komplement-induzierte Lyse, Antikörper-vermittelte...
Zusammenfassung

zelluläre Zytotoxizität, direkte NK Zytotoxizität und Adhäsion von Subpopulationen von humanen Leukozyten; (3) die Analyse des Potentials transgener HLA-E Expression auf pEC zum Schutz vor direkter NK Zytotoxizität.


3. Introduction

3.1. Xenotransplantation

Xenotransplantation, the transplantation of cells, tissues, and organs between members of different species, may represent a potential alternative to allotransplantation which suffers from the lack of human donors for transplantation medicine. Interest in xenotransplantation has varied over the past centuries [1-3]. The pioneers of xenotransplantation performed xenotransfusions, i.e. the transfusion of sheep blood to patients, as early as in the 16th century. In the early 20th century, the first cell and tissue xenotransplantations were reported. In the 1930s, the French physician Serge Voronoff became famous for transplanting testes from apes to humans. During the 1960s, before allotransplantation became a clinical reality, a number of xenografts were transplanted from primates to humans. In 1964, Reemtsma performed one of the most successful clinical xenotransplantations: in one patient a chimpanzee kidney survived with adequate function for nine months [4]. However, the failure to achieve long-term success during this period and the new source of allogeneic organs achieved by the acceptance of the brain death concept dampened enthusiasm for further clinical trials of xenotransplantation. Therefore, the 1970s and 1980s were relatively quiet years in the history of xenogeneic transplantation, while allogeneic transplantation of heart, lung, liver, kidney, and pancreas became fully established. Since the early 1950s, when solid organ allotransplantation was first successfully performed, great advancement has 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 after allotransplantation. However, the success of allotransplantation with first year survival rates above 90% for most procedures resulted in a shortage of human organs in transplantation medicine. In recent years, the supply of organs available for transplantation has fallen far below the number of patients waiting for a transplant. Today, the waiting lists for new organs are long and many patients die while waiting for a transplant. Therefore, the need for an alternative source of donor organs has again focused attention on xenotransplantation. Moreover, the recent years have brought enormous progress in understanding rejection mechanisms and suggesting possible solutions to overcome the barriers preventing successful xenotransplantation. Two different types of xenotransplantation might be clinically applied. The first includes the transplantation of vascularized organs such as heart or kidney, the second uses non-vascularized tissues and cells such as islets, neural cells, or bone marrow.
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For a long period, primates were considered as the best potential donors for xenotransplantation because they are closely related to humans and, therefore, immunologically relatively compatible. However, due to difficulties in breeding primates in large numbers, their small size, and, in particular, the ethical concerns, primates cannot be considered as an optimal xenograft donor. Instead, miniature pigs represent an attractive candidate source for xenografts for several reasons [5,6]. Pigs breed rapidly and in large numbers, they can be housed in pathogen-free environments, and their organs share anatomic and physiologic similarities with human organs. Therefore, the latest period of interest in xenotransplantation has focused on pigs as an organ source. However, immunological barriers in pig-to-primate xenotransplantation are considerable and not easily overcome. Soon it became clear that the conventional treatment applied to allograft transplant recipients, e.g., with immunosuppressive drugs mainly directed against T-cell responses, does not provide sufficient protection from xenograft rejection. Two mechanisms of porcine xenograft rejection will be outlined in the following chapters: antibody-mediated hyperacute rejection and human innate immune responses in acute vascular rejection.

3.2. The Human Innate Immune System

The immune system is a complex network of specialized cells, tissues and organs. It has evolved in vertebrates to protect them from hazardous agents such as bacteria, viruses, fungi, and parasites. The basic principle of the immune system is the ability to distinguish between danger and non-danger or self and non-self, a fact which leads to severe problems in transplantation medicine. Several different cell types are involved in the immune response to dangerous agents or rejection of allogeneic and xenogeneic transplants. Both the activation and inhibition of immune responses are dependent on the interplay between different cell types and soluble factors secreted by them. Whereas the innate immune system is characterized by a prompt non-antigen-specific response, antigen-specific responses are mediated by the adaptive immune system. Innate immunity is composed of many non-antigen-specific factors such as physical barriers, phagocytic cells, and biologically active substances. In addition, cellular mediators including polymorphonuclear neutrophils, macrophages, as well as natural killer cells belong to the innate immune system. In contrast, the major cell types participating in acquired immunity are T and B lymphocytes which carry highly variable antigen receptors allowing specific interactions with epitopes derived from
Introduction

pathogens. In the following chapters, three particular aspects of the innate immune system will be discussed in detail: natural antibodies, the complement system, and natural killer cells.

3.2.1. Natural Antibodies

Naturally occurring antibodies develop in most healthy individuals soon after birth and remain throughout life. They are produced by CD5+ pre-B lymphocytes which account for most of the B-cell repertoire in the fetus and neonate. Natural antibodies (NAb) are frequently polyreactive with low affinity for multiple self-antigens, and are generally encoded by rearranged antibody genes that have not undergone somatic mutation [7,8]. Because of their ability to bind a variety of exogenous antigens, including those on bacteria and viruses, NAb play a major role in the primary line of defense against infections. They have been found to protect against bacterial as well as viral pathogens, and to play an important role in the clearance of endotoxin (reviewed in [9]). In addition, NAb are important in the recognition of apoptotic cells, and of nuclear and cytoplasmic components released from cells. In transplantation medicine, NAb play an important role in the ABO blood group system. Despite the minor differences between the blood group antigens A and B, anti-A and anti-B antibodies have precisely defined specificities, e.g. donors of blood group A do not produce anti-A antibodies and conversely, donors of blood group B lack the expression of anti-B antibodies. In contrast, most blood group donors have NAb recognizing the porcine blood group homologue galactose-α(1,3)galactose (Gal). Anti-Gal NAb make up a considerable amount of the antibodies circulating in the human blood and do not seem to be a result of an antigen-specific immune response to infection. Presumably, these NAb are produced in response to the colonization of the gut by bacteria and to the exposure to viruses, protozoa, or components in food carrying Gal antigens. The binding of NAb to target cells can activate the complement cascade and induce antibody-dependent cell-mediated cytotoxicity as discussed below.

3.2.2. The Complement System

Complement was originally discovered as a heat-labile component of normal plasma that augments the opsonization of bacteria by antibodies and allows antibodies to kill certain bacteria. Today, it is well established that the complement system is part of the innate immune system and conducts three main physiologic tasks: defense against bacterial infections, bridging innate and adaptive immunity, and clearance of immune complexes as
well as products of inflammatory injury [10]. The complement system consists of about 30 plasma proteins that function as enzymes or binding proteins and react with each other. There are three distinct pathways through which complement can be activated: The classical, the alternative, and the lectin pathway. These pathways depend on different molecules for their initiation, but they converge to generate the same set of effector molecules finally leading to the formation of a so-called membrane attack complex which ultimately leads to target cell destruction. The complement system utilizes two main mechanisms for discrimination between self and non-self, but also between danger and non-danger. Certain foreign cell surface structures lead to direct activation of the alternative complement pathway, whereas others are recognized by NAb which in turn activate the classical complement pathway via Fc-complement interactions. Therefore, the binding of anti-porcine NAb can induce a strong complement response as discussed later in this thesis. The complement system has developed an elegant manner of regulating itself. A series of secreted and membrane regulatory proteins exist to prevent autologous complement activation and to prevent host cells from accidental complement attack. In xenotransplantation, regulation of complement activation is a key component to overcome rejection mechanisms.

3.2.3. Natural Killer Cells

Natural killer (NK) cells are a heterogeneous population of large granular lymphocytes comprising approximately 5-15% of peripheral blood mononuclear cells (PBMC). In humans, NK cells are identified by the surface markers CD16 (Fcy receptor III) and CD56, and by the lack of T- and B-cell receptors [11]. NK cells are closely related to cytotoxic T lymphocytes and arise from the same progenitor cells [12]. However, while T cells require the thymus for their development, development of NK cells occurs primarily extrathymically, with critical steps taking place in the bone marrow [13]. The production of interleukin (IL)-15 by stromal cells in the bone marrow is crucial for the development of functional NK cells. In addition to CD16 and CD56, CD2, CD8, and CD57 are expressed on subpopulations of NK cells. However, NK cells are negative for CD3, except for the ζ-chain which is crucial for signal transduction. Cytokines including IL-2, IL-6, IL-10, IL-12, IL-15, interferon (IFN) α/β, tumor growth factor β, and chemokines influence both differentiation and activation of NK cells [11,14,15]. NK-cell effector functions include direct and indirect cytotoxicity, such as antibody-dependent cell-mediated cytotoxicity (ADCC) via CD16 [16], as well as secretion of cytokines. Cytotoxic functions are mediated by perforin/granzyme B secretion and Fas/Fas
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ligand interactions [17]. Upon activation, NK cells also release cytokines such as IFNγ, tumor necrosis factor (TNF) α, and granulocyte-macrophage colony-stimulating factor, and chemokines which induce inflammatory responses. NK cells are responsible for the majority of the potent lymphokine-activated killing activity of PBMC after stimulation with IL-2. In general, NK cells are divided into two subsets based on their cell-surface density of CD56. CD56^{bright} and CD56^{dim} NK cells exhibit distinct phenotypic and functional properties and, therefore, distinct roles in the human immune response. The CD56^{dim} subset of NK cells is mainly cytotoxic and expresses higher levels of Ig-like NK receptors and CD16 than the CD56^{bright} NK-cell subset. In contrast, the CD56^{bright} subset has the capacity to produce abundant cytokines, but has low natural cytotoxicity as compared to CD56^{dim} NK cells [18].

NK activity is tightly regulated by a delicate balance between activating and inhibitory signals, in order to protect healthy cells from NK cytotoxicity [11,19-22]. Two major checkpoints control target cell susceptibility to NK cytotoxicity. First, the expression of ligands for NK activating receptors, and second, the presence of major histocompatibility complex (MHC) class I molecules interacting with inhibitory NK receptors [23-25]. Therefore, NK cytotoxicity occurs when target cells express ligands for activating receptors and, in addition, lack sufficient levels of self-MHC class I molecules, a mechanism originally proposed by Kärre as the "missing self" hypothesis [26,27]. This hypothesis was based on mouse studies where an H-2^{b/b} homozygous bone marrow transplant was rejected by NK cells of H-2^{b/b} hosts, while H-2^{b/b} mice did not reject H-2^{a/b} transplants. In the former case, the transplanted graft (H-2^{b/b}) failed to express one H-2 class I allele of the host (H-2^{a}). This finding proposed the existence of inhibitory self-reactive receptors. Induction is abrogated when inhibitory NK-cell receptors specific for MHC class I molecules interact with their ligands on target cells, *i.e.* NK cells are inhibited when potential targets express normal levels of MHC class I, and kill if they do not receive an inhibitory signal [14,28]. The role of NK cells is therefore complementary to that of cytotoxic T lymphocytes, as they kill MHC class I-deficient cells that cannot be recognized by cytotoxic T lymphocytes.

While NK cells were initially identified by their ability to lyse certain tumor or virally infected cells without prior sensitization [11], more recent work has demonstrated that they have a crucial role in the initial defense against pathogens and are particularly important in responding to viral infections (reviewed in [21]). NK cells are responsible for a rapid response to infected or transformed cells either by direct lysis of abnormal cells or by releasing immunomodulatory chemokines and cytokines such as IFNγ. The chemokines and cytokines released by NK cells influence the initiation and development of the subsequent adaptive
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The type I interferons (IFNα and IFNβ) elicited in response to certain viral infections induce NK-mediated cytotoxicity against virus-infected cells. Furthermore, the production of the type II interferon IFNγ by activated NK cells triggers a number of important pathways associated with direct anti-viral functions. Thus, NK cells are transmitters of several pathways promoting anti-viral defense, including those dependent on either their cytotoxic or cytokine-producing functions. Downregulation of surface MHC class I molecules appears to be a mechanism used by many viruses to escape recognition by cytotoxic T lymphocytes. Accordingly, several gene products were found in human cytomegalovirus interfering with MHC class I expression on infected cells. Therefore, the downregulation of MHC class I by viruses may render the cells susceptible to NK-mediated cytotoxicity.

Furthermore, NK cells may also play an important role in the recognition of tumor cells. The activating NK receptor NKG2D binds to inducible ligands on tumor cells resulting in cell lysis. On the other hand, downregulation of MHC class I on tumor cells or NK receptor-ligand mismatches can also activate NK cytotoxicity against tumor cells. Ruggeri et al. showed that donor-versus-recipient NK-cell alloreactivity in MHC-mismatched hematopoietic stem cell transplantation for acute myeloid leukemia could prevent leukemia relapse and graft rejection [29]. The rationale behind Ruggeri's study was based on the identification of the molecular regulation of NK-cell activity which is negatively regulated by MHC class I-specific inhibitory NK receptors [26,30-32]. In addition, since the early 1980s, many studies have documented decreased NK-cell numbers and impairment of NK-cell function in the peripheral blood of patients with autoimmune diseases such as multiple sclerosis, systemic lupus erythematosus, Sjögren's syndrome, rheumatoid arthritis, and type I diabetes (reviewed in [33,34]).

Whereas the role of NK cells in immunity was neglected by a large part of the scientist community, the discovery of several families of NK receptors in the past 20 years induced appreciable attention. Determination of structures of NK-cell receptors and their ligands has led to an increased understanding of the mechanisms behind the activation and ligand recognition by these receptors, as well as the role of NK cells in innate immunity. Functionally, NK-cell receptors are divided into two groups, the inhibitory and the activating receptors, which will be described in detail in the next two chapters.

3.2.3.1. Inhibitory NK-cell receptors

As proposed by Kärre in the early 1980s, NK cells express receptors which negatively regulate their cytotoxic function. Since the 1990s, several murine and human MHC class I-
specific NK inhibitory receptors have been cloned and identified (reviewed in [35-37]). Lack of engagement of such inhibitory receptors induces target cell lysis, while recognition of MHC class I molecules by inhibitory NK receptors represents a dominant negative signal for NK cytotoxicity [38,39]. All mature NK cells express at least one and in most cases multiple clonally distributed inhibitory receptors specific for self-MHC. These receptors are of limited diversity and recognize distinct MHC class I supertypic epitopes, shared by a large number of MHC alleles [19]. No individual MHC allele appears to provide complete protection from polyclonal NK populations. The inhibitory receptors on NK cells ensure that NK cells do not exert effector functions against cells expressing normal levels of self-MHC class I molecules and are, thus, important in maintaining tolerance of NK cells to self. As shown in Figure 1, inhibitory receptors belong either to the immunoglobulin (Ig)-like superfamily or the C-type lectin superfamily [14].

Ig-like inhibitory receptors include the killer-cell Ig-like receptors (KIR), which recognize the classical human leukocyte antigen (HLA) class I molecules HLA-A, -B, and -C, and the Ig-like transcripts (ILT, also named leukocyte Ig-like receptors, LIR), which recognize the non-classical class I molecule HLA-G [40]. In general, KIR are expressed in a clonally distributed fashion on NK cells and a subset of T cells. They are not only expressed at various levels on single NK cells but are also highly polymorphic and polygenic, i.e. the gene content of the KIR cluster varies from individual to individual [41]. KIR can be subdivided into two different groups: the first has two extracellular Ig-like domains (KIR2D), and the second has three Ig-like domains (KIR3D). KIR2D recognize HLA-C molecules, while KIR3D recognize HLA-A and -B molecules [42]. Both groups have members with either a long (indicated by “L”) or a short (indicated by “S”) cytoplasmic tail. KIR with long cytoplasmic tails contain immunoreceptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic domain, which, upon engagement, transmit inhibitory signals leading to the general shutdown of NK-cell effector functions [43]. Cross-linking of inhibitory receptors results in phosphorylation of a tyrosine residue, which is central to the ITIM. ITIM phosphorylation leads to the recruitment and activation of phosphatases including SHP-1 and SHP-2 [44]. This phosphatase activity interrupts NK-cell activation pathways by dephosphorylating essential signaling intermediates [45].

C-type lectin receptors are a family of calcium-dependent carbohydrate binding proteins. CD94 and members of the NKG2 receptor family belong to the C-type lectin superfamily. Two members of this superfamily have inhibitory functions, the heterodimers CD94/NKG2A and CD94/NKG2B. They are specific for the non-classical MHC class I
molecule HLA-E which binds leader peptides from other MHC class I molecules. The inhibitory signal is mediated via two ITIM in the cytoplasmic domains of NKG2A and -B. Theoretically, NK lectin receptors might also bind the porcine disaccharide Gal which is abundantly expressed on porcine cells, as discussed below.

![Figure 1: Inhibitory human NK-cell receptors.](image)

**Figure 1: Inhibitory human NK-cell receptors.** Inhibitory NK receptors belong either to the Ig-like superfamily or the C-type lectin superfamily. The Ig-like superfamily consists of killer-cell Ig-like receptors (KIR) and Ig-like transcripts (ILT). All receptors bind MHC class I molecules as indicated. One or two ITIM sequences in the cytoplasmic domain deliver an inhibitory signal. CD94 has to be associated with NKG2A or NKG2B to transmit an inhibitory signal.

### 3.2.3.2. Activating NK-cell receptors

Whereas MHC class I-specific inhibitory receptors have been known for several years, the nature of activating NK receptors has remained largely unknown for a long period. The currently known activating NK receptors are shown in Figure 2. Beside inhibitory receptors, the human KIR family also contains activating isoforms. In line with their inhibitory counterparts, activating KIR contain either two or three extracellular Ig-like domains (KIR2D or KIR3D, respectively). However, their cytoplasmic tails are short and lack ITIM sequences. In contrast, a positively charged amino acid in the transmembrane region provides a docking site for the activating adapter molecule DAP12 which contains immunoreceptor tyrosine-based activating motifs (ITAM) [46-48].

CD94/NKG2C and CD94/NKG2E belong to the activating isoforms of the C-type lectin superfamily [49]. They associate with the ITAM-containing molecule DAP12 to deliver an activating signal to the NK cell. Both receptors recognize and bind HLA-E, as do their inhibitory counterpart CD94/NKG2A and -B. However, the inhibitory CD94/NKG2A receptor was shown to have higher binding affinity for HLA-E than the activating receptors, thereby favoring inhibition. In addition, the C-type lectin NKG2D is expressed as a
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homodimer on all NK cells and associates with the ITAM-bearing signaling adaptor molecule DAP10. NKG2D does not recognize MHC class I but interacts with stress-inducible proteins such as MIC-A and MIC-B (MHC class I chain-related proteins) or ULBP 1-4 (UL16-binding proteins) [20]. MIC-A and -B are expressed after infection by certain viruses, e.g. human cytomegalovirus.

Figure 2: Activating human NK-cell receptors. Activating NK receptors belong either to the Ig-like superfamily or the C-type lectin superfamily. The Ig-like superfamily consists of killer-cell Ig-like receptors (KIR) and natural cytotoxicity receptors (NCR). While KIR recognize classical MHC class I molecules, the ligands of NCR are still unknown. The C-type lectin superfamily consists of the CD94/NKG2C and CD94/NKG2E complexes which bind the non-classical HLA-E molecule as well as NKG2D, which recognizes MIC-A/-B and ULBP 1-4. All activating NK receptors signal through ITAM-containing adaptor molecules such as DAP10, DAP12, or CD3ξ.

Only recently, three non-MHC-specific activating NK receptors were discovered, collectively termed natural cytotoxicity receptors (NCR): NKp30, NKp44, and NKp46 [25,50]. NCR belong to the Ig-superfamily and signal through the ITAM-bearing signal-transducing molecule CD3ξ. They are exclusively expressed on NK cells and play a major role in NK-mediated killing of tumor cells. NKp30 and NKp46 are expressed on all NK cells, whereas NKp44 is selectively expressed on activated NK cells [51]. There is a direct correlation between the surface expression of NCR and the ability of NK cells to kill various tumor cells that do not express MHC class I molecules [52]. Intriguingly, no cellular ligands for NCR have been identified so far. However, NKp44 and NKp46 have been shown to recognize viral ligands [53,54] and might therefore contribute to NK cell-mediated anti-viral defenses.

All above mentioned activating receptors mediate NK cytotoxicity through direct interactions with ligands on the target cell surface. However, another NK activating receptor called CD16 (Fcy receptor III) specifically binds the Fc portion of immunoglobulin molecules
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bound to antigens on target cells. CD16 ligation induces a signal transduction cascade which eventually leads to ADCC as well as release of various cytokines [55]. Furthermore, other surface molecules function as co-stimulatory molecules to the major activating receptors on NK cells. These co-receptors include 2B4 and NTB-A, two members of the CD2 subfamily [56,57], as well as LFA-1, CD28, CD69, NKp80, and NKR-P1.

The general concept of the major inhibitory and activating NK receptors is based on an exact discrimination between self and non-self, or healthy and altered cells. There are several reasons why nature has created such an elaborate system of NK-cell regulation. Clearly, NK-cell functions must be carefully regulated to prevent damage of normal tissues or the indiscriminate release of cytokines resulting in inappropriate activation of the adaptive immune system. In addition, fine-tuning of anti-viral and anti-tumor responses, e.g. detection of the loss of only one allele by some NK-cell subsets, provides excellent controlling mechanisms.

3.3. Rejection Mechanisms in Xenotransplantation

Two phases of xenograft rejection are discussed in the following chapters, hyperacute rejection and acute vascular rejection. Whether or not xenotransplants are vascularized determines the nature of the immune response elicited in the recipient. This thesis will concentrate on the mechanisms leading to rejection of vascularized xenografts, i.e. solid organ xenografts, in particular the interactions between porcine endothelial cells (pEC) and components of the human innate immune system including NAb and NK cells. Since the endothelium of a solid xenograft represents the first site of contact with the recipient's circulation, it is appropriate to study the early events between the human innate immune system and pEC in order to develop strategies to protect xenografts from rejection.

3.3.1. Hyperacute Rejection

As proposed by Calne, xenotransplantation can be divided into concordant (e.g. nonhuman primate-to-human) and discordant (e.g. pig-to-primate) species combinations [58]. Discordant xenotransplantation is characterized by the presence of preformed antibodies (Ab) in the recipient which recognize donor antigens. In these combinations, vascularized organ xenografts are lost within minutes to hours by hyperacute rejection (HAR). Differences in cell surface glycosylation between humans and pigs evoke HAR due to the presence of
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xenoreactive NAb in humans, mainly directed against the Gal epitope [59,60]. Binding of xenoreactive NAb to pEC leads to a so-called type I endothelial cell (EC) activation, *i.e.* non-transcriptional activation [61]. This activation is very rapid and leads to EC retraction, expression of P-selectin and von Willebrand factor, secretion of platelet activating factor and loss of heparan sulfate [61,62]. 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 Gal cross-linking. Eventually, these mechanisms lead to an initiation of the complement and coagulation cascades. The histology of xenografts rejected by HAR is marked by extensive intravascular thrombosis, edema, and extravascular hemorrhage as shown in Figure 3 [1].

![Figure 3: Hyperacute rejection of porcine xenografts.](image)

Figure 3: Hyperacute rejection of porcine xenografts. HAR is initiated by the binding of human anti-Gal IgM and IgG to Gal epitopes on the porcine endothelium. This leads to complement activation and finally results in intravascular thrombosis, edema, and extravascular hemorrhage.

