



Immunological response in cynomolgus macaques to porcine α -1,3 galactosyltransferase knockout viable skin xenotransplants—A pre-clinical study

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Abstract

Background: Allogeneic skin recovered from human deceased donors (HDD) has been a mainstay interim treatment for severe burns, but unfortunately risk of infectious disease and availability limitations exist. Genetically engineered α -1,3 galactosyltransferase knockout (GalT-KO) porcine source animals for viable skin xenotransplants may provide a promising clinical alternative.

Methods: Four cynomolgus macaque recipients received full-thickness surgical wounds to model the defects arising from excision of full-thickness burn injury and were treated with biologically active skin xenotransplants derived from GalT-KO, Designated Pathogen Free (DPF) miniature swine. Evaluations were conducted for safety, tolerability, and recipient immunological response.

Results: All skin xenotransplants demonstrated prolonged survival, vascularity, and persistent dermal adhesion until the study endpoint at post-operative day 30. No adverse outcomes were observed during the study. Varying levels of epidermolysis coincided with histologic detection of CD4+ and CD8+ T cells, and other cellular infiltrates in the epidermis. Recipient sera IgM and IgG demonstrated significant antibody immune response to non- α -1,3-galactose porcine xenoantigens. Separately, specific wound healing mediators were quantified. Neither porcine cell migration nor PERV were detected in circulation or any visceral organs.

Conclusions: These results provide a detailed analysis of vital skin xenotransplants utilizing a non-human primate model to predict the anticipated immunological response of human patients. The lack of adverse rejection even in the presence of elevated Ig indicates this is a prospective therapeutic option. The findings reported here directly supported regulatory clearance for a first-in-man, Phase I xenotransplantation clinical trial.

KEYWORDS

α -1,3 galactosyltransferase, GalT-KO, human deceased donor allografts, pig, porcine endogenous retrovirus, porcine xenograft, rejection, skin xenotransplant

1 | INTRODUCTION

Each year, burn injuries claim the lives of 180 000 victims worldwide,¹ and 100 000 Americans are hospitalized.² The ramifications of severe burns are profound for the patient, including long-term metabolic disturbances. When skin is significantly damaged, patients rapidly lose fluid, causing internal pH and homeostatic imbalances. If uncorrected, this leads to multi-organ failure in 50% of non-survivors of severe burns.³ Further, the sequelae of infection, sepsis, and inhalation injury combine to account for the death of three-quarters of all patients with injuries covering 40% or more of the total body surface area (TBSA).²

Therapeutic options depend on the severity of the burn. Autografts are the ideal treatment as they create an effective barrier, cause minimal risk of infectious disease, and are immunologically compatible.

However, autografts can be clinically contraindicated for patients with burns covering 20% or more TBSA.^{4,5} In such scenarios, human deceased donor (HDD) allografts are an effective mainstay in the treatment of severe burns.⁶ HDD allografts are highly effective in creating a barrier for wound closure and thus in preventing infections. This involves formation of a fibrin seal which provides effective wound closure, and a critical bacteriostatic effect that is essential in the treatment of large burns.^{7,8}

However, clinicians have long sought alternative treatment options that address the severe shortcomings of allograft material^{6,9-11} while providing the same fundamental mechanism of action that achieves wound closure and temporary restoration of barrier function.

For decades, the field of xenotransplantation has represented a promising, but unrealized, solution to the global shortage of skin and other transplantable organs.¹² Until the 1970's, xenografts derived from wild-type porcine source animals were used as temporary wound dressings and are still used commonly today in some parts of the world.^{13,14} However, classical xenografts provide wound coverage—not wound closure. Preformed antibodies in humans to wild-type porcine skin lead to antibody mediated, hyperacute rejection, considerably limiting adequate graft adherence to the wound and anastomosis with host vessels for meaningful durations.¹⁵ This hyperacute rejection is due principally to a unique antigen on porcine endothelial cells, the α -1,3-galactose (α -1,3-gal) epitope, which is recognized as foreign by preformed human antibodies.

Extensive research has provided a solution to this rapid rejection of porcine donor tissue in human recipients through genetic modification of source animals, made considerably easier with recently available technologies and gene editing techniques.^{16,17} One of the most common and well-studied modifications is the removal of the α -1,3-gal epitope in genetically engineered α -1,3 galactosyltransferase knockout (GalT-KO) porcine source animal.¹⁸⁻²⁰ As evidenced by pre-clinical studies, these GalT-KO skin xenotransplants delay the recipient's immune response²¹ and exhibit prolonged xenotransplant survival.^{12,22,23}

Immune rejection is not the only barrier to clinical xenotransplantation. Foremost, reduction of associated infectious disease concerns by using Designated Pathogen Free (DPF) donors will be essential to promulgating its large-scale use.^{24,25} However, to date, there has not been in vivo evidence of PERV transmission in human patients exposed to pig cells or tissues.^{24,26,27} Long-term monitoring of potential PERV transmission and/or porcine cell microchimerism has also failed to indicate transmission of the virus from swine to human, even those cases followed for 34 years.¹⁵

The following pre-clinical study, performed under Good Laboratory Practices (GLP) conditions, was conducted in support of a US Food and Drug Administration (FDA) Investigational New Drug (IND) application. Safety and efficacy were both primary considerations of the study, and the findings reported here directly supported regulatory clearance for a Phase I clinical trial.

In our first article describing this pre-clinical study,²⁸ the primary focus was reporting the clinical observations that all xenotransplants demonstrated survival, adherence, and vascularity until post-operative day 30. Here, we present an extended analysis specific to the immunological response in recipients of vital skin xenotransplants, with a focus on the immune response to non- α -1,3-galactose porcine xenoantigens and presence of various immune mediators associated with wound healing.

