EJT European Journal of Transplantation

2023;1:217-225
DOI: 10.57603/EJT-311

Review

LUNG XENOTRANSPLANTATION: CURRENT STATUS 2023

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Summary

Xenotransplantation, the transplantation of organs or tissues between different species, has emerged as a potential solution to address the organ shortage. Introduction of genetically modified pigs with knockout of known xeno-antigens and addition of human complement and coagulation pathway regulatory proteins as well as anti-inflammatory and human 'self-recognition' molecules has enabled significant progress particularly for heart and kidney xenografts. Lung xenotransplantation presents unique challenges due to the lung's complex anatomic structure and associated immunological landscape: a very large surface-area interface between blood and the external environment is defended by an array of innate and adaptive immunocytes. Although recipient survival of up to 31 days has been achieved, formidable obstacles still need to be addressed before clinical lung xenograft application can be attempted with reasonable prospects for therapeutic efficacy. Nonetheless, lung xenotransplantation holds great potential as a future alternative for patients with end-stage lung disease, and ongoing research efforts continue to pave the way for its translation into clinical practice.

Key words: transplantation, heterologous, xenotransplantation,lung transplantation, swine, bioengineering

Received: July 2, 2023
Accepted: July 31, 2023

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How to cite this article: Chaban R, Ileka IS, Pierson III RN. Lung xenotransplantation: current status 2023. EJT 2023;1:217-225. https://doi.org/10.57603/EJT-311

INTRODUCTION

Xenotransplantation, the transplantation of tissues or whole organs from one species to another, has the potential to transform the modern transplantation medicine [1]. Despite huge advances recently, there are many challenges to overcome [2]. Issues such as immune rejection, minimal differences in physiology, infection and ethical concerns are still matter of active research and discussion. Recent progress to human medicine, including the first pig-to-human kidney [3], and heart [4], transplants into brain-dead human recipients, and a transiently successful life-supporting pig-to-human heart transplant [5], have reinvigorated interest in xenotransplantation. This article discusses the current state of lung xenotransplantation research, including the latest developments and remaining challenges. Ethical concerns and predictions regarding the likely path for lung xenotransplantation are also considered.

HISTORY OF THE LUNG XENOTRANSPLANTATION

Successful lung xenotransplantation has not been achieved in human

patients yet (up to the time of writing this manuscript in Summer 2023). Lung allotransplantation is itself a complex technical procedure with unique immunologic and physiologic challenges that contribute to cause inferior long-term outcomes relative to other solid organs. Blood circulates through the lung at relatively low pressures across a very large surface area capillary bed required for efficient gas exchange. The lung has an innate immune defense system that is teleologically 'designed' to protect this complex anatomical structure from infectious organisms. The lung's array of macrophages, eosinophils, and other immunocytes are highly efficient to trigger an inflammatory response, which, in the context of the large vascular bed in close proximity with adjacent airways, makes the lung especially sensitive to the loss of vascular barrier function associated with lung xenograft injury from any cause, including xeno-triggered mechanisms. The pig lung in particular contains specialized immune cells, such as pulmonary intravascular macrophages, which, in addition to alveolar macrophages, and non-T-cell leukocytes, play crucial roles in recognizing and responding to pathogens in the airways. Our work to date suggests that these cell populations are also centrally involved in the robust innate immune activation events observed in discordant lung xenograft rejection, such as when human blood perfuses a pig lung. Preclinical animal studies have occasionally achieved recipient survival of days to weeks [6]. By way of comparison, survival for months and even years have been reported in primates carrying life-supporting pig hearts and kidney, respectively.

