Chapter 16

Xenotransplantation

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Additional information is available at the end of the chapter

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Abstract

Xenotransplantation, defined as the transfer of cells, tissues or organs between species, has been a subject of significant interest for decades as a response to the increasing demand for biological materials to treat patients. In this review, the history and recent progress in xenotransplantation research will be discussed, including the immunological challenges that need to be overcome and the molecular biological methods which are required to allow the complex genome engineering to meet the critical need for organs.

Keywords: xenotransplantation, immune rejection, swine, transgene, gene editing, gene knockout, tolerance

1. Introduction

Over the course of the past 100 years, the rapid progress in drug development and surgical techniques has created a paradox for the area of transplantation medicine. Surgical protocols have become more successful and medicines have overcome many mechanisms of chronic rejection and allowed increased survival of transplant patients. However, the number of organs available for transplant has remained essentially constant. In addition, not all organs available through donation are viable for transplant. Organs such as lung, which are more prone to damage due to trauma, disease or deterioration, are available in drastically reduced numbers compared with heart or kidneys. Therefore, while there is an increasing number of patients who would survive and thrive long-term after organ transplantation, the limited number of organs available means a smaller percentage of eligible patients can actually undergo transplant surgery.

A hard truth about human organ donation is that even with exponential increases in donor numbers, it is unlikely that the organ shortage would be relieved. The diversity of the human species, paired with the efficiency of the immune system, significantly reduces the chances that a given organ will be compatible with the patients in greatest need. Although immunosuppressive drugs can enhance length of survival, chronic rejection remains a risk the greater the mismatch between organ and patient. Furthermore, the donor geographic proximity, organ size and timing of availability of a compatible organ with a matching patient may be limiting. Thus, even as human organ donation continues to be optimized, there remains an immense need for additional organs above and beyond the availability of human donors.

In order to address the above concerns and provide sufficient numbers of compatible organs, a number of approaches, both biological and mechanical, are being actively explored. Use of animal organs provides solutions to the challenges of availability and function. Multiple mammalian species possess organs which may substitute effectively for their human analogs and, in the case of agricultural species, can be rapidly bred in sufficient numbers to overcome organ shortages. Through use of controlled facilities, production of animals can be regulated and disease exposure eliminated. Additionally, careful breeding schedules can provide organs of appropriate size for any given patient on a predictable schedule for optimal timing of surgery. Finally, recent advancements in DNA sequencing and assembly and genome engineering technologies, paired with the advanced understanding of the cellular and molecular immunology responses in transplant rejection, allow the creation of animals which could provide an unlimited supply of rejection-free organs.

2. Early beginnings

Examples of xenotransplantation can be found recorded as early as the seventeenth century, in which the transfusion of blood from animals into human patients was described [1]. In the eighteenth century, more complex tissues such as skin were tested as grafts in human patients [2]. In 1905, Princeteau transferred rabbit kidney sections into a child with immediate positive results, however, after 16 days the child died of pulmonary complications [3]. Soon thereafter, two kidney xenotransplants were attempted, with one patient receiving an organ from goat, the other from pig. Unlike Princeteau's experiment, neither organs functioned and both apparently failed due to thrombosis [4]. Similarly, an attempt by Unger in 1910 to transplant kidneys from a chimpanzee into humans led to failure due to thrombosis in about a day [5]. In 1923, Neuhof transplanted a kidney from a lamb into a human patient, allowing the patient to survive 9 days [6].

In the early twentieth century, an odd offshoot of xenotransplantation was created due to interest in "rejuvenation" via transplant of animal testis in human males, as demonstrated by Voronoff in Russia [7] and Brinkley in the US [8] using chimpanzee or goat testis, respectively. So popular was the use of goat testis in the US, an entire radio empire was built around advertising the services, with many patients claiming enhanced fertility and sexual function [8].

The field of immunology developed in parallel with surgical approaches to xenotransplantation. As the mechanisms of immune rejection were better defined, the enormity of the challenges facing transplant of organs between members of the same species were recognized.

During and after WWII, pharmaceutical companies created a series of increasingly effective immunosuppressive drugs which could inhibit some rejection responses, renewing interest in xenotransplantation.

3. First attempts at human xenotransplantation with primate organs

During 1963–1964, Reemstma carried out a series of transplants into 13 human patients using chimpanzee kidneys, with one patient surviving 9 months after transplant surgery [9]. The need for these experiments was driven in part by the desperate human organ shortage and lack of alternatives. Cadaveric organs often proved insufficient in quality, and volunteer human kidney donation, high risk at the time, was untenable for ethical and legal reasons. Although chronic dialysis had been demonstrated by the early 1960s, it was not widely available for patient treatment [10]. Therefore, despite the risks, xenotransplantation was considered a potentially viable solution.

Reemstma was not alone in exploring xenotransplantation as a means to overcome critical organ shortages. Hume attempted transplanting a chimpanzee kidney into a human, but the organ failed to show renal function [11]. Hardy and team focused on heart, observing survival for only 2 hours after transplanting a chimpanzee heart into a human patient [12]. Starzl carried out a series of transplants in human patients with baboon kidney [13] and livers, with variable results [14]. These seminal attempts at xenotransplantation showed that although surgical techniques and immunosuppressive drug treatments had greatly improved, they were insufficient to address the multitude of challenges in overcoming the xenorejection response. Indeed, it was nearly a generation later before Bailey used a baboon heart for transplantation into an infant, who survived several weeks after receiving the organ [15].