3.3.1.1. The GaLα(1,3)Gal Epitope, Natural Antibodies and Complement

In 1984, Galili et al. described naturally occurring antibodies in humans directed against the carbohydrate epitope Gal [63]. Gal is a terminal disaccharide linked to N-acetyllactosamine on glycoproteins and glycolipids by the enzyme α(1,3)-galactosyltransferase (α1,3GT). While α1,3GT is present in New World monkeys and nonprimate mammals such as pigs, it is absent in man and Old World monkeys [64]. The α1,3GT gene in man and Old World monkeys contains a frame shift and nonsense mutations that result in gene inactivation [65]. Therefore, humans do not express the Gal epitope on their cells, but instead produce anti-Gal NAb circulating in their blood. It is believed that these NAb arise as a result of the colonization of the gut by bacteria and of the exposure to viruses, protozoa, or components in food carrying Gal antigens [66]. The Gal antigen is structurally closely related to the ABO blood group antigens in humans.
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Using \textit{in vitro} models, Cooper et al. showed that xenoreactive NAb were the prime initiators of HAR following pig organ transplantation into humans or nonhuman primates [67]. In some baboons, frequently used as experimental models for humans, all naturally occurring anti-pig Ab are directed against Gal, whereas most humans also possess a minority of NAb directed against non-Gal porcine antigens [60]. Anti-Gal NAb in humans comprise 1-2\% of total serum IgG and 4-8\% of total serum IgM, and approximately 1\% of human circulating B cells produce anti-Gal Ab [68-70]. Binding of NAb to porcine cells results in pEC and complement activation, the main mediators of HAR [65,71]. The complement cascade is activated by the classical pathway.

3.3.1.2. Prevention of Hyperacute Rejection

Immunosuppressive drugs such as cyclosporine, which successfully inhibit T-cell responses in allotransplantation, are not able to control the massive induction of immune responses in pig-to-nonhuman primate xenotransplantation models. However, the following strategies have been shown to effectively avert HAR (Figure 4): (1) Removal of xenoreactive NAb by plasmapheresis or immunoabsorption using columns carrying solid phase Gal oligosaccharides [72,73]. (2) Blocking of anti-Gal NAb by the administration of soluble synthetic Gal molecules. (3) Inactivation or depletion of complement, \textit{e.g.} by treating the xenograft recipient with soluble complement receptor I [74], cobra venom factor (only possible for rodents and baboons), and monoclonal antibodies (mAb) directed against various complement factors [75]. Moreover, several human complement regulatory proteins such as CD46, CD55, or CD59 have been successfully expressed on the endothelium in transgenic pigs and have proven to be effective in preventing HAR [76-79]. The generation of pigs being transgenic for CD55, CD46, or CD59 resulted in a maximal survival of kidney xenografts up to three months. (4) Another way to prevent HAR is the elimination of Gal expression on porcine cells. In an initial study, the level of Gal in transgenic pigs was reduced by the expression of an \(\alpha_1,2\)-fucosyltransferase [80]. In 2002, two groups reported the production of cloned pigs in which one allele of the \(\alpha_1,3\)GT gene had been eliminated [81,82]. However, more importantly, in 2003 new cloning and nuclear transfer technologies enabled the production of Gal-deficient pigs due to elimination of both functional alleles of the \(\alpha_1,3\)GT gene [83,84]. Using hearts and kidneys of these animals in pig-to-baboon models leads to a significantly prolonged xenograft survival even in the presence of complement and NAb [82-87]. Therefore, HAR is no longer considered as a major hurdle for xenotransplantation. The availability of Gal-deficient cells now allows the investigation of other antigenic targets, in
particular the role of non-Gal human anti-porcine NAb, as well as the investigation of cellular immune responses in the absence of Gal.

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Plasmapheresis, Immunoabsorption
Addition of soluble Gal as blocking agent
Expression of human C regulatory proteins, Addition of soluble C inhibitors
Generation of Gal knockout pigs ⇒ only binding of non-Gal anti-porcine NAb

Figure 4: Prevention of hyperacute rejection. Strategies to prevent HAR include (1) removal of anti-Gal antibodies, (2) blocking of anti-Gal antibodies, (3) prevention of complement activation, and (4) elimination of Gal expression.

3.3.2. Acute Vascular Rejection

When HAR is prevented by the use of organs from Gal-deficient animals, discordant organ xenografts are usually lost due to acute vascular rejection (AVR). This rejection process may begin within 24 hours of reperfusion and generally destroys the graft over a period of days to weeks. Leventhal et al. were the first to describe AVR in a guinea pig-to-rat transplantation model [88]. In general, AVR is well characterized in small animal models, however, the incidence of AVR in pig-to-nonhuman primate studies is not completely obvious due to possible changes induced by immunosuppressive protocols. Histologically, AVR is characterized by EC swelling, ischemia, a diffuse microvascular thrombosis with fibrin deposition, and cellular infiltrates consisting of NK cells, monocytes/macrophages, and neutrophils (Figure 5) [61,88,89]. A key factor in the pathogenesis of AVR is the so-called type II EC activation, i.e. transcriptional activation, characterized by increased transcription of genes encoding cell adhesion molecules (E-selectin/CD62E, vascular cell adhesion molecule-1/VCAM-1/CD106, intercellular adhesion molecule-1/ICAM-1/CD54), as well as cytokines and chemokines (IL-1, IL-6, IL-8, monocyte chemoattractant protein/MCP-1). Furthermore, type II EC activation leads to decreased expression of thrombomodulin and increased expression of pro-thrombotic molecules such as tissue factor [90,91]. These EC changes will promote leukocyte recruitment, platelet aggregation and loss of thromboregulation. The triggering events leading to AVR are still a matter of debate and different mechanisms that may initiate AVR are discussed below.
Introduction

Figure 5: Acute vascular rejection of porcine xenografts. AVR may be triggered by anti-porcine Ab inducing a type II pEC activation resulting in infiltration of human leukocytes (NK cells, monocytes/macrophages, neutrophils) into the graft. The histopathology of AVR is characterized by pEC swelling, diffuse microvascular thrombosis with fibrin deposition, and infiltrates of innate immune cells.

3.3.2.1. Antibodies and Acute Vascular Rejection
Complement-independent type II EC activation induced by the deposition of xenoreactive Ab is believed to be important for the initiation of AVR and there are several findings supporting this hypothesis. For instance, in guinea pig hearts transplanted into rats, AVR developed in complement-depleted recipients in the presence of anti-donor Ab [88]. Furthermore, baboons transplanted with transgenic pig organs which expressed human CD55 and CD59 did not develop AVR following removal of circulating anti-Gal Ab by extracorporeal perfusion [92,93]. These data indicate that anti-Gal Ab may indeed mediate AVR.

3.3.2.2. Cellular Infiltration during Acute Vascular Rejection
Cellular infiltrates observed in xenografts during AVR suggest an important role for the cellular innate immune system. Cytokine-induced activation of pEC by NK cells [94,95], neutrophils [96] and monocytes [97] has been reported earlier. The current model for leukocyte extravasation into inflamed tissues can be divided into four phases: capture, rolling, firm adhesion, and transendothelial migration [98]. In the recruitment of human leukocytes to human EC, each step is a prerequisite for the following step to occur. Many receptor-ligand pairs are involved and only a brief description of the most important molecules will be presented here. L-, E- and P-selectin mediate capture and rolling of leukocytes. L-selectin is expressed on leukocytes, whereas E- and P-selectin are found on EC. The selectins bind to the carbohydrate sialyl-Lewis$^x$ and CD162. The subsequent firm adhesion is carried out by $\beta_1$- and $\beta_2$-integrins. $\beta_1$-integrins include VLA-4 (CD49d/CD29), whereas $\beta_2$-integrins include
LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18). These integrins are expressed on the cell surface of human leukocytes. The endothelial counter receptors for LFA-1 and Mac-1 are ICAM-1, -2 and -3, whereas VLA-4 binds to VCAM-1 on the endothelium. The fourth step, extravasation, is mediated by PECAM-1 (CD31) which is especially abundant in areas of cell-cell contact. During the past years, other molecules involved in transendothelial migration, such as CD99 and junctional adhesion molecules, have been identified. In addition, chemokines produced by EC or the underlying tissue are also important in the recruitment of leukocytes to a site of inflammation or infection [98].

A prerequisite for human leukocytes to infiltrate porcine xenografts is functional compatibility between cell adhesion molecules and chemokines with their respective ligands or receptors. The compatibility between human cell adhesion molecules and their porcine counter-receptors is still incompletely understood, but most ligand-receptor pairs investigated so far seem to be compatible [99-101].

3.3.2.3. Role of Natural Killer Cells in Acute Vascular Rejection

It has long been believed that human NK cells do not play an important role in the rejection of allogeneic transplants [102]. However, recent data suggest that NK cells might play an important role in allogeneic bone marrow and stem cell transplantation (reviewed in [103]). In contrast, the role of NK cells in the rejection of both concordant and discordant xenografts has been demonstrated in various studies (reviewed in [104]). A potential role for NK cells in pig-to-human xenograft rejection was initially suggested by the observation that human NK cells are able to lyse pEC in vitro [94,95,104-108], and by the presence of NK-cell infiltrates in pig organs perfused with human blood ex vivo [109-111]. In vivo, NK-cell infiltrates were detected in rejected organs following pig-to-nonhuman primate xenotransplantation [112-114] and in small animal models of both concordant and discordant xenotransplantation [115-118]. In particular, the studies by Waer suggest an important role for NK cells in the rejection of hamster-to-nude rat xenografts [116-118]. In addition, another recent study demonstrated the involvement of NK cells during xenograft rejection, mediated by antibody-dependent mechanisms, in a mouse model [60,119-121]. The initial interactions between circulating NK cells and pEC are characterized by adhesion and chemotaxis (Figure 6). The adhesion might be enhanced by pEC injury related to ischemia and the techniques involved in organ preservation and surgery.
Introduction

Figure 6: Molecular mechanisms involved in the recruitment of human NK cells to pEC. Following recruitment of NK cells from the circulation, rolling with a predominant role for human CD49d and porcine CD106, and with a minor contribution of human CD62L and CD11a, takes place. The subsequent firm adhesion mainly depends on interactions between CD49d and CD106 and between CD11a and porcine ICAM molecules. The soluble molecules that induce chemotaxis, as well as the receptors involved in transmigration, remain to be determined.

In vitro, the molecular structures involved in early recognition of discordant vascular pEC by human NK cells rely on receptor-ligand interactions that are intact over the species barrier [101,104,110,122]. Birmele et al. reported that approximately 50% of human peripheral blood lymphocytes adhering to pEC were NK cells [123]. This adhesion process was enhanced in the presence of xenoreactive human NAb and by cytokines such as human TNFα, whereas it was reduced by antibodies against CD11a, CD49d, or CD62L on NK cells or CD106 on pEC [101,124]. While some of the receptor-ligand pairs known to be involved in allogeneic adhesion also seem to be intact over the species barrier, others, such as porcine and human CD31 [125], are functionally incompatible. In addition, it was proposed that Gal may directly mediate adhesion of human NK cells [108], whereas more recent studies do not support this notion [126]. Direct cell contact between human NK cells and pEC results in pEC activation leading to further chemokine secretion and upregulation of adhesion receptor expression [94].

The damage caused by NK cells in the xenogeneic graft may occur by at least two different mechanisms. First, NK cells bind directly to pEC and cause damage by the release of lytic components. The cytolytic mechanism in the human-to-pig combination depends rather on the perforin/granzyme B pathway than on the induction of apoptosis [127]. Second, NK cells mediate lysis of pig target cells in the presence of anti-porcine Ab, which interact with CD16 on NK cells resulting in ADCC [55]. Unstimulated human NK cells lyse pEC by
Introduction

ADCC in the presence of either human plasma or serum containing Ab or purified anti-Gal Ab [60,105,107,110,119]. In this process, NK recognition of xenoreactive human IgG Ab bound to Gal and other so far unknown antigens leads to a rapid lysis of porcine target cells. In the absence of xenoreactive antibodies, freshly isolated human PBMC and purified naïve NK cells exhibit only little or no direct cytotoxicity against pEC [95,105,106,119]. Notably, considerable variation in human anti-pEC cytotoxicity exists among individual donors and target cells, possibly reflecting differences in the degree of pig-to-human xenogeneic molecular incompatibilities such as different expression levels of porcine NK-cell ligands [128].

3.3.2.4. Prevention of Acute Vascular Rejection

Strategies to prevent AVR include EC protection by induction of accommodation or inhibition of endothelium activation. Accommodation refers to the acceptance of a graft despite the presence of anti-donor Ab in the blood circulation of the recipient. Such acceptance was already observed in ABO-incompatible allografts after a temporary depletion of anti-donor Ab in the recipient [129]. So far, there is limited evidence that accommodation occurs in pig-to-primate xenografts. The mechanisms leading to accommodation following transient depletion of xenoreactive Ab from the recipient are poorly understood. It may reflect a change in the Ab repertoire, a change in antigen expression on the endothelium, or an acquired resistance by the endothelium to humoral immune injury. Studies in rodents have suggested that acquired resistance of EC may be especially important [130]. Endothelial cells of xenografts that accommodate express protective genes that are not expressed, or expressed to a lesser extent, in rejected grafts [130]. These include genes originally described by their anti-apoptotic properties such as Bcl-XL and Bcl-2. Overexpression of these genes in pEC in vitro blocks activation of the transcription factor NF-κB and, thereby, suppresses induction of pro-inflammatory genes associated with pEC activation [131]. Another protective gene expressed by pEC of xenografts that have undergone accommodation is the stress-responsive gene haeme oxygenase-1 [132], which promotes resistance to NK-cell recognition by removing triggering ligands. Additionally, the expression of NK inhibiting ligands might also prevent AVR [133-135]. Finally, expression or treatment with protective molecules such as heparan sulfate proteoglycan analogs may help to prolong xenograft survival [136].

Given the complexity of the mechanisms regulating the expression of protective genes, it is difficult to speculate which events will trigger their expression in accommodated grafts. An interesting hypothesis is that binding of anti-graft Ab to xenograft pEC could trigger the
expression of these genes. This is supported by the observation that Ab directed against Gal can protect pEC from subsequent lysis by Ab and complement [137,138]. These studies do not define which protective genes are expressed on these pEC. However, they do show that anti-pEC Ab can trigger protective responses in pEC, which mimic the responses observed in pEC of xenografts undergoing accommodation [130]. In accordance with these findings, cross-linking of the Gal epitope with an anti-Gal-directed lectin on pEC upregulates the expression of several heat shock proteins, including one of the protective genes expressed on accommodated xenografts, haeme oxygenase-1.
4. Aim of the Study

The early rejection of xenografts can be divided in two phases: HAR mediated by xenoreactive NAb and complement, and AVR, the mechanisms of which are currently not fully understood. Several lines of evidence suggest that NK cells might play an important role during xenograft rejection. Controversial reports whether Gal is a target molecule for NK cells, and insufficient knowledge of the role of non-Gal anti-porcine NAb, requested further analysis. In order to develop strategies to protect the porcine endothelium from NAb- and NK cell-mediated damage, the following aims were outlined for this thesis:

1. Establishment of an *in vitro* model to investigate the role of Gal and non-Gal epitopes in immune responses mediated by human NAb and NK cells. For this purpose, immortalized pEC were knocked-out for Gal expression *in vitro* and used to study the interactions between NAb, NK cells, and pEC. Functional assays included analysis of NAb binding, complement lysis, NK-mediated ADCC, and direct NK cytotoxicity. In addition, adhesion of human NK cells to pEC was analyzed.

2. Subsequently, primary pEC were obtained from Gal-deficient pigs *ex vivo*, enabling us to confirm the results achieved with the *in vitro*-generated immortalized Gal-deficient pEC. Thereby, we expanded our studies to include higher numbers of human sera with defined blood group and additional porcine cell types such as porcine lymphoblastoid cells and erythrocytes. In addition to the analysis of NAb binding, complement lysis, and ADCC, a detailed analysis of xenoreactive IgG subclasses was performed. Furthermore, the influence of Gal deficiency on the adhesion of various human leukocyte subsets including PBMC, PMN, and purified NK cells was studied.

3. Since the lack of Gal did not provide protection from direct human NK cytotoxicity, the potential of the non-classical MHC class I molecule HLA-E to protect porcine cells from human NK cytotoxicity was further explored. This possibility had already been investigated in previous studies with conflicting results. Therefore, immortalized pEC and porcine lymphoblastoid cells were transfected with different gene constructs of HLA-E and appropriate leader peptides. The influence of peptides on HLA-E surface expression was analyzed. In addition, the effect of external peptide pulsing and IFNy treatment on HLA-E surface expression was investigated. Functional assays
Aim of the Study

included direct NK cytotoxicity mediated by polyclonal NK populations as well as by NK clones with defined NK-cell receptor repertoires.

The materials and methods used in this thesis are described in detail in each paper or manuscript. The experimental work is based on different in vitro models aimed at mimicking the in vivo situation of xenograft rejection as closely as possible. In vitro models have a great potential to study specific, isolated interactions that are integrated parts of complex biological processes such as xenograft rejection, and reduce the use of animals. However, data have to be interpreted with caution since the in vivo situation is more complex, and in vitro data may thus not be directly applicable in the clinical situation.
5. Results

5.1. PART I

Lack of Galα(1,3)Gal expression on porcine endothelial cells prevents complement-induced lysis but not direct xenogeneic NK cytotoxicity

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Running Title: NK cell reactivity to αGal-negative porcine endothelial cells.

Keywords: Human, NK Cells, Transplantation, Transgenic/Knockout, Endothelial Cells.

Abbreviations used in this paper: αGal, galactose-α-1,3-galactose; NAb, natural antibodies; pEC, porcine endothelial cells; HAR, hyperacute rejection; ADCC, antibody-dependent cellular cytotoxicity; α1,3GT, α-1,3-galactosyltransferase; KO, knockout; MFIR, geometric mean fluorescence intensity ratio.
Results – PART I

5.1.1. Abstract

The galactose-α-1,3-galactose (αGal) carbohydrate epitope is expressed on porcine but not human cells and therefore represents a major target for preformed human anti-pig natural antibodies (NAb). Based on results from pig-to-primate animal models, NAb binding to porcine endothelial cells (pEC) will likely induce complement activation, lysis, and hyperacute rejection (HAR) in pig-to-human xenotransplantation. Human natural killer (NK) cells may also contribute to innate immune responses against xenografts, either by direct recognition of activating molecules on target cells or by Fcγ receptor III receptor-mediated xenogeneic antibody-dependent cellular cytotoxicity (ADCC). The present study addressed the question whether the lack of αGal protects pEC from NAb/complement-induced lysis, direct xenogeneic NK lysis, NAb-dependent ADCC, and adhesion of human NK cells under shear stress. Homologous recombination, panning, and limiting dilution cloning were used to generate an αGal-negative porcine endothelial cell line, PED2*3.51. NAb/complement-induced xenogeneic lysis of PED2*3.51 was reduced by an average of 86% compared to the αGal-positive phenotype. PED2*3.51 resisted NK cell-mediated ADCC with a reduction of lysis ranging from 30% to 70%. However, direct xenogeneic lysis of PED2*3.51, mediated either by freshly isolated or IL-2-activated human NK cells or the NK cell line NK92, was not reduced. Furthermore, adhesion of IL-2-activated human NK cells did not rely on αGal expression. In conclusion, removal of αGal leads to a clear reduction in complement-induced lysis and ADCC, but does not resolve adhesion of NK cells and direct anti-porcine NK cytotoxicity indicating that αGal is not a dominant target for direct human NK cytotoxicity against porcine cells.

5.1.2. Introduction

Pig-to-human xenotransplantation may resolve the severe shortage of human organs for transplantation [5]. However, differences in cell surface glycosylation between humans and pigs leading to immunological responses constitute major hurdles for xenotransplantation [59,139]. Vascularized organs transplanted from pigs to nonhuman primates undergo hyperacute rejection (HAR) which is caused by the binding of preformed xenoreactive natural antibodies (NAb) to porcine endothelial cells (pEC) [60,65,71]. Beside mediating complement and endothelial cell activation, non-complement-fixing NAb may play an important role by initiating tissue damage in xenotransplants through antibody-dependent...
cell-mediated cytotoxicity (ADCC) [60,105,119]. The majority of these NAb interacts specifically with the galactose-α-1,3-galactose (αGal) carbohydrate structure, an epitope that is synthesized by the α-1,3-galactosyltransferase (α1,3GT) and abundantly expressed on the cell surface of all mammals except humans, apes, and Old World monkeys. Several approaches may prevent HAR, including the temporary removal of xenoreactive antibodies by plasmapheresis and specific extracorporeal immunoadsorption and the inhibition of complement by the expression of human complement regulatory proteins in transgenic pigs [78]. However, xenotransplantation performed after removal of NAb induces the production of even larger amounts of antibodies against αGal [140]. These induced anti-αGal antibodies contribute to a delayed xenograft rejection, also referred to as acute vascular rejection, which severely limits organ survival notwithstanding the presence of human complement regulatory proteins. Immunosuppressive drugs that efficiently suppress allograft rejection fail to prevent the production of anti-αGal antibodies [141]. Alternatively, the administration of soluble αGal glycoconjugates reduces the levels of circulating anti-αGal antibodies and the number of cells that secrete anti-αGal antibodies. Some studies report a diminished antibody production for several weeks after such treatments [142,143]. However, up to date, no therapy has prevented the return of xenoreactive antibodies in pig-to-primate models. Therefore, several attempts have been made to modify αGal expression on porcine cells. Competitive inhibition of the α1,3GT in α-1,2-fucosyltransferase and N-acetylglucosaminyltransferase-III transgenic pigs or the intracellular expression of single chain Fv antibodies against α1,3GT all have resulted in only partial reduction in epitope numbers and failed to substantially prolong graft survival in primates [144-146]. The yet most promising strategy to overcome xenograft rejection concentrates on the elimination of the αGal epitope by knocking-out the α1,3GT locus in pigs. Whereas for many years embryonic stem cell technology essential for the generation of gene knockout (KO) animals was restricted to mice, the advent of new cloning and nuclear transfer technologies [147,148] led to the recent production of several pigs lacking one allele of the α1,3GT locus [81,82] or being completely knocked-out for this enzyme [83]. Currently, preclinical pig-to-nonhuman primate trials are being carried out to test whether organs derived from αGal KO animals confer permanent and complete protection from antibody-mediated rejection. The availability of αGal KO cells, however, should also enable a more systematic and rapid investigation of other antigenic targets involved in immune rejection of porcine xenografts.
Antibody-independent mechanisms also contribute to the high susceptibility of porcine cells to damage inflicted by the human immune system. The finding that human NK cells are able to lyse pEC in vitro suggested that they may participate in the direct rejection of pig-to-human xenografts [104,122]. This hypothesis was supported by NK cell infiltrates found in pig organs perfused with human blood ex vivo [110,111] as well as in pig-to-nonhuman primate xenografts [114] and in small animal models of both concordant and discordant xenotransplantation [115,118]. In agreement with these observations, several adhesion receptors are functional across the human-porcine species barrier, providing the basic requirements for human leukocytes to interact with pEC [101,149].