2 | MATERIALS AND METHODS

2.1 | Ethics

This study was conducted in accordance with US-FDA GLP regulations and guidance documents (21 CFR Part 58.351), the US Department of Agriculture's (USDA) Animal Welfare Act (9 CFR Parts 1, 2, and 3), the *Guide for the Care and Use of Laboratory Animals*²⁹ and the *Guidance for Industry documents: "Source Animal, Product, Preclinical and Clinical Issues for the Use of Xenotransplantation Products in Humans" and GFI 187, "Regulation of Intentionally Attended Genomics DNA in Animals"*. The study surgical procedures, protocols, and guidelines for animal care were independently reviewed and monitored by a standing institutional animal care and use committee (IACUC) committee. Lastly, the final study results were audited by an independent reviewer for quality assurance.

2.2 | Xenotransplant recipients

Two male, non-naïve and two female, naïve cynomolgus monkeys (*Macaca fascicularis*) of Chinese origin were assigned as transplant recipients. This subject choice was necessary, as only humans and non-human primates possess preformed antibodies to the α -1,3-galactose porcine epitope, and thus reject wild-type porcine tissues in a similar manner.^{30,31} Furthermore, cynomolgus monkeys are well established in previous literature as scientifically appropriate subjects for such studies.^{32,33} Non-naïve animals were included as

recipients in this study due to limited subject availability and to most ethically and prudently steward the use of research animals. These animals had previously been involved in pharmacokinetic studies that involved administration of drugs intended to reduce relapsed alcohol consumption and for treatment of cocaine abuse. Pharmacokinetic studies of this type were determined to have no known long-term immunological impact or likely interference with the experimental studies presented here. Pre-study physical examination by the testing facility's veterinary staff did not indicate any health issues that would preclude the animals from the study. Animal health, including clinical observations, body weight, body condition, food consumption, cardiac monitoring, respiratory rate, body temperature, neurological, and pathological examinations were monitored/conducted at pre-determined, regular intervals throughout the duration of the experiment under veterinary supervision.

2.3 | Procurement and preparation of skin xenotransplants

Xenotransplants were obtained from source animals originating from a closed colony of DPF GalT-KO miniature swine originally developed by Sachs et al at Massachusetts General Hospital (Boston, MA).^{34,35} The DPF designation signifies that the animals were raised under prescribed isolation conditions: raised from birth via Cesarean section; confirmed porcine cytomegalovirus (PCMV) negative; hand-fed by gowned staff with sterilized food and water; housed separately in a Biological Safety Level 2 (BSL-2), positive pressure, temperature-controlled room with restricted access and no exposure to pigs outside of the housing area; vaccinated against normal swine pathogens and monitored on a quarterly basis for external swine pathogens to ensure the absence of several specific adventitious agents in line with the indicated guidance and ethics. One GalT-KO miniature swine from the closed colony was selected as the donor source animal for all eight skin xenotransplants used in this study.³⁶ The preparation and procurement of the skin xenotransplants from the donor were previously described in detail.²⁸ The skin was procured under stringent aseptic conditions and sterile environment via air-driven Zimmer dermatome (Medfix Solution, Inc, Tucson, AZ). The harvested split-thickness porcine skin xenotransplants were inspected for quality, verified for thickness (0.55 mm/0.022 inches), and trimmed to form, measuring approximately 25 cm². This was followed by additional processing under sterile conditions, which carefully removed commensal skin flora and achieved United States Pharmacopeia (USP) <71> Sterility Standards³⁷ while retaining cellular viability. Skin xenotransplants were incubated in media containing a proprietary combination of antibiotics and antimycotics, rinsed in saline, rolled in nylon mesh, and placed in a threaded-seal cryovial. CryoStor CS5 media, 5 mL, (BioLife Solutions) was added to the vial, which was then sealed.

Vials were placed into a controlled-rate freezer, slow-cooled at a rate of 1°C per minute to -40°C, then rapidly cooled to -80°C before being transferred to a -80°C freezer. The cryopreservation

method used in this study was evaluated in a previous study,³⁶ comparing identically prepared porcine skin grafts cryopreserved for different lengths of time. The results demonstrated no significant differences in clinical or histologic outcomes, or in cellular viability as assessed with 3-4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assays. Combined, these specific practices retain the essential metabolic activity in porcine skin xenotransplants after cryopreservation and storage for extended durations.

The skin xenotransplants used in this study were cryopreserved for six months prior to use. Skin xenotransplants were thawed by immersing the sealed vials in a 37°C water bath for approximately one minute, followed by three one-minute aseptic serial washes in sterile normal saline with gentle agitation. Thawed skin xenotransplants were taken to the surgical field for use at ambient temperature.

2.4 | Surgical transplantation of skin xenotransplants

Transplantation surgery was performed on each of the four non-human primate (NHP) recipients via four sequential, independent surgical procedures. Animals were sedated with Ketamine (~15 mg/kg) and pre-medicated with Atropine (0.04 mg/kg IM). The animals were intubated and maintained on Isoflurane anesthesia (~1.0-2.5%, oxygen 2.0 liters). Prior to surgery, animals were given Buprenorphine (0.03 mg/kg IM) and Cefazolin (20 mg/kg IM). Hair was clipped from the dorsal and lateral thorax. The surgical site was prepared for surgery using three alternating scrubs of either povidone iodine or chlorhexidine scrub solution and sponges soaked in 70% isopropyl alcohol. The animals were moved to the operating table and positioned in sternal recumbency. Heart rate, respiratory rate, blood pressure (as applicable), end-tidal carbon dioxide, and body temperature were continually monitored throughout the procedure and recorded at least every 15-20 minutes as applicable. The surgical site was then prepared for aseptic surgery by wiping or spraying the surgical site with 70% isopropyl alcohol followed by an application of Dura-PrepTM. A local anesthetic line block of 0.25-0.5 mL Lidocaine (or Bupivacaine) per site was performed prior to the procedure.