4. A shift in species

Although the close evolutionary relationship between non-human primates and humans would suggest an advantage in using chimpanzee or baboon organs for xenotransplantation, clinical, practical and ethical considerations prevent them from being a viable option. Non-human primate organs do indeed function almost identically to human organs, but are subject to a variety of diseases which are readily transmissible to humans [16]. Given the relatively fragile state of patients receiving multiple immunosuppressive drugs, the risk of primate zoonoses is too great. In addition, chimpanzees, baboons and many other non-human primates are impractical for large scale breeding. The low numbers of progeny of non-human primates limits the production of large numbers of animals by natural breeding or *in vitro* fertilization compared with agricultural species. Finally, use of non-human primates as organ donors faces insurmountable ethical barriers.

A much more viable approach is the use of pig organs for xenotransplantation. Porcine organs are structurally and physiologically close to humans, and therefore can functionally substitute for analogous human organ functions. Unlike non-human primates, pigs are more evolutionarily distant from humans and thus have a greatly reduced risk of transmission of diseases to human patients, which can be essentially eliminated through genetic manipulation [17].

Husbandry techniques for pigs are extremely well-understood, with large litter sizes and rapid cycle times, allowing production of populations that could overcome organ shortages in a much shorter timeframe than possible with non-human primates. Furthermore, a suite of genome engineering technologies is available for use in pigs to make critical changes to enhance survival and function of the pig organ, while avoiding the human immune rejection response. In fact, the complex engineering approaches now available may actually provide organs with advantages over even closely-matched human organs.

5. Current status and challenges for xenotransplantation

A variety of academic, clinical and industrial institutions have made substantial progress in recent years in the understanding of the molecular mechanisms of the xenorejection response and the genetic modification of pigs to overcome these mechanisms [18]. Professional organizations are working with the FDA to develop guidelines for clinical use [19], with several groups indicating their intention to initiate clinical trials in the near term with porcine organs [20].

For xenotransplantation to become a viable routine human therapeutic option, there are a number of challenges that still need to be overcome. These challenges fall into two broad categories; the biology of the xenorejection responses and the engineering technologies needed to restructure the porcine genome to overcome these responses.

6. The immune system and rejection

The immune system is an evolutionarily ancient collection of structures, mechanisms and cells that detect and eliminate harmful organisms from the host. In older texts, the immune system is often described as distinguishing "self" from "non-self," but more recent research demonstrates that there are a variety of roles for the microbiome ("non-self" micro-organisms resident within, on or around the host) in maintaining the health of the host organism. Thus, the host immune system must be able to not only identify and eliminate harmful pathogens, it must also tolerate the presence of a variety of beneficial bacteria, fungi and yeast [21]. Because transplantation of cells, tissues and organs is an unnatural situation created through deliberate medical intervention, the human immune response uses incredible precision to identify even closely related human cells as "non-self" and efficiently removing them, a process referred to as "immune rejection." In general, the strength of the response is proportional to the degree of difference between the host and donor materials, therefore, when exposed to materials from an animal, the rejection response is much faster and stronger, increasing the challenge in controlling the immune response.

7. Xenotransplant rejection

Xenorejection is a much more exaggerated and rapid form of the allorejection response. Four overlapping and inter-related reactions occur temporally; hyperacute rejection (HAR), acute vascular rejection/acute humoral xenograft rejection (AVR/AHXR), acute cellular rejection (ACR) and chronic rejection (CR) (**Figure 1**) [22]. Although these processes can be

characterized as distinct stages based on histological and clinical data, each is due to highly interconnected pathways and mechanisms that are challenging to separate. In fact, these responses were defined as pathologic observations prior to development of more detailed analyses of the molecular immunology mechanisms.

HAR is primarily due to immediate binding of pre-existing host natural antibodies specific for xenoantigens expressed by the donor tissues. Antibody binding can activate the endothelial cells, causing the release of immune activators, as well as inducing the complement-mediated destruction of the endothelial layer, reducing the barrier function and allowing host cells to infiltrate the organ. Cell debris released by the damage to the endothelium and products of the complement cascade also stimulate coagulation and the innate inflammatory response. These pathways synergize during rejection to create stronger responses that are more pathogenic and can be less amenable to control.

AVR/AHXR, like HAR, is also mediated by host antibodies. However, instead of pre-existing natural antibodies, AVR/AHXR is often the result of humoral responses which lead to production of antigen-specific antibodies. The AVR/AHXR is delayed due to the time it takes to induce