Pigs have emerged as the primary source of xenograft organs due to their widespread availability, size compatibility, rapid maturation, prolific breeding, and short gestation period, which allow for shorter development and testing cycles [7]. *Ex-vivo* experiments, in which pig lungs were perfused with human blood under physiological conditions and ventilation, were first reported by Bryant et al. in 1968 [8], and have since yielded valuable knowledge about lung xenotransplantation [9]. The Pierson lab improved this model by using fresh human blood in a side-by-side paired model to evaluate mechanisms of lung xenograft injury, including the contribution of leukocytes and platelets [10,11]. Those trials were particularly important in identify the features of 'hyperacute rejection' that occur within the range of the first 6-8 hours after perfusing the pig lung graft with fresh human blood [12].

In vivo experiments using baboons as the recipient of pig organs have been conducted intensively in the field of xenotransplantation research owing to the anatomical and immunological similarities between baboons and humans [13]. Baboons have been used to cross-circulate pig livers as an *ex-vivo* liver xenograft models [14], as recipients of non-life-supporting heterotopic heart xenografts, as life-supporting kidney and heart xenograft recipients [15-18], and as recipient of left-sided lung xenotransplantation [10]. In the *in vivo* lung xeno model, measuring flow and pressures in the pulmonary artery as well as cardiac output and gas exchange before chest closure generated a large amount of valuable data regarding lung physiology and function [10,19].

LUNG XENOGRAFT INJURY

Perfusing a wild pig lung with human blood results within minutes in fulminant inflammatory response, known as hyperacute lung rejection (HALR) [11,20]. HALR is manifested by rapid endothelial injury, platelet adhesion, complement and coagulation systems activation, elevation of pulmonary vascular resistance (PVR), loss of vascular barrier function and lung edema, and eventually ischemic damage of the pig lung [21]. HALR is attributed primarily to naturally occurring antibodies in human blood against pig carbohydrates (Tab. I): *Galactose- a1,3-galactose* (a-Gal, 80-90%) [22], *N-Glycolylneuraminic acid* (Neu5Gc, 5-15%, encoded by the *CMAH* gene) [23] and the *SID blood group* (sda, 1-5%, encoded by the b4Galactosyl transferase gene, or b4GalNT2) [24]. Almost all mammals, except for humans and old-world primates express these antigens on most of their cells and are directly targeted by preformed human antibodies upon contact with them [25,26]. Early works focused merely on the elimination of these preformed antibodies utilizing different methods and succeeded in delaying the rejection [27,28]. Advances in genetical engineering and the emerging of newer technologies [29], have enabled precise editing and the removal of the coding genes of the above mentioned antigens, and resulted in the introduction of the Gal-free (GalT-knockout, GTKO) [30], double (GTKO plus CMAH or b4Gal KO), and triple knockout swine that lacks all the three above mentioned carbohydrates [23,31,32]. Available evidence strongly suggests that all three of these antigens will be important to exclude from pig lungs intended for clinical use, since the few long-surviving lung xenografts in baboons all failed in association with high levels of anti-a4Gal antibody [6], and the Neu5Gc antigen elicits an immune response for other cell and organ xenografts. Importantly, there is evidence for unveiling of a *"fourth" xenoantigen* that creates a positive cross-match against CMAH+GalT KO or TKO cells and organs in NHP (but NOT in humans). Although results of triple knockout experiments in primate are promising [33], we strongly believe that transplant results achieved in primates with cells or organs that include the CMAH KO are likely to inaccurately predict (underperform) clinical results due to the *fourth antigen* and associated positive crossmatch in NHP's.

Table I. Known carbohydrate xenoantigens expressed on pig cells.

Carbohydrate antigen (abbreviation)	Responsible enzyme	Gene-knockout pig
Galactose- 1,3-galactose (Gal)	1,3-galactosyltransferase	GTKO
N-glycolylneuraminic acid (Neu5Gc)	Cytidine monophosphate-N-acetylneuraminic acid hydroxylase	CMAH-KO
Sd[a]	-1,4N-acetylgalactosaminyl-transferase 2	4GalNT2-KO

Out of proportion to surgical blood loss, anemia has been observed after *in vivo* xeno lung transplantation in *ex vivo* models [34] and following in vivo pig-to-baboon lung xenotransplantation [6]. We have shown that sialic acid on human erythrocytes binds to pig sialoadhesin [34], and causes RBC fragmentation during *ex vivo* organ perfusion in lung [35] and heart (unpublished) models. The addition of a monoclonal antibody (41D3) directed against pig sialoadhesin was found effective to inhibit the process *in vitro* [37]. Genetic modifications that humanize the pig sialoadhesin or silence its expression may address this issue in future xenotransplantation [34].

