Strategies to induce natural killer cell tolerance in xenotransplantation

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Eliminating major xenoantigens in pig cells has drastically reduced human antibody-mediated hyperacute xenograft rejection (HXR). Despite these advancements, acute xenograft rejection (AXR) remains one of the major obstacles to clinical xenotransplantation, mediated by innate immune cells, including macrophages, neutrophils, and natural killer (NK) cells. NK cells play an ‘effector’ role by releasing cytotoxicity granules against xenogeneic cells and an ‘affecter’ role on other immune cells through cytokine secretion. We highlight the key receptor-ligand interactions that determine the NK cell response to target cells, focusing on the regulation of NK cell activating receptor (NKG2D, DNAM1) and inhibitory receptor (KIR2DL1-4, NKG2A, and LIR-1) signaling pathways. Inhibition of NK cell activity may protect xenografts from cytotoxicity. Recent successful approaches to reducing NK cell-mediated HXR and AXR are reviewed, including genetic modifications of porcine xenografts aimed at improving pig-to-human compatibility. Future directions to promote xenograft acceptance are discussed, including NK cell tolerance in pregnancy and NK cell evasion in viral infection.

Keywords
NK cells, NK cell tolerance, xenotransplant, xenotransplantation, tolerance

Abbreviations: αGal, galactose-α(1,3)galactose; AXR, Acute xenograft rejection; βGalNT2, Beta-1,4-N-Acetyl-Galactosaminyltransferase 2; CMAH, Cytidine monophospho-N-acetylneuraminic acid hydroxylase; GGTαI, Alpha-1,3-galactosyltransferase; HLA, Human leukocyte antigen; HXR, Hyperacute xenograft rejection; MHC, Major histocompatibility complex; Neu5Gc, N-glycolylneuraminic acid; NK Cells, Natural Killer Cells; PBMC, Peripheral blood mononuclear cells; pEC, Porcine endothelial cells; SLA, Swine leukocyte antigen; 5GKO, GGTαI/CMAH/βGalNT2/SLA-Ia/β2-microglobulin KO.
Introduction

The persistent lack of transplantable organs has resulted in the death of thousands of patients every year. There have been several proposed solutions to address this issue, including manufacturing bioartificial organs (1), 3D printing human organs (2), and transplanting organs from different species into humans, a practice known as xenotransplantation. Xenotransplantation represents one of the most promising approaches. Elimination of major xenointigens on xenografts by gene-editing tools has proven to be an effective approach to preventing hyperacute xenograft rejection (HXR) (3–6). Earlier this year, the first pig-to-human heart transplantation was performed and supported the patient’s life for two months. In this xenotransplant, HXR was successfully prevented with 10-gene modification, particularly with three major xenoantigens (αGal, Neu5Gc, and Sda) removal in the xenograft (7). Despite this exciting success, xenotransplantation must overcome other barriers before becoming a widespread clinically viable solution. As a result of current advances, the field has shifted towards addressing the next major immunologic barrier: acute and chronic xenograft rejection.

Natural killer (NK) cells are a subset of lymphocytes that not only constitute the innate immune system’s first line of defense but also play a significant role in regulating adaptive immunity (8, 9). NK cells can destroy target cells either directly or via antibody-dependent cellular cytotoxicity (ADCC) in the absence of antigen priming (10). NK cell-mediated cytotoxicity may initiate robust adaptive immune responses via CD8+ T cell priming, antigen-specific CD4+ T cell response, and humoral responses (11). NK cells also secrete cytokines and chemokines, which regulate dendritic cells, macrophages, and neutrophils, as well as antigen-specific T cell and B cell function (9, 12, 13).

NK cells express various activating and inhibitory receptors that interact with the ligands on target cells (9). The balance between activating and inhibitory signals of NK cells determines NK cell activation or tolerance (14). In classical education (also known as NK licensing), naive hyporesponsive NK cells learn to recognize MHC class I molecules as "self" (15). This knowledge of "self" allows NK cells to activate when target cells are missing MHC ligands. Killer cell immunoglobulin-like receptors (KIR) are a major group of human NK inhibitory receptors for HLA class I molecules. Interaction of NK inhibitory cell receptors KIR2DL4 and CD94 (NKGA2A) with non-classical class I molecules HLA-G and HLA-E at the fetomaternal interface results in maternal immune tolerance during pregnancy (16) (Figure 1). Activating human NK cell receptors include members of KIR family, NKG2D, natural cytotoxicity receptors such as Nkp30, NKP44, Nkp46, and the nectin/nectin-like binding receptors DNAM-1 and CRTAM, which are responsible for initiating activating signals (17, 18) (Figure 1).

NK cells play a crucial role in influencing immune responses to solid organ allografts. Activated NK cells can kill allogeneic target cells and secrete immunomodulatory chemokines and cytokines, contributing to either rejection or tolerance (19).