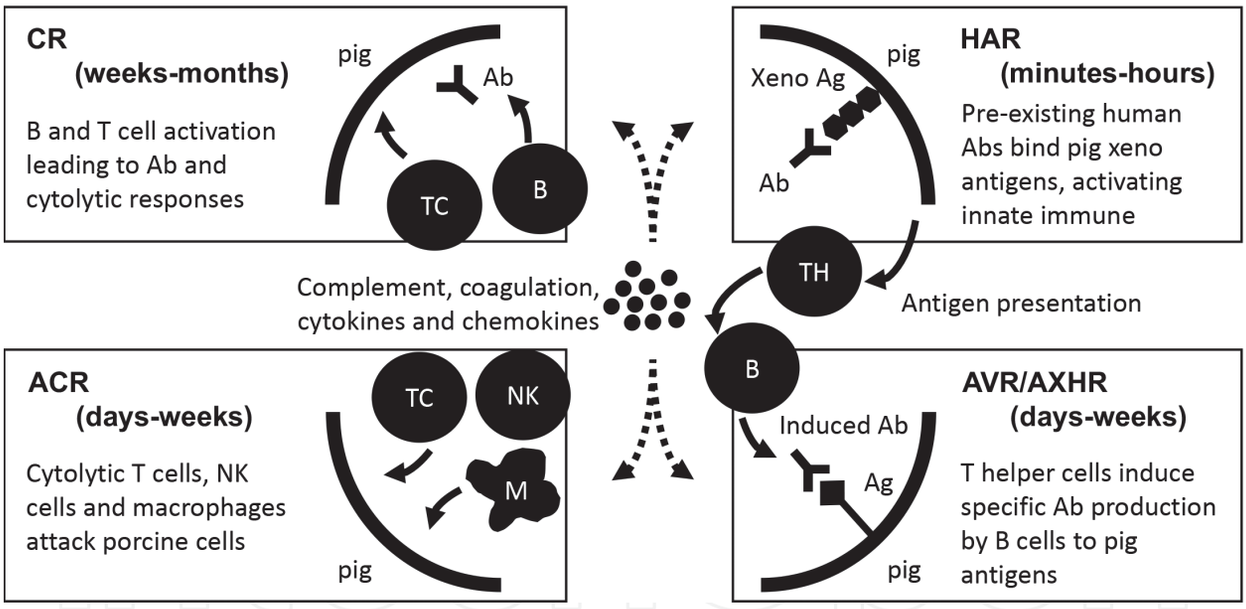

Figure 1. Overview of the xeno-rejection response. The human immune response to xeno-organs initiates within minutes to hours with hyperacute rejection (HAR, upper right), in which pre-existing antibodies (Ab) in human serum bind to xenoantigens (xeno Ag) on the surface of the pig cells. This results in cell destruction and presentation of porcine antigens to human helper (TH) and cytolytic (TC) T cells, as well as groups of pro-inflammatory and pro-immunity cytokines and other soluble mediators. Activation of human helper T cells (TH) stimulates human B cells (B) over the course of days to weeks, resulting in production of induced antibodies (induced Ab) as part of the acute vascular rejection/acute humoral xenograft rejection (AVR/AHXR, lower right) response. These secondary antibodies are often more specific and higher affinity than the pre-existing human serum antibodies, and also cause cellular destruction of the xenograft. in parallel with AVR/AHXR, the acute cellular rejection response (ACR, lower left) is carried out by human NK cells (NK), macrophages (M) and the xenograft-specific cytolytic T cells (TC) recruited to the xeno-organ within days to weeks. The activated cells express a variety of molecules to attack the porcine cells, as well as secrete additional cytokines to recruit more human immune cells. After weeks to months, the human immune response may be again induced to react to the xeno-organ during chronic rejection (CR, upper left), leading to specific antibody (Ab) responses from B cells (B) or cytolytic T cell (TC) destruction. A large collection of cytokines, chemokines, complement and coagulation factors (center) play a key role in regulating the complex set of reactions occurring in every aspect of rejection.

an adaptive immune response via germinal center reactions, typically days to weeks. Much like natural antibodies, the induced antibodies recognize components of the xeno-organ and, similar to HAR cause activation of the endothelial cells and their destruction via the complement system. The specific antibody binding also attracts multiple elements of the cellular immune system, such as NK cells and phagocytes, creating further damage of the target tissues and secreting soluble factors, such as cytokines and chemokines, which further enhance immune responses.

ACR includes predominantly cellular responses to the graft, such as T cell activation, which occur within days to weeks of organ transplant. Although ACR is well-established in allotransplant, the importance of ACR in the xenorejection response is not entirely clear. This may be due to either the more rapid activation of hematopoietic populations in HAR and AVR/AHXR compared with allotransplantation. However, some groups have proposed that reduction of the HAR and AVR/AHXR in earlier stages of xenorejection would unmask ACR which would otherwise be unnoticed amidst the earlier more pathogenic responses. In either case, ACR is expected to be substantially similar between allo- and xenotransplantation and thus more readily controlled by immunosuppressive drugs already in use for allotransplant.

CR is longer term, occurring within months or even years after transplantation. CR can be due to complications due to other immune activity, such as infection, or escape of humoral or cellular responses from immunosuppressive drug control. CR is well-understood in allotransplant and effective treatments are available for control and reversal of CR.

HAR and AVR/AHXR are the most unique and most critical to address in xenotransplantation. These earlier reactions can greatly enhance later reactions, with some of the mechanistic elements of the xenorejection response initiated even before the transplant surgery itself occurs. Therefore, it is essential to control the initiating events as early as possible in order to reduce the course of later responses. Much like the layers of an onion, removing one layer reveals the next, but as each layer is removed the overall size may be diminished.

The latter two responses, ACR and CR, are mechanistically similar between xeno- and allorejection responses [23]. Use of currently-available immunosuppressive drugs are believed to be able to control both responses as evidenced by the extensive data from allotransplants in humans. However, the speed and violence of the HAR and AVR reactions against xeno-organs can greatly accelerate and strengthen ACR and CR. Thus, even well-established treatments for allorejection may need to be reviewed as xenotransplantation proceeds toward clinical trials.

8. Innate and adaptive immunity in xenotransplantation

The immune system has two inter-related arms; the innate and the adaptive immune systems, both of which contribute to the rejection of xenotransplanted cells, tissues and organs. Although often described as separate, the systems have a large network of connections which are interdependent, and thus are not completely distinct. Both systems utilize multiple mechanisms to protect the host, creating a series of defense layers of increasing specificity. When functioning properly, a given layer may not be 100% efficient, but in aggregate will capture the overwhelming majority of pathogens. In addition, the ability to detect subtle differences between highly

related cells has the potential benefit of identifying and eliminating cells with oncogenic mutations, preventing tumors before they have a chance to establish themselves [21].