Sequestration of leukocytes and platelets is a hallmark of *ex-vivo* pig lung perfusion experiments [37]. Pig interleukin-8 was found to be elevated in pig-to-human *ex-vivo* lung models and was effective in stimulating human neutrophils and increasing their adhesion [38]. Blocking of IL-8 receptor with Reparixin prevented neutrophil activation [38]. Activated pig endothelium was found to express high amount of P- and E-selectin facilitating human neutrophils rolling and tethering [39]. TNF-a, IL-4, DDAVP, histamine and thrombin are recognized as potent upregulators of various selectins [39]. The importance of DDAVP lung pretreatment, and by inference vWF/GPIB interactions, has been confirmed by the Korean group [40]. Selectin antagonists (GM1271 for E-selectin and rPSGL-1 for P-selectin) were effective in inhibiting neutrophil rolling [39]. For these reasons, we have incorporated Reparixin, DDAVP, an anti-GPIB monoclonal Fab (6B4), and E- and P-selectin inhibitors in our *ex-vivo* and *in vivo* lung xenotransplantation treatment protocols.

The complement system is an essential part of the immune system that plays a crucial role in host defense against pathogens and foreign invaders, as well as in the regulation of immune responses. To prevent an excessive activation and potential damage to the host tissues, the complement system is tightly regulated by regulatory proteins, such as decay accelerating factor (DAF_CD55); membrane cofactor protein (MCP_CD46) and membrane-attack-complex-inhibitory protein (MACIP_CD59), which collectively are known as complement pathway regulatory proteins (CPRPs) [41,42]. Whether due to molecular incompatibilities between species or differences in quantitative expression on endothelium, porcine CPRPs seem to exhibit reduced efficiency in controlling activation of the human complement system, which explains why complement-mediated xenograft injury is prominent in preclinical xenotransplantation studies of the lung [11, 42], and other organs [44,45]. Early xenotransplantation experiments in the lung and other organs evaluated complement pathway antagonizing agents, such as C1- esterase inhibitor, soluble complement receptor 1 (sCR1), and FUT-175, or depleting agents (eg, cobra venom factor - CoVF), and were partially successful [46-48]. Advances in gene editing technology have enabled introduction of human complement pathway regulatory proteins (CPRPs) into xenografts, such as CD46, CD55, CD59, etc. [49] in the context of the GalTKO, DKO, or TKO background. Expression of hCPRP 'transgenes' is closely correlated with reduced complement deposition, and with decreased platelet activation and delayed graft injury in xeno lung [50-52], as also shown for heart [53], and kidney [54].

Cluster of differentiation CD47 is ubiquitously expressed on hematopoietic cells and plays a critical role in the regulation of immune responses, particularly in the context of immune evasion and tissue protection by acting as a "self-identification" signal, when binding to its receptor, the signal regulatory protein alpha (SIRP), on phagocytic cells [55,56]. This binding transmits an inhibitory signal and prevents the engulfing and clearing of cells displaying CD47 [57]. Yamada and colleagues have shown that lung expression of CD47 is associated with prolonged duration of engraftment of pig bone marrow (particularly when injected directly into the bone marrow compartment) and prolonged lung xenograft recipient survival; life-supporting lung function was not interrogated in those studies [58].