In this review, we focus on (i) the dual function of NK cells in rejection and tolerance in allotransplantation, (ii) the state of

**FIGURE 1**
Genetic Modifications that Reduce NK Cell-directed Cytotoxicity. Top left: Recruitment occurs due to adhesive interactions between endothelial ligands and NK cell receptors. Transmigration is mediated by interactions between CD99 and unknown ligands on porcine endothelial cells. Top middle: Antibody-dependent cellular cytotoxicity (ADCC) present upon NK cell recognition of preformed IgG antibodies directed against the xenoantigens αGal, Neu5Gc, and Sda. Bottom left: Failed self-recognition due to non-homology between SLA I and HLA I molecules. Bottom middle: NK cell receptor activation results from interactions with unknown porcine ligands. Right: Summary of current genetic modification proposed to reduce NK cell-mediated cytotoxicity.
current research regarding genetic modifications to promote NK cell tolerance in xenotransplantation, and (iii) promising future directions to advance xenotransplantation to the clinical reality.

**NK cells in allotransplantation**

Within days of solid organ transplantation, NK cell infiltration has been observed in allografts (20). Historically, acute rejection episodes have been characterized by an increased number of circulating cytotoxic NK cells (21). NK cells are primarily responsible for augmenting the immune response by secreting key pro-inflammatory cytokines, such as TNF-α and INF-γ (22) and recruiting activated lymphocytes (23). Although T cells are the dominant cell type in allograft rejection, fully activated NK cells have been implicated in allograft rejection in the absence of T cells and B cells in mice (24). The early recruitment of immune cells to the graft via NK cell cytokine secretion can propagate the acute rejection process, linking the innate and adaptive immune responses (25). NK cell facilitation of rejection is evidenced by simultaneously and significantly elevated expression of NKG2D and its ligands during acute cardiac allograft rejection in mice (26).

Beyond acute rejection, the role of NK cells in chronic rejection has been uncovered in multiple studies. For example, depletion of mouse NK cells by anti-NK1.1 monoclonal antibodies prevented early phase cardiac allograft vasculopathy (CAV) (27). In a RAG1−/− murine model (deficient in T and B cells), NK-induced CAV lesions were restored when wild-type helper T (Th) cells were transferred to the recipient, suggesting that NK cells may play a role in chronic rejection in a Th cell-dependent manner (19, 28). A similar role in chronic rejection was noted in a murine lung transplant model as the use of an anti-NK1.1 monoclonal antibody attenuated bronchiolitis obliterans, a form of chronic lung transplant rejection (29). In a retrospective immunogenetic study of human cadaveric kidney allografts, lacking inhibitory combinations of functional recipient KIR and donor HLA ligand led to a higher risk of chronic rejection (30). Collectively these findings support the conclusion that the presence of NK cells promotes chronic allograft rejection. Thus, inhibiting NK cell activation could be beneficial in improving allograft survival.

Additionally, NK cells promote kidney graft rejection independently of immunosuppressant cyclosporine A therapy, which blocks T cell activation through IL-2 inhibition (31). Due to the varying mechanisms of action of immunosuppressants, uniform influence on NK cell cytotoxicity is not seen. Immunosuppressants limit NK cell functions, such as IFN-γ production, expression of activation/inhibitory and adhesion markers, antibody-dependent cell-mediated cytotoxicity (ADCC), and NK cell proliferation in different manners to different degrees (32). However, identifying potent inhibitors of NK cell functions (methylprednisolone and intravenous immunoglobulin) has important implications for their use in promoting xenotransplant acceptance (32).

The role of NK cells in allograft tolerance is more complex, especially given that both recipient and donor NK cells contribute to allograft rejection and tolerance (33–35). Depletion of recipient NK cells has been reported to prolong allograft survival in a murine liver transplantation model (23). Conversely, in a mouse skin allograft model, recipient NK cells were shown to target and destroy donor antigen-presenting cells, ultimately leading to improved skin allograft tolerance (36). A human lung allotransplantation study also showed these opposing actions of rejection and tolerance (37). Given the array of NK cell functions in acute and chronic allograft rejection, a thorough understanding of their precise roles will be essential to designing an immunologically optimized porcine xenograft.

**NK cells in xenotransplantation**

Direct contact of human blood with porcine vascular endothelial cells leads to pig endothelial cell injury and secretion of cytokines and chemokines (6, 38, 39). Adhesion molecules, including platelet endothelial cell adhesion molecule, E-selectin (CD62E), P-selectin (CD62P), and vascular cell adhesion molecule-1 (VCAM-1), are upregulated on activated porcine endothelial cells (pEC), and function in recruiting human NK cells (6, 40). Studies showed that interactions of porcine VCAM-1 with human very late antigen-4 (VLA-4) facilitated human NK cell adhesion to pEC (41, 42), and later transendothelial migration was mediated CD99 (43). Manipulating the VCAM-1/integrin adhesion pathway is impractical as the deletion of VCAM-1 line has been shown to significantly suppress ADCC (50, 51). Additional elimination of N-glycolyneuraminic acid (Neu5Gc) (52) and Sda (product of SDA-1) xenoantigen in pECs may further inhibit NK cell-mediated ADCC (51). The pivotal work in reducing NK cell hyperacute xenograft rejection is well summarized by Puga Yung et al. (6).