8.1. The innate immune system and xenorejection

The innate immune system is evolutionarily ancient, with related mechanisms found in both plants and animals. The innate immune system consists of relatively invariant mechanisms for the identification of pathogens, and, although less specific, is extremely rapid and strong in response. The rapidity of the innate immune system provides an immediate barrier to pathogen infiltration and infection of the host, limiting the pathogen burden and giving the adaptive immune system time to develop more specific responses [24].

The use of physical barriers is one of the most critical elements of innate immunity. Although organ transplant bypasses the skin as a protective layer, the endothelium of the blood vessels, which connect the organ to the host circulatory system, remains as the main interface between the human hematopoietic system and xeno-organ tissues. As such, many of the immediate mechanisms of the innate response are greatly influenced by the interactions between the human immune cells and the porcine endothelial cells. Once the human innate immune system attacks the porcine endothelium, the barrier function is quickly lost, followed by rapid influx of human immune cells, pro-inflammatory infiltrates and edema, and then necrosis and destruction of the xeno-organ. It is important to note that the endothelium is an extremely active part of the immune response, which responds to soluble factor and cellular interactions to induce a variety of immune and inflammatory responses. Therefore, any efforts to improve the engraftment of xeno-organs must take into account the functional role of the endothelium in regulating the rejection response [25].

8.2. Inflammation

Inflammation is one of the earliest innate responses, driven by pattern recognition receptors found on human immune cells which recognize damage-associated molecular patterns (DAMPs). The binding and signaling of DAMPs causes the immediate secretion of proinflammatory mediators, such as cytokines and chemokines, which attract additional innate immune cells and induce a variety of local responses which would be highly beneficial during an infection but destructive to xeno-organs. For example, vasodilation and increased vascular permeability, which would normally allow host immune cells greater access to tissue to rapidly eliminate pathogens, instead causes the xeno-tissue to be more quickly infiltrated by human innate immune cells, which in turn leads to more inflammation and destruction. Similarly, there are blood-borne proteinaceous biochemical cascades activated by inflammation, such as the coagulation and the complement systems, which further degrade xeno-organ function and survival [26].

8.3. Xenoantigens

The genes encoded by the porcine genome can encode proteins that are substantially different from their human counterparts or may carry post-translational modifications which are not present in humans. Interestingly, some of these molecules, referred to as “xenoantigens”,

are recognized by pre-existing natural antibodies found in human serum. One subset of these antigens is the swine leukocyte antigens (SLA), which are the physical and functional equivalent of the human leukocyte antigens (HLA). Much like the case for human allotransplant, the SLA genes are highly diverse and individual patients will have a variable level of cross-reactive antibodies in their serum for a given set of SLA genes [27]. A separate group of xeno-antigens are glycan molecules, such as Gal alpha (1,3) Gal and Neu5Gc, which are expressed in porcine, but not human, cells [28].

Although specific induced antibodies are produced by B cells as part of the adaptive response, the presence of pre-existing antibodies in human serum contributes to the innate response. The specific reasons for the existence of these human natural antibodies are not entirely clear. In the case of glycan structures, one hypothesis is that the molecules are related to those found in pathogens, and that the natural antibodies are cross-reactive to each. Alternately, consumption of porcine materials in the human diet may induce antibody formation. Regardless of the specific source in human serum, xenotransplantion of porcine cells and tissues in humans leads to binding of these pre-existing natural antibodies, activation of complement and eventual destruction of target cells carrying the xenoantigens.

Several approaches have been taken to address xenoantigens, including cross-matching donors and recipients for reduced immunoreactivity, removal or modification of the xenoantigen from the donor pig, or the reduction of the ability of the antibodies to induce the complement cascade. In the first case, typing of patients and porcine donors to find the best matches would be very similar to the current system used for determining allotranplant cross-reactivity [29]. Use of gene targeting or editing technologies can eliminate the genes encoding SLA or the enzymes required for expression of the relevant glycan. This has been proven to be highly effective for ablating the genes GGTA1 (the gene encoding alpha 1,3-galactosyltransferase essential for Gal alpha (1,3) Gal), CMAH (cytidine monophosphate-N-acetylneuraminic acid hydroxylase critical for Neu5Gc biosynthesis) and B4GALNT2 (beta 1,4 N-acetylgalactosam inyltransferase). In each case, the elimination of the glycan leads to greatly reduced recognition of porcine cells by natural antibodies in human serum, and reduction in complement-mediated destruction [28]. Unfortunately, as the number of antibody targets increases there is a risk that one or more of the xenoantigens alone or in combination may have essential functions which cannot be eliminated without damaging the development or function of the pig. Therefore, efforts to introduce more subtle mutation in SLA which remove immunogenic epitopes while leaving critical antigen-presentation functions intact, or even replacement of SLA with HLA, may be more effective.

The second approach, which is often used in combination with the first, is to reset the threshold at which the complement cascade is activated, making it more difficult for the binding of human natural antibodies to targets on porcine cells to induce the complement cascade. There are a series of "complement regulatory proteins" (CRPs), such as CD46, CD55 and CD59, expressed on the cell surface which prevent complement activation by the inadvertent non-specific binding of human antibody to human cells [30]. By overexpressing one or more of the CRP molecules on the porcine endothelium, the amount of antibody binding required for complement activation is increased, which reduces the amount of antibody-mediated cell destruction due to human natural antibodies [31].

8.4. Coagulation

Inflammation and vascular leakage, due to loss of endothelial barrier function, both induce coagulation, which normally is required to repair localized endothelial damage. In the case of xenotransplantion, the attack of the endothelium is rapidly occurring at multiple sites, therefore, coagulation spreads throughout the blood vessels in the xeno-organ and can overcome the normal control mechanism. The thrombosis produced by the procoagulant environment leads to occlusion of the vessels within the graft, known as thrombotic microangiopathy (TM). The lack of blood flow results in hypoxia and tissue damage and necrosis, further complicating transplant function. The relatively greater amount of endothelial injury and coagulation in xenotransplant therefore creates more frequent and extensive TM and contributes to the more rapid destruction of the graft [32].