CD39 and CD73 act as an anti-inflammatory mediators by converting the extracellular pro-inflammatory and vasoconstrictive ATP into AMP [59,60]. Low levels of expression of the pig homologues of this pathway on pig endothelium may contribute to the increased vascular resistance and proinflammatory environment seen in lung (and other organ) xenografts; we speculate that xenograft injury might be inhibited by introducing the human CD39 or CD73, or enhancing expression of these pig genes, in gene-edited pigs [9].

Pig major histocompatibility complex (MHC), also known as *swine leukocyte antigen* (SLA), exhibits weak binding

to the inhibitory receptors expressed by human NK cells. Consequently, this interaction leads to the in vitro killing of pig cells by NK cells [61]. The introduction of transgenic expression of human leukocyte antigen E (HLA-E) into gene-edited pig offers protection against xenograft injury caused by human NK cells [62,63]. Remarkably, expression of HLA-E on GalTKO.hCD46 pig lungs is associated with a significant protective effect, illustrating the importance of NK cells to initial injury of lung and other xenografts [64].

Diffuse microvascular thrombosis and consumptive coagulopathy were reported weeks to months after transplanting kidney and heart xenografts from transgenic pigs that lack the pig carbohydrates and additionally express multiple human CPRPs [64]. This phenomenon revealed that incompatibilities between pig coagulation factors and human thromboregulatory molecules [65,66], constitute a substantial additional obstacle to successful xenotransplantation for the lung and other primarily vascularized organs. Non-physiologic interactions between pig von Willebrand Factor (vWF) and human GPIB and between human coagulation pathway molecules and pig thromboregulatory proteins have been implicated in this phenomenon.

Pig aortic endothelial cells (PAEC) were found to be a potent activator of the human prothrombin [67] and platelets [66,68]. von Willebrand Factor (vWF) is a large multimeric protein that is found in endothelium, platelets and megakaryocytes and is involved in initiating platelet adhesion to the endothelium, after being activated by shearing force and binding to GPIb receptors [69]. Pig vWF non-physiologically aggregates and activates human platelets through aberrant interaction with the human glycoprotein receptor GPIb [70,71]. In our previous work, pre-depleting pig vWF from pig endothelium before organ harvesting, by using DDAVP, coupled with blood treatment with a GPIb antagonist was sufficient to attenuate platelet activation in *ex-vivo* lung perfusion experiments [72]. Humanizing pig von Willebrand factor (by partially replacing the gene region that encodes the glycoprotein Ib-binding site of the pig vWF with the human analogue) in GalTKO.hCF46 pig lungs corrected the non-physiological human platelet aggregation and sequestration within the pig lung and liver [73]. This modification could prove pivotal to accomplishing safe, effective lung xenotransplantation, and may prove valuable to enable other organ and cell xenotransplant applications.

Pig thrombomodulin (TBM) can bind to human thrombin, but it is less potent in activating protein C, with only 1-10% the activity of the human counterpart [74,75]. Its cofactor; pig Endothelial protein C receptor (EPCR); is also less efficient in activating protein C. As a consequence, pig endothelium does not efficiently regulate (physiologically constrain) amplification of coagulation by human coagulation factors [74,76]. Pig Tissue factor pathway inhibitor (TFPI) is a direct inhibitor of the activated extrinsic coagulation pathway factors and is suspected to be inefficient in regulating the human tissue factor-initiated coagulation [77]. In support of this hypothesis, expression of human TFPI (and human CD47) was found to be associated with less neutrophil activation and hence protection against xeno injury in GalTKO pig lungs [78].

Innate immune activation is a prominent feature of pig lung perfusion with human blood. Early elaboration of thromboxane and histamine were identified in *ex-vivo* pig lung perfusion experiments and are believed to arise mainly from the pulmonary intravascular macrophages, and mediate the elevation in pulmonary vascular resistance and loss of vascular barrier function (pulmonary edema and intra-alveolar hemorrhage) observed in these experiments [50]. Treating galactosyltransferase gene knocked out (GalTKO) pig lungs with 1-Benzylimidazole (1-BIA), a strong selective thromboxane inhibitor, combined with histamine receptor blocker (famotidine, and H-2 receptor blocker, or diphenhydramine, which is non-selective), significantly attenuated PVR elevation and delayed the loss of vascular barrier function [79]. These findings illustrate the important role of these primitive innate immune activation pathways in lung xenograft injury.