The function of NK cells is regulated by a concert of activating and inhibitory signals [19,150]. After the binding of NK cells to potential target cells, several receptor-ligand interactions take place that determine the fate of the latter. Recognition of self-MHC class I molecules by inhibitory NK receptors represents a dominant negative signal for NK cytotoxicity [38]. However, in the absence of sufficient inhibitory signals, ligation of activating receptors induces NK cytotoxicity. The reported susceptibility of pEC to human NK cytotoxicity may be explained by the failure of human NK inhibitory receptors to recognize xenogeneic porcine MHC class I molecules. This hypothesis is supported by the fact that expression of human MHC class I molecules on pEC provides partial protection from human NK cytotoxicity [133,134]. The nature of the potential activating NK ligands on porcine cells is largely unknown at present, however, some groups reported a direct recognition of αGal by human NK cells [108,151,152]. Apart from this direct NK cytotoxicity, the expression of Fcγ receptor III (CD16) on human NK cells mediates ADCC against porcine target cells [60,105,119]. In this process, NK recognition of xenoreactive human IgG antibodies bound to αGal and other so far unknown epitopes on pEC leads to a rapid lysis of these pig cells.

The aim of the present study was to examine whether the lack of αGal protects pEC from human NAb/complement-induced lysis, direct human NK-mediated xenogeneic lysis, ADCC, and adhesion of human NK cells under physiological shear stress. We demonstrate that the lack of αGal protects pEC from complement-induced cytotoxicity, from ADCC but not from adhesion and direct lysis mediated by human NK cells.
5.1.3. Materials and Methods

Cells
The SV40-immortalized aortic pEC line PEDSV.15 was established and characterized in our laboratory [153] and the αGal-negative cell line PED2*3.51 was generated as described below. Both porcine cell lines were cultured in DMEM (Invitrogen AG, Basel, Switzerland) supplemented with 10% FCS (PAA Laboratories, Luzern, Switzerland), 1 mM sodium pyruvate, 2 mM L-glutamine, non-essential amino acids (100x), and 20 mM HEPES (all Invitrogen), PED2*3.51 were repeatedly exposed to 400 μg/ml of G418 (Life Technologies, Gaithersburg, MD). For induction of VCAM-1 expression pEC were stimulated with porcine IFNγ (Innogenetics, Zwijndrecht, Belgium) for 24 h (100 ng/ml). The isolation of human PBMC, the purification of NK cells, and the generation of polyclonal NK populations have been described previously [134]. After isolation, NK cells were either used directly or activated by culture in RPMI medium (Invitrogen AG, Basel, Switzerland) containing 100 U/ml of human IL-2 (Chiron, Palo Alto, CA). The human clonal NK cell line NK92 [154] was obtained from the American Type Culture Collection (Rockville, MD) and maintained in MyeloCult™ H5100 medium (StemCell Technologies, Vancouver, Canada) containing 500 U/ml of human IL-2.

Targeting vector construction
A genomic library was constructed from DNA of the same d/d haplotype partially inbred miniature swine line used for isolation of the pEC line PEDSV.15 [153,155]. DNA from overlapping genomic clones was assembled into a contiguous 23 kbp segment of the α1,3GT gene locus (GGTA1), beginning downstream of exon 7 and continuing 10.5 kbp beyond exon 9. In-frame termination codons were introduced near the beginning of exon 9, which should result in translational termination N-terminal to the catalytic domain of the enzyme. This mutated segment was cloned into a pOCUS II-based vector containing a G418 resistance gene under the control of the murine phosphoglycerol kinase (PGK) promoter (Novagen, San Diego, CA). The resulting construct, pGallaway, was linearized at a unique Xho I site near exon 8 for use as an insertion-type targeting vector.

Gene targeted immortalized endothelial cell line isolation
PEDSV.15 cells were electroporated at 260 V and 960 μFD in 0.8 ml of HEPES buffered saline containing 0.5 pmol/ml of Xho I-linearized pGallaway DNA assuring isogenicity of vector and target locus. Thirteen pools of transfected PEDSV.15 cells were selected for two
weeks in 400 μg/ml G418. Based on analysis of smaller pools, these thirteen pools were expected to contain approximately 7500 G418-resistant clones, 1% of which (75) would be targeted at the α1,3GT gene locus. In order to select for spontaneous null mutant cells arising from heterozygously targeted PEDSV.15 cells, each pool was subjected to multiple rounds of lysis with affinity-purified baboon antibodies against αGal and complement (Kolber-Simonds et al., submitted). Following five rounds of selection, one of the 13 pools gave rise to a cell line that was totally resistant to subsequent selection with antibody and complement. This line was designated PED2*3. RNA from this line was analyzed by RT-PCR using a forward primer upstream of the 5' end of the targeting vector and a downstream primer within exon 9 downstream of the introduced termination codons. Only RNA containing the artificial stop codons was readily detected in the PED2*3 line. Further limiting dilution cloning by standard methods resulted in the stable αGal-negative cell line PED2*3.51 as demonstrated by flow cytometry.

**Human serum samples**

Human sera were obtained from healthy adult volunteers. Decomplementation by heat-inactivation was carried out at 56°C for 30 min. Samples were stored at 4°C for short periods or aliquoted and stored at -20°C. Binding of human immunoglobulins to pEC was analyzed by incubation with different dilutions of human serum for 30 min at 4°C and subsequent flow cytometry as described below.

**Affinity purification of αGal-reactive NAb and ELISA**

Affinity purification of αGal-reactive NAb and αGal ELISA were performed as described elsewhere [156]. In brief, a 1/1 mixture of synthetic αGal (Bdi) and Galα(1-3)Galβ(1-4)Glc (Tri6) in the form of flexible, hydrophilic polyacrylamide conjugates covalently coupled to Fast-Flow Sepharose was used as immunoabsorbent. The purity of the anti-αGal NAb-depleted serum and the eluated human IgM and IgG antibodies specific for αGal were tested by ELISA using plates coated with Bdi and Tri6. Immunoabsorbsents and coating antigens were kindly provided by N. Bovin (Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia).

**Cell staining and flow cytometry**

Surface expression of αGal molecules on pEC was analyzed on a FACScan (Becton Dickinson, Basel, Switzerland) by direct and indirect immunofluorescence using either the
FITC-labelled isolectin B4 from Bandeiraea simplicifolia [157] (BS-IB4, Sigma, Buchs, Switzerland) or the mouse anti-αGal IgM mAb M86 [158] (Alexis Corporation, Lausen, Switzerland). For the latter a secondary FITC-conjugated goat anti-mouse Ig antibody (Boehringer Mannheim, Rotkreuz, Switzerland) was used. Porcine EC cells were resuspended after trypsinization (0.25% trypsin, Life Technologies, Switzerland) at 5x10^5 cells per tube in staining buffer (PBS, 0.1% BSA) and incubated for 30 min at 4°C with saturating concentrations of IB4 lectin or the mAb M86. For the expression of adhesion molecules on pEC the following mAb (all IgG1) were used: 3F4 (anti-VCAM-1, kindly provided by Alexion, New Haven, CT), 1.2B6 (anti-E-selectin, Serotec, Kidlington-Oxford, UK) and 12C5 (anti-P-selectin, kindly provided by D. Haskard, Hammersmith Hospital, London, UK). Binding of human immunoglobulins to pEC was analyzed by incubating pEC with different dilutions of human serum, anti-αGal NAb-depleted serum or purified anti-αGal NAb for 30 min at 4°C. For detection a secondary FITC-conjugated goat anti-human IgM (Sigma, Buchs, Switzerland) or a mouse anti-human IgG (Zymed, Basel, Switzerland) antibody were used. Phenotypic analysis of NK cells was carried out by direct immunofluorescence using FITC-UCHT1 (anti-CD3), PE-B73.1 (anti-CD16), and PE-B159 (anti-CD56) mAb (all from Pharmingen, San Diego, CA). Irrelevant, isotype-matched mAb or FACS buffer alone (for BS-IB4) were used as controls and propidium iodide gating to exclude dead cells in all experiments. To compare the levels of surface expression the geometric mean fluorescence intensity ratios (MFIR) were calculated by dividing the mean fluorescence intensity of staining with the mAb of interest with the mean fluorescence intensity of the control mAb.

Cytotoxicity assays
The cytotoxic activity of polyclonal human NK populations and the NK line NK92 was tested in 4 h ^51^Cr-release assays in serum-free AIM-V medium as described previously [105]. Briefly, target cells were added to triplicate samples of serial twofold dilutions of freshly isolated or IL-2-activated NK cells in round-bottom 96-well plates at E:T ratios ranging from 40:1 to 5:1. To determine the effects of complement-induced xenogeneic lysis and ADCC, human serum containing NAb and complement, decomplemented human serum, decomplemented anti-αGal NAb-depleted serum, or purified αGal-reactive NAb were added to ^51^Cr-labelled target cells. Concentrations ranging from 25% to 3% serum or 50μg/ml to 6μg/ml NAb were used in the absence or presence of freshly isolated NK cells at an E:T ratio of 40:1. After incubation for 4 h at 37°C the release of radioactive ^51^Cr was analyzed on a gamma counter and the percentage of direct specific lysis was calculated. For ADCC the
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percentage of relative lysis was calculated as the difference in NK cytotoxicity against pEC in the presence or absence of anti-porcine NAb.

Adhesion assay

Adhesion of human NK cells on pEC was analyzed under shear stress as previously described [124]. Briefly, pEC were grown to confluency in 30 mm culture Petri dishes and cultured for 24 h in the presence or absence of 100 ng/ml porcine IFNγ. The resulting monolayers were washed and overlayed with 10^6 purified IL-2-activated NK cells in a volume of 100 μl. The dishes were then rotated at 64 rpm in a prewarmed (37°C) horizontal shaker-incubator (Infors AG, Bottmingen, Switzerland). After 10 min the assay was stopped by rapidly placing the dishes on ice and by prefixing the cells for 2 min with 1% paraformaldehyde in PBS. The monolayers were then gently washed, fixed, and finally protected with a glass coverslip. For quantification, four fields of 0.16 mm^2 were defined at a distance of 0.6 cm from the center of rotation and the number of adhering NK cells was counted by light microscopy.

5.1.4. Results

Generation of the αGal KO porcine endothelial cell line PED2*3.51

A combined approach of gene targeting and panning was used to generate the αGal-negative PED2*3 line. RNA from the PED2*3 line was analyzed by RT-PCR and only RNA containing the artificial stop codons was detected (data not shown). This apparent lack of transcription of the wild type α1,3GT genes in the PED2*3 line indicated that one allele was successfully targeted and the second allele silenced. Explanations could include a chromosome loss, promoter deletion, gene rearrangement, splicing mutation, epigenetic downregulation or other unknown mechanisms. The fast growing and stable PED2*3.51 subline was established by limiting dilution cloning. The lack of αGal surface expression on PED2*3.51 was demonstrated by staining with the specific mAb M86 [158]. Flow cytometry revealed an MFIR of 20.4 on the paternal PEDSV.15 line and 1.1 on PED2*3.51 (Fig. 1a). In addition, the BS-IB4 lectin stained PEDSV.15 and PED2*3.51 with an MFIR of 541.1 and 3.8, respectively (Fig. 1b). A possible explanation for the faint positive staining of PED2*3.51 with BS-IB4 lies within the cross-reactivity of this lectin to other oligosaccharides expressed on porcine cells including Galα1,6Gal [159]. As a negative control for αGal expression primary human aortic endothelial cells were found to be completely negative for staining with M86 (Fig. 1c) or BS-IB4 (Fig. 1d). Repetitive analysis of M86 binding throughout the study
confirmed that the PED2*3.51 line does not express an active α1,3GT. Additional phenotyping of PED2*3.51 cells with antibodies directed against CD14, CD31, CD44, CD49e, CD62E, CD62P, CD86, and SLA class I d/d revealed an expression pattern (data not shown) similar as for the paternal PEDSV.15 line [153].

**FIGURE 1. Absence of αGal on PED2*3.51.** Cell surface expression of αGal on PEDSV.15 (black histogram) and PED2*3.51 (grey histogram) cells was analyzed using the αGal-specific mAb M86 (a) or the lectin BS-IB4 (b) in flow cytometry. As negative control, staining of human aortic endothelial cells with M86 (c) or BS-IB4 (d) is shown (white histograms, solid line). Data are representative of three (a), seven (b), and two (c and d) independent experiments, respectively. Dashed lines depict background staining using an isotype-matched control antibody (for M86) or FACS buffer alone (for BS-IB4) on PEDSV.15 cells which was representative for PED2*3.51 background staining.


d
t

**Binding of human NAb to porcine endothelium is strongly reduced by the lack of αGal**

To determine the influence of αGal expression on the binding of NAb, pEC were incubated with different concentrations of human serum, anti-αGal-depleted serum, and purified anti-αGal NAb. As detected by flow cytometry the binding of human IgM and IgG NAb in non-absorbed serum was about ten times lower on αGal KO PED2*3.51 cells than on the paternal line PEDSV.15 (Fig. 2a and d). In contrast, when anti-αGal-depleted human serum was used, binding of IgM and IgG molecules was similar to both cell lines (Fig. 2b and e). Moreover, the level of binding observed in the latter experiments was similar to the level of binding of non-absorbed serum to the αGal KO PED2*3.51 cells. These results suggested, firstly, that the higher amount of NAb binding to PEDSV.15 using non-absorbed serum is due to αGal-specific antibodies and, secondly, that the remaining antibodies binding to PED2*3.51 are of
non-\(\alpha\)Gal-specific nature. Flow cytometry after incubation of PED2*3.51 cells with affinity-purified anti-\(\alpha\)Gal antibodies revealed virtually no binding thus confirming our previous negative staining using the anti-\(\alpha\)Gal mAb M86 (Fig. 2c and f). However, there was a weak staining with IgG at very high antibody concentrations (\( \geq 25\mu g/ml \)) probably due to small amounts of contaminating non-\(\alpha\)Gal-specific IgG NAb or unspecific binding due to supersaturating conditions.

**FIGURE 2.** Binding of human NAb to porcine endothelium is strongly reduced by the lack of \(\alpha\)Gal. Shown is the IgM (a-c) and IgG (d-f) binding of non-depleted human serum (a and d), anti-\(\alpha\)Gal-depleted serum (b and e), and purified anti-\(\alpha\)Gal NAb (c and f) on PEDSV.15 (filled squares) and PED2*3.51 (empty squares). Depicted are MFIR. Data shown were obtained with serum from donor KH and are representative of three independent experiments using sera from three different donors.

**Strong reduction of xenogeneic NAb/complement lysis of PED2*3.51**

It is well known that binding of human xenoreactive NAb to pEC activates the complement cascade resulting in lysis of the cells. These data were confirmed in our study by the incubation of PEDSV.15 cells with human serum. A strong dose-dependent lysis of
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PEDSV.15 cells was observed with small amounts of serum (6%) derived from the donor MS inducing a lysis over 40% (Fig. 3). In contrast, αGal KO PED2*3.51 cells were highly resistant to NAb/complement-mediated lysis even in the presence of up to 25% of MS serum. The degree of complement-induced xenogeneic lysis of PED2*3.51 and PEDSV.15 was donor-dependent probably due to different levels and reactivities of the xenoreactive NAb present in the respective sera. However, when sera from five different donors were analyzed, lysis of PED2*3.51 was reduced by an average of 86% as compared to PEDSV.15 as shown in Table 1. A similar effect was seen with human plasma (data not shown).

![Graph showing lysis of PEDSV.15 and PED2*3.51 cells](MS_Serum.png)

**FIGURE 3. Reduction of xenogeneic NAb/complement-mediated lysis by lack of αGal on PED2*3.51 cells.** Lysis of PEDSV.15 (squares) and PED2*3.51 cells (triangles) was analyzed by the addition of NAb/complement-containing serum from donor MS. Data are shown as percentage of specific lysis and are representative of two independent experiments.

*Direct cytotoxicity of human NK cells and NK92 is independent of αGal expression*

Porcine EC are susceptible to human NK cytotoxicity and there have been controversial reports about a direct involvement of αGal as a target molecule. To test the role of αGal in xenogeneic NK cytotoxicity, PEDSV.15 and αGal KO PED2*3.51 cells were used as targets for freshly isolated or IL-2-activated human NK cells in standard 4 h 51Cr-release assays. Confirming previous reports, the specific lysis of pEC mediated by fresh NK cells was less than 20%, whereas the killing by IL-2-activated NK cells ranged between 50% and 100% at the highest E:T ratios. Both target cell lines, PEDSV.15 and αGal KO PED2*3.51, were equally susceptible to human NK cytotoxicity using NK effector cells from nine different human donors. Shown is the lysis by SH NK cells being representative for all nine NK lines (Fig. 4a). In addition, the human NK line NK92 also exhibited the same level of cytotoxicity against both targets (Fig. 4b). These findings indicate that αGal is either not directly recognized by human NK cells or does not play a dominant role in triggering xenogeneic cytotoxicity in NK cells.
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FIGURE 4. Direct NK cytotoxicity is not reduced by a lack of αGal expression. The cytotoxic activity of NK cells purified from donor SH (a) and the NK line NK92 (b) against PEDSV.15 (squares) and PED2*3.51 (triangles) cells is shown. NK cells were either freshly isolated (filled symbols) or activated with IL-2 (open symbols). Cytotoxicity was determined at four different E:T ratios (40:1 to 5:1) in standard 4 h ⁵¹Cr-release assays and expressed as percentage of specific lysis. Data are representative of three (a) and four (b) independent experiments, respectively.

Reduction of xenogeneic ADCC against αGal KO PED2*3.51

As previously shown in vitro, NAb-dependent ADCC of pEC mediated by human NK cells is a very efficient mechanism of lysis and may also represent an important in vivo mechanism leading to graft rejection. Here, we investigated whether the lack of αGal on the surface of pEC protects against human NK cell-mediated ADCC. Therefore, freshly isolated NK cells were added to PEDSV.15 and αGal KO PED2*3.51 target cells at an E:T ratio of 40:1 in the presence of decomplemented human serum, anti-αGal-depleted serum and purified αGal-NAb. In general, the level of xenogeneic ADCC correlated with the amount of anti-pig NAb present in the serum of the three donors tested. As shown in Figure 5, ADCC of PED2*3.51 cells was strongly reduced as compared to PEDSV.15. The level of ADCC of PED2*3.51 was similar when using non-depleted or anti-αGal-depleted serum, suggesting that the remaining non-αGal-specific NAb are responsible for this lysis. However, using anti-αGal-depleted serum the level of ADCC was still clearly higher against PEDSV.15 than against PED2*3.51 cells possibly due to small amounts of remaining anti-αGal antibodies after depletion as detected by ELISA (data not shown). When using purified anti-αGal NAb, ADCC against PED2*3.51 cells was almost completely abrogated. These findings indicate an important role
for αGal-specific antibodies in xenogeneic ADCC whereas non-αGal-specific antibodies seem to be of minor importance.

**FIGURE 5. Reduction of xenogeneic ADCC by a lack of αGal expression.** Effects of ADCC were measured by the addition of freshly isolated KHNK cells to PEDSV.15 (black bar) and PED2*3.51 (white bar) cells at an E:T ratio of 40:1 in combination with decomplemented KH serum, anti-αGal NAb-depleted serum, or purified anti-αGal NAb. Serum concentrations ranged from 25% to 6% and anti-αGal concentrations from 50 ng/ml to 12 μg/ml. The percentage of ADCC is the difference between the lysis induced by serum/NAb plus NK cells and the lysis induced by NK cells alone. Data shown are representative of two independent experiments.

**Adhesion of human NK cells to PED2*3.51 cells under physiological shear stress is not dependent on αGal expression**

Before target cell lysis *in vivo*, NK cells have to adhere to pEC, a process that includes rolling and, subsequently, firm adhesion. Since αGal expression was reported to be involved in adhesion of human NK cells to pEC in an earlier report [108], this question was also addressed in the present study. Adhesion of IL-2-activated human NK cells derived from different healthy donors was almost absent on PED2*3.51 cells. However, VCAM-1 which is crucial for the adhesion of human NK cells to pEC [124] was only detected on PEDSV.15 but not on PED2*3.51 unless stimulated with porcine IFNγ (Fig. 6a and b). When IFNγ-treated PED2*3.51 cells with upregulated VCAM-1 expression were used in dynamic adhesion assays, human NK cells adhered to PED2*3.51 cells (Fig. 6d). However, the expression pattern of VCAM-1 on IFNγ-stimulated PED2*3.51 cells was heterogeneous compared to PEDSV.15, a likely explanation for the lower numbers of adherent cells. Of note, the expression of αGal on PED2*3.51 cells was not affected by treatment with porcine IFNγ remaining completely negative (Fig. 6c). We therefore conclude that αGal is not crucial for the adhesion of human NK cells to porcine endothelial cells.
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FIGURE 6. Adhesion of human NK cells on pEC does not depend on αGal expression. Expression of VCAM-1 (a and b) and αGal (c) was analyzed on untreated (empty histograms, solid line) and porcine IFNy-stimulated (grey histograms) PEDSV.15 and PED2*3.51 cells using the mAb 3F4 and M86, respectively. Dashed lines depict background staining using an isotype-matched control antibody. Data are representative of three independent experiments. (d) Absolute numbers ± SEM of adherent IL-2-activated KH NK cells on pEC. Bars depict adhesion to untreated (black) and IFNy-stimulated (white) PEDSV.15 and PED2*3.51 cells. Data are representative of three independent experiments.

5.1.5. Discussion

The vigorous antibody response to porcine carbohydrate antigens, mainly to the αGal epitope, has to be conquered before significant progress in clinical xenotransplantation can be made. It has been estimated that anti-αGal NAb comprise about 80% of all xenoreactive NAb in human serum [160], 1.0% to 2.4% of total IgG and 1.0% to 8.0% of total IgM [63,69,70]. Since a key role of anti-αGal antibodies in xenograft rejection has been clearly demonstrated, great expectations are associated with the generation of pig organ donors that do not express αGal. First results of pig-to-baboon kidney or heart transplantation using organs of α1,3GT KO pigs suggest that HAR can be overcome even without extracorporeal immunoabsorption.
and/or treatment with cobra venom factor [161,162]. Consequently, the contribution of NAb with alternative non-αGal specificities and the study of subsequent cellular immune responses to xenografts such as acute vascular rejection are of growing importance.