In addition to physiological pathways induced by human innate immune responses, there are non-physiological activities caused by mismatches between porcine and human constituents of the coagulation cascade [33]. For example, porcine von Willebrand factor (vWF) has been shown to bind more avidly to the human GP1b receptor and activate human platelets, leading to coagulation and rapid loss of platelets from the circulation [34]. Ongoing efforts seek to engineer porcine vWF to eliminate the inappropriate interactions with GP1b, while maintaining normal coagulative phenotypes. In addition, porcine proteins which provide positive and negative feedback to control the coagulation cascade do not function as efficiently upon the human coagulation targets, leading to dysregulation of the cascade. The targeting the porcine genome to express human regulatory proteins in porcine cells has been shown to help control human coagulation in response to exposure to the modified porcine materials [35].

8.5. Innate immune cells

Macrophages and neutrophils are two of the earliest host cell types to infiltrate xeno-organs. Both cell types are instrumental in the phagocytosis and destruction of pathogens during infection. During a xenorejection response, the damaged porcine cells release a variety of DAMPs which are recognized by the human innate cells, inducing phagocytic functions which further damage the xeno-organ and increasing production of additional proinflammatory and other immune mediators which attract more innate immune cells [36, 37].

Similar to the molecular mismatch described above for vWF and coagulation, macrophages express the SIRPA receptor, which must interact with the surface receptor CD47 to prevent the target cell destruction by the macrophage. Thus, the CD47 receptor expressed on the cell surface binds to SIRPA to instruct the macrophage not to consume the target cell. In the case of porcine CD47, the interaction with human SIRPA appears to be unproductive and cannot inhibit the macrophage activity. Expression of the human form of CD47 in porcine cells has been shown to greatly reduce human macrophage activity directed against the porcine cells [38].

NK cells are functionally analogous to cytolytic T cells, and even share some mechanistic pathways for targeted cell destruction. NK cells express a collection of stimulatory and inhibitory receptors on the cell surface, which engage conserved targets on the surface of target cells. The balance of activation and inhibition via combinatorial signaling determines whether

the NK cells are stimulated to kill or ignore the target cell. The target cell receptors, such as HLA-E, may be perturbed by pathogens or tumorigenesis, which is detected by the NK cells and the target cells eliminated [39].

In the case of xenotransplantation, the porcine cell receptors, although expressed normally, are not sufficiently well-conserved with their human counterparts and thus cannot inhibit NK cell attack. By expressing on porcine cells the human versions of receptors which stimulate the inhibitory receptors on NK cells, the damage may be averted. With careful genetic modification, the normal mechanisms for detection of infection or other dysfunction may be maintained, allowing normal NK functions while eliminating the xeno-specific destruction [40, 41].

8.6. Resolution of innate immune responses

There are a variety of mechanisms used to resolve innate immune reactions. Many of the soluble mediators of innate immunity have extremely short half-lives which allows them to dissipate quickly. In addition, immune receptors become increasingly desensitized to further stimulation during the course of the innate response, reducing reactions. A variety of negative regulators are also produced to further inhibit the innate effectors. All of these mechanisms are in place to prevent over-reaction of the immune system and the destruction that it can cause once the pathogenic threat has been eliminated [42]. In the case of a xenorejection response, however, the "threat" that is recognized comes from every porcine cell and thus the innate response is never fully resolved without intervention. It may be possible to take advantage of these resolution mechanisms to create porcine cells with an enhanced ability to curtail or end a human inflammatory response through careful genetic modification.

8.7. The adaptive immune system and xenorejection

The adaptive immune system is comprised of cellular and antibody components which recognize pathogens and develop highly specific responses, which can increase in specificity and effectiveness over time and exposure. The adaptive immune response also creates immunological "memory" to allow more rapid reactions should similar pathogens be encountered in the future. Because of the time required to develop specific responses, the adaptive immune system generally becomes more critical after the initial innate immune response [21].

8.8. Antigen presentation and T cells

Antigen presentation is a crucial mechanistic part of the adaptive immune response and plays a major role in the decision between immunity and tolerance for a given target. There are two main routes for antigen presentation to the immune system, reflective of the different classes of pathogen antigens, intracellular or extracellular.

Intracellular antigens, either natural cellular proteins or those derived from viral or bacterial infection of cells, are enzymatically cleaved into peptides which bind to the ubiquitously expressed class I human lymphocyte antigens (HLA). The peptide-HLA class I complex is displayed on the cell surface where it can be surveyed by the binding of cytolytic T cells expressing T cell receptors (TCR) and CD8 co-receptors on the T cell surface. Similar to antibodies, TCRs are assembled combinatorially, creating a diversity of specificities for HLA-peptide

complexes, with only a small subset of TCRs binding to a given complex. Should a given CD8 T cell be activated by the HLA-peptide complex, it will express a series of cytolytic molecules which kill the target cell. This system works due to the efficient T cell selection mechanisms applied during T cell development. After initial production of a rearranged TCR, the nascent T cell is tested in the thymus for inappropriate reactivity against cellular antigens. If the T cell survives the selection process, it exits to the body and theoretically will only be activated when it encounters and antigen that does not naturally exist in body, such as a peptide from a pathogenic organism, or a mutant peptide from an oncogenic cell [43].