Wound beds were prepared in two stages, first by preparing partial-thickness wound beds using an air-driven Zimmer dermatome. These wound beds were then surgically converted into full-thickness wound beds using a scalpel, removing all tissue superficial to the underlying fascia. This resulted in two separate full-thickness 9 cm² wounds on the dorsal aspect of each subject, between the inferior aspect of the scapulae and superior to the iliac crests, centered medially along the spine. Split-thickness skin xenotransplants were trimmed to fit the wound bed and uniformly sutured in place, covering the entire wound, using simple, interrupted, 3-0 nylon sutures. In total, eight (n = 8) skin xenotransplants were placed across the four subjects (n = 4).

2.5 | Post-operative care and wound dressing of recipient subjects

Post-operative care for the recipients was described in detail by Holzer et al²⁸. Briefly, all wounds and overlying skin xenotransplants were covered with pressure dressings, VetRap, and primate jackets.

Animals received Buprenorphine (0.03 mg/kg, IM) immediately on post-operative day 0 (POD-0) and every other day thereon as required. Clinical observations were performed at least twice daily. At no time during this study was immunosuppressive therapy administered to the transplant recipients.

Gross assessment of the wound sites occurred on POD-7, 14, 21, and 30 (end of study). During each observation, primate jackets and dressings were removed, the wounds were cleaned, the wound sites were evaluated for duration of survivability (ie, time to immune-mediated rejection) of the skin xenotransplants and were redressed as previously described. At each dressing change, peripheral blood was obtained for later evaluation. At the conclusion of the study, all xenotransplant recipients were euthanized and a complete necropsy was performed.

2.6 | Post-operative histopathological assessment

Approximately 5 mm³ pieces of major tissues (the spleen, liver, kidney, lung, and heart) were obtained from each animal at necropsy. These major tissues and wound sites were collected and fixed in 10% neutral buffered formalin (NBF). The major tissues were then transferred to 70% ethanol after approximately 72 hours (\pm 4 hours). Wound sites, major tissues, and additional organs were shipped to Alizée Pathology (Thurmont, MD) for histologic processing and evaluation. On receipt, wound sites were trimmed, processed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E).

Immunohistochemistry was performed on the wound sites to look for the presence of CD4⁺ and CD8⁺ T cells. Paraffin-embedded tissues were cut at 4 μ m thickness, mounted on glass slides and incubated at 60°C for 30 minutes, followed by deparaffinization in xylene and rehydration in graded alcohol into water then tris-buffered saline (TBS)/Tween. Antigen retrieval was performed by Diva Decloaker (Biocare) for 15 minutes. Endogenous blocking was done with normal goat serum and avidin for 20 minutes. The tissues were incubated with 1:20 dilution of CD4 mouse monoclonal antibody (Biocare ACI3148A Clone 4B12) or 1:20 dilution of CD8 mouse monoclonal antibody (Biocare ACI3160A, Clone CD8/144B) in van Gogh diluent (Biocare) at 4°C overnight. After washing, slides were incubated via the avidin-biotin complex method for 20 minutes.³⁸ This was followed by 4 + Biotinylated Universal Goat Link Polymer horse radish peroxidase, (Biocare, Agilent) probe, and polymer each for 20 minutes at room temperature. After washing, DAB (3,3'-diaminobenzidine) reagent (DAKO; Agilent) was added with monitoring for 5 minutes. Counterstaining

was performed using Harris hematoxylin. Slides were briefly dehydrated and then mounted with Cytoseal Mounting Media (Life Technologies).

2.7 | Post-operative assessment of recipient immune response

Peripheral blood (PB) was obtained for isolation of serum and peripheral blood mononuclear cells (PBMCs) on POD-0, 7, 14, 21, and 30. Isolated serum was aliquoted and placed into a -80°C freezer until analyzed. PBMCs were harvested from PB samples by lysing the red blood cells in RBC lysing solution twice and then washing in phosphate-buffered saline. The resulting PBMCs were pelleted, lysed, and frozen at -80°C until use.

To evaluate the inflammatory and anti-inflammatory wound healing cytokines and chemokines, characteristic of initial wound healing processes, recipient sera was analyzed using a Luminex 23-plex assay, Milliplex Map Non-Human Primate Cytokine Magnetic Bead Panel (Millipore Sigma, PRCYTOMAG-40K, Merck KGaA). The multiplexed assay panel was specific for cross-reactivity with NHP samples. The panel of 23 mediators measured in this assay included the following: interleukin-1ra (IL-1ra), IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12/23(p40), IL-13, IL-15, IL-17, IL-18, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 α (MIP-1 α), macrophage inflammatory protein-1-beta (MIP-1 β), soluble CD40 ligand (sCD40L), transforming growth factor- α (TGF- α), transforming growth factor-beta (TGF- β), vascular endothelial growth factor (VEGF), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), and interferon-gamma (IFN-gamma).

In addition, binding of recipient serum IgM and IgG antibodies to non- α -1,3-galactose porcine xenoantigens in the skin xenotransplants was measured by flow cytometry. PBMCs from GalT-KO swine were isolated from the buffy coat of porcine peripheral blood and counted using a Coulter MD II Hematology Analyzer. Serum samples were de-complemented in a 56°C dry heat bath for 30 minutes and serially diluted at 1:2, 1:10, 100, 1,000, and 10,000 in Flow Activated Cell Sorting Media (FACS) (1X Hanks Balanced Salt Solution with Ca²⁺ and Mg²⁺, 0.1% Bovine Serum Albumin (BSA), and 0.1% Sodium Azide). 10 μ L of serially diluted serum samples were incubated with 1.5×10^5 cells in 100 mL FACS buffer for 30 minutes at 4°C. Cells were washed twice and stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgM and phycoerythrin-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories Inc) for 30 min at 4°C. Cells were washed twice with FACS buffer, and data acquisition was performed with an ACEA NovoCyte Flow Cytometer. Appropriate compensation, Limit of Blank (LOB) (which is the mean fluorescent intensity of cells using only secondary antibody in the absence of serum), and Fluorescence Minus One (FMO) controls were run. Binding of IgM and IgG was assessed using mean fluorescence intensity (MFI) and relative MFI values which were obtained as follows: Relative MFI = Actual MFI value/LOB.

