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Long-Term IgG Response to Porcine Neu5Gc Antigens without Transmission of PERV in Burn Patients Treated with Porcine Skin Xenografts

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Acellular materials of xenogenic origin are used worldwide as xenografts, and phase I trials of viable pig pancreatic islets are currently being performed. However, limited information is available on transmission of porcine endogenous retrovirus (PERV) after xenotransplantation and on the long-term immune response of recipients to xenoantigens. We analyzed the blood of burn patients who had received living pig-skin dressings for up to 8 wk for the presence of PERV as well as for the level and nature of their long term (maximum, 34 y) immune response against pig Ags. Although no evidence of PERV genomic material or anti-PERV Ab response was found, we observed a moderate increase in anti-αGal Abs and a high and sustained anti–non-αGal IgG response in those patients. Abs against the nonhuman sialic acid Neu5Gc constituted the anti–non-αGal response with the recognition pattern on a sialoglycan array differing from that of burn patients treated without pig skin. These data suggest that anti-Neu5Gc Abs represent a barrier for long-term acceptance of porcine xenografts. Because anti-Neu5Gc Abs can promote chronic inflammation, the long-term safety of living and acellular pig tissue implants in recipients warrants further evaluation.
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The prospect of clinical xenotransplantation could result in a medical revolution in the near future (1). Clinical xenotransplantation trials involving pig cells and tissues are imminent and range from the cornea to islet cells for diabetes and to brain cells for neuronal diseases, such as Parkinson disease. For example, a clinical trial of islet xenotransplantation is ongoing (2, 3). Transient xenograft transplantation for fulminant organ failure (e.g., heart, liver) has been used as a bridge to allotransplantation (4). In addition, devitalized animal tissues including heart valves, skin, and tendons, are commonly implanted in patients (5–7). Likewise, vital pig skin (PS) has been used widely as a dressing for burns (8, 9). In these clinical settings, where patients are exposed to xenogeneic Ags and pathogens in unnatural fashions, microbial safety (10) and immunologic effects (11, 12) are of critical importance and require extensive assessments.

The porcine endogenous retrovirus (PERV) has been regarded as the major potential risk of zoonoses in xenotransplantation (10). A limited number of studies on patients who received xenotrans-

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Abbreviations used in this article: BMC-Prague, Burn Medicine Clinic, Faculty Hospital of Královské Vinohrady, Prague; BP-CTRL, control burn patient; BP-PS, burn patient treated with pig skin; GalKO, α1-3GT knockout; HC, healthy control; KO, knockout; Neu5Gc, *N*-glycolylneuraminic acid; PAEC, porcine aortic endothelial cell; PERV, porcine endogenous retrovirus; PS, pig skin; ROC, receiver operating characteristic; vRNA, viral RNA; WT, wild type.

plantation found no evidence of PERV infection (13–19). However, retroviruses in other species often establish latent infection and cause chronic immunologic or neurologic diseases as well as cancer. Therefore, although the relevance of PERV is debated (20), it is still necessary to monitor for PERV infection (21). Thus, further testing of xenograft recipients for chronic PERV infection is warranted.

An additional risk in xenotransplantation stems from the well-known xenoreactive Abs that can cause rejection of xenografts (22–25). Humans, apes, and old world monkeys are defective in the *GGTA1* gene encoding the α1-3-galactosyl-transferase enzyme (α1-3GT) and produce high levels of anti-αGal Abs (25–27), largely because of continuous exposure to αGal-expressing bacteria in the gastrointestinal normal flora. These Abs can cause hyperacute rejection of porcine organ xenografts (25, 28, 29). To prevent hyperacute rejection, pigs with knocked out α1-3GT have been generated and are currently being investigated (30–35). However, pig grafts express many non-αGal Ags (36, 37), and induction of other xenoreactive Abs has also been observed (11, 38–40). *N*-Glycolylneuraminic acid (Neu5Gc) is a dietary, nonhuman sialic acid that incorporates into diverse human glycoconjugates. Such modified glycoconjugates then become immunogenic and lead to the generation of anti-Neu5Gc xeno-autoantibodies (37, 41–44). However the major difference between anti-αGal and anti-Neu5Gc Abs lies in the fact that Neu5Gc Ags can incorporate metabolically into normal human tissues, in contrast to the αGal Ag, which cannot (43). Furthermore, it was shown that the persistent presence of anti-Neu5Gc Abs can result in chronic inflammation because of the concomitant presence of Neu5Gc-antigens, likely leading to exacerbation of vascular diseases and cancer (42, 44–46).