Extracellular antigens can be any molecule taken up by a cell from its environment and degraded in lysosomes intracellularly. The resulting peptides are then loaded onto HLA class II molecules which, unlike HLA class I, are expressed on only a subset of immune-related cells. The class II HLA-peptide complex is recognized by a different T cell subset expressing TCRs with the co-receptor CD4. The CD4 T cell subset also undergoes thymic selection as observed with CD8 T cells, to eliminate recognition of self-antigens [44]. However, CD4 T cells can be induced to create different phenotypes once they specifically recognize class II HLA-peptide complexes. A large variety of T cell subsets have been described, including production of helper T cells, which participate in the activation of B cells for the production of antigen-specific antibodies, or regulatory T cells, which act to inhibit the immune response [45]. The choice of outcomes is driven by the soluble mediators, such as cytokines, found in the local environment, and the collection of co-receptors expressed on the antigen presenting cells.

HLA itself is a significant direct contributor to rejection responses outside of its role in antigen presentation. As described above, T cells are selected for lack of recognition of self-antigens. This not only includes the recognition of self-peptides bound to HLA molecules, but of the HLA molecules themselves. Normally, T cells bearing TCRs with inappropriately high affinity for binding HLA molecules, even in the absence of peptide, are eliminated early in T cell development. Because the human T cells have not been exposed to, or selected by, the class I or II swine lymphocyte antigens (SLA), a subset of human TCRs will bind to SLA and induce strong T cell activation, regardless of the peptide presented in the SLA [46]. As porcine cells are attacked by the human immune system, donor peptides are efficiently presented by human cells via HLA to human T cells as part of the normal human adaptive response. Conversely, depending upon the organ transplanted, there can also be donor T cells and antigen presenting cells transferred which result in donor immune responses against the host tissues, referred to as graft versus host disease (GvHD) [47]. In all cases, the immune cells are responding normally, but in the setting of xenotransplantation can be extremely pathogenic due to the artificially high concentration of immunogenic targets present.

Because HLA matching is part of organ selection in allotransplantation, a frequent question is whether introduction of human HLA in place of porcine SLA would help overcome rejection. Although SLA ablation may be helpful in averting antibody-dependent damage, this approach does not resolve some of the challenges related to antigen presentation in xenorejection responses [29]. It is true that the human T cell binding directly to pig SLA could be eliminated by substitution of SLA with HLA, however, the HLA genes are highly polymorphic, hence the need to HLA match human patients. This means that for a given patient, a donor pig would need to be engineered to specifically express the HLA homologous to that patient, which would be limiting given the timelines necessary for production and validation

of genetically-modified pigs. In addition, in the normal situation human cells display human peptides in the HLA, the overwhelming majority of which will be conserved between a human donor and recipient, and thus much less likely to induce a response. If pigs were engineered to express human HLA which is perfectly matched to the patient, the donor porcine cells could now be significantly more efficient at displaying porcine peptides to the human immune system and more rapidly induce T cell activation. Therefore, introduction of human HLA in place of porcine SLA may not provide a benefit without additional engineering.

Humans possess a number of pre-existing antibodies specific for porcine antigens which can contribute to the xeno-organ damage during HAR. As the donor tissue is damaged, the antigens are released and presented to T cells as described above, causing the activation of helper T cells. These T cells interact with B cells in lymphoid organs, inducing the activation of any B cells which express antibodies specific for the xeno-antigens. This initiates the germinal center reaction, in which antigen-specific B cells rapidly proliferate and mutate their antibody sequences and are then progressively selected for improved antibody function. The resulting B cells expressing the affinity-matured antibodies exit the germinal center and can differentiate further to plasma cells, which act as factories that can produce extraordinarily high levels of serum antibody [48]. These induced antibodies, like natural antibodies, further amplify AVR/AHXR and contribute to the destruction of the xeno-organ.

The *de novo* production of antibodies can be quite rapid and are a risk for the lifetime of the transplant whether for allo- or xenotransplantation. There are a number of drugs available for the control of B cell reactions. One of the most effective approaches is the depletion of B cells using antibody therapeutics such as Rituxan, specific for the CD20 surface molecule [49]. However, the constitutive ablation of host B cells will create long term immunosuppression and could be prohibitively expensive. Although highly related to CR in allotransplant, the B cell responses in xenotransplant are stronger and more challenging and likely to require more stringent therapeutic control.

9. Immune suppression and tolerance in xenotransplantation

Advancements in understanding of immune mechanisms in immune rejection have elucidated a number of targets and pathways for intervention, and discovered a variety of small molecule and protein therapeutics for the suppression and manipulation of the immune system. However, the restraint of the immune system required to prevent xeno-organ rejection places the patient at significant risk of infections, tumors and other diseases which are preventable by an intact immune system. Therefore, there is a growing interest in the application of immune tolerance mechanisms in the transplant setting.

Immune tolerance is the natural unresponsiveness of the immune system to targets which may otherwise create an immune response. As mentioned previously, there are many mechanisms used by the immune system to identify non-self-antigens to prevent autoimmune diseases. As the body of literature regarding the molecular basis of immune tolerance has grown, interest in testing tolerance mechanisms in xenotransplantation has also increased [50].

Mixed chimerism is one route that has shown significant promise in both allo- and xenotransplant settings. This approach combines the transfer to the recipient of both the organ and hematopoietic cells from the donor. Typically, the patient is pre-treated with radiation or drugs to allow hematopoietic cell engraftment prior to the organ transplant. The combination of hematopoietic cells from host and donor allows cross-tolerance of host immune cells to donor tissue as well as donor immune cells to host tissue. Therefore, the resulting immune system is a combination of the donor and host, or a "mixed chimera," which recognizes the donor organ and host tissue as "self" despite the differences in genetic origin [51].