2.8 | Post-operative assessment of porcine endogenous retrovirus (PERV)

Samples of liver, spleen, kidney, lung, residual skin xenotransplant, and underlying wound bed were obtained at necropsy. These, along with lysed PBMCs and sera obtained concomitant with each clinical assessment, were dedicated for assessment of post-operative transmission of PERV. DNA was isolated from the PBMCs and wound beds using the Genra Puregene kit (Qiagen). RNA was isolated from tissue samples using the RNeasy mini kit (Qiagen). Viral RNA was isolated from serum using the Viral RNA mini kit (Qiagen). DNA PCR assays for PERV and microchimerism (porcine centromeric DNA) were performed as previously described.¹⁵ For viral RNA, each reaction was spiked with Taqman exogenous internal positive control (Applied Biosystems). For total RNA, reactions included 18S RNA as a reference using probe and primers from the 18S rRNA control kit (Eurogentec). This reference was used to avoid false negatives due to the absence of RT-PCR failures and/or PERV RNA.

2.9 | Statistical analysis

Simple mean and standard deviation were used exclusively. A student t-test was used to compare changes in cytokine/chemokine levels to baseline, and a paired t-test was used to compare the change in IgG and IgM binding from POD-0 to POD-30.

3 | RESULTS

3.1 | Post-operative survival and tolerability assessment

All xenotransplant recipients tolerated the initial surgical procedure and placement of bilateral skin xenotransplants without issue. All four subjects survived to the scheduled end of study, POD-30, without experiencing any adverse events. Each recipient lost weight following the surgical procedure, but maintained a body condition score ranging between 2.5 (lean) and 3 (healthy). Percent reduction in body weight for each recipient, between pre-operative evaluation and the

end of study (POD-30), was -3.8% (subject 2001), -8.2% (subject 2002), -10.0% (subject 2101), and -9.4% (subject 2102). These values are within acceptable ranges following a surgical event of this nature.

3.2 | Post-operative clinical assessment

As previously reported,²⁸ all eight skin xenotransplants adhered completely to the underlying wound bed; zero technical failures were observed. Over the course of the study, each xenotransplant independently demonstrated prolonged survivability and vascularity relative to previous studies that utilized similar models and study designs,^{22,39-41} based on the color, texture, capillary refill, and overall clinical appearance.

At no time was sloughing of the xenotransplant clinically visible or exposure of the underlying wound bed observed. Epidermolysis (mild to moderate) was first noted on POD-14, but the dermis at all eight sites remained completely adherent. By POD-21, progression to complete epidermolysis was noted. Later, assessments also revealed wound bed granulation and signs of re-epithelialization, such that by POD-30, varying degrees of re-epithelialization (up to 100% in some cases) had occurred in all four subjects (Figure 1; Table 1).

3.3 | Post-operative histopathological assessment

There were no systemic histopathological differences observed between POD-0 and POD-30, following a comprehensive examination which included assessment of the external body and all orifices, as well as the cranial, thoracic, abdominal cavities, and their contents. Histologic examination of kidney, lung, liver, spleen, and heart samples demonstrated no evidence of toxicity related to the xenotransplants.

As previously reported,²⁸ hematoxylin and eosin (H&E)-prepared sections of skin xenotransplants and wound beds obtained at the end of study were microscopically evaluated by a blinded pathologist. H&E staining showed minimal to moderate inflammatory response. Ulceration of the epithelia (epidermolysis) was observed in four out



FIGURE 1 Skin xenotransplants on dorsal surface of Animal 2001 at POD-30. (A) Low power image of Wound Site 1. (B) High power image of Wound Site 1

TABLE 1 Banff grades and pathologic component scores^a of skin xenotransplants at POD-30

Animal	Wound Site	Surgeon assessment	Banff Grade	pc ^b	pa ^c	ei ^d	e ^e	v ^f	c ^g	cav ^h
2001	1	100% re-epithelialized	III	3	3	3	1	0	1	0
2001	2	100% re-epithelialized	III-IV	3	3	3	2	0	1	0
2002	1	30% re-epithelialized	III-IV	3	3	3	3	0	0	0
2002	2	30%re-epithelialized	III-IV	3	3	3	3	0	0	0
2101	1	40% re-epithelialized	II	3	3	2	3	0	0	0
2101	2	40% re-epithelialized	III-IV	3	3	3	2	0	0	0
2102	1	20% re-epithelialized	III-IV	3	3	3	3	0	0	0
2102	2	20% re-epithelialized	II-III	3	3	3	3	0	0	0

^aPathologic Component Scores developed by Rosales, et al⁴²

^bpc = perivascular cells—number of cells surrounding dermal vessels (venules, capillaries, and arterioles) in deep and superficial dermis; scored on the most involved vessels; pc3 > 50 cells/vessel.

^cpa = perivascular dermal infiltrate area—percent area occupied by the most involved dermal vessels at 40x magnification; pa3 > 75%

^dei = epidermal infiltrate—total number of mononuclear cells per four 20x fields; ei3 = transepidermal infiltrate, ei2 > 20 cells

^ee = epidermal injury and necrosis—presence of keratinocyte apoptosis and necrosis; e3 = sloughed, e2 = focal necrosis, e1 = apoptosis

^fv = endarteritis—mononuclear cells underneath arterial endothelium; scored on the most involved artery; v0 = none.