In this study, we established an αGal-negative porcine endothelial cell line, PED2*3.51, to investigate the influence of αGal on the cytotoxicity mediated by xenoreactive human NAb and NK cells. Our approach included targeting of one allele of the α1,3GT gene and a subsequent panning procedure. Recent reports demonstrated that beside α1,3GT another enzyme, isoglobotriaosylceramide-3 (iGb3) synthase, is able to synthesize αGal in rats [163,164]. Theoretically, a similar enzyme may also exist in pigs. However, since we could not detect any binding on PED2*3.51 cells using either the monoclonal anti-αGal antibody M86 or purified anti-αGal NAb we conclude that no other functional enzymes synthesizing αGal are present. There is only one other description of porcine cells, primary fetal fibroblasts, lacking the α1,3GT gene [165]. In extensive FACS analysis the α1,3GT KO fetal pig fibroblasts were negative for staining with BS-IB4 and several different anti-αGal antibodies. However, surprisingly the cells still gave a positive FACS signal using two other mouse anti-αGal mAb, including the mAb that was used for the panning procedure to eliminate αGal expression. Therefore, it remains to be investigated whether this positive signal was due to a lack of antibody specificity, technical problems such as supersaturating conditions or whether pig cells express αGal residues in the absence of α1,3GT which are only detected by a subset of antibodies.

The removal of αGal led to a strong reduction of complement-induced xenogeneic lysis. Moreover, NK cell-mediated xenogeneic ADCC was reduced but not totally absent when αGal-negative pEC were used as targets. In line with these results, binding of human serum IgM and IgG molecules was strongly reduced but not absent on αGal-negative pEC. This strong reduction of binding was observed for all donors tested, however, the magnitude of reduction varied considerably. The remaining NAb binding on αGal-negative pEC, most probably due to non-αGal-reactive antibodies including those reactive with N-glycosylneuraminic acid [166], represent an additional hurdle to xenotransplantation [160]. Moreover, it has been suggested that the elimination of αGal from porcine cells could lead to the generation of new epitopes such as terminal β-galactosyl residues or the N-acetyllactosamin disaccharide which are recognized by preformed human xenoreactive antibodies [167]. Surprisingly, the levels of ADCC of αGal-positive PEDSV.15 and αGal KO PED2*3.51 cells using anti-αGal-depleted serum differed more than expected. We therefore
assume that non-αGal antigens recognized by NAb inducing ADCC are possibly expressed to a lesser extent on αGal KO PED2*3.51 as compared to PEDSV.15 cells. A possible explanation for this difference might be the extensive panning procedure using anti-αGal antibodies affinity-purified from baboon serum which might have led to the elimination of other non-αGal antigens on the αGal KO line by contaminating non-αGal antibodies. In contrast to ADCC, non-αGal-specific NAb were not very effective in inducing xenogeneic complement lysis. The amount of bound non-αGal antibody required to induce complement lysis was rather high compared to ADCC. This finding correlates with an earlier study indicating that the threshold for NAb/complement-induced cytotoxicity may be higher than the threshold to induce ADCC [168].

An important but still controversial question is whether carbohydrate determinants, in addition to proteins, can act as target molecules for NK cells. While αGal is likely to be the most important carbohydrate in xenotransplantation, other carbohydrates could also have an impact on NK cell biology as in response to tumors or viral infections. So far, only little is known about ligands for lectin-like receptors on NK cells and their effects on NK cell functions [169]. Whereas direct recognition of αGal by human NK cells leading to adhesion and cytotoxicity was reported by earlier studies [108,151,152,170] our results suggest that elimination of αGal on pEC does not resolve NK adhesion and direct anti-porcine NK cytotoxicity. Porcine EC lacking αGal were equally lysed by human NK cells as pEC expressing high αGal levels, and the adhesion of NK cells to PED2*3.51 expressing the important adhesion molecule VCAM-1 [124], but still lacking αGal, was detected on these cells. Therefore, we conclude that there is no direct correlation between the absence of αGal and VCAM-1 and that αGal does not play a crucial role in the adhesion process. In line with our findings, naïve human NK cells adhered, activated and lysed pEC independently of αGal [126], and no differences regarding NK adhesion and lysis were observed upon introduction of αGal in human aortic endothelial cells [171]. In contrast, using α1,3GT-transfected COS-7 cells, soluble carbohydrates and F(ab')2 of xenoreactive NAb, Inverardi et al. showed a role for αGal in antibody-independent NK adhesion to and destruction of pEC [108]. In addition, a partial decrease in NK cell-mediated direct cytotoxicity was reported when αGal epitopes were blocked by the mAb M86, the GSI lectin or after treatment of pEC with α-galactosidase [152]. Nevertheless, the level of NK killing was very low and no ADCC was demonstrated in this study. α-galactosidase treatment of pEC had not reduced NK killing in an earlier study [167]. Finally, genetical modification of the αGal epitope by expressing α-1,2-
fucosyltransferase also resulted in a decreased susceptibility to human NK lysis against porcine endothelial cells [151] and fibroblasts [170]. The apparent lack of congruence in analyzing the studies on the role of αGal in xenogeneic NK responses is hard to explain. Differences in the experimental protocols including the grade of activation of the NK effector cells and the source of porcine target cells may in part be responsible. The present study is the first to test NK cytotoxicity against pEC in the absence of α1,3GT gene expression and without the need to treat the cells with neither α-galactosidase nor blocking reagents. We therefore conclude that αGal does not act as a dominant cytotoxicity-inducing NK target molecule. Our results do not exclude a role for αGal in NK recognition, it may be necessary but not sufficient to interfere with αGal in order to overcome xenogeneic NK responses.

In summary, the results presented here show that a modification of glycosyl epitopes such as the lack of αGal expression on porcine cells provides an effective protection from αGal antibody-mediated rejection including complement lysis and ADCC. Non-αGal antibody-mediated damage of and direct NK cytotoxicity against porcine targets, however, is not resolved by knocking-out the α1,3GT gene. These data extend the current knowledge of the mechanisms leading to xenograft rejection, may prompt further preventive and therapeutic strategies and underline that only a combination of approaches addressing both antibody- and cell-mediated mechanisms of xenograft rejection may facilitate clinical pig-to-human xenotransplantation.

5.1.6. Acknowledgments

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5.2. PART II

Endothelial cells derived from pigs lacking Galα(1,3)Gal: no reduction of human leukocyte adhesion and NK cytotoxicity

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Keywords: NK Cells, Endothelial Cells, Xenotransplantation, Transgenic/Knockout.

Abbreviations used in this paper: α1,3GT, α(1,3)galactosyltransferase; BS-IB4, Bandeiraea simplicifolia isoelectin B4; E:T ratio, effector:target ratio; Gal, galactose-α(1,3)galactose; IL-2, interleukin-2; mAb, monoclonal antibody; MFIR, geometric mean fluorescence intensity ratio; NK, natural killer; PAEC, porcine aortic endothelial cells; PBMC, peripheral blood mononuclear cells; PI, propidium iodide; PMN, polymorphonuclear neutrophils; TNFα, tumor-necrosis factor-α.
Results – PART II

5.2.1. Abstract

Background. The expression of galactose-α(1,3)galactose (Gal) on porcine cells represents a major barrier to xenotransplantation. The generation of Gal− pigs to overcome this barrier redirected the focus of research to other rejection mechanisms including cellular immunity. The present in vitro study investigated (1) the adhesive interactions between human leukocyte subsets and primary endothelial cells derived from inbred Gal− and Gal+/+ pigs, and (2) the susceptibility of such Gal− porcine endothelial cells to human natural killer (NK) cytotoxicity.

Methods. Primary porcine aortic endothelial cells (PAEC) were isolated from Gal− (PAEC-Gal−) and Gal+/+ (PAEC-Gal+/+) pigs. Human peripheral blood mononuclear cells (PBMC), polymorphonuclear neutrophils (PMN), and NK cells were isolated from healthy volunteers and tested in functional adhesion and cytotoxicity assays.

Results. Adhesion of human PBMC, PMN, or purified NK cells on PAEC-Gal− cells was not different from that on PAEC-Gal+/+ cells. Comparing the different leukocyte subsets of PBMC a preferential adhesion of NK and B cells on both PAEC-Gal− and PAEC-Gal+/+ was detected. Tumor-necrosis factor-α (TNFα)-stimulation of PAEC-Gal− and PAEC-Gal+/+ induced an upregulation of CD62E and CD106 expression and increased cellular adhesion, in particular of PMN. The lack of Gal expression on PAEC-Gal− cells did not prevent xenogeneic human NK cytotoxicity mediated by freshly isolated or interleukin-2 (IL-2)-activated NK cells.

Conclusions. Neither human leukocyte adhesion nor xenogeneic NK cytotoxicity against porcine endothelial cells are impaired by the lack of Gal indicating that Gal is not a dominant target of cellular rejection.

5.2.2. Introduction

The recent development of α(1,3)galactosyltransferase (α1,3GT) knockout pigs by means of nuclear transfer technologies represents a new era in xenotransplantation research [5,60,83]. Overcoming the barrier of anti-galactose-α(1,3)galactose (Gal)-mediated rejection reveals other immunological hurdles including those being mediated by the cellular immune system.
These rejection mechanisms are characterized by recognition, infiltration, and damage of grafts by various leukocyte subsets. The finding that human natural killer (NK) cells adhere to and lyse porcine cells in vitro suggests a role in the rejection of pig-to-human xenografts [104,122]. This hypothesis is supported by NK cell infiltrates found in pig organs perfused with human blood ex vivo [111] as well as in pig-to-nonhuman primate xenografts [114] and in small animal models of xenotransplantation. Recently, the importance of NK cells in vascular xenograft rejection was clearly demonstrated in a mouse model [120]. The function of NK cells is regulated by a concert of inhibitory and activating signals [19] and the reported susceptibility of porcine cells to human NK cytotoxicity may be explained by a failure of human NK inhibitory receptors to recognize xenogeneic porcine MHC class I molecules. In contrast, the nature of the potential activating NK ligands on porcine cells is largely unknown at present. Whereas some groups reported a direct recognition of Gal by human NK cells [108,151,152,170], we and others did not observe any differences in NK cytotoxicity comparing Gal-positive and -negative porcine cells [126,171,172]. In our previous study we used an immortalized Gal-negative porcine endothelial cell line that was generated in vitro by gene targeting, panning and limiting dilution cloning. Moreover, the significance of the adhesion data obtained with this Gal-negative cell line was compromised by a lack of CD106 expression, a molecule important for adhesion of peripheral blood mononuclear cells (PBMC) to endothelial cells [172]. Therefore, the aim of the present study was to confirm our previous findings using primary porcine aortic endothelial cells (PAEC) derived from Gal" pigs with no obvious phenotypic differences compared to Gal" PAEC. Expanding the previous analysis, the question of whether the lack of Gal expression on primary PAEC impairs the binding of other human leukocyte subsets including PBMC and polymorphonuclear neutrophils (PMN) was addressed in a detailed analysis of adhesion under shear stress.

5.2.3. Materials and Methods

Cells

α1,3GT null pigs were generated by gene targeting, panning, and nuclear transfer technologies [84]. The primary aortic endothelial cell lines PAEC-Gal" and PAEC-Gal" were isolated from normal and α1,3GT null pigs following standard procedures [105] and cultured in DMEM (Invitrogen AG, Basel, Switzerland) supplemented with 10% FCS (PAA Laboratories, Luzern, Switzerland), 1 mM sodium pyruvate, 2 mM L-glutamine, non-essential amino acids (100x), essential amino acids (50x), and 20 mM HEPES (all Invitrogen). The
isolation of human PBMC, PMN, and NK cells, and the generation of polyclonal NK populations activated by culture in AIM-V medium (Invitrogen AG, Basel, Switzerland) containing 300 U/ml of human IL-2 (Chiron, Palo Alto, CA) have been described previously [105,173]. The human clonal NK cell line NK92 [154] was obtained from the American Type Culture Collection (Rockville, MD) and maintained in MyeloCult™ H5100 medium (StemCell Technologies, Vancouver, Canada) containing 500 U/ml of human IL-2.

**Cell staining and flow cytometry**

Surface expression of Gal molecules on PAEC was analyzed by direct and indirect immunofluorescence using either the FITC-labelled isoelectin B4 from Bandeiraea simplicifolia (BS-IB4, Sigma, Buchs, Switzerland [157]) or the mouse anti-Gal IgM monoclonal antibody (mAb) M86 (Alexis Corporation, Lausen, Switzerland [158]) as previously described [172]. The expression of adhesion molecules on PAEC was tested using the following unlabelled mAb (all IgG1): 3F4 (anti-VCAM-1/CD106, kindly provided by Alexion, New Haven, CT), 1.2B6 (anti-E-selectin/CD62E, Serotec, Kidlington-Oxford, UK), and 12C5 and 19C7 (anti-P-selectin/CD62P and anti-ICAM-1/CD54, respectively, both kindly provided by D. Haskard, Hammersmith Hospital, London, UK). A secondary FITC-conjugated goat anti-mouse Ig (Boehringer Mannheim, Rotkreuz, Switzerland) was used for detection. Porcine ICAM-2/CD102 expression was analyzed with polyclonal rabbit antibodies (kindly provided by P. Cowan, University of Melbourne, Victoria, Australia [174]) and secondary FITC-conjugated goat anti-rabbit Ig.

Phenotype and purity of PBMC, PMN, and NK cells was analyzed by direct immunofluorescence using FITC-UCHT1 (anti-CD3), FITC-MφP9 (anti-CD14), PE-B73.1 (anti-CD16), PE-B43 (anti-CD19), PE-B159 (anti-CD56) mAb (all from BD-Pharmingen, San Diego, CA), and VIMC6-FITC (anti-CD15, Caltag Laboratories, Hamburg, Germany). Isotype-matched control mAb or FACS buffer alone (for BS-IB4) were used as controls and propidium iodide (PI) gating to exclude dead cells in all experiments. All cells were analyzed on a FACScan (Becton Dickinson, Basel, Switzerland). To compare the levels of surface expression the geometric mean fluorescence intensity ratios (MFIR) were calculated by dividing the mean fluorescence intensity of staining with the mAb of interest with the mean fluorescence intensity of the control mAb.
Results – PART II

Adhesion assays
Adhesion of human PBMC, PMN, and freshly isolated or IL-2-activated NK cells on PAEC was analyzed under shear stress as previously described [124]. Briefly, PAEC were grown to confluency on an area of approximately 3 cm² and cultured for 4 h in the presence or absence of 100 U/ml human TNFα (Sigma). The resulting monolayers were washed and overlayed with 10^6 leukocytes in a volume of 100 μl. The dishes were then rotated at 64 rpm at 37°C for 10 min and washed. Adherent leukocytes were counted by light microscopy on four fields of 0.16 mm² per dish at a distance of 0.6 cm from the center.

Alternatively, PAEC were grown to confluency in 9.6 cm² petri dishes, washed twice and overlayed with 5x10^6 freshly isolated PBMC in 0.5 ml Weissmann buffer containing 5.55 mM glucose and 5 mg/ml human albumin. After 20 min of incubation in a shaker-incubator at 37°C and 64 rpm the dishes were washed twice to remove nonadherent leukocytes. Firmly adherent PBMC and PAEC were detached with trypsin, washed, and incubated with isotype control-FITC vs isotype control-PE, CD45-FITC vs isotype ctrl-PE, CD3-FITC vs CD56-PE, and CD14-FITC vs CD19-PE. Samples were then washed, stained with PI and analyzed on a single laser flow cytometer (FACScan). For each mixed and original PBMC population, a sample incubated with CD45-FITC, isotype control-PE, and PI was used to define a size (FSC) vs granularity (SSC) region (= R3) of PBMC, by double gating on PI-negative cells in an FSC vs PI plot (= R1) and on CD45-positive cells in a CD45 vs SSC plot (= R2). The fractions of T cells, monocytes, B cells, and NK cells were then determined from CD3-FITC/CD56-PE/PI and CD14-FITC/CD19-PE/PI samples after gating on R1 and R3.

Cytotoxicity assay
The cytotoxic activity of polyclonal human NK populations and the NK line NK92 was tested in 4 h ⁵¹Cr-release assays in serum-free AIM-V medium as described previously [105]. Briefly, target cells were added to triplicate samples of serial twofold dilutions of naïve or IL-2-activated NK cells in round-bottom 96-well plates at effector-to-target (E:T) ratios ranging from 40:1 to 5:1. After incubation for 4 h at 37°C the release of ⁵¹Cr was analyzed on a gamma counter and the percentage of specific lysis was calculated.

Statistical analysis
ANOVA analysis was used to analyze differences in adhesion of leukocyte subsets to PAEC. For subsets with a significant difference paired Student's t-testing was used to compare the
adhesion of this subset to PAEC-Gal$^{+/+}$ and PAEC-Gal$^{-/-}$, either left untreated or stimulated with TNFα. P values < 0.05 were considered to be statistically significant.

5.2.4. Results

Characterization of Gal$^{-/-}$ primary PAEC

Gal surface expression on PAEC-Gal$^{+/+}$ and PAEC-Gal$^{-/-}$ was analyzed by staining with the specific mAb M86 and the Gal-specific lectin BS-IB4 by flow cytometry. As shown in Figure 1, PAEC-Gal$^{+/+}$ do not express Gal on their surface, M86 staining revealed an MFIR of 2.9 on PAEC-Gal$^{+/+}$ and 1.0 on PAEC-Gal$^{-/-}$ cells (Fig. 1A), whereas BS-IB4 stained PAEC-Gal$^{+/+}$ and PAEC-Gal$^{-/-}$ with an MFIR of 417.7 and 1.2, respectively (Fig. 1B). Phenotypic analysis did not reveal significant differences between PAEC-Gal$^{+/+}$ and PAEC-Gal$^{-/-}$ cells regarding the expression intensities of adhesion molecules including CD62E, CD62P, CD54, CD102, and CD106 on untreated (Fig. 1C) and TNFα-stimulated (Fig. 1D) PAEC. Additional phenotyping of PAEC with antibodies directed against CD14, CD31, CD49e, CD86, and SLA class I d/d also demonstrated comparable expression patterns (data not shown).

![Figure 1: Cell surface expression of Gal and adhesion molecules on PAEC-Gal$^{+/+}$ and PAEC-Gal$^{-/-}$](image)

FIGURE 1. Cell surface expression of Gal and adhesion molecules on PAEC-Gal$^{+/+}$ and PAEC-Gal$^{-/-}$. Gal expression was analyzed using the Gal-specific mAb M86 (A) or the lectin BS-IB4 (B). Expression of CD62E, CD62P, CD54, CD102, and CD106 was analyzed on untreated (C) and TNFα-stimulated (D) PAEC. Dashed lines depict staining with an isotype-matched control mAb (A, C, and D) or FACS buffer alone (B) on PAEC-Gal$^{+/+}$ cells, being representative also for PAEC-Gal$^{-/-}$ background staining. Data are representative of three independent experiments.
Adhesion of human PBMC, PMN, and NK cells to PAEC is not dependent on Gal expression

Adhesion of human leukocyte subsets to primary PAEC plays an important role in the activation of the endothelium and precedes organ infiltration and target cell lysis in vivo. Therefore, we investigated the adhesion of freshly isolated human PBMC, PMN, and NK cells and IL-2-activated NK populations to PAEC under physiological shear stress. Figure 2 shows the adhesion of all leukocyte subsets on PAEC-Gal$^{+/+}$ and PAEC-Gal$^{-/-}$ cells, either left untreated or stimulated with TNFα. The adhesion of PMN was significantly increased on TNFα-stimulated PAEC (P = 0.001 for PAEC-Gal$^{+/+}$ and PAEC-Gal$^{-/-}$). In addition, the adhesion of IL-2-activated NK populations was also significantly increased on TNFα-stimulated PAEC (P = 0.045 for PAEC-Gal$^{+/+}$ and P = 0.002 for PAEC-Gal$^{-/-}$). We also observed an increase in adhesion of freshly isolated PBMC and NK cells after TNFα-treatment of PAEC that was not statistically significant. Furthermore and importantly, no significant differences in the adhesion of PBMC, PMN, and freshly isolated or IL-2-activated NK cells were detected comparing PAEC-Gal$^{+/+}$ and PAEC-Gal$^{-/-}$ cells.

To investigate whether individual PBMC subsets preferentially adhere to PAEC-Gal$^{-/-}$ and/or PAEC-Gal$^{+/+}$, the proportions of T cells, NK cells, B cells, and monocytes in adherent PBMC were compared to the original PBMC fractions (Fig. 3). After adjusting the total percentage of T cells, NK cells, B cells and monocytes to 100% for each population, the mean percentage of NK cells increased from 16.5% in the original PBMC population to 34.9% and 39.9% of adherent PBMC on untreated, and 33.2% and 39.9% on TNFα-stimulated PAEC-Gal$^{+/+}$ and
PAEC-Gal<sup>+</sup> and PAEC-Gal<sup>−</sup>, respectively, revealing more than a doubling of the NK fraction. A strong preferential adhesion was also recorded for B cells, whereas the T-cell portion of PBMC was halved on both untreated and stimulated PAEC-Gal<sup>++</sup> and PAEC-Gal<sup>−</sup>. The proportion of adherent monocytes varied extensively between the donors.

**FIGURE 3. Human NK cells and B cells preferentially adhere to PAEC.** Freshly isolated PBMC were cocultured with PAEC under rotation. Adherent cells were collected, stained, and analyzed by flow cytometry to determine percentages of T cells, monocytes, B cells, and NK cells. Shown is PBMC gating in CD45 vs SSC plots, CD3 vs CD56 staining, and CD14 vs CD19 staining of original PBMC (upper panel, donor BB shown) and of a mixed population of PBMC and PAEC after an adhesion assay (lower panel, donor BB PBMC + TNFα-stimulated PAEC-Gal<sup>−</sup> shown). The data of PBMC adhesion on TNFα-stimulated PAEC-Gal<sup>−</sup> were also representative for PBMC adhering on untreated PAEC-Gal<sup>−</sup> and on stimulated and untreated PAEC-Gal<sup>++</sup>. Data are representative of three independent experiments.

**Cytotoxicity of human NK cells is independent of Gal expression**

PAEC are lysed by human NK cells in vitro and there have been controversial reports about a direct involvement of Gal as target recognition molecule. To test the role of Gal in xenogeneic NK cytotoxicity, PAEC-Gal<sup>++</sup> and -Gal<sup>−</sup> cells were used as targets for freshly isolated or IL-2-activated human NK cells in 4 h 51Cr-release cytotoxicity assays. The specific lysis of PAEC mediated by freshly isolated NK cells was up to 40%, whereas the killing by IL-2-activated NK populations ranged between 40% and 90% at the highest E:T ratio. At 40:1, the mean percentage of specific lysis using IL-2-activated NK cells from eight different donors was 66 ± 17% for PAEC-Gal<sup>++</sup> and 70 ± 15% for PAEC-Gal<sup>−</sup>. In addition, the mean percentage specific lysis using the human NK line NK92 in three independent experiments was 73 ± 8% for PAEC-Gal<sup>++</sup> and 76 ± 1% for PAEC-Gal<sup>−</sup>. Thus, both Gal<sup>++</sup> and Gal<sup>−</sup> primary porcine endothelial cells were equally susceptible to human NK cytotoxicity. Shown
is the lysis mediated by activated VN NK cells (Fig. 4A), by freshly isolated GS NK cells (Fig. 4B), and by the NK line NK92 (Fig. 4C). These findings indicate that Gal is either not directly recognized by human NK cells or does not play a dominant role in triggering xenogeneic cytotoxicity in NK cells.