In this study, we sought to investigate these two major long-term risk factors in xenotransplantation, especially in light of the anticipated future increase in recipients of xenografts (or animal-derived medical devices). For this purpose, we took a retrospective approach by using clinical samples of xenograft-treated patients. The use of skin harvested from commercial pigs (vital PS) was demonstrated to be effective in treating patients with severe burns by limiting wound secretions and facilitating granulation and epithelialization of the affected areas (47, 48). We tested long-term immunologic effects of exposure to PS in blood and serum samples collected from burn patients up to 34 y after treatment with PS compared with age-matched control burn patients without PS treatment. We present compelling evidence for a lack of PERV infection in PS recipients, but persistent and high anti-Neu5Gc IgG response to porcine Neu5Gc Ags.

Materials and Methods

Burn patients and control groups

This study was approved by the Ethical Committee of The University Hospital Královské Vinohrady, Prague (No. EK/25/2007). The study was also conducted in compliance with the requirement of the Ethics Committee of the European Commission, which supported the study.

A total of 220 burn patients among more than 15,000 patients who received PS treatment (BP-PS) at Burn Medicine Clinic, Faculty Hospital of Královské Vinohrady, Prague (BMC-Prague) between 1973 and 2005 were invited to participate in this study. These patients were selected for their burn injury severity and recent hospital contact records. Fifty-four patients responded positively and provided their informed consent and blood samples. Blood samples were collected and de-identified, and serum and PBMCs were prepared. No specific chronic or unexplained illness potentially related to PS treatment was noted in the patient record or in the interview by the clinician prior to sampling. One individual was still undergoing treatment for her injuries at the time of sampling (1 mo after xenograft). Samples from control burn patients not treated with PS (BP-CTRL) were also collected at BMC-Prague (n = 7) or Centre Hospitalo Universitaire, Nantes (n = 7); average duration between injury and sampling was 113.7 mo for BP-PS and 45.8 mo for BP-CTRLs. Burn wounds of these BP-CTRL patients were tailored by removing necrotic tissues and healed by autografting. Healthy controls (HC; n = 27) were mainly voluntary laboratory workers at the Institute of Molecular Genetics, Prague.

Clinical characteristics

BP-PS were admitted with full-thickness and deep dermal burns and 10–80% total body surface area burns equating to second- and third-degree burns between 1976 and 2005. Patient age ranged from 18 mo to 71 y at the time of treatment. This group included 4 children aged 18 mo, 8 y, 15 y, and 17 y. Prior to the use of PS, strips were immersed in an antibiotic solution containing streptomycin, chloramphenicol, and furantoina for 20 min and then assessed for microbial contamination. Viability of skin was determined by assessing glucose metabolism and then either snap frozen in cryoprotective media containing 15% glycerol for storage in liquid nitrogen or used fresh (8). Patients were exposed to porcine xenografts up to 8 wk and a maximum of 18 xenograft changes ranging 2–5 d apart. At the time of the injury, the use of fresh porcine skin xenografts at BMC-Prague was considered the best lifesaving treatment. All cases were historical, and blood was collected at an average of 4 y after injury, with the exception of patient number 27 who had a more recent burn injury and was sampled 1 mo after treatment.

PS-CTRLs were age matched and presented similar burn lesion grading. Burns were treated by removal of necrotic tissue and auto grafts.

In vitro transmission of PERV

Blood samples were taken from the commercial herd (Large White x Landrace x Goland Poland) used for collection of skin for xenografts (n = 10). PBMCs were isolated as described in (49, 50), cocultured with porcine fibroblasts (ST-Iowa) and human epithelial (293) cells, and assayed for PERV production as described previously (13, 51).