Preformed human anti-pig antibodies binding to xenoantigens on porcine vascular endothelium initiates the hyperacute xenograft rejection cascade (45, 46). ADCC is a major mechanism for NK cell-mediated cytotoxicity through Fc-receptors (CD16a) binding to IgG1 and IgG3 on pEC and releasing lytic granules (47–49). The αGal-knockout (KO) in pEC line has been shown to significantly suppress ADCC (50, 51). Additional elimination of N-glycolyneuraminic acid (Neu5Gc) (52) and Sda (product of β-1,4 N-acetylgalactosaminyl transferase) (53, 54) carbohydrates and SLA-I xenoantigen in pECs may further inhibit NK cell-mediated ADCC (51). The pivotal work in reducing NK cell hyperacute xenograft rejection is well summarized by Puga Yung et al. (6).

Several studies have been conducted to evaluate the role of NK cells in acute xenograft rejection. In a pig-to-nonhuman primate (NHP) preclinical cardiac xenotransplantation with antibody depletion, Itescu et al. observed greater levels of NK cell and
Future directions

Xenotransplantation differs from allotransplantation in that preventing rejection can be achieved through genetic modification of the donor pig, improving donor-recipient compatibility. A thorough understanding of the NK cell tolerance mechanisms is necessary for developing novel strategies to mitigate NK cell activation in xenotransplantation. To overcome NK cell-mediated acute xenograft rejection, future directions may require emulating the tolerance strategies used at the maternal-fetal interface and the evasion mechanisms employed by viruses (Table 1).

Introducing inhibitory ligands in pig organs

An example of natural human tolerance is maternal acceptance of the fetus during pregnancy. NK cells constitute the largest proportion of immune cells present in the post-conception endometrium and play a vital role in establishing maternal-fetal immune tolerance. Unique co-expression of fetal HLA-C, HLA-E, and HLA-G in extravillous trophoblasts (EVT) provides inhibitory signals to NK cells (Figure 2) (22). Previous studies demonstrated that reduced expression of inhibitory receptors (i.e., KIR2DL1, KIR2DL2, and KIR2DL3) was associated with recurrent spontaneous abortion (62) and that disruption of the NKG2A/HLA-E axis can lead to adverse fetal outcomes in a murine model (63). This specific in-utero environment provides a model for xenotransplantation as the presence of MHC Class I molecules induce an immunotolerant phenotype of maternal NK cells.

Genetically-modified pECs expressing HLA-E demonstrated partial protection from human NK cytotoxicity, and this effect was most pronounced in NK cells with high NKG2A expression (64). This protection was later reproduced in pECs isolated from a double HLA-E B2M transgenic pig (65), confirming the utility of this genetic modification. In an ex vivo porcine limb perfusion study with human blood using transgenic pigs co-expressing HLA-E and human CD46 (HLA-E/hCD46), decreased NK cell tissue infiltration was observed (66). Moreover, in vitro NK cell-

MACROPHAGE INFILTRATION IN XENOGRAFTS THAN IN ALLOGRAFTS WHEN ANTIBODIES WERE DEPLETED (55), SUGGESTING XENOGRAFTS EXPERIENCE A MORE ROBUST CELLULAR IMMUNE RESPONSE EVEN IN THE ABSENCE OF ANTIBODY-MEDIATED REJECTION. IN A GUINEA PIG-TO-RAT CARDIAC XENOTRANSPLANTATION STUDY, CONTINUOUS DELETION OF NK CELLS CAN PROLONG XENOGRAFT SURVIVAL (56), FURTHER INDICATING THAT THE ABSENCE OF NK CELLS MAY PROMOTE LONG-TERM XENOGRAFT ACCEPTANCE. AN EARLY AND TANGENTIALLY ELEVATED IFN-γ WAS RECOGNIZED IN PIG-TO-NHP HEART AND KIDNEY XENOTRANSPLANTATION WHEN RETROSPECTIVE ANALYSES OF NHP SERA WERE PERFORMED. FURTHER STUDY INDICATED THAT NHP NK CELLS ARE THE SOURCE OF EARLY IFN-γ UPON THE STIMULATION BY BOTH WILD-TYPE AND αGal-KO pEC IN VITRO, WHICH COULD AMPLIFY INFLAMMATION (57). TAKEN TOGETHER, THESE FINDINGS SUGGEST THAT XENOGRAFTS EXPERIENCE PROFOUND CELLULAR REJECTION, AND NK CELLS PLAY A PIVOTAL ROLE IN BOTH EXECUTING AND AMPLIFYING THIS RESPONSE.