FIGURE 4. Human NK cytotoxicity against porcine endothelial cells is not reduced by lack of Gal expression. The cytotoxic activity of IL-2-activated NK cells purified from donor VN (A), freshly isolated NK cells from donor GS (B), and the NK line NK92 (C) against PAEC-Gal+/+ (black squares) and PAEC-Gal−/− (white squares) is shown. Cytotoxicity was determined at four different E:T ratios in standard 4 h 51Cr-release assays and expressed as percentage of specific lysis. Data are representative of three (A), two (B), and four (C) independent experiments.

5.2.5. Discussion

The generation of pig organ donors devoid of Gal has raised great expectations in the field of xenotransplantation, since a key role for anti-Gal antibodies in xenograft rejection has been clearly demonstrated [60]. First results of pig-to-baboon kidney or heart transplantation using organs of Gal−/− pigs suggest that hyperacute rejection can be overcome even without extracorporeal immunoabsorption and/or treatment with cobra venom factor resulting in improved survival times over 100 days [175]. Therefore, the study of subsequent cellular immune responses against xenografts is of growing importance. In the present study, primary PAEC derived from Gal−/− pigs were used to investigate the influence of Gal on the adhesion of human PBMC, PMN, and NK cells, and the cytotoxicity mediated by NK cells. The question whether carbohydrate determinants, in addition to proteins, can act as NK target molecules is still a matter of debate. Using an in vitro-generated immortalized Gal-negative cell line we recently showed that the lack of Gal on PAEC does not reduce NK adhesion and anti-porcine NK cytotoxicity [172]. In contrast to the Gal-negative immortalized porcine endothelial cell line used in the latter study, there were no phenotypic differences between primary PAEC derived from Gal−/− and Gal+/+ pigs used in the present study. Since Gal had no influence on the lysis of these primary PAEC mediated by human NK cells, we were able to confirm our results. In addition, expanding our previous findings with NK cells, the adhesion
of PBMC, PMN, and NK cells to primary PAEC-Gal$^{+}$ was not different compared to PAEC-Gal$^{-}$. Comparing the different leukocyte subsets, a preferential adhesion of NK cells and B cells was detected. In fact, NK cells constituted the major cell subset among adherent PBMC on both PAEC-Gal$^{-}$ and PAEC-Gal$^{+}$, whereas a clear dominance for monocytes was found in PBMC adhering to activated human endothelial cells (own unpublished observations). These findings are in line with a previous study showing that naïve human NK cells adhered, activated, and lysed PAEC independently of Gal [126]. Due to the possibility of redundant mechanisms our results do not formally rule out a role for Gal in NK recognition. Theoretically, it may be necessary but not sufficient to interfere with Gal to overcome NK responses due to other triggering factors present on porcine endothelial cells. However, He et al. recently showed that there were no differences regarding NK adhesion and cytotoxicity upon introduction of Gal in human aortic endothelial cells [171]. Taken together with our ex vivo results, the possibility of Gal acting as an independent activation signal for NK cells is thus virtually excluded. It seems very unlikely that a reduction of xenogeneic cellular responses due to the elimination of Gal was compensated by newly exposed xenoantigens recognized by pattern recognition receptors and resulting in responses of similar strength. The lack of congruence with the conclusions of other studies [108,151,152,170] may be explained by differences in the experimental in vitro protocols, in particular the indirect methods to remove or block Gal that were used in these studies.

Beside the finding that genetic deletion of Gal does not reduce leukocyte adhesion and NK cytotoxicity it is also noteworthy that the Gal modification did not result in an increase in any of the examined potentially deleterious actions of leukocytes. As shown by Shinkel et al., elimination of Gal in α1,2-fucosyltransferase transgenic mice generated new xenoantigens or exposed cryptic antigens on the cell surface which influenced the binding of lectins and human natural antibodies [176]. In contrast, inactivation of the α1,3GT gene resulted in relatively minor changes in the cell surface glycosylation pattern other than the elimination of Gal [176]. Genetic deletion of Gal therefore did not induce non-Gal-specific human natural antibodies and cellular responses. In this context, the binding of human xenoreactive antibodies to Gal-negative porcine endothelial cells is of great interest and is currently under investigation.

In conclusion, lack of Gal expression on porcine endothelial cells, the major target for xenoreactive antibody-mediated responses, does neither seem to protect against human leukocyte adhesion and NK cytotoxicity nor trigger Gal-independent cellular human anti-pig responses. This finding underlines our view that only a combination of approaches,
addressing both antibody- and cell-mediated mechanisms of xenograft rejection, may eventually facilitate clinical pig-to-human xenotransplantation.

5.2.6. Acknowledgments

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5.3. PART III

Reactivity of human natural antibodies to endothelial cells from
Galα(1,3)Gal-deficient pigs

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Running Title: Human antibodies binding to Gal-deficient porcine cells.

Keywords: Human, Endothelial Cells, Antibodies, Natural Killer Cells, Transplantation.

Abbreviations used in this paper: ADCC, antibody-dependent cell-mediated cytoxicity; BS-
IB4, Bandeiraea simplicifolia isolectin B4; DFP, diisopropylfluorophosphat; Gal, galactose-
α(1,3)galactose; GT, α(1,3)galactosyltransferase; HAR, hyperacute rejection; IVIG,
intravenous immunoglobulin; MFIR, geometric mean fluorescence intensity ratio; NAb,
natural antibodies; PAEC, porcine aortic endothelial cells; pEC, porcine endothelial cells;
pLCL, porcine lymphoblastoid cells; pRBC, porcine red blood cells.
5.3.1. Abstract
Xenoreactive human natural antibodies (NAb) are predominantly directed against galactose-\(\alpha(1,3)\)galactose (Gal). Binding of NAb to porcine endothelial cells (pEC) initiates hyperacute rejection of xenografts by activating the complement system. In vitro, non-complement-fixing IgG NAb induce human NK cell-mediated lysis of pEC by antibody-dependent cell-mediated cytotoxicity (ADCC). The role of non-Gal anti-porcine NAb for complement lysis and ADCC remains unknown. The present study examined the levels and functional properties of anti-porcine NAb using porcine red blood cells (pRBC), lymphoblastoid cells (pLCL), and pEC derived from control or Gal-deficient pigs. When sera from 120 healthy human donors were analyzed for the presence of anti-porcine NAb by flow cytometry, human IgM binding to pRBC was found in 93% and IgG binding in 86% of all samples. The correlation of NAb binding to Gal-positive pEC and pRBC was excellent, but weak between pEC and pLCL. Binding of non-Gal anti-porcine NAb to pEC comprised 13% of total IgM and 36% of total IgG anti-pig NAb binding. In line, NAb/complement-induced xenogeneic lysis and ADCC of Gal-deficient compared to Gal-positive pEC were 21% and 29%, respectively. Both anti-Gal and non-Gal anti-porcine IgG NAb were predominantly of the IgG2 subclass. The generation of Gal-deficient pigs has overcome anti-Gal-mediated xenograft rejection in non-human primates. As our data demonstrate, this approach also leads to a substantial reduction of human anti-pig NAb binding, complement lysis, and ADCC. Nevertheless, non-Gal anti-porcine NAb still represent a relevant immunological hurdle and may induce xenograft rejection in vivo.

5.3.2. Introduction
Pig-to-human xenotransplantation is currently viewed as a possible solution to overcome the persistent shortage of human organs available for transplantation medicine [5]. However, differences in cell surface glycosylation between humans and pigs evoke strong immunological responses due to naturally occurring antibodies (NAb) directed against porcine cells [59,139]. These antibodies predominantly recognize the galactose-\(\alpha(1,3)\)galactose epitope (Gal), a terminal disaccharide synthesized by the \(\alpha(1,3)\)galactosyltransferase (GT) and abundantly expressed on the cell surface of all non-primate mammals. It is well known that binding of anti-Gal NAb activates porcine endothelial cells (pEC) and thereby initiates hyperacute rejection (HAR) of porcine organs transplanted.
into non-human primates. HAR is characterized by activation of both the complement and coagulation system [60]. Moreover, non-complement-fixing NAb may play an important role in the initiation of tissue damage in xenotransplants through antibody-dependent cell-mediated cytotoxicity (ADCC). IgG NAb bound to Gal and other so far unknown epitopes on the surface of pEC are recognized by FcγRIII on human NK cells leading to rapid lysis by ADCC [60,105,119]. The frequency of anti-Gal NAb in humans has been described to comprise 1-2% of total IgG and 4-8% of total IgM, and 1% of the circulating B lymphocytes produce anti-Gal antibodies [68,70]. In preclinical models, HAR can be largely prevented by complement inactivation or removal of anti-donor NAb from the recipient. Recently, new cloning and nuclear transfer technologies have enabled the production of Gal-deficient pigs due to complete elimination of both GT alleles [83,84]. Prolonged xenograft survival times up to 83 and 179 days using kidneys and hearts from GT-knockout pigs in pig-to-baboon xenotransplantation models were reported [86,87]. The availability of Gal-deficient cells from such pigs facilitates the investigation of other antigenic targets involved in immune rejection of porcine xenografts and in particular the potential role of non-Gal anti-porcine NAb.

Similar to HAR in xenotransplantation, NAb against ABO blood group antigens induce a complement-mediated acute rejection of allogeneic ABO-incompatible organs [177]. Compared to the levels of anti-Gal NAb present in human serum, anti-A/B NAb levels are much lower, approximately one fourth of anti-Gal NAb, and occur in the majority of healthy individuals according to their ABO blood group [68,70,178]. Since the Gal epitope is structurally strongly related to human ABO blood group antigens, an individual’s blood group might influence the formation of Gal-specific antibodies [179]. In particular, the B antigen differs only by one fucosylation site from the Gal antigen and a number of studies have related the B and AB blood groups with lower levels of anti-Gal NAb [180-182], however, with controversial results.

As previously reported, NAb-induced complement lysis and ADCC of Gal-deficient pEC were significantly lower as compared to Gal-positive pEC, whereas adhesion and direct cytotoxicity of human NK cells did not differ [172,183]. To expand these results, the current study examined the levels and functional properties of anti-porcine NAb in the sera of 120 healthy blood donors using porcine red blood cells (pRBC), lymphoblastoid cells (pLCL), and pEC derived from control or Gal-deficient pigs. In particular, human sera obtained from donors of all four ABO blood groups were tested for individual and group-specific differences in the levels and functional properties of anti-Gal and non-Gal anti-porcine NAb. In addition, NAb IgG subclasses and the correlation between NAb binding and complement lysis were
analyzed. In summary, the results presented here indicate that (1) non-Gal anti-porcine NAb still represent a relevant immunological hurdle for xenotransplantation, and that (2) pretransplant testing using quantitative and functional methods will be required prior to pig-to-human xenotransplantation due to considerable differences in the NAb repertoire among human individuals.

5.3.3. Materials and Methods

**Cells**

GT-knockout pigs were generated by gene targeting, panning, and nuclear transfer technologies as described elsewhere [84]. Porcine aortic endothelial cells, PAEC-Gal+7+ and PAEC-Gal−7−, were isolated from control or GT-knockout pigs following standard procedures [105] and either used as primary cells up to passage 25 or as SV40-immortalized lines. The SV40-immortalized aortic PEDSV.15 line was generated and characterized in our laboratory as described earlier [153]. All pEC were cultured in DMEM (Invitrogen AG, Basel, Switzerland) supplemented with 10% FCS (PAA Laboratories, Luzern, Switzerland), 1 mM sodium pyruvate, 2 mM L-glutamine, non-essential amino acids (100x), and 20 mM HEPES (all Invitrogen). The immortalized porcine lymphoblastoid cell line 13271.10 (pLCL) was a kind gift of G. Waneck (Massachusetts General Hospital, Boston, MA [184]) and cultured in RPMI 1640 (Invitrogen) supplemented with 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, non-essential amino acids (100x) and 50 μM 2-mercaptoethanol (Invitrogen). Porcine RBC and serum were obtained from the local slaughterhouse. To prevent agglutination, pRBC were fixed according to a previously described method [178,185]. Briefly, pRBC were incubated for 20 min at 4°C in Karnovsky buffer, containing formaldehyde and glutaraldehyde, and washed thereafter in PBS, containing 0.6% BSA (Fluka Biochemicals, Buchs, Switzerland). After fixation pRBC were stored in PBS at 4°C and used for up to 6 weeks without changes in antibody binding levels. The isolation of NK cells from healthy blood donors has been described previously [134].

**Human serum samples**

Human serum was obtained from 120 healthy blood donors from the local blood bank (38 blood group O, 28 blood group A, 28 blood group B, and 26 blood group AB) and additional healthy volunteers. Statistical analyses of donor age, rhesus blood group and gender did not reveal significant differences among the ABO blood groups. Samples were stored at -20°C for...
long periods or at 4°C for short periods. Decomplementation by heat inactivation was carried out for 30 min at 56°C. Moreover, to exclude unspecific binding due to antibody oligomerization after heat inactivation, complement inactivation was also performed by addition of 5 mM diisopropylfluorophosphat (DFP, Fluka Biochemicals). DFP irreversibly inactivates serine proteases and thereby specifically inhibits the complement system [186]. In addition, commercially available pooled human intravenous immunoglobulin (IVIG) preparations used for clinical applications were tested for the presence of xenoreactive NAb at a final concentration of 500 μg/ml (Endobulin® S/D, Baxter, Volketswil, Switzerland).

**Affinity purification and ELISA of anti-Gal antibodies**

Affinity purification of anti-Gal NAb was performed as described elsewhere [156] and tested in a Gal-specific ELISA. In brief, a 1/1 mixture of synthetic Gal (B-disaccharide, Galα(1,3)Gal) and Galα(1,3)Galβ(1,4)glucose (linear B-trisaccharide type 6) in the form of flexible, hydrophilic polyacrylamide conjugates covalently coupled to Fast-Flow Sepharose was used as immunoabsorbent. The purity of the anti-Gal NAb-depleted serum was tested by ELISA using plates coated with B-disaccharide and linear B-trisaccharide type 6. Immunoabsorbents and coating antigens were kindly provided by N. Bovin (Shemyakin Institute of Bioorganic Chemistry, Moscow, Russia).

**Cell staining and flow cytometry**

Fixed pRBC and trypsinized (0.25% trypsin, Life Technologies, Switzerland) pEC were diluted to 10^5 cells per tube in staining buffer (HANKS, 0.1% BSA). Porcine LCL were resuspended in RPMI 1640 containing 1% FCS. Surface expression of Gal molecules on porcine cells was analyzed on a FACScan (Becton Dickinson, Basel, Switzerland) by direct and indirect immunofluorescence using either the FITC-labeled isolectin B4 from Bandeiraea simplicifolia (BS-IB4, Sigma, Buchs, Switzerland [157]) or the mouse anti-Gal IgM mAb M86 (Alexis Corporation, Lausen, Switzerland [158]). For the latter a secondary FITC-conjugated goat anti-mouse Ig antibody (Boehringer Mannheim, Rotkreuz, Switzerland) was used. Cells were incubated for 30 min on ice with saturating concentrations of IB4 lectin or the mAb M86. Irrelevant, isotype-matched mAb or staining buffer alone (for BS-IB4) were used as negative controls and propidium iodide gating to exclude dead cells in all experiments.
Results – PART III

Binding of xenoreactive NAb to porcine cells was analyzed by incubating $10^5$ cells with 10% heat-inactivated or DFP-treated human serum or human IVIG preparations for 30 min on ice. Secondary FITC-conjugated polyclonal goat anti-human IgM (Sigma, Buchs, Switzerland) and monoclonal mouse anti-human IgG detecting all subclasses (Zymed, Basel, Switzerland) were used for isotype-specific NAb detection. IgG subclasses were stained with secondary biotinylated mouse anti-human IgG1, IgG2, IgG3, or IgG4 antibodies (all Zymed) and Streptavidin-FITC conjugate (Pharmingen, San Diego, USA). Porcine serum (for binding to pRBC) or staining buffer (for binding to pEC and pLCL) were used as negative control. To compare the levels of surface expression the geometric mean fluorescence intensity ratios (MFIR) were calculated by dividing the mean fluorescence intensity of the sample of interest with the mean fluorescence intensity of the negative control.

Phenotypic analysis of NK cells was carried out by direct immunofluorescence using FITC-UCHT1 (anti-CD3), PE-B73.1 (anti-CD16), and PE-B159 (anti-CD56) mAb (all from Pharmingen, San Diego, CA). NK purity (CD37CD16+CD56+) was greater than 95% in all experiments.

Hemagglutination
Anti-porcine hemagglutination titers were measured with a microplate hemagglutination assay as described elsewhere [178]. Serially diluted serum was incubated with a 1% suspension of pRBC for 30 min at room temperature in 96-well U-bottom plates (Nunclon Surface, NUNC, Roskilde, Denmark). Thereafter, the plates were centrifuged for 2 min at 300g and placed in an upright position for 10 min. Agglutination was judged positive if the pRBC remained clotted and did not run down the well of the 96-well plate. Titers were expressed as the greatest dilution that caused agglutination. All experiments were performed twice, in cases of more than one titer difference, a third experiment was performed, and the median value was taken for further analysis.

Complement lysis
The anti-porcine cytotoxicity of human serum before and after immunoabsorption was analyzed by flow cytometry. Briefly, porcine cells were incubated in staining buffer containing heat-inactivated human serum at concentrations ranging from 5-25% for 60 min at 37°C and washed with staining buffer. Thereafter, cells were incubated with HANKS containing 15% rabbit serum (Biotest, Rupperswil, Switzerland) for 30 min at 37°C.
Complement-induced xenogeneic lysis was determined by flow cytometry by propidium iodide uptake analyzing the percentage of dead cells.

**Antibody-dependent cell-mediated cytotoxicity**

To determine the effects of xenogeneic ADCC, PAEC monolayers in fibronectin-coated 96-well tissue culture plates (BD Biosciences, Allschwil, Switzerland) were labeled with $^{51}$Cr (4 μCi per well) for 90 min at 37°C. Labeled cells were washed and incubated with 10-20% heat-inactivated human serum for 60 min at 37°C. After two washing steps NK cells were added at an E:T ratio of 2.5:1. NK cells were freshly isolated from different donors and were used along with the serum of the same donor (autologous ADCC) or not (allogeneic ADCC). Incubation with NK cells in the absence of serum and serum alone were used as negative controls. All experiments were performed in triplicates. After incubation for 4 h at 37°C the release of radioactive $^{51}$Cr was analyzed on a gamma counter and the percentage of specific lysis was calculated as follows:

\[
\text{% specific lysis} = \left(\frac{(E - S)}{(M - S)}\right) \times 100
\]

where E represents the experimental release (cpm in the supernatant from target cells incubated with serum and/or effector cells), S is the spontaneous release (cpm in the supernatant from target cells incubated with medium alone), and M is the maximum release (cpm released from target cells lysed with 1% NP-40). The percentage of ADCC was calculated as the difference in NK cytotoxicity against PAEC in the presence or absence of human serum.

**Nephelometry**

Concentrations of total IgM, IgG, and IgG subclasses for eleven sera were analyzed by nephelometry (BN II nephelometer, Dade Behring, Düdingen, Switzerland) after staining with antisera to human IgM, IgG, and IgG subclasses (all Dade Behring).

**Statistical analysis**

All statistical analyses were performed with SPSS software (SPSS for Windows, version 11.0). The groups were compared by Student’s t-test or non-parametric tests as appropriate. Standard deviation (SD) or range were used as estimates of variance. Spearman’s rank test ($r_s$) was performed for correlation analyses. All reported p-values are two-sided, and $p<0.05$ was considered to be statistically significant.
5.3.4. Results

Gal expression on porcine cells

Cell surface expression of Gal on pEC, pLCL, and pRBC was analyzed by flow cytometry using the Gal-specific mAb M86 and lectin BS-IB4. As shown in Figure 1, Gal expression levels on different porcine cell types varied considerably using BS-IB4, whereas M86 staining revealed comparable levels. The lack of Gal expression on ex vivo immortalized PAEC-Gal<sup>−/−</sup> is clearly shown by both M86 and lectin staining, the same finding was obtained with primary PAEC-Gal<sup>−/−</sup> (data not shown). The expression of Gal on fixed pRBC was about three times reduced compared to unfixed cells (data not shown).

![Graph showing flow cytometry results](image)

FIGURE 1. Gal surface expression on porcine cells. (A) Immortalized PAEC, (B) pLCL, and (C) pRBC were analyzed by flow cytometry using the lectin BS-IB4 (left column) and the monoclonal anti-Gal Ab M86 (right column). Parental Gal-positive cells are depicted as black histograms and Gal-deficient cells as grey histograms. Histograms for an isotype-matched control antibody (for M86) or staining buffer only (for lectin) are shown as empty histograms with dashed lines. Numbers indicate MFIR of Gal expression and are representative of three independent experiments.

Levels of human natural antibodies against porcine red blood cells

Binding of xenogeneic NAb to pRBC was determined by flow cytometry in 120 healthy blood donors. As described in the original publication, IgM and IgG were considered to be positive if the MFIR was greater than 3.7 and 2.0, respectively [178]. Anti-porcine IgM antibodies were positive in 112 of 120 samples (93%) and anti-porcine IgG antibodies in 103 of 120
samples (86%). Mean MFIR of anti-porcine IgM was 37.3 (range: 1.4-234.4) and 10.9 for anti-porcine IgG (range: 1.3-52.2). As shown in Figure 2, there was a high variability in IgM and IgG anti-porcine NAb levels among individuals of the same ABO blood group. Blood group B and AB sera contained significantly lower anti-porcine NAb levels as compared to blood group A and O (p=0.01 for IgM and <0.001 for IgG, Mann-Whitney-U test). Hemagglutination showed a high correlation with the FACS analysis of anti-porcine IgM and a weak correlation with anti-porcine IgG (r_s=0.689 with p<0.001 for anti-porcine IgM; r_s=0.336 with p<0.001 for anti-porcine IgG). Similar to the results obtained by FACS analysis, lower levels of anti-porcine IgM were observed in blood group B and AB sera as compared to A and O in the hemagglutination assay (data not shown).

**FIGURE 2. Levels of xenoreactive anti-pRBC NAb in 120 healthy human blood donors.** Anti-pRBC IgM (A) and IgG (B) were measured in 120 healthy blood donors by flow cytometry using FITC-labeled anti-human IgM and IgG antibodies as secondary antibodies. Shown are MFIR values calculated using porcine serum as negative control. Boxplots represent median values of MFIR and the 25 and 75 percentile. Results are calculated as the mean of two independent experiments.

**Correlation of human natural antibody levels with porcine endothelial, lymphoblastoid, and red blood cells**

Binding of anti-porcine NAb on pEC, pRBC, and pLCL was compared using sera from 40 different healthy blood donors (10 donors of each blood group). An excellent correlation of anti-porcine NAb binding to the immortalized Gal-positive pEC lines PAEC-Gal^+^ and PEDSV.15 was found (r_s=0.963 for IgM and r_s=0.904 for IgG), whereas the correlations between PAEC-Gal^+^ and pRBC (r_s=0.849 for IgM and r_s=0.732 for IgG), and between PEDSV.15 and pRBC (r_s=0.902 for IgM and r_s=0.814 for IgG) were weaker but still significant. In contrast, binding results with pLCL did not closely correlate with the results obtained with pRBC or pEC (data not shown). Taken together, these findings suggest that
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NAb bind to additional and/or other antigenic structures expressed on lymphoblastoid cells as compared to endothelial and red blood cells.