A further refinement of mixed chimerism includes transplant of donor thymus into the recipient, allowing selection of host T cells via donor antigen presentation [52], suggesting that tolerance is T cell dependent. A large body of evidence points to the role of regulatory T cells (Treg) as a driver of immune tolerance. Treg cells are antigen-specific but upon binding of the specific HLA-peptide complex on antigen-presenting cells will produce a variety of immune inhibiting and tolerogenic factors. The Treg cells may be derived from either thymus selection (central tolerance) or selection in tissues (peripheral tolerance), with central tolerance believed to be more durable, and the conceptual basis for donor thymus transplantation in mixed chimerism [53].

A critical factor in the maintenance of tolerance is the balance between Treg and effector T cells over time. Any imbalance that increases the number of effector T cells can rapidly lead to immune rejection. If indeed the Treg population is the main active component of immune tolerance, then it may be desirable to specifically bolster the numbers of Treg cells transferred to the recipient to more greatly ensure that the balance is biased firmly toward tolerance. A number of groups have established protocols for the generation of Treg cells that are specific for xeno-organs and tissues through *in vitro* selections and expansions [54]. While this has been shown to have positive effects in allograft tolerance, the durability is variable and, worse, some studies have described conversion of Treg to effector T cells which then contribute to rejection [55]. Despite these concerns, mixed chimerism, with or without Treg supplementation, remains a potentially valuable approach to immune tolerance.

10. Genome engineering to improve xenotransplantation

The progress of xenotransplantation research in recent times has closely paralleled the advancement in genome engineering technologies. As the complexity of the engineering toolsets has increased, so too has the complexity of porcine genomic manipulations increased to address the immunological challenges described in previous sections.

Complex mammalian genome engineering has advanced much more rapidly in mice than in virtually any other species, including pigs. The reason for the rapid progress in mice is the availability of embryonic stem (ES) cells which can be maintained in culture for extended periods time and undergo extensive transfection/transduction protocols and drug selection without losing the ability to produce large numbers of fertile progeny via blastocyst injection [56]. Although several labs have made strides in this area, similarly manipulable and viable ES cells are not currently available for routine use in the generation of cloned pigs [57].

The most common approach for production of genetically-modified pigs is very similar to the protocol described in the creation of "Dolly the sheep." Briefly, the nucleus from a pig cell carrying the desired genome changes is extracted and introduced into a pig oocyte, which has previously had its own nucleus removed, and then induced to initiate embryogenesis using electrical and chemical induction, a process referred to as somatic cell nuclear transfer (SCNT). The newly-created cells are implanted into surrogate female pigs and allowed to develop to birth. Compared to genetically-modified mouse production, this process is significantly less efficient and more costly, limiting the number of facilities capable of effectively carrying out this complex process [58].

A key factor in the success of SCNT is the source of the donor nucleus. These cells are typically primary cells derived from fetal sources. Extended culture, transfection, or drug selection of these donor cells can all cause a significant loss of viability for subsequent productive SCNT. Therefore, the approaches commonly used for mouse ES cell manipulation such as multigenic targeting and selections with various drugs over long periods in culture would not allow for production of modified pigs using SCNT. Similarly, any genome manipulations of pig cells must also maintain the viability of the cells for SCNT, which alters the approaches available compared with mice.

10.1. Gene knockouts

One of the earliest genome engineering approaches applied to pigs was introduction of gene knockouts (KO). For any given gene, mutations which remove or disrupt the coding sequence can eliminate the expression of the gene and, provided that the KO is not lethal, create an organism which is entirely missing the gene product. The introduction of gene KO technology has been a key factor in the rapid advancement of the field of xenotransplantation [59].

As discussed above, there are several glycan molecules present in pigs which are absent in humans. These glycans are recognized by antibodies present in human serum which leads to rapid and extensive antibody-mediated damage to the porcine cells. Therefore, the elimination of the specific carbohydrate structures should help prevent human antibody recognition of the pig tissues. Unlike protein antigens which are directly coded by the DNA, glycosylation is due to the action of enzymes which create post-translational modifications of a variety of proteins produced by the cell. Therefore, glycosylation pathways must be examined to identify the key enzyme that creates the immunogenic glycan while otherwise leaving cellular metabolism intact.

The GGTA1 gene is responsible for creating the Gal alpha (1,3) Gal epitope in pigs. Although the specific reasons for this are unclear, human patients can express high levels of antibody specific for the Gal alpha (1,3) Gal epitope, presenting a major challenge to xenotransplantation [60]. The KO of the GGTA1 gene is one of the earliest genetic modifications of pigs for application in xenotransplantation, and results in greatly reduced human antibody recognition of porcine cells [61]. However, elimination of the GGTA1 gene alone has been shown to be insufficient due to a variety of other xenoantigens present in pig cells which are recognized by antibodies present in human serum. Generation of KO of CMAH [62], B4GALNT2 [28] and other xenoantigen genes have further decreased the reactivity of porcine cells to human serum. However, it is important to keep in mind that the greater the number of gene KO, especially when made in combination, may lead to detrimental effects on pig health.

10.2. Gene insertions

The use of gene KO approaches is highly useful for eliminating xenoantigens but does not address the need for expression of human or synthetic versions of genes necessary for control or proper function of dysregulated pathways. This requires the ability to permanently introduce heterologous DNA into the genome in a manner which maintains gene function.