^gc = capillaritis—maximum number of cells per capillary cross section; scored on most involved capillaries; c1 = 2-4/capillary, c0 = 0-1/capillary

^hcav = chronic allograft vasculopathy—intimal thickening with luminal reduction; scored as percent luminal reduction; cav0 = none.

of eight treated sites. The wound sites were initially viewed at a lower magnification to identify the border of the host tissue and remaining tissue of the skin xenotransplant (Figure 2). Wound defects were characterized histopathologically by the presence of a mature dermal collagen network distinct in appearance from the host dermis bordering the wound site, interpreted to be dermis of the xenotransplant,

surrounded by a variable layer of new collagen (Figure 2). Features of dermal fibrosis, focal thinning of the epidermis and granulation tissue were also noted. Edema was minimal and considered within normal range. No additional staining was performed to confirm this (Additional, confirmatory staining methods for the presence of porcine material are in development and the results will be later reported).

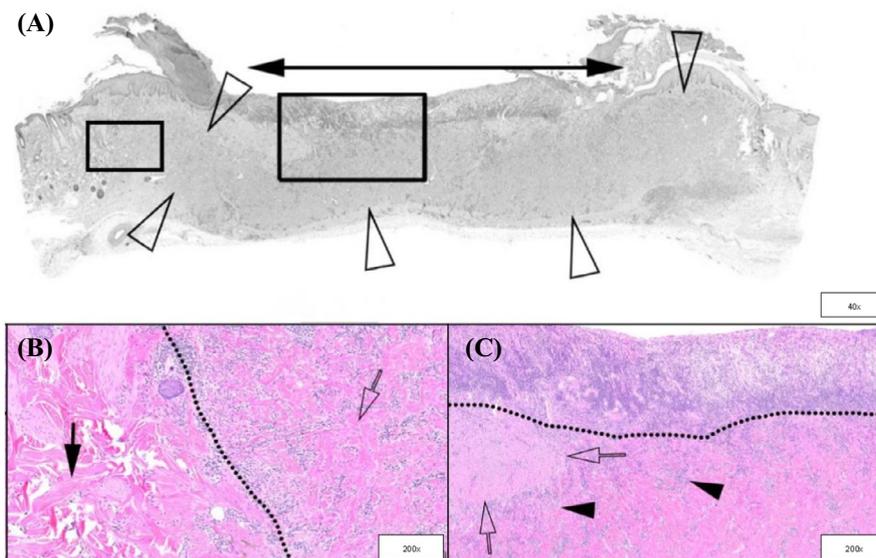


FIGURE 2 Wound Site 1, Animal 2002 at POD-30. (A) 40x power image of the skin xenotransplant wound site. The double-headed (closed, top) arrow indicates incomplete epithelial coverage (region of ulceration or epidermolysis). The open arrowheads indicate areas of new collagen formation surrounding the residual tissue from the skin xenotransplant. (B) 200x power image of small inset box (left) in Figure A. To the left of the dotted line (closed arrow) is native host dermis, while residual dermal tissue from the skin xenotransplant dermis is located the right of the dotted line (open arrow). Note the distinct difference in collagen morphology between the two regions. (C) 200x power image of large inset box (right) in Figure A. Prominent inflammatory infiltrates attributed to the region of ulceration at the surface of the skin xenotransplant is visible in the region above the dotted line. Below the dotted line, most of the tissue consists of skin xenotransplant with relatively less inflammation (closed arrowheads) as well as evidence of new collagen (open arrows)

A second pathologist further assessed the skin xenotransplants for cellular infiltrations and other microscopic indicators of immunological rejection via two methods.⁴² The Banff Classification for skin-containing composite tissue allograft pathology was used to categorize xenotransplant rejection. A component scoring system designed to complement the Banff system was also used to identify variations in cellular infiltration and other pathologic lesions. These results are shown in Table 1. The Banff Grades,⁴³ based on the level of epidermal apoptosis, epidermal infiltrates, and perivascular/dermal infiltrates, ranged from II (epidermal infiltration) to IV (necrotizing acute rejection), with most showing Grade III (severe). Multiple foci of perivascular inflammation and epidermal infiltrates with apoptosis were observed. Cellular infiltrates included macrophages, lymphocytes, neutrophils, eosinophils, and plasma cells. CD4+ and CD8+ T cells were identified in perivascular and epidermal infiltrates (Figure 3). Multinucleated giant cells associated with granulomatous inflammation were also present (not shown).

3.4 | Post-operative assessment of recipient immune response

Twelve of the 23 cytokines/chemokines assayed were consistently below the level of detection throughout the entire study period: TNF- α , IFN- γ , TGF- β , G-CSF, GM-CSF, IL-1- β , IL-4, IL-5, IL-10, IL-13,

IL-17, IL-18, and MIP-1- α . VEGF exceeded the level of detection at only 3 of 20 individual time points, and levels of MIP-1-beta were discernable only once (data not presented).

Nine mediators detected over the period of the study were observed to increase above background at POD-7, the first day of sampling, and are listed in Table 2. IL-2, IL-8, MCP-1, and TGF- α peaked at POD-7 and decreased over time. IL-15 and IL-12/23 (p40) peaked at POD-14, while sCD40L, IL-1ra, and IL-6 had an elevated peak at POD-21. In general, all detectable mediators showed a return to normal by POD-30 with the exception of sCD40L, which remained elevated at POD-30. Of interest, levels of IL-12/23 (p40) were nearly absent until conspicuously elevated on POD-14, gradually reducing in concentration over the remainder of the study.

In addition, the binding of recipient serum anti-porcine IgM and IgG to non- α -1,3-galactose porcine xenoantigens from GalT-KO donors was assessed by flow cytometry. Serum IgM and IgG antibody levels from each recipient, at POD-0 and POD-30, were analyzed by flow cytometry. In Table 3, the relative mean fluorescent intensity (MFI) and fold increase in binding are summarized for each recipient. An increase in anti-porcine IgM and IgG antibodies was detected in all animals. From levels measured pre-transplant to those detected at the end of study, IgM anti-porcine antibodies increased between 1.4- and 4.9-fold ($P = .0095$), and IgG anti-porcine antibodies increased between 28.7- and 70.8-fold ($P = .0003$).