**IgG subclass analysis of human anti-porcine natural antibodies**

IgG subclass antibodies are known to have different functional properties. We therefore measured binding of IgG subclasses to primary PAEC-Gal⁺⁺ and PAEC-Gal⁺⁺. Serum donors were selected by middle to high binding of total anti-porcine IgG NAb to PEDSV.15. The mean Ig levels as determined by nephelometry were all within normal ranges and as follows: 1.2 g/L for IgM, 10.5 g/L for total IgG, 6.4 g/L for IgG₁, 3.7 g/L for IgG₂, 0.4 g/L for IgG₃, and 0.5 g/L for IgG₄. As shown in Figure 3, anti-Gal IgG NAb were predominantly of the IgG₂ subtype with lower levels of IgG₁ and IgG₃. All samples tested were negative for IgG₄. Donors with high total IgG levels had also high IgG₂ titers. Similarly, IgG₂ was the predominant subclass when using IVIG preparations (data not shown). Binding of IgG subclasses to PAEC-Gal⁺⁺ and PAEC-Gal⁺⁺ demonstrated a comparable distribution pattern with IgG₂ being also the predominant subclass of non-Gal anti-porcine NAb. The mean levels of non-Gal anti-porcine NAb as compared to the total amount of xenoreactive IgG NAb were 32% for IgG₁, 24% for IgG₂, and 23% for IgG₃. Moreover, binding of IgG subclasses to pLCL and pRBC showed similar results as compared to PAEC-Gal⁺⁺ cells (data not shown), confirming that the predominant anti-pig IgG NAb belong to the IgG₂ subclass.

![Diagram showing IgG subclass distribution](image)

**FIGURE 3.** IgG₂ is the predominant subtype of non-Gal anti-porcine NAb. Binding of IgG subclasses from sera of eight blood donors (two of each blood group) to primary PAEC-Gal⁺⁺ (black diamonds) and PAEC-Gal⁺⁺ (white diamonds) was analyzed by flow cytometry. Shown are MFIR values, data are representative of two independent experiments.
Levels of human anti-Gal and non-Gal anti-porcine natural antibodies against endothelial cells

To determine the influence of Gal expressed on pEC, the binding of anti-Gal and non-Gal anti-porcine NAb to immortalized PAEC-Gal\(^{+/−}\) and PAEC-Gal\(^{+/+}\), respectively, was analyzed by flow cytometry. As shown in Figure 4, binding of human NAb present in sera from randomly chosen 40 blood donors to PAEC-Gal\(^{+/−}\) was significantly lower as compared to PAEC-Gal\(^{+/+}\). The levels of non-Gal anti-porcine NAb comprised 13% of total IgM and 36% of total IgG xenoreactive NAb. Similar results were obtained using DFP-treated sera excluding false positive signals caused by Ig oligomerization after heat inactivation (data not shown). However, again there was a considerable variability of binding reactivity among individual blood donors and different blood groups. In contrast to the reduced levels of anti-porcine NAb in blood group B and AB sera shown in Figure 2, where pRBC were used, there was no reduction of anti-pEC NAb levels in blood group AB sera. The reduction of IgM binding to Gal-deficient PAEC as compared to Gal-positive PAEC ranged from 67-97% and the reduction of IgG binding from 0-98%, respectively. In general, higher MFIR were found for IgM than for IgG NAb binding, but direct comparisons are difficult to interpret due to the use of different secondary antibodies in the FACS assays, which might have different binding affinities and FITC-labeling intensities.

**FIGURE 4.** Strongly decreased human NAb binding to Gal-deficient PAEC. Anti-Gal and non-Gal anti-porcine NAb levels were analyzed by flow cytometry using sera of 40 randomly selected donors (10 of each blood group). Binding of human IgM (A) and IgG (B) NAb was analyzed on immortalized PAEC-Gal\(^{+/−}\) (white boxplots) and PAEC-Gal\(^{+/+}\) (grey boxplots). Boxplots represent median values of MFIR and 25 and 75 percentiles. Data are representative of three independent experiments.
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Binding of anti-Gal-depleted and non-depleted sera to porcine cells

To investigate the levels of non-Gal anti-porcine NAb, sera of three donors were depleted of anti-Gal NAb by column absorption and analyzed by flow cytometry using different porcine cell types. As shown in Table I, anti-Gal depletion resulted in a strong decrease of NAb binding to PAEC-Gal⁺⁺, pRBC, and pLCL (ranges: 75-87% for IgM and 52-95% for IgG), whereas binding to PAEC-Gal⁻⁻ was similarly low for both anti-Gal-depleted and non-depleted serum. In contrast to the reduction of specific anti-Gal NAb after anti-Gal depletion, there were only minor changes of the absolute Ig levels as measured by nephelometry after immune absorption. The mean IgG level was 11.6 g/L in non-depleted and 10.7 g/L in anti-Gal-depleted serum (8% reduction), and 1.7 g/L and 1.6 g/L for IgM (6% reduction), respectively.

<table>
<thead>
<tr>
<th>Anti-Gal Depletion</th>
<th>Anti-Porcine IgM¹</th>
<th>Anti-Porcine IgG¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Primary PAEC-Gal⁺⁺</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>Primary PAEC-Gal⁻⁻</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Immortalized PAEC-Gal⁺⁺</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>Immortalized PAEC-Gal⁻⁻</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Porcine RBC</td>
<td>159</td>
<td>31</td>
</tr>
<tr>
<td>Porcine LCL</td>
<td>38</td>
<td>8</td>
</tr>
</tbody>
</table>

¹Data represent mean MFIR values from three different donors.

Table I: Comparison of anti-porcine NAb on different cell types using non-depleted and anti-Gal-depleted serum.

Strong reduction of xenogeneic complement lysis of Gal-deficient porcine endothelial cells

Binding of human xenoreactive NAb to pEC leads to activation of the complement cascade and consequently to cell lysis. Complement lysis of pEC was analyzed in 50 healthy donors using heat-inactivated human serum and rabbit serum as a source of complement. The mean specific lysis of PAEC-Gal⁻⁻ was 21% as compared to PAEC-Gal⁺⁺ (Fig. 5). Complement lysis of PAEC-Gal⁻⁻ was significantly lower in blood group B and AB as compared to blood group A and O (p= 0.01, Student’s t-test). Table II shows the correlation between NAb MFIR levels of these donors and the corresponding specific complement lysis. While the level of non-Gal anti-porcine NAb in blood group O was identical compared to the other blood groups, the level of complement lysis of Gal-deficient cells was higher: The relative complement lysis of PAEC-Gal⁻⁻ was similar using non-depleted and anti-Gal-depleted serum of three donors and comprised 17% of the lysis of PAEC-Gal⁺⁺ (data not shown). This result strongly correlated with the complement lysis and binding data shown in Figure 4 and Table
II and indicates that non-Gal anti-porcine NAb are responsible for human anti-pig NAb binding and complement lysis.

![FIGURE 5. Strong reduction of xenogeneic complement lysis using Gal-deficient porcine endothelial cells. Complement lysis of primary PAEC-Gal''/+(black bars) and PAEC-Gal''/(white bars) was measured using sera of 50 healthy blood donors and is shown as the mean of two serum concentrations (10 and 20%). Rabbit serum was used as complement source. Data are representative of two independent experiments.]

<table>
<thead>
<tr>
<th>Blood group</th>
<th>PAEC-Gal''/+</th>
<th>PAEC-Gal''/</th>
<th>PAEC-Gal''/+</th>
<th>PAEC-Gal''/</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (n=13)</td>
<td>61 30 6 4</td>
<td>65 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B (n=11)</td>
<td>26 23 5 3</td>
<td>44 11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O (n=13)</td>
<td>57 37 6 2</td>
<td>65 23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB (n=13)</td>
<td>67 20 3 6</td>
<td>53 8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Median MFIR
2 Percentage of specific complement lysis using 10% human serum

Table II: Comparison of anti-porcine NAb levels and complement lysis.

**Antibody-dependent cell-mediated cytotoxicity against porcine endothelial cells**

ADCC of pEC mediated by NAb and human NK cells is an efficient mechanism of lysis and might represent an important *in vivo* mechanism leading to xenograft rejection. Therefore, we analyzed autologous and allogeneic ADCC against PAEC-Gal''/+ and PAEC-Gal''/ using sera of eleven different donors. ADCC was observed in preliminary experiments using both allogeneic and autologous NK cells, for practical reasons of donor availability and serum measurements in other assays only allogeneic data are presented here. Allogeneic ADCC against PAEC-Gal''/+ using non-depleted serum was observed using freshly isolated NK cells from eight out of eleven donors at an E:T ratio of 2.5:1 with a mean of 29% specific lysis
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(range: 7-42%). In comparison, ADCC against PAEC-Gal⁺ was 70% lower with a mean of 8% specific lysis (range: 3-12%). To demonstrate the individual differences, ADCC data obtained from two allogeneic donors are shown in Figure 6, one with an apparent ADCC response induced by non-Gal anti-porcine NAb and another with no ADCC response against neither Gal-positive nor Gal-deficient pEC. Taken together, although the levels of ADCC varied considerably among different NK donors and sera, our findings confirm that non-Gal anti-porcine NAb are able to induce xenogeneic ADCC.

![Graph showing ADCC reduction using Gal-deficient porcine endothelial cells.](image)

**FIGURE 6. Reduction of xenogeneic ADCC using Gal-deficient porcine endothelial cells.** Levels of ADCC were measured by adding NK cells at an E:T ratio of 2.5:1 in combination with allogeneic human sera to PAEC-Gal⁺ (black squares) and PAEC-Gal⁻ (white squares). Shown is the percentage of specific lysis using 10 and 20% human serum. Data are representative of two independent experiments.

5.3.5. Discussion

The importance of human NAb directed against Gal in the rejection of porcine xenografts is well recognized. Prevention of NAb-induced reactions is mandatory for further progress in xenotransplantation, thus, the recent generation of GT-knockout pigs has raised great expectations. Indeed, first reports of pig-to-baboon kidney or heart transplantations using organs of GT-knockout pigs are encouraging. Hyperacute rejection was prevented without the need for extracorporeal immunoabsorption and/or treatment with cobra venom factor [86,87]. In contrast, transplants from pigs expressing low levels of Gal were still hyperacutely rejected supporting the pivotal role of Gal in HAR. Despite this major breakthrough the immunological hurdles of xenotransplantation are still not overcome. In the pig-to-baboon heart transplant model acute humoral xenograft rejection occurred in 5 out of 8 transplanted organs [86,87]. This type of rejection is potentially associated with the presence of non-Gal anti-porcine NAb. Moreover, the anti-pig NAb repertoire might differ considerably between humans and baboons used in preclinical models. Thus, one aim of the current study was to analyze the role and functional properties of human anti-Gal and non-Gal anti-porcine NAb.
binding to endothelial cells derived from control or GT-knockout pigs. Intriguingly, when anti-porcine NAb binding of 120 human sera to pRBC was analyzed, a minority of 8 (7%) and 17 (14%) samples did not comprise any anti-porcine IgM and IgG NAb, respectively. In a previous study using an \textit{in vitro}-generated Gal-deficient pEC line we showed that non-Gal anti-porcine NAb are able to induce complement lysis and ADCC. Confirming and extending these results using Gal-deficient primary pEC derived from GT-knockout pigs, we show here that the reduction of NAb binding levels correlated with the reduction of complement lysis and ADCC. Furthermore, the remaining NAb binding, complement lysis, and ADCC underlines the potential importance of non-Gal anti-porcine human NAb.

Non-Gal anti-porcine NAb have been estimated to comprise approximately 20% of all xenoreactive human NAb and are directed against a variety of mostly unknown porcine antigens \cite{160,166}. As suggested by Zhu et al, the majority of human non-Gal anti-porcine NAb is specific for carbohydrate structures carrying terminally linked N-glycolyneuraminic acid. Most animals including pigs and baboons express this antigen, however, humans do not express it and thus may have NAb directed towards this structure. Since baboons do not have anti-N-glycolyneuraminic acid antibodies, it is not possible to study the effect of such antibodies in \textit{in vivo} experiments using pig-to-baboon models \cite{166}. Using flow cytometry we found 13\% non-Gal anti-porcine IgM and 36\% IgG NAb binding to pEC derived from GT-knockout pigs compared to Gal-positive pEC. Moreover, a similar amount of non-Gal anti-porcine NAb was demonstrated in three donors by anti-Gal-depletion with Gal-specific immunoabsorption columns and when using Ig preparations (IVIG) reflecting the antibody repertoire of several hundreds of healthy human donors. However and more importantly, there was a considerable variability of non-Gal anti-porcine NAb between different individuals. The levels of anti-porcine IgM and IgG binding to Gal-deficient pEC as compared to Gal-positive pEC ranged from 3-23\% and 2-100\%, respectively. Furthermore, for unknown reasons, not all individuals have non-Gal anti-porcine IgM and IgG NAb in their serum. This finding highlights the importance of screening for the presence of non-Gal anti-porcine NAb in potential recipients of xenotransplants.

The levels of human anti-porcine NAb were also analyzed on porcine lymphoblastoid and red blood cells. Binding of anti-porcine NAb to pRBC and pEC was comparable, suggesting that these cell types express similar amounts of Gal and non-Gal antigens on their surface. In contrast, levels of NAb binding to pLCL were fairly different as compared to those on pEC and pRBC, indicating differential antigenic patterns. Because Gal expression levels were not different between pEC and pLCL (Fig. 1) and the observation of both high binding
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to pEC together with low binding to pLCL and vice versa, the usage of trypsin to detach pEC from culture plastic ware does not seem to have reduced the surface expression levels of antigens recognized by human anti-porcine NAb. This finding has potentially important implications for functional pretransplant screening assays for future patients awaiting a xenograft. Moreover, the observed different NAb binding profiles to pEC and pLCL also indicate that the antibody crossmatching prior to clinical allotransplantation using human lymphocytes might not be sufficient to detect all alloreactive antibodies. Whereas the majority of HLA-specific antibodies are readily detected, this method misses alloreactive antibodies directed against other antigens. Consequently, new methods need to be introduced to detect alloreactive antibodies, most importantly those directed against non-HLA epitopes expressed on endothelial cells.

In the majority of donors, the levels of non-Gal anti-porcine NAb and complement lysis corresponded fairly well and there was a considerable variability between individual sera in the reduction of complement lysis ranging from 24% to 100%. Thus, complement lysis is caused by both anti-Gal and non-Gal anti-porcine NAb. In line with these findings, recent data have shown that non-Gal anti-porcine antibodies are able to induce endothelial cell activation in vitro and in vivo deposition of xenoreactive antibodies in the transplanted organ [187]. Moreover, infusion of synthetic Gal in pig-to-baboon heart and kidney transplantation models prevented HAR, but acute vascular rejection ascribed to non-Gal anti-porcine antibodies occurred after the initial posttransplant period despite further suppression of anti-Gal antibodies [188,189]. Taken together, these reports and our findings suggest a potentially important role of complement lysis caused by non-Gal anti-porcine antibodies for the pathogenesis of acute vascular rejection of porcine xenografts.

In line with data reported earlier by several laboratories including ours [60,105,119,167] human anti-pig ADCC against Gal-positive pEC was observed using human sera and NK cells obtained from several donors. However, only two out of six NK populations tested and eight out of eleven sera were able to induce ADCC. This finding indicates that the respective allogeneic combination of NK cell and serum is important for the induction of ADCC. Lysis of Gal-deficient pEC by ADCC was reduced by 70% as compared to control pEC corresponding approximately to the levels of non-Gal anti-porcine NAb present in the respective serum. In addition, for three donors, anti-Gal-depleted serum was tested for the ability to induce ADCC. The levels of ADCC against PAEC-Gal<sup>+</sup> were similar when using non-depleted and anti-Gal-depleted serum, suggesting that non-Gal anti-porcine NAb are responsible for this lysis (data not shown). In conclusion, NK cell-mediated ADCC
is a potential mechanism of endothelial cell damage in xenotransplantation, but not all donors are able to mount xenoreactive ADCC responses.

Several but not all studies have shown that the levels of anti-porcine IgG NAb depend on the ABO blood group of the serum donor, with lower levels in blood group B and AB sera [181,182]. We could confirm this observation by flow cytometry in a large pool of sera with different ABO blood groups. Moreover, in contrast to previous reports, we also found significantly lower levels of anti-porcine IgM NAb in blood group B and AB sera as compared to blood group A and O, which was also reflected by a decrease in the agglutination capacity and complement lysis of Gal-positive pEC by these sera. The reason for this observation remains unclear, however, in contrast to the A antigen the structure of the B antigen differs only by one fucosylation site from Gal. An association of anti-Gal and anti-A/B antibodies was recently also described in human ABO-incompatible allotransplantation [190]. The immune response elicited by A or B antigens on ABO-incompatible kidney allografts resulted in the activation of anti-Gal B cells producing anti-Gal antibodies.

In conclusion, the present study shows that complement lysis and ADCC due to non-Gal anti-porcine NAb still represent a potentially relevant immunological hurdle for xenotransplantation. Considerable inter-individual differences in the levels of non-Gal anti-porcine NAb as well as complement lysis and ADCC indicate the need for individual testing of non-Gal anti-porcine NAb using quantitative and functional methods for potential recipients of xenotransplants. Some human transplant candidates might even lack any anti-porcine NAb.

5.3.6. Acknowledgments

The authors thank Katja Matozan for performing absorption and ELISA experiments, Rolf Dubs for nephelometry data, Jörg Halter for the IVIG preparations, the local blood bank for providing human sera and the Zurich slaughterhouse for porcine blood samples. Ian Cummings is recognized for technical assistance and Hans U. Lutz for helpful discussions, technical assistance and critical reading of the manuscript.
5.4. PART IV

HLA-E expression on porcine cells: protection from human NK cytotoxicity depends on peptide loading

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5.4.1 Abstract

Human NK cells lyse porcine cells and may play an important role in the cell-mediated rejection of pig-to-human xenografts. Lysis is probably a consequence of the failure of human MHC-specific killer inhibitory receptors to recognize porcine MHC class I molecules. A majority of activated human NK cells express the HLA-E-specific inhibitory receptor CD94/NKG2A. The aim of this study was therefore to test the hypothesis that stable surface expression of HLA-E on porcine cells protects against xenogeneic NK-mediated cytotoxicity. Porcine lymphoblastoid (13271) and endothelial (pEC) cell lines were transfected with constructs coding for HLA-E together with the leader sequence of HLA-B7 or -A2. HLA-E was correctly expressed on 13271 cells while pEC required peptide-pulsing and/or IFN-γ stimulation to express the HLA-E complex on the cell surface. HLA-E-expressing porcine cells were partially protected from lysis mediated by human polyclonal NK populations and completely protected from killing by NKG2A^bright^ NK clones. In conclusion, the capability of different porcine cell types to express HLA-E on the cell surface can differ considerably depending decisively on the availability of peptides. These findings are important for the applicability of transgenic HLA-E expression as an approach to protect porcine tissues from human NK cytotoxicity.

5.4.2. Introduction

NK cells have the potential to lyse cells with downregulated or altered MHC class I expression, leading to the elimination of transformed and virally infected cells [22]. The role of NK cells in allogeneic and xenogeneic immune responses, however, needs further evaluation. A variety of molecules that deliver either activating or inhibitory signals regulate NK cell activity [23-25]. After binding to potential target cells several receptor-ligand interactions between molecules on the surface of NK and target cells occur. Interactions between natural cytotoxicity receptors (NCR) expressed on NK cells and their corresponding ligands on target cells activate the killing machinery of NK cells. To avoid lysis of “healthy cells” expressing low levels of NCR ligands, NK cells express different families of inhibitory receptors that are specific for MHC class I molecules. This "missing self" theory was first put forward by Ljunggren and Kärre in 1990 and later confirmed by the discovery of several NK receptor families [23,27]. One of these NK receptors, the CD94/NKG2 heterodimer, is widely
expressed on human NK cells and binds HLA-E, a monomorphic MHC class I molecule expressed at weak levels on most tissues [191-194].

Two alleles (HLA-E*0103 and *0101) have been described for HLA-E. The single difference at amino acid position 107 results in a higher cell surface expression level of HLA-E*0103 as compared to HLA-E*0101 [195]. HLA-E is expressed on the surface as a trimeric complex constituted by HLA-E, β2-microglobulin (β2m), and a peptide derived from the leader sequence of some MHC class I molecules [196], from the CMV protein UL40, and from heat shock proteins (hsp60) [197-199]. The signal sequence of HLA-E itself, as well as those from several HLA-A and -B gene products, generate peptides that are not loaded on HLA-E. Consequently, the surface expression of HLA-E but also the binding affinity to CD94/NKG2 dimers and the resistance to NK-mediated lysis depend on the nature of the peptide [200]. The HLA-B7-derived leader peptide shows the strongest effect regarding surface expression followed by the HLA-A2 and -G-derived peptide. Some MHC-derived peptides (HLA-B15, -Cw0402, -Cw7) stabilize the cell surface expression of both alleles but do not confer resistance to NK lysis [195,197,201,202]. Individual members of the HLA-E-specific NKG2 receptor family with activating (NKG2-C, -E, -H) or inhibitory (NKG2-A, -B) features [192] form disulfide heterodimers with the invariant CD94 chain. The activating CD94/NKG2C receptor binds to combinations such as the complex between the HLA-G-derived peptide and HLA-E with at least 10-fold lower affinity than to the inhibitory CD94/NKG2A receptor [202-204].

The notion that NK cells might play a role in the rejection of pig-to-human xenografts is based on findings in rodent models of both concordant and discordant xenotransplantation, on human NK cell infiltration in pig organs perfused with human blood ex vivo and on NK cells found in rejected porcine hearts transplanted into baboons [109,111,113,114,116,118,205]. Human NK cells have further been reported to lyse porcine target cells in vitro, especially after activation and in the presence of human serum containing xenoreactive natural antibodies [133-135]. This susceptibility of pEC to human NK cytotoxicity may be explained by the failure of swine leukocyte antigen (SLA) class I molecules to interact with human NK inhibitory receptors. In line with this view, we previously demonstrated that transgenic expression of HLA-Cw3 and HLA-G on pEC provides partial protection from human NK cytotoxicity [133,134,206]. However, polymorphic HLA-C expressed on pig cells triggers allore cognition by T cells, which can be prevented by genetical modification of HLA-C [206,207]. Conversely, the wide expression of HLA-E-specific inhibitory receptors of the CD94/NKG2 family on IL-2-activated NK cells
proposes HLA-E as an attractive candidate to prevent NK cell-mediated damage in xenotransplants by transgenic expression. Although this concept has been reported earlier by others, conflicting results were obtained [208,209]. Additional studies are needed to resolve this discrepancy and to understand the potential of HLA-E to protect porcine cells from human NK cytotoxicity.

The aim of the present study was therefore to stably express HLA-E on the surface of porcine endothelial and lymphoblastoid cells, to test whether the expression of HLA-E depends on peptide loading, and to determine the protective effect of HLA-E expression on human xenogeneic NK cytotoxicity.