Determination of PERV in patient samples

Both total RNA and viral RNA (vRNA) was extracted from fresh whole blood (RNeasy Kit; Qiagen, Surrey, U.K.) and serum (Qiagen Viral RNA Kit; Surrey, U.K.), respectively, according to the manufacturer's instructions. The presence or absence of PERV RNA was confirmed by quantitative RT-PCR using the following primers and probe P218F 5′-CCGGCTCTCATCCTGATCA-3′ and P315R 5′-TCTTGGTTTATTTAGCCATGGTTTAA-3′ and probe P241T 5′-FAM-CCCTATATCCTTACGTGGCAAGATTTGGCA-TAMRA-3′ targeting the pol region. Reactions were performed according to the manufacturer's instructions using TaqMan one-step RT-PCR master mix (Applied Biosystems, Warrington, U.K.), 0.5 μM of each primer, 1 μM of probe, and 3 μl of RNA sample. The cycling conditions were 48°C for 30 min, and 95° C for 10 min followed by 40 cycles of 95°C for 15 s, 53°C for 30 s, and 60°C for 30 s (ABI 7500 Fast; Applied Biosystems). For vRNA, each reaction was spiked with Taqman exogenous internal positive control (Applied Biosystems). For total RNA, reactions included 18S RNA as a reference using primers and probe from the 18S rRNA control kit (Eurogentec, Hampshire, U.K.) to avoid false negatives owing to the absence of PERV RNA, RT-PCR failures, or both.

DNA was prepared from WBCs collected from the patients using the Nucleon Bacc2 kit (GE Healthcare, Buckinghamshire, U.K.) according to the manufacturer's instructions. Patient DNA was tested for the presence of PERV DNA with the same conditions as above using 1 μg of DNA and Gene Expression Master Mix (Applied Biosystems). Reactions were performed according to manufacturer's instructions with 0.5 μM of each primer and 1 μM of probe. Absolute quantification was used to define results, and samples were compared against a plasmid standard expressing the gag-pol region of PERV (52). Patient DNA was also tested for the presence of pig centromeric DNA to determine whether patients displayed microchimerism according to the method and sensitivity described previously (13).

Validation of these assays showed that the detection limit of this quantitative PCR assay for PERV DNA was one copy of PERV per 1 μg of DNA (300,000 cells) (Supplementary Fig. 1C), which gave a confidence level of >99.9% of detecting ≥1 copies and a <0.01% chance of a false negative. The sensitivity of the vRNA PCR was 5 copies per 3 μl of vRNA preparation, and validation of this assay showed that we could consistently detect 475 viral particles per milliliter of serum.

Neutralizing Abs to PERV

A total of 11 xenograft recipients and 4 control samples were tested for seroneutralization of PERV. The recombinant PERVA/C virus 14/220 (49) was replicated in 293T cells, cell-free supernatant containing virus was recovered, divided into aliquots, and stored at −80°C. The stock virus was titrated by immunostaining on 293T cells. The human sera were inacti-

vated for 30 min at 56˚C. Thirty microliters of the serum was incubated with 30 μl of the virus dilution containing 120 focus-forming units of PERVA/C virus for 1 h at 37˚C. Next, 50 μl of the mixture was added in duplicate to 293T monolayers in 96-well plates and incubated for 1 h at 37˚C. Viral inocula were replaced with culture medium, and the cells were incubated for 48 h and then fixed with methanol-acetone. Viral Ags were detected by immunostaining using a rabbit anti-capsid serum and counting foci as described previously (50).

Anti-αGal IgG ELISA

The ELISA for detection of human IgG Abs specific for the Galα1–3Gal disaccharide epitope was adapted from a previously described method (53). In brief, polystyrene microtiter plates (NUNC Maxisorp; NUNC AB, Roskilde, Denmark) were coated with 100 μl per well of 5 μg/ml Galα1–3Gal-polyacrylamide conjugate (PAA-Bdi; Syntesome, Munich, Germany) in 0.1 M carbonate buffer (pH 9.6) overnight at 4˚C. The plates were then washed and saturated with a 0.5% solution of fish gelatin (Sigma-Aldrich) diluted in PBS. Sera diluted in PBS-Tween 0.1% were incubated for 2 h at 37˚C in triplicate. Instead of serum, PBS-Tween was used as a blank. Goat anti-human IgG Abs (diluted 1:1000; Jackson ImmunoResearch), and a TMB substrate was used to reveal bound Abs. After a 5-min revelation and addition of H2S04, OD values were read at 405 nm.