GENERAL PIG LUNG XENOTRANSPLANTATION CONCERNS

Clinically important differences exist in the anatomy of the pig compared to that of humans. Pig lungs have seven lobes compared to five in humans, with four lobes on the right (cranial, middle, accessory and caudal lobes) and three on the left (cranial, middle and caudal) [80]. The right cranial lobe is of particular interest for human transplantation, as the bronchus for this lobe arises directly from the trachea (the so called *pig bronchus*), which arises a significant distance above the carinal bifurcation. This prevents performance of the conventional end-to-end bronchial anastomosis on the right side [7]. In principle a tracheoplasty could be performed to the right lateral aspect of the recipient airway, but a long anastomosis might be vulnerable to healing challenges, particularly if the microvascular collateral circulation to the pig lung is compromised due to inflammation or immunologic insults.

Infection, impaired wound healing, and cancer are significant complications commonly observed in organ allograft recipients, particularly in association with intensive, long-term immunosuppressive drug treatment using high doses of the early generations of contemporary 'conventional' immunosuppressive drugs (steroids,

azathioprine or mycophenolate mofetile, calcineurin inhibitors) [81]. It is anticipated that future lung xenotransplantation endeavors may encounter similar challenges, although costimulation pathway blockade appears particularly promising to block xenograft rejection with a very acceptable safety profile in NHP. Wild and commercially raised pigs are susceptible to a wide range of common opportunistic infections (e.g. campylobacter, E. coli), and traditional zoonosis (e.g. toxoplasma gondii, strongyloides) that can be efficiently transmitted to humans, as well as to species specific pathogens (e.g. porcine CMV, adenovirus) for which infection is harmful only to the pig [82,83].

The recent report of porcine cytomegalovirus (pCMV) infection in a pig-to-human heart transplant performed at the University of Maryland in 2022 has raised concerns regarding the screening process for this virus and its potential role in the eventual failure of the transplanted organ [84]. Porcine CMV is endemic in pig populations worldwide and is transmitted through nasal secretion and in utero [85]. The prevention of cytomegalovirus (CMV) transmission to piglets is achievable through a combination of caesarean section to minimize the exposure to CMV-infected birth fluids and barrier rearing by maintaining strict biosecurity measures and physical separation between infected and uninfected animals [86]. New more sensitive screening to detect pCMV and other potential infections are subject of ongoing research.

Another concern is the theoretical risk of porcine endogenous retroviruses (PERVs) being transmitted to humans that were raised in the late 1990s [87]. Encouragingly, PERV infection of humans has never been observed in clinical circumstances following transplantation of living tissues (skin) or cells (splenocytes, islets) from pigs [88]. PERV transmission to primates has not been detected in preclinical trial either [89]. Should PERV infect a xenograft recipient, with or without causing disease, the risk is low, and should be manageable with existing antiviral drug treatments [90]. In addition, advances in genetic editing have enabled the creation of PERV-deleted swine by utilizing CRISPR-based technology. All 62 copies of the porcine endogenous retrovirus (PERV) have been successfully inactivated [91,92]. From these animals, xenografts have been successfully propagated, and could be bred under strictly isolated laboratory conditions from designated pathogen free herds (DPF) [93]. This combination of PERV-KO and DFP housing will in principle be making the cumulative risk of infection potentially lower than that of an allograft from another human, whose exposure to a wide variety of known pathogens such as Hepatitis virus, HIV, etc, is often not well-defined [94].