5.4.3. Materials and Methods

Cells

The bone marrow-derived microvascular 2A2 and the aortic PEDSV.15 (PED) pEC lines were described previously [153]. The immortalized porcine lymphoblastoid cell line 13271.10 (13271) was a kind gift of G. Waneck (Massachusetts General Hospital, Boston, MA) [184,207]. The human 721.221-AEH cell line stably transfected with an HLA-E/A2 hybrid gene where the promoter and first exon of HLA-A2 are fused to intron 1 of HLA-E*0101 was described previously [210]. The isolation of NK cells from PBMC of healthy donors and the generation of polyclonal and monoclonal NK cells was reported in a previous paper [134]. The human NK cell line NK92 was obtained from American Type Culture Collection (Rockville, MD).

Transfection

The HLA-E*01033 gene (HLA-E/B7) and the HLA-E*01033 cDNA (HLA-E/A2) in which sequences corresponding to the first exon (coding for the leader peptide) were replaced by the equivalent exons of HLA-B7 and HLA-A2, were used for transfection and designated E/B7 and E/A2, respectively [211]. E/B7-expression was driven by the genomic HLA-E promoter while the CMV promoter (pcDNA3) was used for E/A2. 2A2-E/B7 and PED-E/B7 cells were obtained by calcium phosphate transfection [134] with the HLA-E/B7 expressing construct. PED-E/B7 cells were superperfected with human β2m cDNA and selected using hygromycin B at a concentration of 0.1 mg/ml (Life Technologies, Gaithersburg, MD). Porcine 13271-E/B7 and 13271-E/A2 transfectants were generated by electroporation (Gene Pulser, BioRad Laboratories) using standard procedures (250V, 975 μF in PBS) and selected by adding 0.2
mg/ml hygromycin B or 1 mg/ml G418. After antibiotic selection stably transfected cells expressing HLA-E were further purified by flow cytometry sorting or by limiting dilution cloning.

**Immunostaining and flow cytometry**

Surface expression of MHC class I on transfected cells was analyzed by indirect immunofluorescence using secondary FITC-conjugated goat anti-mouse Ab (Boehringer Mannheim, Indianapolis, IN) on a FACScan (Becton Dickinson, Basel, Switzerland). Cells were resuspended at $5 \times 10^5$ per tube in staining buffer (PBS, 0.1% BSA) and incubated for 30 min at 4°C with saturating concentrations of the following mouse mAb: DX17 (anti-HLA class I, IgG1, L. Lanier, University of California, San Francisco, CA), W6/32 (anti-HLA class I, IgG2a, Sigma, Buchs, Switzerland), 3D12 (anti-HLA-E, IgG1, D. Geraghty, Fred Hutchinson Cancer Research Center, Seattle, WA), 2.27.3A (anti-SLA class I, IgG2a, J.S. Arn, Harvard Medical School, Boston, MA), and B1G6 (anti-human β2m, IgG2a, Immunotech, Marseille, France). The 3D12 mAb recognizes both HLA-E free chain and complex [210], while pan HLA class I-reactive mAb (W6/32 and DX17) recognize a conformational epitope dependent on association between heavy chain, β2m, and peptide [212]. Phenotypic analysis of NK cells was carried out by immunofluorescence using FITC-UCHT1 (anti-CD3), PE-B73.1 (anti-CD16), and PE-B159 (anti-CD56) mAb (BD Pharmingen, San Diego, CA), HP-3B1 (anti-CD94, IgG2a) and Z199 (anti-NKG2A-B/CD94, IgG2a) mAb (Beckman Coulter, Nyon, Switzerland) and DX22 mAb (anti-CD94, IgG1, L. Lanier). Irrelevant isotype-matched mAb were used as controls and propidium iodide gating to exclude dead cells in all experiments. The percentage of NKG2A- or CD94-positive NK cells in PBMC was determined by direct, tricolor immunofluorescence using N-901 (anti-CD56) and 3G8 (anti-CD16), both PC-5-conjugated, PE-labelled Z199 (anti-NKG2A) or DX22 (anti-CD94), and FITC-labelled UCHT1 (anti-CD3), purchased from Beckman-Coulter. NKG2A and CD94 analysis was done on CD3<sup>-</sup>CD56<sup>+</sup>-gated cells. The geometric mean fluorescence intensity (MFI) ratios (MFIR) were calculated by dividing the MFI of staining with the Ab of interest by the MFI of the control Ab.

**Cytotoxicity assays**

The cytotoxicity of polyclonal human NK cells and clones against 13271, PED, and 2A2 cells and their transfecteds was tested in 4 h $^{51}$Cr-release assays in serum-free AIM-V medium as described previously [134]. To perform antibody blocking experiments porcine cells and NK
cells were preincubated with 20 μg/ml of the F(ab')2 fragment of DX17, and with HP-3B1, DX22, and Z199 mAb or an isotype control mAb for 30 min at 4°C. Antibodies were also present in the culture medium during the cytotoxicity assay at a concentration of 10 μg/ml.

**Peptide loading of HLA-E molecules on porcine cells**

Porcine cells expressing HLA-E/B7 were cultured at 26°C for 16 h in DMEM medium supplemented with 10% FCS in the presence of the following synthetic peptides solubilized in H2O-10% DMSO at a concentration of 100 μM: VMAPRTVLL (B7 peptide) and RRIKEIVKK (control peptide). Both HPLC-purified peptides were obtained from Bachem (Bubendorf, Switzerland). Following the incubation with B7 peptide at 26°C, the cells were used for analysis of surface expression by flow cytometry or as targets in cytotoxicity assays.

**5.4.4. Results**

**Analysis of HLA-E expression on transfected porcine cells**

The expression of HLA-E depends on loading with peptides derived from the leader sequence of specific HLA class I proteins. As shown by flow cytometry using both anti-HLA-E and pan anti-HLA class I mAb, HLA-E was efficiently expressed on the surface of stably transfected 13271-E/A2 and 13271-E/B7 cells (Figure 1A and B). Neither untransfected (data not shown) nor mock-transfected 13271 (Figure 1C) were stained by anti-HLA mAb (W6/32, DX17, 3D12) indicating that these mAb do not cross-react with porcine surface molecules. As positive control, HLA class I-negative human 721.221 cells transfected with HLA-E/A2 were positively stained by all anti-HLA mAb (data not shown). Two pEC lines, stably transfected either with HLA-E/B7 alone (2A2-E/B7) or in combination with human β2m (PED-E/B7), were also stained by 3D12, but surprisingly, not by W6/32 and DX17 (Figure 2A). We next tested whether porcine IFN-γ stimulation and peptide pulsing was able to improve HLA-E surface expression. Stimulation of gene expression in PED-E/B7 cells by IFN-γ induced a 5-fold increase of surface HLA-E expression as detected by 3D12 staining, whereas W6/32 and DX17 binding was still low (Figure 2B). IFN-γ-stimulated PED-E/B7 cells expressed HLA-E with an MFIR of 13.4 ± 2 (n=4) when stained with 3D12, while W6/32 and DX17 stained very weakly. The failure of DX17 and W6/32 to bind HLA-E on PED-E/B7 cells suggested that these cells do not assemble the trimeric complexes on the cell surface. We speculated that processing and loading of the endogenously produced B7 peptide within HLA-E-transfected
pEC might be impaired. Consequently, IFN-γ-stimulated PED-E/B7 cells were pulsed with the B7 peptide. As shown in Figure 2B, HLA-E expression was readily detectable by anti-HLA class I mAb W6/32 and DX17 after peptide pulsing. When 2A2-E/B7 cells were stimulated with porcine IFN-γ HLA-E expression became detectable with W6/32 and DX17, and exogenous peptide pulsing further enhanced HLA-E surface expression. HLA-E expression on PED-E/B7 cells pulsed with a control peptide was unchanged (data not shown) indicating that an external source of HLA-E-specific peptide enhances and/or stabilizes HLA-E surface expression on pEC.

**FIGURE 1:** Cell surface expression of HLA-E on transfected porcine lymphoblastoid cells. 13271 cells were transfected with (A) HLA-E/A2, (B) HLA-E/B7, or (C) the empty vector. Cells were analyzed by indirect immunofluorescence using pan anti-HLA class I (W6/32 and DX17) and HLA-E-specific (3D12) mAb (filled histograms). Histograms for an isotype-matched control antibody are also depicted (empty histograms). Numbers indicate the MFI of HLA-E expression and are representative of three independent experiments.

**HLA-E protects porcine lymphoblastoid cells from xenogeneic human NK cytotoxicity**

Cytotoxicity mediated by polyclonal human NK cells against 13271-E/A2 and 13271-E/B7 cells was partially reduced compared to the efficient lysis of untransfected 13271 cells (Figure 3A). The percentage of inhibition of NK cytotoxicity against 13271-E/A2 and 13271-E/B7 cells varied between 27% and 62% (mean: 44 ± 14%), and 14% and 36% (mean: 24 ± 10%), respectively (Table 1). NK cytotoxicity against HLA-E-transfected 13271 cells in the presence of blocking anti-HLA class I, anti-CD94, and anti-NKG2A mAb was consistently higher than that of untreated or control mAb-treated target cells (Figure 3B and C). Thus, the
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restoration of NK cytotoxicity by blocking mAb demonstrated a direct inhibitory effect of HLA-E mediated by CD94/NKG2A.

![Graph showing cell surface expression of HLA-E](image)

**FIGURE 2:** Cell surface expression of HLA-E on transfected porcine endothelial cells. (A) Untransfected (empty histograms) and HLA-E-transfected PED-E/B7 and 2A2-E/B7 cells (filled histograms) in the absence of IFN-γ stimulation and peptide pulsing. (B) PED-E/B7 and 2A2-E/B7 cells incubated with porcine IFN-γ in the absence (solid lines) or presence (dotted lines) of synthetic B7 peptide. Staining of untransfected PED and 2A2 cells was used as control (dashed lines). Numbers indicate the MFI of HLA-E expression and are representative of four independent experiments.

<table>
<thead>
<tr>
<th>NK Donor</th>
<th>13271-E/A2</th>
<th>13271-E/B7</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH</td>
<td>62 ± 5 %</td>
<td>36 ± 15 %</td>
</tr>
<tr>
<td>MS</td>
<td>53 ± 22 %</td>
<td>32 ± 15 %</td>
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<tr>
<td>PF</td>
<td>38 ± 10 %</td>
<td>22 ± 15 %</td>
</tr>
<tr>
<td>GS</td>
<td>39 ± 12 %</td>
<td>15 ± 15 %</td>
</tr>
<tr>
<td>RM</td>
<td>27 ± 18 %</td>
<td>14 ± 15 %</td>
</tr>
</tbody>
</table>

Table 1: Percentage of inhibition of human NK cytotoxicity against HLA-E-transfected porcine lymphoblastoid cells. The percentage of inhibition of NK lysis against 13271-E/A2 and 13271-E/B7 cells ± standard deviation, as compared to the lysis of the respective intraassay control target 13271 cells was calculated from three independent experiments at four different E:T ratios (40:1 to 5:1).
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FIGURE 3: HLA-E expression on porcine lymphoblastoid cells inhibits xenogeneic human NK cytotoxicity. (A) NK cytotoxicity of two donors (upper panel: KH; lower panel: MS) against 13271 cells transfected with HLA-E/A2 (left column) and HLA-E/B7 (right column) compared to the lysis of untransfected 13271 cells. Results are depicted as percentage of specific lysis and were obtained at four different E:T ratios. Data are representative of three independent experiments. Open triangles represent HLA-E-transfected 13271 cells, filled diamonds represent untransfected 13271 cells. (B) Increase of NK cytotoxicity against 13271-E/A2 cells in the presence of purified F(ab')2 fragments of anti-HLA class I mAb DX17 as compared to treatment with isotype control mAb (open triangles). (C) Restoration of NK cytotoxicity against 13271-E/A2 cells (right panel) in the presence of anti-CD94 (grey bar) or anti-NKG2A mAb (white bar), but not with isotype control mAb (black bar). The presence of anti-CD94 and control mAb did not change the lysis of untransfected 13271 cells (left panel).

The presence of the HLA-E complex on the surface of porcine endothelial cells inhibits human NK cytotoxicity

Lysis of PED-E/B7 and untransfected PED cells by IL-2-activated human NK cells was equivalent while lysis of human 721.221 cells was inhibited by the expression of HLA-E (Figure 4A). NK cytotoxicity was also analyzed in more detail using a panel of NK clones with defined NK receptor repertoires. In line with the failure of pan anti-HLA class I mAb to recognize HLA-E expression on PED-E/B7 cells, xenogeneic killing mediated by CD94-positive NK clones against PED-E/B7 cells was not inhibited (Figure 4B). Next we tested whether IFN-γ stimulation and external pulsing with synthetic B7 peptide provided protection from NK cytotoxicity via enhancement and/or stabilization of HLA-E surface expression on transfected PED-E/B7 cells. The low level of HLA-E on IFN-γ-stimulated PED-E/B7 cells was able to provide moderate protection (32 ± 12%, n=5) (Figure 5A). This finding indicated
FIGURE 4: Incomplete HLA-E expression on porcine endothelial cells does not inhibit human NK cytotoxicity. (A) NK cytotoxicity of two donors (left panel: PF; right panel: ER) against PED-E/B7 and PED (mean of four E:T ratios, 40:1 to 5:1). Lysis of PED-E/B7 was not reduced compared to PED, whereas HLA-E-transfected human 721.221 cells were protected from NK lysis under the same conditions. (B) No difference in lysis mediated by five different CD94-positive NK clones derived from donor CM against PED-E/B7 and PED.

that small amounts of trimeric HLA-E on the surface of potential target cells are sufficient to partially protect them from NK cytotoxicity. The additional increase of HLA-E expression on IFN-γ-stimulated PED-E/B7 induced by peptide pulsing (Figure 2B) further improved the resistance of these pig cells to human NK-mediated lysis (47 ± 7% protection). Pre-incubation of NK cells with anti-CD94, but not with control anti-CD56 mAb reversed the protection of B7 peptide-pulsed PED-E/B7 cells demonstrating a direct effect of HLA-E/CD94 interactions (Figure 5B). In contrast, lysis of untransfected PED cells was not changed by incubation of NK cells with anti-CD94 (data not shown). In parallel to the results obtained with PED-E/B7 cells, expression of HLA-E on IFN-γ-stimulated 2A2-E/B7 cells provided partial protection (52%) from human NK cytotoxicity (data not shown). Since it has been argued that human NK cells may be inhibited by porcine SLA class I molecules, although weakly [213], we also tested the levels of SLA class I expression on HLA-E-transfected pEC pulsed or not with B7 peptides. No differences in SLA class I expression were found (data not shown) and therefore, we conclude that the observed protection from NK lysis upon peptide pulsing was not related to changes in SLA class I surface levels.

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FIGURE 5: IFN-γ stimulation and peptide pulsing of PED-E/B7 provides partial protection from human polyclonal NK lysis. (A) NK cytotoxicity of donor PF and the cell line NK92 against PED (∆) and PED-E/B7 incubated in the presence (●) or absence (■) of B7 peptide. Target cells were incubated with porcine IFN-γ for 2 days prior to the cytotoxicity assay. NK cytotoxicity was partially inhibited following IFN-γ stimulation and peptide pulsing. (B) HLA-E-mediated partial protection of PED-E/B7 from human NK cytotoxicity was reversed upon blocking of CD94 on NK cells. IFN-γ-stimulated PED-E/B7 pulsed (right panel) or not (left panel) with exogenous B7 peptide were used as targets in 51Cr-release assays with PF effector NK cells. Blocking with anti-CD94 mAb (∆) increased NK cytotoxicity, while anti-CD56 (Δ) or isotype-control mAb (●) had no effect. All experiments were performed at four different E:T ratios and are representative of at least three independent experiments.

Correlation between NKG2A expression and protection from xenogeneic NK cytotoxicity by HLA-E expression

The failure of HLA-E to protect porcine cells completely from lysis mediated by polyclonal human NK cell populations may be explained by a heterogeneous expression pattern of the NKG2A receptor on NK cells. To answer this hypothesis, the frequency of NKG2A on polyclonal NK cells isolated from healthy donors was measured by tricolor flow cytometry analysis. NKG2A was present on the cell surface of 53 ± 17% (range: 24 - 73%, n=8) of freshly isolated NK cells. Activation of NK cells with IL-2 during in vitro culture induced the expression of NKG2A on a larger percentage of cells (mean: 88 ± 4%, range: 82 - 91%, n=8) as well as an increase of the level of NKG2A expression as compared to naïve NK cells (MFIR 42 ± 17 versus 20 ± 6). The percentage of CD94-positive NK cells increased only slightly after IL-2-induced activation (from 80 ± 9% to 90 ± 7%). The presence of NKG2A-negative subpopulations in polyclonal NK cells might well explain the observed partial inhibition of NK cytotoxicity by HLA-E. Therefore, we generated a panel of NK clones from two donors and determined both CD94/NKG2A surface expression as well as cytotoxicity against IFN-γ-stimulated 2A2-E/B7 cells exhibiting substantial HLA-E surface expression.
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(Figure 2B). Regarding NKG2A expression three groups of NK clones were identified showing either bright (MFIR 48 ± 14, n=5, group I), low (MFIR 9 ± 4, n=2, group II), or missing expression (MFIR ≤ 1; n=2, group III). The level of NKG2A expression on a given NK clone correlated well with the potential of HLA-E to inhibit cytotoxicity (Figure 6A-C). Xenogeneic NK cytotoxicity was either completely (group I) or partially (group II) inhibited by the expression of HLA-E while NKG2A-negative NK clones (group III) lysed 2A2-E/B7 target cells to the same extent as untransfected 2A2 cells. The protection of 2A2-E/B7 cells from cytotoxicity mediated by NKG2A bright NK clones was partially reversed in the presence of blocking anti-NKG2A mAb but not in the presence of a control mAb (Figure 6D). Parallel results were obtained when 13271-E/A2 cells were used as targets (data not shown).

![FIGURE 6: NKG2A bright NK clones are completely inhibited by the expression of HLA-E on porcine endothelial cells. Cytotoxicity of NK clones against IFN-γ-stimulated 2A2 and 2A2-E/B7 depends on NKG2A expression. (A) Lysis of 2A2-E/B7 (D) mediated by a NKG2A bright NK clone was completely inhibited, while (B) lysis mediated by a NKG2A low NK clone was only partially inhibited compared to control 2A2 targets (A). (C) Cytotoxicity of a NKG2A low NK clone against 2A2 cells was unaffected by HLA-E expression. (D) HLA-E-mediated protection of 2A2-E/B7 from human NK cytotoxicity mediated by an NKG2A bright NK clone was partially restored upon blocking of NKG2A on NK cells. NK cytotoxicity against 2A2-E/B7 (open symbols) was almost absent compared to the lysis of control 2A2 (▲). Blocking of NKG2A (▲) increased the lysis significantly while an isotype control mAb had no effect (□). All experiments were performed at four different E:T ratios and are representative of two independent experiments.]

5.4.5. Discussion

The potential of HLA-E to protect porcine cells from human NK cytotoxicity was tested in previous studies with conflicting results [208,209]. In line with our results, Matsunami et al reported negligible HLA-E expression on pEC stably transfected with genomic HLA-E along with the human β2m gene. Surface expression was markedly increased in the presence of
exogenous HLA-A2-derived leader peptides (MFIR 12.9). Low HLA-E expression levels were found after transfection with modified gene constructs containing the leader peptide of HLA-A2 or HLA-G1. Unfortunately, the potential of HLA-E expression to inhibit NK cytotoxicity cannot be estimated accurately from this study since PBMC were used as effector cells achieving very low percentages of target cell lysis. In contrast, transient expression of HLA-E on pEC were reported by Sasaki et al at very low levels in the absence of an endogenous or exogenous source of HLA class I-derived peptides [209]. HLA-E provided partial protection against human NK cytotoxicity via CD94 interactions. It is difficult to explain the discrepant results, but a different pig cell line, potentially expressing endogenous HLA-E-binding peptides, and different NK purification methods were used in the latter study. In agreement with Matsunami et al, our data clearly demonstrate that HLA class I-derived peptide supply is crucial for the correct formation of trimeric HLA-E on the surface of pEC in order to provide stable surface expression and consistent protection from human NK cytotoxicity by HLA-E. Taken together, our observations suggest that pEC which can easily express HLA class I molecules from other loci may downregulate HLA-E gene expression post-translationally, e.g. by limiting peptide supply. Therefore, protection from human NK cytotoxicity will be difficult to achieve using standard HLA-E-transgenic pigs as organ source. The usage of single chain trimer constructs consisting of HLA-E, human ß2m, and an HLA-derived signal peptide might circumvent the observed insufficient HLA-E expression levels [214].

What is the reason for the observed differential expression pattern between HLA-E-transfected 13271 cells and pEC? The observed lack of correct HLA-E surface expression on pEC was a consistent finding using two different endothelial cell lines and after several independent rounds of transfection. Theoretically, the origin of ß2m, i.e. human, porcine (intracellular pool) or bovine (FCS-derived), could be important during the formation of trimeric HLA-E [215]. However, HLA-E-transfected 13271 cells and pEC were both cultured in the presence of FCS and superfection of PED-E/B7 with human ß2m did not increase HLA-E surface expression levels (data not shown). Stimulation of PED-E/B7 with porcine IFN-γ and pulsing with exogenous B7 peptide both stabilized surface HLA-E expression. IFN-γ might increase the synthesis of HLA-B7 leader peptides and consequently the expression of trimeric HLA-E [216]. Alternatively, HLA-E expression augmented following IFN-γ stimulation by loading with peptides derived from porcine proteins dependent on IFN-γ induction, improved cleavage of the signal peptide, or increased TAP or proteasome activity [217]. In contrast to pEC, expression of trimeric HLA-E was detected on lymphoblastoid
13271 cells without IFN-γ stimulation or peptide pulsing, suggesting a cell type-specific effect. However, since only three cell lines were tested an assertion of a tissue-specific phenomenon cannot be made. The observed difference in HLA-E expression between pEC and 13271 was not addressed experimentally, therefore we can only speculate on potential explanations. The monoclonal anti-HLA-E Ab used, 3D12, may bind a complex composed of HLA-E, β2m, and an endothelial-specific porcine peptide on HLA-E-transfected pEC that is not recognized by W6/32 or DX17 due to an altered conformation [218]. Alternatively, the lack of HLA-E surface expression on transfected pEC could result from dissociation of β2m and/or weakly bound peptides from HLA-E chains in transfected pEC leaving only monomeric heavy chains on the surface. Finally, the intracellular pathways involved in the processing of HLA-B7 leader peptides may not be efficient in pEC resulting in a modest production of the nonameric B7 peptide [210,219-221].