Cell binding

The level of xenoreactive Abs was assessed in 53 burn patients who had received PS xenografts (BP-PS), 14 BP-CTRLs, and 27 HC samples. FACS Ab binding assays were performed on porcine aortic endothelial cells (PAEC) isolated from wild type (WT) or α1-3GT knockout (GalKO) pigs (with no expression Gal epitope). Cells were isolated, phenotypically characterized, and grown as described previously (54). An aliquot of a pool of normal male AB blood group sera (n = 250) was used as an internal control in every experiment. IgM xenoreactivity was assessed using undiluted sera on both WT and GalKO PAECs. In contrast, for the measurement of IgG xenoreactivity, sera were assessed on WT PAECs at a 1:32 dilution and on αGT-KO PAECs at a 1:4 dilution. Heat-inactivated sera were incubated for 30 min at 4˚C with 1 to 2 × 10^5 PAECs, cells were washed twice in cold FACS buffer, and PAECs were incubated separately for 30 min at 4˚C with FITC-labeled anti-human IgM goat Abs (Jackson ImmunoResearch, West Grove, PA) or FITC-labeled anti-human IgG goat Abs (Beckman Coulter, Indianapolis, IN). After staining and washing steps, cells were harvested in PBS/paraformaldehyde 2% and analyzed using a Canto-II Flow cytometer (Becton Dickinson, San Diego, CA, USA) with DIVA (Becton Dickinson) and FlowJo software (Tree Star, Ashland, OR). The difference of the median fluorescence intensity between test samples and negative staining controls (average of quadrupled experiments with secondary FITC-labeled Abs only) were presented as median fluorescence intensity shifts. Statistical analyses were performed with the Mann–Whitney nonparametric test and the Kruskal–Wallis test.

To assess the proportion of anti-Neu5Gc IgG among IgG binding to GalKO PAECs, selected serum samples were incubated for 2 h at 4˚C with Neu5Ac or Neu5Gc at various concentrations: 0, 2.5, 5, and 7.5 mM in FACS buffer. Next, for each selected sera, these different conditions were tested by the FACS binding assay on GalKO PAECs for the IgG as described above.

Detection of anti-Neu5Gc Abs by EIA and ELISA

Anti-Neu5Gc Abs were detected in 1:100 diluted human serum samples by ELISA inhibition assay (EIA method) on WT mouse sera, as described previously (55). To demonstrate Neu5Gc-specific binding, diluted human sera were preincubated for 2 h on ice in EIA buffer (55) supplemented with 5 mM Neu5Gc or control 5 mM Neu5Ac prior to testing binding to coated wells. To demonstrate sialic acid–specific binding, the coated ELISA plates were either pretreated with mild periodate or mock treated (55).

Detection of anti-Neu5Gc Abs by sialoglycan microarray

Arrays were fabricated by KAMTEK (Gaithersburg, MD) on Epoxide-derivatized slides (Corning, Thermo Fisher Scientific) with eight full subarrays per slide as described (array 1) (56). Anti-Neu5Gc Abs were detected in 1:250 diluted human serum by sialoglycan microarray version 13 at 200 μl per subarray and developed as described (56).

Results

Burn patients and control samples cohort

Blood samples from 55 BP-PS, 14 BP-CTRLs, 27 HCs were obtained. Table I shows the demography of patients and controls.

In vitro transmission of PERV from porcine peripheral blood mononuclear cells

To assess potential risk of infectious PERV in pigs used for burn patient treatments, samples from the animals of a commercial herd that had been used for PS treatment at Burn Medicine Clinic (Faculty Hospital of Královské Vinohrady) were tested for PERV transmission in vitro by coculture. Of the 10 animals tested, 4 were found to transmit PERV to porcine cells, and none transmitted PERV to human 293 cells (Supplementary Fig. 1A, 1B).

Analysis of PERV in PS-treated patient and control samples

Sensitive quantitative PCR-based PERV assays were validated according to previous xenotransplantation studies (13). Of the 55 patient samples tested, one produced a false-positive result, yielding an overall false positive rate of 0.83%. However, RNA was extracted again from this sample and confirmed to be negative in a duplicate. We also received a new sample from the patient and confirmed that it was negative. Test results for microchimerism (i.e., the presence of circulating pig cells) were also negative.