New strategies in immunosuppressive treatment are being developed for allo- and xenotransplantation with the aim of minimizing the side effects associated with 'conventional' immunosuppression and improving long-term patient outcomes. Some of these strategies are likely to enable bringing lung xenotransplantation into clinical reality. Specifically, immunosuppressive agents that selectively inhibit the CD154/CD40 and CD28/B7 costimulatory pathways are currently being investigated by the Pierson lab and others, with encouraging initial results for heart allografts [95,96], and kidney allografts [97] and for heart and kidney xenografts [5,98-100].

A long-term strategy would be the use of immunomodulatory therapies that promote "immune tolerance" and allow for controlled immune response to the transplanted organ [101]. This approach aims to establish a state of immune balance where the immune system recognizes the transplanted organ as "self" and avoids mounting an aggressive immune response. Multilineage chimerism has been achieved successfully in preclinical trail through bone marrow transplantation [102]. However, achieving survival of xenogeneic bone marrow across species has proven challenging [103], and as a consequence accomplishing immune tolerance to xenografts remains a significant challenge.

FUTURE CONSIDERATIONS AND ETHICAL CONCERNS

The organ shortage is a significant challenge to accomplishing the potential therapeutic impact of lung transplantation [104]. The number of patients added to the waiting list for lung transplantation grew steadily in the United States to 3153 patients in 2022 (Organ Procurement and Transplantation Network -OPTN) [105]. The demand for organs far exceeds the supply, resulting in long waiting lists and a substantial gap between the number of patients in need of a transplant and the available organs for transplantation [106]. Lung allotransplantation still suffers from technical issues regarding the limitation imposed by the restricted time window of the harvesting and transplantation process. Mechanical devices that supplement pulmonary function in cases of severe respiratory failure such as veno-venous (or veno-arterial) extracorporeal membrane oxygenation (ECMO) or mechanical ventilators cannot provide a permanent solution for end-stage lung failure. These devices are primarily used as temporary measures to support pulmonary function while the underlying condition is treated or until the patient's condition improves. New approaches, such as lung bioengineering (whole organ decellularization/recellularization, 3-D organ printing, and stem-cell-based regenerative medicine), are still in their early stages. Tissue engineering has been used to generate human skin, bladder and vascular grafts [107], but creating functional vascularized solid organs, and particularly the lung, has proven very difficult.

As described above, lung xenotransplantation holds the potential to address the critical shortage of donor organs and provide a practically unlimited supply of safe, readily available, and optimally functioning lungs for transplantation. Future lung xenotransplantation will be carried on under elective conditions giving both the surgeons and the patients enough time to conduct the necessary preparation avoiding the burden of waiting for a donor organ. Ethical concerns regarding prioritizing patients on waiting lists will be decompressed by ready access to healthy, available donor organs. If initial results of lung xenotransplantation are less durable than allo transplantation, the former may still be useful as bridging treatment until an allo lung becomes available, presuming that receipt of the lung xenograft does not sensitize the patient to alloantigens and thus complicate their path to a subsequent allograft.

CONCLUSIONS

In summary, xeno lung transplantation holds great promise as a potential solution to the shortage of donor lungs for transplantation. In our view, predictable infections are likely manageable based on what is currently known, or can be treated with available tools at hand; we will need to be prepared to diagnose and treat unpredicted infections if they arise. While there are still significant challenges to overcome, such as the formidable known innate immune barriers, the unknown risk of adaptive immune injury (conventional cellular or humoral 'rejection'), and other known and as-yet-unidentified non-immunological incompatibilities, ongoing research and technological advancements appear likely to allow us to overcome these hurdles. With continued research to accomplish predictable advancements in the field, a future where safe, effective pig lungs can be readily available to save countless lives is within reach.

Conflict of interest statement

The authors declare no conflict of interest.

Funding

Ryan Chaban is supported by the Benjamin Research Fellowship from the German Research Foundation (DFG).

Author contributions

RC, II: literature review; RC, II, RP: manuscript writing; RP: critical review.

Ethical consideration

Not applicable.

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