GIVEN THE IMPORTANCE OF NK CELLS IN ACUTE XENOGRAFT REJECTION, THE INTERSPECIES RECEPTOR-LIGAND INTERACTION HAS BEEN INVESTIGATED WITH THE GOAL OF MANIPULATING PORCINE LIGANDS TO PREVENT HUMAN NK CELL CYTOTOXICITY. THREE HLA CLASS I MOLECULES WERE EXPRESSED IN AN IMMORTALIZED PORCINE BONE MARROW-DERIVED ENDOTHELIAL CELL LINE. HLA-Cw3 PROVIDED SUBSTANTIAL PROTECTION AGAINST CYTOTOXICITY OF SEVERAL NK CLONES, BUT HLA-A2 AND HLA-B27 PROVIDED NO PROTECTION (58). THE CO-EXPRESSION OF HLA-Cw3 AND HLA-Cw4 DID NOT OFFER ADDITIONAL BENEFIT THAN WHEN EACH WAS EXPRESSED ALONE (59).

THE INHIBITORY ROLE OF SWINE LEUKOCYTE ANTIGEN-I (SLA-I) TOWARDS HUMAN NK CELLS HAS BEEN INVESTIGATED IN THE PAST WITH MIXED FINDINGS. SULLIVAN ET AL. REPORTED THAT SLA-I IS UNABLE TO EFFICIENTLY TRANSMIT INHIBITORY SIGNALS TO HUMAN NK CELLS BECAUSE AMINO ACID RESIDUES CRITICAL FOR THE BINDING TO HUMAN NK CELL INHIBITORY RECEPTORS ARE ALTERED IN SLA-I WHEN COMPARED TO HLA CLASS I (60). IN CONTRAST, KWiatkowski ET AL. FOUND THAT INDUCTION OF SLA-I EXPRESSION ON PORCINE ENDOTHELIUM BY TNF-α REDUCED HUMAN NK CELL-MEDIATED CYTOTOXICITY (41). HEIN ET AL. REPORTED THAT DECREASED SLA-I EXPRESSION (SLA-I LOW) DID NOT DECREASE NK CELL ACTIVATION (61). A RECENT STUDY DEMONSTRATED THAT SLA-I IS NOT AN INHIBITORY LIGAND FOR HUMAN NK CELLS USING AN IMMORTALIZED pEC WITH SLA-I GENE DISRUPTION (5). THE ABSENCE OF INHIBITORY LIGANDS ON pEC TRIGGERS HUMAN NK CELL DESTRUCTION.

TABLE 1 Proposed Directions to Reduce NK Cell-Mediated Rejection.

| Model                        | Target                                      | Expected Outcome               |
|------------------------------|---------------------------------------------|--------------------------------|
| Pregnancy                   | Co-expression of HLA-E and HLA-G            | Addition of inhibitory signal  |
| Viral evasion                | UL40 co-expression with HLA-E               | Promotion of inhibitory signals|
|                               | gpUL18 decoy expression                     |                                 |
| Deletion of porcine activating ligands | pULBP1, pCD58, and pCD112 deletion         | Deletion of activating ligand   |
| Identification of unknown porcine activating ligands | NKG2D, CD2, NKp44, and DNAM-1 ligands      | Identify additional targets for deletion |

Future strategies to reduce NK cell-mediated acute xenograft rejection include replicating natural mechanisms of NK cell tolerance in pregnancy and evasion by viruses. Further work is required to identify unknown porcine activating ligands for genetic modifications.

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mediated lysis of pECs from HLA-E/hCD46 transgenic pig was significantly reduced compared to pECs from both wild-type and hCD46 alone (66). As a result of transgenic HLA-E expression, ex vivo perfusion models have also demonstrated increased survival of porcine lungs (67) and improved function of porcine hearts (68). The ability of HLA-E expression to improve graft compatibility across different organs suggests a generalizable mechanism of immune protection, making it a particularly useful genetic modification for optimizing organ donor pigs.

Expressing HLA-G in porcine aortic endothelial cells inhibits xenogeneic human NK cell-mediated cytotoxicity (69). Dose-dependent soluble HLA-G1 has also been shown to reduce human NK cell-mediated cytotoxicity towards pEC (70). In another study, co-transfection with human β2M enhanced the level of HLA-G1, which led to a significant reduction of NK cell-mediated pEC lysis compared to the HLA-G3 isoform (71).

Given the substantial evidence that both HLA-E and HLA-G expression reduce human NK cell-mediated cytotoxicity, co-expression of HLA-E and HLA-G in xenografts may provide immunoprotection and should be further studied (72). Matsunami et al. discussed the effects of co-transfection of HLA-E and HLA-G into pECs while evaluating NK cell response. Though differences were noted across leader peptides and expression rate, substantial NK cell inhibition was noted in HLA-E and HLA-G co-expression compared to either alone. This synergistic relationship between HLA-E and HLA-G demonstrates the potential use in xenografts (73).