Ab testing against PERV elements was also performed in these patients. Consistent with previous studies (13), most of the reactivity observed was due to background cross reactivity and was considered to be negative for Ab to PERV in comparison with normal HC individuals. In addition, no significant neutralization of PERV infection was detected (Supplementary Fig. 1D).

These results provided no evidence of PERV transmission or presence of specific anti-PERV Abs in these patients, consistent with previous studies, one with 15 BP-PS recipients (13), and additional other studies (13, 15, 16, 19).

Xenoantibodies binding to porcine cells

There is limited information on long-term Ab responses and the nature of non-αGal Abs in recipients (11). We first examined the

Table I. Summary of patient histories

	Average	Median	Minimum	Maximum
55 BP-PS (48 male, 7 female)				
Age at time of sampling	47.6	45	10	92
Age at time of injury	38.2	35	1.5	71
Months since burn	113.7	88	1	408
Burn %	32.3	30	5	87
14 BP-CTRL (8 male, 6 female)				
Age at time of sampling	44.2	46	23	66
Age at time of injury	40.4	42.5	7	65
Months since burn	45.8	24.5	2	240
Burn %	31.6	24.5	7	90
27 HC (14 male, 13 female)				
Age at time of sampling	34.1	29	23	79

anti-αGal Ab level using a previously established ELISA-based Ab binding assay (53) (Fig. 1A, 1B). Higher anti-αGal IgG levels were observed for BP-PS samples at low serum dilutions compared with BP-CTRL samples (both 1/40 and 1/160 dilutions) and HC samples at 1/40 dilution (not significant at higher dilutions of 1/640 and 1/2580, Supplementary Fig. 2). Next, we established cell binding assays to detect anti-pig cell Abs by FACS analyses (see Supplementary Fig. S3A–C for typical FACS profiles). WT PAECs were used to detect both anti-αGal and anti–non-αGal Ig (Fig. 1C), whereas PAEC derived from αGal-deficient pigs (α1-3GT knock out; GalKO) were used specifically to detect anti–non-αGal Ig (Fig. 1D). BP-PSs had significantly higher levels of IgG that bound to both WT and GalKO PAECs compared with HC and BP-CTRL and their levels are on average 2–3-fold (WT PAEC) and 4–6-fold (GalKO PAEC) higher than in the controls (Fig. 1). This increased response is evidenced in PS-treated patients at an average of 9.4 y (range, 1–408 mo; Table I) following exposure to PS. Although several BP-PS recipients had strikingly high levels of anti–non-αGal Abs (5–30-fold higher than the average of BP-CTRL), approximately one third of BP-PS recipients remained in the control range (Fig. 1D). We have also examined IgM binding to these porcine cells (Supplementary Fig. 3D, 3E). No significant difference was observed between BP-PS and BP-CTRL in IgM binding to both WT and GalKO PAECs. These results raised a question of the nature of these non-αGal Ags that are recognized by IgG in BP-PSs. Neu5Gc might be an important xenoantigen as recent data on Gal/CMAH double-knockout (KO) pigs (57) suggests that it is a major target for preformed human anti-pig Abs.

Blocking of IgG binding to Gal KO PAEC by Neu5Gc

To estimate the proportion of anti-Neu5Gc IgG binding to GalKO PAECs in sera of BP-PS, cell binding FACS assays were performed in the presence of Neu5Gc or Neu5Ac at various concentrations (Fig. 2). At high concentrations of Neu5Gc, but not Neu5Ac, IgG binding reached a plateau and was reduced from 10% to 50%, especially for the samples that had higher binding to GalKO PAECs in the absence of Neu5Gc. These results suggest that Neu5Gc glycans are major non-αGal Ags that induced long-term IgG responses in BP-PSs, although other non-αGal Ags might also be involved.

High levels of anti-Neu5Gc IgG in sera of BP-PS long after PS treatment

Humans can produce xeno-autoantibodies against various Neu5Gc epitopes, and pig tissues and cells bear such Neu5Gc Ags (58). We therefore characterized the anti-Neu5Gc response in 10 representative BP-PSs that showed significantly higher levels of IgG binding to GalKO PAECs, as well as in 14 BP-CTRLs and 10 HCs. To detect anti-Neu5Gc Abs, we used a newly developed sensitive ELISA that allows detection of overall anti-Neu5Gc response in human sera to multiple Neu5Gc Ags in a single assay (55). Compared with HCs or BP-CTRLs, serum samples of BP-PSs had significantly higher levels of anti-Neu5Gc IgG or IgM, but not IgA (Fig. 3A). Pretreatment of coated sialoglycan Ags with mild periodate (that truncates the sialic acid side chain) abrogated Sia-specific binding of Abs (46, 59) both in BP-PS and BP-CTRL; this suggests Sia-specific binding in both groups, albeit higher in