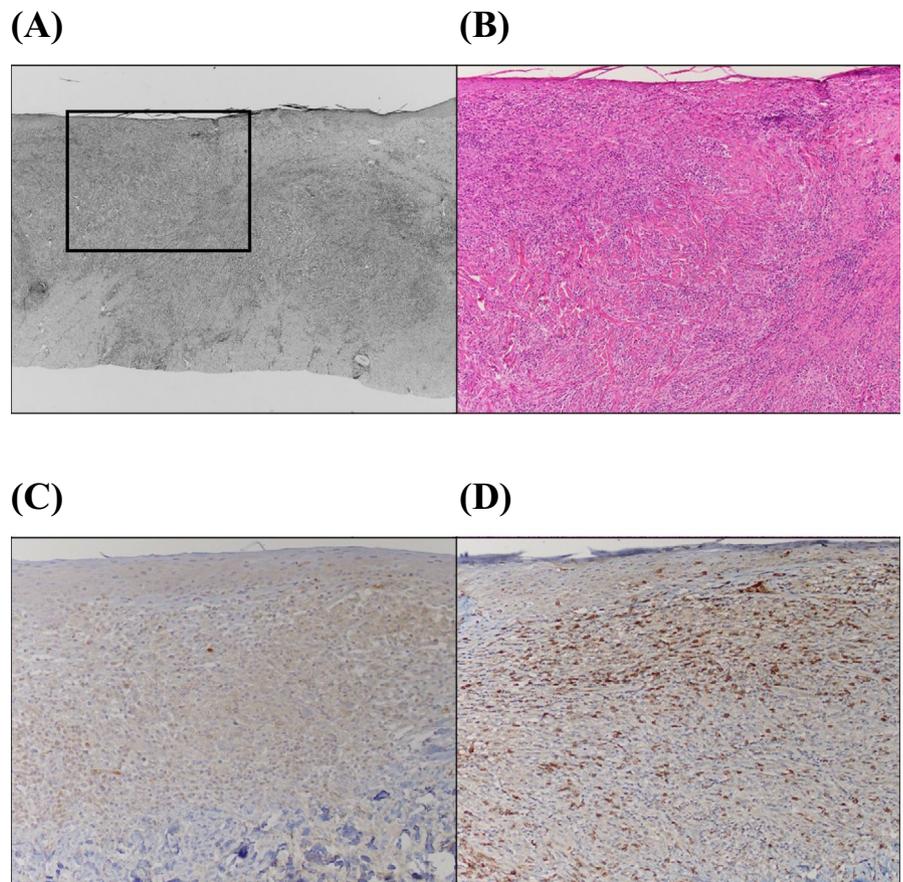


FIGURE 3 Wound Site 1, Animal 2001 at POD-30. (A) 40x power image of H&E staining of the wound bed demonstrating epidermal infiltrates into the skin xenotransplant at necropsy on POD-30. (B) 200x power image of inset box in Figure A. Multiple foci of perivascular inflammation and epidermal infiltrates with apoptosis (Banff Grade III). Features of dermal fibrosis, focal thinning of the epidermis, and tissue granulation were also noted. (C) 200x power image of the epidermal infiltrates stained for CD4+ T cells. (D) 200x power image of the same section stained for CD8+ T cells. Eosinophils, neutrophils, and plasma cells were present in perivascular and epidermal infiltrates, in Figures C and D

TABLE 2 Changes in serum cytokines and chemokines after skin xeno transplantation (pg/mL)

Cytokine/Chemokine	POD-0	POD-7	POD-14	POD-21	POD-30
sCD40L	1900 ± 1000	7900 ± 3100*	7700 ± 3100 ^{a,*}	8600 ± 4000 ^{a,*}	8500 ± 5200 ^{a,*}
IL-1ra	7.6 ± 2.8	50 ± 44*	28 ± 11*	66 ± 83*	24 ± 13*
IL-2	29 ± 11	42 ± 18*	37 ± 11*	41 ± 9*	30 ± 12
IL-6	0.31 ± 0.6	7.3 ± 8.3*	4.1 ± 2.6*	8.5 ± 6.3*	3.3 ± 2.7*
IL-8	2500 ± 1300	4200 ± 3200*	3700 ± 2600*	3900 ± 2300*	2500 ± 2100
IL-12/23 (p40)	0.6 ± 1.0	1.8 ± 2.7	26 ± 22*	16 ± 11*	6.7 ± 7.7*
IL-15	3.1 ± 1.9	6.0 ± 2.0*	7.1 ± 1.3*	5.0 ± 1.3*	6.0 ± 1.5*
MCP-1	360 ± 150	710 ± 540*	420 ± 110*	460 ± 110*	310 ± 120
TGF-α	4.5 ± 4.6	22 ± 11*	16 ± 11*	5.2 ± 3.6	9.9 ± 8.9

Note: Values are means (n = 4) ± SD.

POD, Post-operative day.

^aValues include data at the upper level of detection (12,000 pg/mL).

*Significant datapoints (P < .05) compared to POD 0, student t-test.

TABLE 3 Post-transplant changes in binding of recipient serum IgM and IgG to PBMC targets from GalT-KOswine donors

Recipient	Pre/Post-transplant	IgM		IgG	
		rMFI	Fold change	rMFI	Fold change
2001	Pre	8.51	--	16.28	--
	Post	45.72	4.4	1089.85	65.9
2002	Pre	5.07	--	28.29	--
	Post	30.01	4.9	840.64	28.7
2101	Pre	7.92	--	16.03	--
	Post	22.48	1.8	730.83	44.6
2102	Pre	6.47	--	5.19	--
	Post	15.49	1.4	372.88	70.8

Abbreviations: GalT-KO, α-1,3 galactosyltransferase knockout; IgG, immunoglobulin G; IgM, immunoglobulin M; PBMC, peripheral blood mononuclear cell; POD-0, Pre-transplant; POD-30, Post-transplant; rMFI, relative Mean Fluorescent Intensity.