An additional model to reduce NK cell cytotoxicity is noted in viral proteins that increase inhibitory signaling in efforts to circumvent immune destruction. Herpesviridae, a family which includes herpes simplex virus (HSV) and human cytomegalovirus (HCMV), have adapted several unique mechanisms centered around decreasing activating and increasing inhibitory receptor-ligand interactions to evade immune detection by NK cells (74). HCMV evades NK cell destruction by amplifying inhibitory signaling via the expression of UL40 and gpUL18 in the endoplasmic reticulum of infected cells (75). UL40-bound HLA-E binds to the NK cell inhibitory receptor NKG2A with greater affinity (6-fold more tightly) than HLA-E alone (Figure 3) (76). Similarly, gpUL18, an MHC I decoy ligand, binds to the inhibitory NK cell receptor LIR-1000-fold more tightly than native MHC I molecules, resulting in a significant reduction in NK cell cytotoxicity (Figure 3) (77–79). The combination of enhanced cell-surface expression of HLA-E and tighter binding to NKG2A results in a marked decrease in NK cell cytotoxicity (80, 81). Repurposing the success of HCMV by expressing these unique viral peptides in xenograft cell lines may induce NK cell tolerance via amplification of inhibitory signals (Table 1).

**Eliminating activating ligands in pig organs**

Another strategy to reduce human NK cell cytotoxicity is eliminating ligands that bind to human NK cell activating receptors. Blocking of activating ligands is a strategy used by HSV-infected cells to downregulate the expression of several ligands of the activating NK cell receptor NKG2D, including MICB (major histocompatibility complex class I polypeptide-related sequence B) and UL-16 binding proteins (ULBP1,2,3)
HSV-infected cells transcribe miR-H8, a microRNA that prevents anchoring of NKG2D-activating ligands such as ULBP-2 and ULBP-3 to the cell surface (74–86). HCMV achieves a similar result by expressing the virally encoded UL-16 protein that binds to and sequesters MICB,ULBP1, and ULBP2 (87). Efforts to mimic these mechanisms may provide an approach to promote xenograft acceptance. Nevertheless, findings surrounding these ligand-receptor interactions are still unclear, and further experimentation is necessary.

Lilienfeld et al. confirmed porcine ULBP1 as a ligand for human NKG2D through anti-porcine ULBP1 polyclonal antibody studies (88). Tran et al. subsequently demonstrated additional unknown porcine ligands to NKG2D (89). After generating a ULBP1-KO on a 5GKO pEC line (ULBP1KO/5GKO), our laboratory concluded that porcine ULBP1 is not the dominant ligand for NKG2D as its deletion did not offer a statistically significant decrease in NK cell activation (90). Additional studies are needed to identify functional porcine ligands to human NKG2D in pig cells.

Glycoprotein D of HSV and pseudorabies virus downregulates CD112 expression, a ligand for the activating NK cell receptor DNAM-1, which results in decreased NK cell activation and reduced cytotoxicity (18, 91). Two porcine isoforms of CD112 have been reported (92), but further study is needed to evaluate the role of porcine CD112 in human NK cell activation. Discovery and deletion of porcine activating ligands could promote human NK cell tolerance (Table 1). Expressing viral proteins in pigs to reduce activating ligands may represent a complementary approach to inhibiting NK cell-mediated cytotoxicity.

When evaluating an array of activating NK cell receptors (NKp46, 2B4, CD49d, CD48, CD2, and NKG2D), Kim et al. discovered that only CD2 and NKG2D were involved in both cytotoxicity and cytokine-secreting functions against porcine cells (93). It is important to note that complete protection against NK cell-mediated cytotoxicity was only obtained when combinations of activating receptors (NKp44 and NKG2D or CD2 and NKG2D) were blocked (93). Structural protein modeling of human CD2 and porcine CD58 demonstrated a highly conserved interface (94). In fact, blocking with an anti-porcine-CD58 antibody inhibited lysis of porcine cells (95), indicating porcine CD58 is a potential activating ligand for modification. Genetic modifications aimed at elimination of activating ligands will require extensive research to identify the most potent receptor-ligand interactions. Simultaneously maximizing inhibitory signaling will be important to induce NK cell tolerance in xenotransplantation.

Conclusions

NK cells play a pivotal role in acute xenograft rejection, yet relatively few attempts have been made to overcome this barrier. Inducing human NK cell tolerance by expressing inhibitory ligands and eliminating activating ligands in porcine xenografts via genetic engineering approach may provide a novel way to protect xenografts from NK cell-mediated destruction. Understanding the mechanism of cross-species immune recognition and response, developing a novel
approach to induce local immune tolerance/acceptance, and guiding the engineering of pigs to meet clinical needs will be our future focus in xenotransplantation.

Author contributions

BE and PL provided critical guidance in the concept and design of the review paper. KL and AC-N wrote the initial draft of the manuscript. KL and AC-N designed the figures and tables. All authors participated in a critical review of the manuscript. All authors approved the final manuscript.