HLA-E-transfected porcine cells with detectable surface expression by flow cytometry using W6/32 were partially protected from lysis mediated by human polyclonal NK cell populations. Blocking studies demonstrated a direct effect of interactions between HLA-E expressed on transfected porcine cells and the inhibitory receptor CD94/NKG2A on human NK cells. Complete protection from killing was only observed when NKG2A bright NK clones were used. The lack of complete protection from polyclonal NK populations is therefore most likely explained by the presence of NKG2A-negative effector subpopulations. We also assume that for NK cells with low NKG2A expression inhibitory HLA-E-specific signals are not sufficiently strong to override triggering signals delivered by activating receptors. The apparent heterogeneity of NKG2A expression levels on different NK cell effector populations may further explain the discrepant reports on the effect of HLA-E expression on pEC to prevent human NK cytotoxicity [208,209]. Nevertheless, this study confirms the principle that porcine cells can be protected partially from human NK cytotoxicity if sufficient levels of trimeric HLA-E are expressed on their surface. Since vascularized xenografts from HLA-E transgenic pigs will not be protected completely from human NK cytotoxicity, co-expression of HLA-E with other HLA class I genes on pig cells such as HLA-G or modified HLA-C could have synergistic effects, but remains to be evaluated [133,206,207]. Moreover, the observed HLA-E-mediated protection of porcine lymphoblastoid cells from human NK cytotoxicity indicates a possible use in hematopoietic stem cell xenotransplantation, which has been proposed as a method to induce xenogeneic tolerance [222].

In conclusion, human NK cytotoxicity was partially inhibited by the expression of HLA-E on porcine cells. The capacity of different porcine cell types to load the appropriate
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peptide and express HLA-E in the correct conformation varied considerably. Although the potential of HLA-E to protect porcine cells from human NK-mediated lysis and rejection should be further explored in animal models, a combination with other approaches such as targeting the pathways that activate NK cells seems to be necessary to completely overcome NK-mediated xenograft rejection.

5.4.6. Acknowledgments

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6. General Discussion

Xenotransplantation has the potential to offer a solution to the chronic shortage of human organs available for transplantation medicine. Considerable progress in the field of xenotransplantation has been made in the last years. However, significant immunological barriers still remain and have to be resolved before clinical xenotransplantation can become a reality. The understanding of xenogeneic immune responses progressed significantly with the discovery that Gal is the primary target of human NAb and that complement activation leads to HAR of xenografts. Therefore, the recent generation of pigs lacking Gal expression was a hallmark in the history of xenotransplantation and a breakthrough in overcoming HAR. The first data on xenotransplants performed with homozygous α1,3GT-knockout pigs were published earlier this year. Kuwaki et al. transplanted pig hearts heterotopically into immunosuppressed baboons [86]. The mean survival of six successfully transplanted xenografts was 92 days (median 73 days), with one heart surviving for 179 days. Thrombotic microangiopathy occurred in several xenografted hearts, a problem which needs to be resolved in order to achieve long-term xenograft survival. The exact causes of these forms of graft failure are still not well understood, but may involve Ab directed against targets other than Gal, molecular incompatibilities of coagulation regulation, and possibly cellular innate immune responses including those mediated by NK cells [223]. Yamada et al. transplanted Gal-deficient pig kidneys in combination with thymus into baboons receiving a regimen aimed at tolerance induction [87]. Life-supporting kidney function with normal creatinine levels was achieved up to 83 days. These two studies demonstrate that the usage of organs derived from Gal-deficient pigs has achieved considerable progress in terms of graft survival, but perhaps below expectation. However, in conclusion, HAR is no longer considered as a major problem in xenograft rejection.

One major aim of this thesis was to investigate the influence of Gal deficiency and the role of remaining non-Gal epitopes on the cytotoxicity mediated by human NAb and NK cells. The availability of primary pEC from Gal-deficient pigs enabled us to study the role of non-Gal anti-porcine NAb and NK cells in an optimal in vitro model, while earlier reports were based on more artificial models using Gal-blocking agents or in vitro modifications of the α1,3GT gene. First results of pig-to-baboon heart transplantations using organs of Gal-deficient pigs show that, despite prevention of HAR, acute humoral xenograft rejection still occurred in five out of eight transplanted organs [86]. This type of humoral rejection is potentially associated
with the presence of non-Gal and/or induced xenoreactive anti-porcine Ab. Using primary pEC of Gal-deficient pigs we showed that both complement lysis and ADCC may also be induced by non-Gal anti-porcine NAb [172,183]. The finding that there is considerable variability of non-Gal anti-porcine NAb between different individuals highlights the importance of screening for the presence of NAb in potential recipients of xenotransplants.

In accordance with these findings, recent data showed that non-Gal anti-porcine Ab are able to induce pEC activation in vitro and deposition of xenoreactive Ab in transplanted organs in vivo [187]. Moreover, infusion of synthetic Gal in pig-to-baboon heart and kidney transplantation models prevented HAR, but AVR, ascribed to non-Gal anti-porcine Ab, occurred after the initial post-transplant period despite further suppression of anti-Gal Ab [188,189]. This indicates an important role of complement lysis caused by non-Gal anti-porcine Ab for the pathogenesis of AVR of porcine xenografts. Future studies aiming at avoiding AVR should therefore include the identification of porcine targets recognized by human non-Gal anti-porcine Ab. Such targets may be carbohydrates, proteins, lipids, or any combination of these structures. Identification of the exact epitopes could be achieved using cell lysates of Gal-deficient pEC in Western blots together with purified non-Gal anti-porcine Ab, as well as screening of potential carbohydrate targets using DNA expression libraries. The identification of these targets might be especially important in pig-to-human xenotransplantation, since humans, in contrast to baboons, have higher titers of non-Gal anti-porcine Ab. In addition, induced Ab of non-Gal specificity may arise following xenotransplantation and the understanding of such elicited anti-porcine Ab responses could be important to prevent AVR.

The question whether carbohydrates, in addition to proteins, can act as NK-cell target molecules is a matter of debate [108,151,152,170,171,224]. Our data provide evidence that Gal is not a major target molecule for NK cells and, therefore, the use of organs from Gal-deficient pigs will not circumvent the problem of direct NK-mediated xenograft rejection. The lack of congruence with other studies may be explained by differences in the experimental protocols, in particular the indirect methods to remove or block Gal that were used in earlier reports. In our initial studies, when using in vitro-knocked out pEC, we could not conclusively rule out whether Gal plays a role in the adhesion of human leukocytes to pEC because of the decreased expression of VCAM-1 on these pEC. However, subsequent studies showed that ex vivo primary pEC, from Gal-negative and Gal-positive pigs, with equal
adhesion molecule expression, mediated similar adhesion of human total PBMC, PMN, and purified NK cells. Thus, other strategies will be necessary to prevent human leukocyte adhesion and NK cytotoxicity.

Since direct NK cytotoxicity was not affected by the lack of Gal expression, a further aim was to investigate the protective potential of HLA-E expression on pEC. As shown earlier, HLA class I molecules have the potential to partially protect pEC from human NK cytotoxicity [133-135,206,207,225]. The wide expression of HLA-E-specific inhibitory receptors of the CD94/NKG2 family on IL-2-activated NK cells proposes HLA-E as an attractive candidate for transgenic expression to prevent NK-mediated damage in xenotransplants. The capability of HLA-E to protect porcine cells from human NK cytotoxicity was tested in previous studies with conflicting results [208,209]. Initially, we were confronted with problems regarding the induction of HLA-E surface expression on pEC. In agreement with other studies and in contrast to the use of classical HLA class I alleles, we demonstrated that HLA class I-derived peptide supply is crucial to provide stable expression of HLA-E on the surface of pEC. However, we found that human NK cytotoxicity was only partially inhibited by the expression of HLA-E on porcine cells. Complete protection from NK cytotoxicity was only observed when NKG2A$^{bright}$ NK clones were used as effector cells. Whether the co-expression of HLA-E with other HLA class I molecules on pig cells could have synergistic effects on preventing human NK cytotoxicity remains to be evaluated.

Taken together, these studies indicate that further approaches, such as targeting the pathways that activate NK cells, seem to be necessary to completely overcome xenogeneic NK cytotoxicity. Therefore, identifying the activating human NK receptors such as NKG2D and NCR and their corresponding porcine ligands involved in xenogeneic NK-cell responses might be crucial. The hypothesis behind these studies is that, in the absence of triggering signals mediated through activating NK receptors, human NK-mediated rejection of porcine xenografts will be surmounted. Our unpublished data show that triggering of human NKG2D by porcine ligands contributes significantly to xenogeneic NK cytotoxicity. Porcine NKG2D ligands are unknown, however, comparison of porcine and human genomic sequences suggests the presence of one porcine ULBP gene and one MIC-A/MIC-B-like gene in the pig [226,227]. Cloning and determination of the role of these two porcine genes and their products to induce xenogeneic NK cytotoxicity mediated by NKG2D will allow us to consequently develop preventive strategies. However, blocking of NKG2D signaling does not
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completely inhibit human NK cytotoxicity against pEC. Additional blocking of NKp44 is also crucial to achieve complete protection. So far, neither the human nor the porcine genes coding for putative NKp44 ligands are known. In summary, the identification of porcine ligands for NKG2D and NKp44 may allow the design of strategies to overcome cytotoxicity of human NK cells against porcine cells in a highly specific manner.

In conclusion, based on the data of this thesis, it must be stressed that only a combination of approaches, addressing both antibody- and NK-cell-mediated mechanisms of xenograft rejection, may eventually facilitate clinical pig-to-human xenotransplantation. Additional important aspects about the future perspectives of xenotransplantation are discussed in the next chapter.
7. Future Perspectives

The recent generation of pigs lacking Gal expression was a hallmark in the history of xenotransplantation and a breakthrough in overcoming HAR. Two recent studies demonstrate that the usage of organs derived from Gal-deficient pigs has achieved considerable progress in terms of graft survival and, therefore, HAR is no longer considered a major problem in xenograft rejection. However, despite heavy immunosuppression, long-term xenograft survival was not achieved and thrombotic microangiopathy occurred as a new obstacle. One attempt to overcome remaining rejection processes is the induction of immunological tolerance, which can be divided into peripheral and central tolerance. In general, peripheral tolerance is achieved by induction of anergy or deletion of peripheral T cells. In contrast, central tolerance results from intrathymic clonal deletion of T cells. Mixed chimerism has been proposed as an approach leading to central tolerance [228]. Therefore, bone marrow or purified hematopoietic stem cells are transferred from the potential organ donor to the recipient prior to organ transplantation. Supposedly, the presentation of foreign xenogeneic antigens in the donor thymus leads to a clonal T-cell depletion in the thymus and, therefore, to tolerance. However, this method includes a partial myeloablative regimen and heavy immunosuppressive treatment in order to prevent rejection of the hematopoietic stem cell transplants, as well as graft-versus-host disease. Thus, the induction of chimerism still harbors a major risk for the recipient. More than 10 years ago, it was reported that induction of complete hematopoietic chimerism with a full myeloablative therapy and bone marrow transplantation in humans provided tolerance to subsequently implanted renal allografts of the same donor with no requirement of immunosuppression [229]. However, the infectious and graft-versus-host disease-derived morbidity associated with the myeloablative conditioning precluded the routine application of this approach for the induction of tolerance for patients undergoing solid organ transplantation. In recent years, improvements regarding the minimal requirements for inducing a mixed chimerism have once more moved this approach of tolerance induction towards clinical application. Treatment of recipients pre- and post-transplant with depleting doses of anti-CD4 and anti-CD8 mAb together with cyclosporine A led to an induction of mixed chimerism (reviewed in [230]). This conditioning regimen does not ablate the recipient's hematopoietic system and is hence referred to as non-myeloablative. Clearly, a state of mixed chimerism, in which the presence of donor-derived elements induces tolerance while host type antigen-presenting cells maintain normal immunocompetence, would be preferable. As shown by Sachs and colleagues, the induction of central tolerance in
mice, pigs and monkeys receiving combined kidney/bone marrow transplants without immunosuppression resulted in long-term survival of fully mismatched allogeneic grafts (reviewed in [231]). However, pig hematopoietic chimerism in baboons was never achieved. In another approach, aiming at central tolerance, thymokidney grafts were used. Thereby, implantation of autologous thymic tissue beneath the donor's renal capsule before transplantation induced allogeneic transplantation tolerance across fully MHC-mismatched barriers in pigs, and did also induce tolerance in xenogeneic models [232].

Besides the general problem of xenograft rejection, a major concern in xenotransplantation is the potential of xenozoonosis, i.e. the transmission of pathogens or infectious diseases from animals to humans [233]. Theoretically, viral diseases which are of limited pathogenicity in primates, might cause lethal infections in humans. Pigs do not carry known replicating and therefore potentially infectious retroviruses, however, there are several endogenous retroviral sequences in the pig genome. Endogenous retroviruses are integrated in the genome of all organisms and are harmless in their hosts. In vitro studies have shown that certain porcine endogenous retroviruses (PERV) can infect human cells at a low efficiency and that these PERV are also able to recombine with human viral elements [234]. In addition, SCID mice became infected with PERV after pig-islet transplantation [235,236]. These findings suggest that xenotransplantation might lead to the incorporation of porcine endogenous retroviral sequences into the genome of human recipients. However, no PERV infection was detected in more than 100 patients exposed to porcine tissue [237-241]. In addition, inbred miniature pigs that appear unable to transmit retroviruses to human cells have recently been identified [240]. Nevertheless, the porcine endogenous retroviral sequence may theoretically recombine with human endogenous retroviruses or proviruses, resulting in the generation of new viruses that are both pathogenic and infectious for human beings. Most investigators believe that the risk of generating new pathogens by xenotransplantation procedures is extremely low compared to the significant and identifiable risks associated with allotransplantation. In future clinical trials, the recipients of xenotransplants must be monitored closely in order to immediately detect possible xenozoonosis and to prevent spreading to the general population, as prescribed by the current Swiss law. Chiang et al. generated a specific mAb against the gag protein of PERV [242]. This antibody detects PERV produced by cells in vitro by immunocytochemistry and flow cytometry, and, therefore, may be useful for the development of immunoassays to monitor PERV infection in xenotransplant recipients. Moreover, Shah et al. developed a highly sensitive PCR method for optimal detection of PERV [243]. Other
viruses, including porcine cytomegalovirus, can be removed by pathogen-free breeding of pigs and, thus, should not set any limitations for xenotransplantation [244].

Another important issue to address is the ethics of xenotransplantation [245]. The most evident question is whether humans are allowed to use animals for transplantation at all. However, pigs have been bred for a long time to serve as a food supply for humans, and the part of the community accepting this fact will most likely also approve the use of pig organs for treatment of human diseases. Moreover, the use of pig organs is also accepted by the three major monotheistic religions, Christianity, Judaism, and Islam (reviewed in [245]). Knowledge about the general opinion on xenotransplantation is essential and public perception must always be considered in an overall risk/benefit analysis [246]. Furthermore, clinical trials of xenotransplantation must be scientifically sound and associated with significant expectation of benefit to the transplant recipient. In general, there must be adequate pre-clinical nonhuman primate data to support successful xenotransplantation.

Finally, besides all concerns about potential rejection mechanisms and infection risks, one important remaining question is whether porcine organs will provide physiological function in the human body. Due to the limited clinical experience of pig-to-human organ xenotransplantation, it is not well known whether pig organs will provide adequate function in humans [247]. Extensive studies on pig organ transplantation to nonhuman primates have been performed, and in this context, pig hearts and kidneys have been shown to function well [248,249]. Pig islets transplanted into humans provided short-term, but not long-term function. Xenogeneic livers are believed to function less efficiently due to their inability to meet the metabolic demands of the recipient [250]. The baboon is considered the most reliable nonhuman primate animal model, since baboons reject transplanted pig organs in a hyperacute fashion similar to humans [251]. However, the limited comparability of baboons and human beings must be taken into consideration. In general, even though pig organs would prove to function less well as human organs, they may be useful for short-term transplantation in patients waiting for a suitable human organ.
8. Appendix

8.1. References

Appendix


Appendix


208. Matsunami K, Miyagawa S, Nakai R, Yamada M, Shirakura R. Modulation of the leader peptide sequence of the HLA-E gene up-regulates its expression and down-
Appendix


Appendix


### 8.2. List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1,3GT</td>
<td>α(1,3)-galactosyltransferase</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cell-mediated cytotoxicity</td>
</tr>
<tr>
<td>AVR</td>
<td>Acute vascular rejection</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BS-IB4</td>
<td>Bandeiraea simplicifolia isolectin B4</td>
</tr>
<tr>
<td>C</td>
<td>Complement</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>E:T ratio</td>
<td>Effector-to-target ratio</td>
</tr>
<tr>
<td>Gal</td>
<td>Galactose-α(1,3)galactose</td>
</tr>
<tr>
<td>HAR</td>
<td>Hyperacute rejection</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ILT</td>
<td>Ig-like transcript</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activating motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibitory motif</td>
</tr>
<tr>
<td>KIR</td>
<td>Killer-cell Ig-like receptor</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LFA</td>
<td>Lymphocyte function associated antigen</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MFIR</td>
<td>Geometric mean fluorescence intensity ratio</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIC</td>
<td>MHC class I chain-related protein</td>
</tr>
<tr>
<td>NAb</td>
<td>Natural antibody</td>
</tr>
<tr>
<td>NCR</td>
<td>Natural cytotoxicity receptor</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>PAEC</td>
<td>Porcine aortic endothelial cell</td>
</tr>
<tr>
<td>pEC</td>
<td>Porcine endothelial cell</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PECAM</td>
<td>Platelet/endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PERV</td>
<td>Porcine endogenous retrovirus</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
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<tr>
<td>PMN</td>
<td>Polymorphonuclear neutrophil</td>
</tr>
<tr>
<td>PoLCL</td>
<td>Porcine lymphoblastoid cell</td>
</tr>
<tr>
<td>PoRBC</td>
<td>Porcine red blood cell</td>
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<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SLA</td>
<td>Swine leukocyte antigen</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>ULBP</td>
<td>UL16-binding protein</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VLA</td>
<td>Very late antigen</td>
</tr>
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</table>
8.3. Curriculum Vitae

Name
Bettina Charlotte Baumann

Date of Birth
December 25, 1976

Place of Birth
Zurich, Switzerland

Nationality
Swiss

Citizenship
Zurich

Civil Status
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Education and Work Experience

06/2001 - 05/2005
University Hospital Zurich and ETH Zurich
PhD student in the group of Dr. Jörg Seebach, Laboratory for Transplantation Immunology, under the direction of Prof. Hans Hengartner (ETH Zurich)

09/1999 - 06/2000
University Hospital Zurich and ETH Zurich
Diploma thesis in the group of Prof. Rolf Stahel, Laboratory for Medical Oncology, under the direction of Prof. Kaspar Winterhalter (ETH Zurich)

10/1996 - 04/2001
ETH Zurich
Studies for the degree of “Master of Science” in Biology

02/1996 - 06/1996
Union Bank of Switzerland, Zurich
Temporary job in “Securities Administration”

08/1989 - 01/1996
Kantonsschule Hohe Promenade Zurich
Matura Type B

04/1983 - 07/1989
Primary school in Zurich
### Training and Courses

<table>
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<tr>
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<tr>
<td>10/03 - 12/03</td>
<td>Course: “Basic Scientific Writing”</td>
<td>Center of Medical Research, University Hospital Zurich</td>
</tr>
<tr>
<td>06/03</td>
<td>Workshop: “Presentation - Publication - Communication”</td>
<td>Didactics Center ETH Zurich (3 days)</td>
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### Awards and Grants

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<tr>
<th>Year</th>
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<tr>
<td>2005</td>
<td>Travel Award, 8th International Xenotransplantation Congress, Gothenburg, Sweden</td>
</tr>
<tr>
<td>2004</td>
<td>1st Prize of the Swiss Transplantation Society, Interlaken, Switzerland, for the Scientific Work entitled “Lack of Galα(1-3)Gal expression on porcine endothelial cells prevents complement-induced lysis but not direct xenogeneic NK cytotoxicity”, Journal of Immunology, 2004. (Swiss Transplant Research Award)</td>
</tr>
<tr>
<td>2004</td>
<td>Research Grant from the University of Zurich (1 year) (&quot;Xenotransplantation: Prevention of Antibody- and Cell-Mediated Immune Responses&quot;)</td>
</tr>
<tr>
<td>2004</td>
<td>Young Investigator's Travel Award, 8th Annual Meeting of the Society for Natural Immunity and 20th International Natural Killer Cell Workshop, Noordwijkerhout, The Netherlands</td>
</tr>
<tr>
<td>2002</td>
<td>Young Investigator's Travel Award, 7th Annual Meeting of the Society for Natural Immunity and 19th International Natural Killer Cell Workshop, Puerto Rico</td>
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</table>
Appendix

Publications


Manuscripts in preparation


Forte P, **Baumann BC**, Seebach JD. HLA-C gene expression on porcine cells to overcome xenogeneic human anti-pig NK responses.

Forte P, Lilienfeld BG, **Baumann BC**, Seebach JD. Human NK cytotoxicity against porcine cells is triggered by NKp44 and NKG2D. *(submitted)*
Appendix

Presentations at Scientific Meetings

**Oral presentation** at the 17\(^{th}\) meeting of the Swiss Immunology PhD students in Ermatingen, Switzerland (2005).

**Poster presentation** at the 4\(^{th}\) Day of Clinical Research, University Hospital Zurich, Switzerland (2005).

**Poster presentation** at the annual SSAI * meeting in Bern, Switzerland (2005).

**Oral presentation** at the 3\(^{rd}\) Annual Meeting of the Swiss Transplantation Society, Interlaken, Switzerland (2005).

**Poster presentation** at the 8\(^{th}\) Annual Meeting of the Society of Natural Immunity and 20\(^{th}\) International Natural Killer Cell Workshop, Noordwijkerhout, The Netherlands (2004).

**Poster presentation** at the annual SSAI * meeting in Geneva, Switzerland (2004).

**Oral presentation** at the 16\(^{th}\) meeting of the Swiss Immunology PhD students in Ermatingen, Switzerland (2004).

**Oral presentation** at the 3\(^{rd}\) Day of Clinical Research, University Hospital Zurich, Switzerland (2004).

**Oral presentation** at the 1\(^{st}\) Xenotransplantation Research Meeting in Bern, Switzerland (2004).

**Oral presentation** at the 7\(^{th}\) International Xenotransplantation Association Congress in Glasgow, Scotland (2003).

**Poster presentation** at the 2\(^{nd}\) Day of Clinical Research, University Hospital Zurich, Switzerland (2003).

**Oral presentation** at the 15\(^{th}\) meeting of the Swiss Immunology PhD students in Ermatingen, Switzerland (2003)

**Poster presentation** at the annual SSAI * meeting in St. Gallen, Switzerland (2003).

**Poster presentation** at the 7\(^{th}\) Annual Meeting of the Society of Natural Immunity and 19\(^{th}\) International Natural Killer Cell Workshop, San Juan, Puerto Rico (2002).

**Poster presentation** at the 1\(^{st}\) Day of Clinical Research, University Hospital Zurich, Switzerland (2002).

**Poster presentation** at the annual SSAI * meeting in Lugano, Switzerland (2002).

* SSAI, Swiss Society of Allergology and Immunology
8.4. Acknowledgments

I wish to express my sincere gratitude to everyone who has helped, supported and encouraged me during these years. I am especially grateful to:

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Katja Huggel, for all the help on performing experiments for the natural antibody study.

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