3.5 | Post-operative assessment of systemic porcine cell migration and PERV

Naïve skin xenotransplants from the DPF donor were analyzed for PERV copy number. Each cell contained copies of PERV A (32 ± 1), B (9 ± 0.1), and C (16 ± 0.1). At necropsy, the presence of PERV was found in four of eight wound beds (3/4 recipients) at the site of the xenotransplants. This is likely due to localized porcine cell migration, as evidenced by the positive results from the microchimerism assay (Table 4).

Regardless of the limitations of the animal model for PERV infection, due to evidence of porcine cellular presence, PBMC samples from each of the four recipients were tested for microchimerism (ie, the presence of circulating pig cells) and for PERV. All samples tested negative. Sera from the four recipients were also evaluated for the presence of circulating PERV; all samples

TABLE 4 Data for post-operative analysis of wound beds (Wound Site 1 and 2)

Animal ID	Wound Site	PERV copies/500 ng (SD)	Micro-chimerism ^a	QC ^b
2001	W1	<LOD†	-	+
	W2	1495.6 (±521)	+	+
2002	W1	1518.8(±21)	+	+
	W2	<LOD	-	+
2101	W1	527.1 (±134)	+	+
	W2	137.8 (±16)	+	+
2102	W1	<LOD	-	+
	W2	<LOD	-	+

Abbreviations: LOD, limit of detection; QC, quality control; SD, standard deviation.

^aPorcine microchimerism cannot be accurately quantified due to mixture of cells present in wound bed extraction.

^bAll QC gave a positive Ct, indicating no inhibition.

were found to be negative for PERV *pol* and below the limit of detection. Liver, spleen, kidney, and lung tissues taken at the end of the study (POD-30) were evaluated for PERV expression and were also found to be negative.

4 | DISCUSSION

4.1 | 30-day survival of skin xenotransplant exceeds previous findings in similar models

The skin xenotransplants in this study demonstrated adherence, vascularity, and restoration of barrier function beyond previous published findings, and well beyond those same characteristics demonstrated by acellular, non-vital porcine xenografts, such as aldehyde

cross-linked porcine dermis.⁴⁴ Prior reports using earlier versions of GalT-KO materials^{23,40} reported an average of 10–14 day survival, while true wild-type, GalT+ skin xenotransplants exhibited a clinical appearance of “white grafts” as early as POD-4,^{22,39–41} an indication of ischemic injury caused by preformed antibodies against porcine endothelial cell α -1,3-galactose epitope.²² In contrast, all eight cryopreserved and vital (ie, possessing metabolically active cells) skin xenotransplants evaluated in the present study, sourced from GalT-KO miniature swine from a DPF closed colony, remained visibly adherent at POD-30 without the administration of immunosuppressive agents. These are encouraging findings with promising clinical implications.

The exact cause for the observed prolonged skin xenotransplant survival is a topic of continued investigation. We hypothesize that the use of DPF source animals, particularly those negative for PCMV and other various adventitious agents, and the stringent pre- and post-operative conditions of a third party facility required to meet regulatory compliance, likely had a combined, advantageous impact on the ultimate survival of the skin xenotransplants. PCMV positive porcine organs into non-human primates have been reported to significantly reduce porcine xenotransplant survival time,^{45–47} but it has not been reported for skin xeno transplants. The donor animals used in this study were routinely screened and known to be PCMV-negative. In addition, use of meticulous aseptic procurement and sterile processing methods, which achieved USP <71> levels of sterility for the skin xenotransplants, are novel and have not been previously reported. Combined with optimized freeze-thaw protocols and maximum retention of viable, metabolically active cells, it is possible that collectively these too had a positive effect on the survival of the skin xenotransplants.

In contrast to our preceding study²⁸—wherein skin xenotransplants were shown to perform similarly to skin allotransplants, without discernable impact on subsequent autotransplants—in this study, only xenogeneic materials were employed. In order to isolate the observed immunological effects to those only related to the skin xenotransplants by avoiding unnecessary confounding factors or introduction of potential “bystander” effects, neither allogeneic nor autologous materials were used. Further, the four recipients in the present study had not previously received a xenotransplant.

Lastly, it could be posited that these findings could be attributed to the existence of subtle or nuanced variations of the immunological response to non- α -1,3-galactose porcine xenoantigens between species. In the majority of previous, similar studies,^{22,23,39,40} baboon recipients were used instead of cynomolgus macaques. However, Fujita et al used a cynomolgus monkey model and showed a survival time (nine days) comparable to what was reported in the baboon model.⁴⁸

The key clinical observation from the present study was that the xenotransplant dermis remained adherent to the wound bed until POD-30, and complete sloughing of the graft was not reported as in previous studies.^{39–41} Histologic assessment of xenotransplant rejection using Banff grades ranged from II (epidermal infiltration) to IV

(total epidermal necrosis). In the regions of epidermal necrotization, cellular infiltrates included CD4+, and CD8+ T cells.

4.2 | Post-operative anti-porcine antibodies and cytokine concentrations

For xenotransplantation to be successful, both humoral and cell-mediated immune responses must be adequately mitigated in order to prevent rejection of the foreign transplanted organ. In the present study, measurement of anti-porcine IgM and IgG antibodies demonstrated a significant increase in the humoral response over the 30 day study period, likely due to the presence of non- α -1,3-galactose porcine xenoantigens in the skin xenotransplant.

In a similar model using GalT-KO miniature swine donors and baboon recipients, Albritton, et al³⁹ described an acute onset of anti-porcine IgM antibodies with subsequent decline, later followed by an observable increase in IgG antibodies. The use of GalT-KO donor swine eliminates the α -1,3-galactose porcine epitope as the target antigen, and several additional non- α -1,3-galactose porcine xenoantigens have been identified which are likely the focus of the IgG antibody response.^{15,49,50} These were not evaluated in this study, but it has been demonstrated that remaining non- α -1,3-galactose porcine xenoantigens can be eliminated with additional genetic knockouts introduced to donor source animals,^{49,50} rendering xenotransplants derived from such donors immunologically more similar to allotransplants.