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Conflict of interest

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References

1. Wang X. Bioartificial organ manufacturing technologies. Cell Transplant (2019) 28(1):5–17. doi:10.1080/09636937188099918
2. Murphy SV, Atala A. 3d bioprinting of tissues and organs. Nat Biotechnol (2014) 32(8):773–85. doi:10.1038/nbt.2958
3. Lutz AF, Li P, Estrada JL, Sidher RA, Chihara RK, Downey SM, et al. Double knockout pigs deficient in n-glycolytransferase and galactose-1-phosphate uridyl transferase are non-human primate antibody binding to pig cells lacking Gtf2a1/Cmah/β2galnt2 genes. Xenotransplantation (2015) 20(1):27–35. doi:10.1111/xen.12019
4. Estrada JL, Martens G, Li P, Adams A, Newell KA, Ford ML, et al. Evaluation of human and non-human primate antibody binding to pig cells lacking Gtf2a1/Cmah/β2galnt2 genes. Xenotransplantation (2015) 22(3):194–202. doi:10.1111/xen.12161
5. Li P, Walsh JR, Lopez K, Isidan A, Zhang W, Chen AM, et al. Genetic engineering of porcine endothelial cell lines for evaluation of human-to-pig xenotransplantation. Cell Rep (2021) 11(1):1331. doi:10.1016/j.celrep.2021.04.058
6. Puga Yung G, Schneider MKJ, Seebach JD. The role of nk cells in pig-to-human xenotransplantation. J Immunol Res (2017) 2017:4627384. doi:10.1155/2017/4627384
7. Griffith BP, Goebel C, Singh A, Kothbattl M, Lai CL, Shah A, et al. Genetically Modified Porcine-to-Human Cardiac Xenotransplantation. N Engl J Med (2022) 387(1):35–44. doi:10.1056/NEJMoa2104422
8. Moretta L, Bottino C, Cantoni C, Mingari MC, Moretta A. Human natural killer cell function and receptors. Curr Opin Pharmacol (2001) 1(4):387–91. doi:10.1016/s1471-4892(01)00007-4
9. Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, et al. Innate or adaptive immunity? the example of natural killer cells. Science (2011) 331(6013):44–9. doi:10.1126/science.1198867
10. Lanier LL. Nk cell recognition. Annu Rev Immunol (2005) 23:225–74. doi:10.1146/annurev.immunol.23.021704.115526
11. Krebs P, Barnes MJ, Lampke K, Whitley K, Bahjat KS, Beutler B, et al. Nk-Cell-Mediated killing of target cells triggers robust antigen-specific T-Cell-Mediated and humoral responses. Blood (2009) 113(9):6593–602. doi:10.1182/blood-2009-01-201467
12. Walter T, Dalod M, Robbins SH, Zitvogel L, Vivier E. Natural-killer cells and dendritic cells: “L’union fait la force”. Blood (2005) 106(7):2252–8. doi:10.1182/blood-2005-03-1154
25. Kondo T, Morita K, Watari Y, Auerbach MB, Taub DD, Novick AC, et al. Early increases in bone marrow production and production in murine allogenic skin grafts is mediated by natural killer cells. *Transplantation* (2000) 69(5):969–77. doi: 10.1097/00007890-200005100-00051

26. Feng L, Ke N, Ye Z, Guo Y, Li S, Li Q, et al. Expression of Nkg2d and its ligand in mouse heart allografts may have a role in acute rejection. *Transplant Proc* (2009) 41(3):1329–9. doi: 10.1016/transpro.2009.08.063

27. Hirohashi T, Chase CM, DellaPelle P, Sebastiani D, Farkeh E, Colvin RB, et al. Depletion of T regulatory cells promotes natural killer cell-mediated cardiac allograft vasculopathy. *Transplantation* (2014) 98(8):828–34. doi: 10.1097/TP.0000000000000329

28. Uehara S, Chase CM, Kitchens WH, Rose HS, Colvin RB, Russell PS, et al. Nk cells can trigger allograft vasculopathy: The role of hybrid resistance in solid organ allografts. *J Immunol* (2005) 175(5):3424–30. doi: 10.4049/ immunol.175.5.3424

29. Kawakami T, Ito K, Matsuda Y, Noda M, Sakurada A, Hoshikawa Y, et al. Cytotoxicity of natural killer cells activated through Nkg2d contributes to the development of bronchiolitis obliterans in a murine heterotopic tracheal transplant model. *Am J Transplant* (2017) 17(9):2338–49. doi: 10.1111/aht.14257

30. Littera R, Piredda G, Argiolas D, Lai S, Congeddu E, Ragatzu P, et al. Kid and their hla class I ligands: Two more pieces towards completing the puzzle of chronic kidney allograft rejection by natural killer cells. *J Immunol* (2016) 196(3):491–8. doi: 10.4049/jimmunol.1500003

31. Ashraf MI, Sarwar A, Kühl AA, Hunger E, Sattler A, Aigner F, et al. Lack of the appropriate potential modulation of murine natural killer cell activity and xenogeneic allograft rejection in the absence of xenoreactive antibody in xenotransplantation. *Transplant Proc* (2021) 53(10):3809–100. doi: 10.1016/j.transproceed.2021.09.028