The initial approach to gene insertions was simply random integration of DNA into the target genome. These genes are introduced from elsewhere and thus termed "transgenes" (TG). Once the ability to introduce DNA into mammalian cells was established using a variety of technological approaches, it became clear that over long-term culture a subset of cells could be isolated which have permanently incorporated the heterologous DNA. Because many transgenes do not provide a straightforward means to identify cells which have incorporated foreign DNA from the population that have not, TGs often include genes encoding drug resistance markers. In order for cells to survive drug treatment they must incorporate the resistance gene, greatly reducing the population to be screened, and increasing the chance of identifying cells which incorporate the TG of interest along with the drug resistance gene [63].

The integration of transgenes is rapid but relatively uncontrolled. Although there may be some preferences for integration site based upon chromatin accessibility, these are hard to predict and may be related to DNA breakage sites at which repair mechanisms fortuitously insert the transgene DNA [64]. The random nature of the insertions can create risks. For example, the same transgene inserted at different sites can yield highly variable results in expression. Furthermore, some insertions may be deleterious to cell function, causing them to grow more slowly or die off, or, if these cells are used for generation of animals *in vivo*, there is a possibility of insertions creating mutations, instability or even lethality.

Due to the risks of random integration, significant effort has focused on protocols to create targeted integration, or gene knock-in (KI), of heterologous DNA into the genome. This is accomplished in mice by taking advantage of ES cells which undergo homologous recombination. In this approach, the transgene of interest is flanked by DNA sequences that are identical to regions of the genome to be targeted. After introduction of the heterologous DNA, the regions of DNA sequence identity are aligned with the target sequence and the homologous recombination machinery creates crossover events to switch the endogenous sequence with the heterologous sequence. This approach is much less efficient than random integration of TG, therefore drug selection schemes often need to be employed to identify the relatively rare targeting events [64].

Homologous recombination is well-established for targeting in mice but requires ES cells which express the enzymes necessary for the targeting event. Unfortunately, porcine ES cells are not available that both possess homologous recombination function and can reliably generate cloned animals. For reasons that are not entirely clear, generation of ES cells competent for homologous recombination and cloning seems to be challenging for most species other than mice [57]. Therefore, alternate approaches are required for targeted integration in the pig genome [65].

10.3. Tools for genome engineering

A number of novel enzymatic molecules have been created which help resolve the dilemma of targeted integration in porcine cells. Zinc Finger Nucleases (ZFN), Transcription Activator-Like Effector Nuclease (TALEN) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) are all synthetic molecules based on genuine proteins which allow the precise targeting of genomic DNA based upon sequence [66–68]. In each case there are two functional components, a targeting module which recognizes a specific genomic sequence, and an enzymatic module which introduces a double-stranded DNA break at the target site. In the case of ZFN and TALEN, the targeting module is a complex array of protein sequences which have previously been shown to recognize specific DNA sequences and can be mixed in a modular way to bind to any desired sequence. Although both technologies have shown great success, the effort and cost required to identify a single functional molecule can be significant. In contrast, the relatively more recently recognized CRISPR, and related prokaryotic systems, is much more easily applied in mammalian cells. The DNA binding module in this case is RNA base-pairing to provide sequence specificity. The enzymatic module cleaves DNA, creating a double strand break similar to ZFN and TALEN. When heterologous DNA is present, the cellular repair machinery may use the synthetic DNA to repair the break, inserting the TG at the desired genomic site. It is important to note that all of these systems, ZFN, TALEN or CRISPR, are essentially the same in that they introduce double strand DNA breaks at a selected site in the genome and do not directly affect the rate of DNA insertion. Therefore, it is often necessary to include selection schemes for identification of the modified cells. The greater efficiency and ease of use of these systems, CRISPR in particular, has allowed targeted insertion of DNA into genomes that were not previously able to be modified [69].

Due to the challenges of creating genomic modifications in porcine primary cells while maintaining their viability for SCNT, more efficient engineering methods are desirable. One approach to enhance efficiency is to target a specific region of DNA, called a landing pad, with multiple genes at once. By inserting a DNA vector bearing multiple therapeutic genes at once, a large amount of breeding and testing can be circumvented using a single event. This approach has the added advantage of avoiding inefficient crossbreeding necessary to bring loci from distinct chromosomes together in one lineage. When combined with the use of tools such as ZFN, TALEN and CRISPR more rapid progress in the genetic modification of animals has been greatly facilitated [70].

11. Conclusions and future prospects

The increasing sophistication and accessibility of genome engineering toolsets and deeper understanding of immunological rejection mechanisms has allowed greater advancement in xenotransplantation than ever before. A key question is just how many genetic changes are required in order to make a pig organ suitable for transplantation? While the critical experimental data needed for such an assessment is still accumulating, it is clear that the number of alterations required for one organ may be different from another. For example, xeno-hearts with relatively minimal genetic modifications have demonstrated months to years survival in transplantation studies with non-human primates, whereas xeno-lungs with more extensive modifications have

yet to survive more than a few weeks. This is due to the relative differences in structure and function of organs, the resilience to trauma, and susceptibility to rejection responses. Furthermore, tolerance mechanisms may be able to supplant the need for some genetic modifications, and thus the specific protocols and treatments will govern the ultimate complement of alterations.

The immediate need in xenotransplantation is to define the specific genetics required for xeno-organ survival, however, it is possible to project further enhancements such that porcine organs may be superior to human organs for human transplant. Synthetic biology approaches have created novel genetic circuits which can react in real time to human immune responses, inducing counter-reactions in the porcine cells to circumvent and tolerize the xeno-organ against human rejection. Furthermore, xeno-organs may be engineered to express protein therapeutics to further control human immunity while saving hundreds of thousands of dollars in expensive biotherapeutic treatments. Thus, the first version of pigs appropriate for xenotransplantation are likely to be further refined and improved to create increasingly useful rejection-free organs.

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