Examination of wound healing cytokines and chemokines, characteristic of initial wound healing processes, provided unique insights into underlying immune mechanisms caused as a result of the skin xenotransplants used in this study. Many of the anticipated proinflammatory cytokines prevalent acutely following the precipitating trauma or injury event were not detectable at POD-7 and may have resolved below the level of detection by the first post-operative evaluation.⁵¹ Circulating levels of sCD40L were elevated at POD-7 and remained elevated to the end of study. This observation is consistent with those in surgical trauma patients. Activated platelets release sCD40L, a mediator that links inflammation, hemostasis, and vascular dysfunction, and is associated with tissue/endothelial damage and platelet activation.⁵² It also plays an important role in inflammation by increasing the expression of cell adhesion molecules, cytokines, and matrix metalloproteinase-1 (MMP-1), all of which are associated with neovascularization.⁵² Given the criticality of adequate perfusion of the skin xenotransplant for survival after the initial imbibition phase, the finding of elevated sCD40L levels present at the end of the study is in line with the clinical healing observed at each wound site.

Other wound healing factors such as TGF- α were detected with a peak at POD-7. TGF- α is a mitogenic polypeptide that is a ligand for epidermal growth factor receptors and can stimulate the proliferation and development of epidermal cells. It is produced by platelets, activated macrophages, and keratinocytes at

the wound site. Previously, topical administration of TGF- α in a porcine wound model was shown to improve wound healing of the damaged skin.⁵³ It has also been shown to stimulate the migration of keratinocytes to the wound site for initiation of repair and has been detected in the serum at the wound site.⁵⁴ Similarly, IL-8, a chemotactic factor for neutrophils,⁵⁵ and MCP-1, a chemotactic factor for monocytes/macrophages⁵⁶ were also elevated during the inflammatory response of the wound and repair process, at POD-7.

Elevated serum levels of IL-12/23(p40), which is involved with wound repair⁵⁷ and is produced by activated inflammatory cells such as macrophages, neutrophils, dendritic cells, and keratinocytes,⁵⁸ was notably detected at POD-14. IL-12/23(p40) is composed of two cytokines, IL-12 and IL-23, which have distinct roles associated with CD4+ naïve T cells. The IL-12 portion promotes differentiation to TH₁ effector cells that stimulate natural killer cells and CD8+ T cell production of IFN- γ . The IL-23 portion stimulates generation of TH₁₇ cells, shown to be beneficial in wound repair.⁵⁹ Wound repair studies in IL-12/23(p40) knockout mice demonstrated that IL-12 and IL-23 modulate early inflammatory responses and subsequent angiogenesis.⁵⁷ The transient peak at POD-14 of IL-12/IL-23(p40) is possibly the result of an overproduction during wound healing. Lastly, IL-23 is reported to be associated with regulating the expression of IL-17,⁶⁰ shown to be essential in driving the macrophage population from proinflammatory to pro-repair. Although the results of our study did not detect any IL-17 in serum, it could be postulated that IL-17 was locally confined to, or consumed at, the wound site.

4.3 | No evidence of systemic porcine cell migration or PERV transmission observed in skin xenotransplant recipients

While PERV was present in four of eight wound beds at the site of the skin xenotransplants, the positive results from the microchimerism assay are likely an indicator of localized porcine cell migration, resulting from the intimate contact between the porcine xenotransplant and the perfused wound bed.

No evidence of PERV DNA or RNA was detected in samples of sera, PBMC, spleen, liver, lung, or kidney tissues evaluated from the four subjects at the time of sacrifice. Overall, these results demonstrate that porcine DNA and systemic porcine cell migration into peripheral circulation of the recipients could not be detected. Altogether, these results provided no evidence of PERV transmission, consistent with previous studies.^{15,24,26,27,61,62}

It could be argued that a main caveat of this portion of the present study, with respect to PERV transmission, is the limitation of utilizing NHPs as models of PERV infection. It is well known that the lack of receptors for PERV cause NHPs to be the best available, but not ideal, animal model for such evaluation.⁶³ No preferred alternatives exist.

To be clear, while the animal model used in this study did not provide evidence of a lack of PERV transmission, it is prudent to

evaluate tools to be used in a clinical trial. FDA guidance states that recipient screening is required in clinical applications.⁶⁴ Although to date, there is still no evidence of PERV transmission to human recipients of porcine xenotransplants or circulatory migration of porcine cells,^{24,65-69} we believe that our assessment of porcine cell microchimerism and absence of PERV detection presented here are relevant and satisfy the relevant regulatory guidelines required. While not evaluated here, a follow-up beyond 30 days would have been informative.

4.4 | Applicability of findings to clinical evaluation of skin xenotransplants

The complexity of the immune reaction of a patient with severe burns must be taken into account when designing a study intended to evaluate safety and tolerability of xenotransplants for clinical use. The design of the present study was intended to create an in vivo model that could adequately consider, in a predictive capacity, risk factors associated with the skin xenotransplant combined with those of the injury itself.

A considerable body of existing pre-clinical and non-clinical data demonstrate that such vital, GalT-KO porcine skin xenotransplants offer a promising alternative option to HDD allotransplants for temporary wound closure in and full-thickness burn injuries.^{22,39-41}

This study supports these previous findings and provides further evidence that xenotransplantation represents a promising safe and efficacious clinical alternative. Clinical efficacy combined with the elimination of the risk associated with disease transmission from human through the use of DPF source animal donors, as well as scalability and increased material availability would provide a significant therapeutic benefit to patients with severe burn injuries.

Due in part to these findings, the US-FDA granted clearance to proceed with a Phase I evaluation of safety and tolerability of a vital porcine xenotransplant to provide temporary wound closure of full-thickness burn wounds. Patient enrollment for a first-in-human xenotransplantation trial commenced in 2019, at Massachusetts General Hospital, Boston, MA.

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