32. Pradier A, Papaserafeim M, Li N, Rietveld A, Kaestel C, Gruaz L, et al. Human anti-pig cytotoxicity mediated by activated natural killer cells. *J Immunol* (2011) 187(8):4144–54. doi: 10.4049/jimmunol.12124

33. Kan 2004, 78(2):386–9. doi: 10.1016/j.transproceed.2009.08.060

34. Schneider MK, Ghielmetti M, Rhyner DM, Antsiferova MA, Seebach JD. Human leukocyte transmigration across Galalapha(1,3)Gal-negative pig endothelium is regulated by human Cd18 and Cd99. *Transplantation* (2006) 81(4):491–9. doi: 10.1097/TP.0b013e3181595fb6d

35. Kwiatkowski P, Artrip HJ, John R, Edwards NM, Wang SF, Micheler RE, et al. Induction of swine major histocompatibility complex class I molecules on porcine endothelium by tumor necrosis factor-alpha reduces lysis by human natural killer cells. *Transplantation* (1999) 67(2):211–8. doi: 10.1097/00007890-199901270-00005

36. Zhang XF, Feng MF. Adherence of human monocytes and natural killer cells to human aorta xenografts may have a role in acute rejection. *Transplant Proc* (2000) 32(6):2058–9. doi: 10.1006/trans.2000.5987

37. Weiss EH, Lilienfeld BD, Müller S, Müller E, Herbach N, Kessler B, et al. Human anti-pig natural killer cell cytotoxicity. *Transplantation* (2009) 87(1):35–43. doi: 10.1097/TP.0b013e318191f7c84
of natural killer cells. Bound peptides in bound to a host inhibitory receptor. Xenotransplantation (2017) 24(2):1–8. doi: 10.1111/xen.12294

68. Abicht JM, Sfriso R, Reichart B, Längin M, Gaulle K, Puga Yang GL, et al. Multiple genetically modified Girk/Hla/H2j-Mg porcine hearts are protected from complement activation and natural killer cell infiltration during ex vivo perfusion with human blood. Xenotransplantation (2018) 25(1):1–13. doi: 10.1111/xen.12357

69. Forte P, Matter-Reissmann UB, Strasser M, Schneider MK, Seebach JD. Porcine aortic endothelial cells transfected with hla-G are partially protected from xenogenic human nk cytotoxicity. Hum Immunol (2000) 61(11):1066–73. doi: 10.1016/s0198-8859(00)00202-0

70. Zeng MH, Fang CY, Wang SS, Zhu M, Xie L, Li R, et al. A study of soluble hla-G1 protecting porcine endothelial cells against human natural killer cell-mediated cytotoxicity. Transplant Proc (2006) 38(10):3312–4. doi: 10.1016/j.transproceed.2006.10.179

71. Matsunami K, Miyagawa S, Nakai R, Murase A, Shirakura R. The possible use of hla-G1 and G3 in the inhibition of nk cell-mediated swine endothelial cell lysis. Cell Transplantation (2006) 16(4):295–304. doi: 10.1080/09636920600880798

72. Crew MD. Play it in e or G: Utilization of hla-e and -G in xenotransplantation. Xenotransplantation (2007) 14(3):198–207. doi: 10.1111/j.1399-3089.2007.00395.x

73. 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-regulates natural killer cell-mediated swine endothelial cell lysis. Transplantation (2002) 73(10):1582–9. doi: 10.1097/00007890-200203200-00010

74. Koyanagi N, Kawaguchi Y. Evasion of the cell-mediated immune response by alphaherpesviruses. Viruses (2020) 12(12):1–16. doi: 10.3390/v12121354

75. Prod’homme V, Tomasec P, Cunningham C, Lemberg MK, Stanton RJ, Crew MD, et al. Herpesvirus evasion of early host antiviral responses. J Virol (2008) 82(24):12549–59. doi: 10.1128/jvi.02105-17

76. Ilano M, Lee N, Navarro F, Garcia P, Albar JP, Geraghty DE, et al. Hla-e- Bound peptides influence hla-e recognition by inhibitor and triggering Cd94/Nkg2 receptors: Preferential response to an hla-G-Derived nonamer. Eur J Immunol (1998) 28(9):2854–63. doi: 10.1002/(sici)1521-4149(199809)28:9<2854::aid-eji2854>3.0.co;2-w

77. Reyburn HT, Melabeoum O, Vales-Gomez M, Davis DM, Pazmany L, Strominger JL. The class I mhc homologue of cytomegalovirus inhibits attack by natural killer cells. Nature (1997) 386(6624):514–7. doi: 10.1038/386514a0

78. Cosman D, Fanger N, Borges L, Kuhin M, Chin W, Peterson L, et al. A novel immunoglobulin superfamily receptor for cellular and viral mhc class 1 molecules. Immunity (1997) 7(2):273–82. doi: 10.1016/s1074-7613(03)00529-4

79. Yang Z, Bjorkman PJ. Structure of Ul18, a peptide-binding viral mhc mimic, bound to a host inhibitory receptor. Proc Natl Acad Sci U.S.A. (2008) 105(29):10095–100. doi: 10.1073/pnas.0804551105

80. De Pelsmaeker S, Romero N, Vitale M, Favorolle HW. Herpesvirus evasion of natural killer cells. J Virol (2018) 92(11):1991–1005. doi: 10.1128/jvi.02105-17

81. Ulbrecht M, Martinuzzi S, Grzeschuk M, Hengel H, Ellwatt JW, Pla M, et al. Cutting edge: The human cytomegalovirus Ul40 gene product contains a ligand for hla-e and prevents nk cell-mediated lysis. J Immunol (2000) 164(10):5019–22. doi: 10.4049/jimmunol.164.10.5019

82. Spech D, D’Amato M, Studahl M, Bergström T, Karre K, Berg L. Herpes simplex virus infection downmodulates Nkg2d ligand expression. Scand J Immunol (2006) 65(5):429–36. doi: 10.1111/j.1365-3083.2006.02241.x

83. Tognarelli EL, Palominio TF, Corrales N, Bueno SM, Kaleris AM, González PA. Herpes simplex virus evasion of early host antiviral responses. Front Cell Infect Microbiol (2019) 9:127. doi: 10.3389/fcimb.2019.00127

84. Enk J, Levi A, Weisblum Y, Yaman R, Charpak-Amikam Y, Wolf DG, et al. Hsv1 microtus modulation of gpi anchoring and downstream immune evasion. Cell Rep (2016) 17(4):949–56. doi: 10.1016/j.celrep.2016.09.077

85. Kinoehita T. Biosynthesis and deficiencies of glycosylphosphatidylinositol. Prog Ipn Acad Ser B Phys Biol Sci (2014) 90(4):130–45. doi: 10.2183/pabj.90.130

86. Oshita K, Inoue N, Kinoehita T. Pigs and pig-t, essential for gpi anchor attachment to proteins, form a complex with Gaal and Gpshk EMBO J (2000) 20(15):4088–98. doi: 10.1093/emboj/cd0.15.4088

87. Dunn C, Chalupny NJ, Sutherland CL, Dosh S, Sivakumar PV, Johnson DC, et al. Human cytomegalovirus glycoprotein Ul16 causes intracellular sequestration of Nkg2d ligands, protecting against natural killer cell cytotoxicity. J Exp Med (2003) 197(11):1427–39. doi: 10.1084/jem.20022059

88. Lilienfeld BG, Garcia-Borges C, Crew MD, Seebach JD. Porcine Ul16-binding protein 1 expressed on the surface of endothelial cells triggers human nk cytotoxicity through Nkg2d. J Immunol (2006) 177(7):2416–52. doi: 10.4049/jimmunol.177.7.2416

89. Tran PD, Christiansen D, Winterhalter A, Brooks A, Gorrell M, Lilienfeld BG, et al. Porcine cells express more than one functional ligand for the human lymphocyte activating receptor Nkg2d. Xenotransplantation (2008) 15(3):321–32. doi: 10.1111/j.1365-3089.2008.00489.x

90. Lopez K, Isadan A, Cross-Naja F, Campana G, Zhang W, Esker B, et al. Disrupting porcine ul-binding protein does not significantly reduce human nk cell activation in vitro model. Xenotransplantation (2021) 28:31(2021):3 1.1365-3089.200209

91. Denaeghel S, De Pelsmaeker S, Van Waesberge C, Favorolle HW. Pseudorabies virus infection causes downregulation of ligands for the activating nk cell receptor Nkg2d. Viruses (2021) 13(2):1–14. doi: 10.3390/v13020266

92. Wang L, Zhang W, Wu DA, Chen C, Xu QZ, Zhao B, et al. Molecular cloning, characterization and three-dimensional modeling of porcine nectin-2/ Cdt112. Vet Immunol Immunopathol (2009) 132(2–3):257–63. doi: 10.1016/j.vetimm.2009.05.008

93. Kim TJ, Kim N, Kim EO, Choi JR, Bluestone JA, Lee KM. Suppression of human anti-porcine natural killer cell xenogenic responses by combinations of monoclonal antibodies specific to Cdt2 and Nkg2d and extracellular signal-regulated kinase kinase inhibitor. Immunology (2010) 130(4):545–55. doi: 10.1111/j.1365-2567.2010.02353.x

94. Brossay A, Hubé F, Moreau T, Bardos P, Watier H. Porcine Cdt58: Cdna cloning and molecular dissection of the porcine Cdt58–human Cdt2 interface. Biosichem Biophys Res Commun (2003) 309(4):992–8. doi: 10.1016/j.bbrc.2003.08.099

95. Crosby K, Yatko C, Dersimonian H, Pan L, Edge AS. A novel monoclonal antibody inhibits the immune response of human cells against porcine cells. Identification of a porcine antigen homologous to Cdt58. Transplantation (2004) 77(8):1288–94. doi: 10.1097/01.tp.0000120377.57543.d8