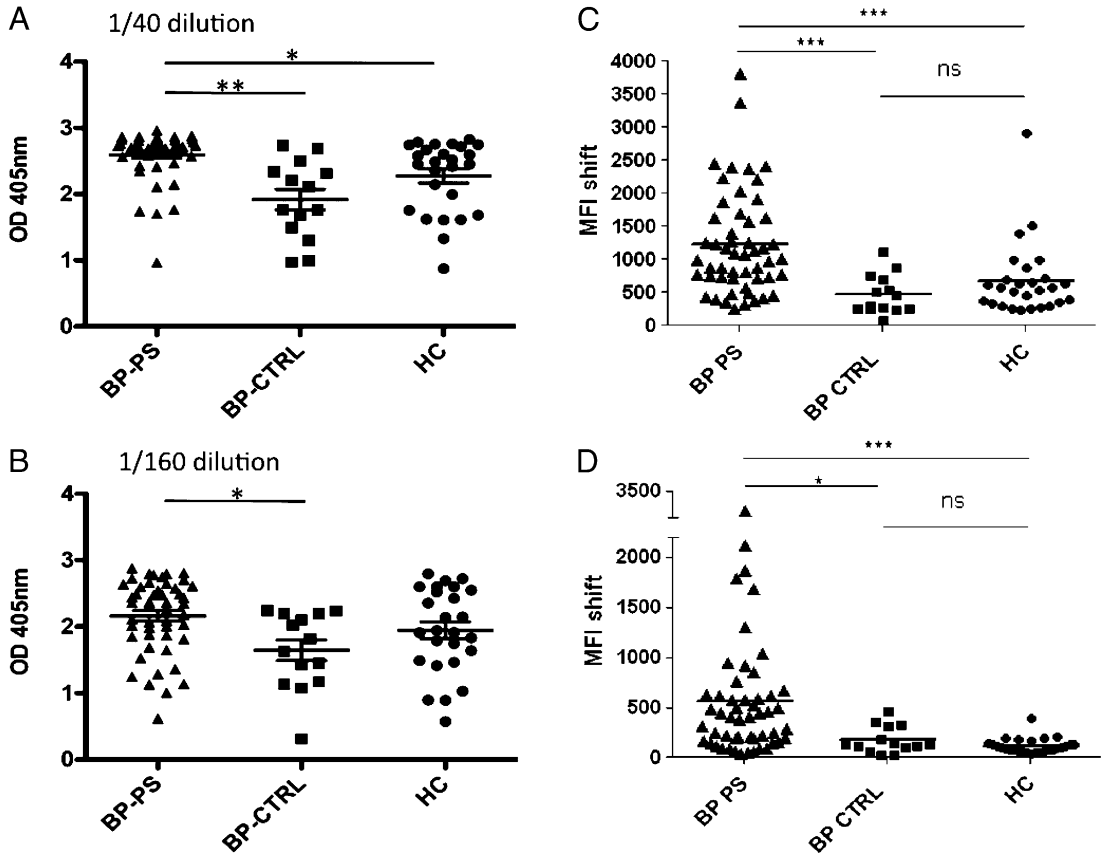


FIGURE 1. Assessment of xenoreactive IgG Abs. Dilutions at 1/40 (**A**) and 1/160 (**B**) of serum from healthy volunteers (●, HC; $n = 26$), burn patients treated conventionally (■, BP-CTRL; $n = 14$), or those who received a PS plaster (▲, BP-PS; $n = 49$) were reacted against Galα1–3Gal-polyacrylamide conjugate and revealed with anti-human IgG Abs by ELISA. Serum xenoreactivity of healthy controls (●, HC; $n = 27$), control burn patients (■, BP-CTRL; $n = 14$) and PS-treated burn patients (▲, BP-PS; $n = 53$) was assessed by flow cytometry. IgG serum reactivity was assessed against WT PAECs at a 1/32 dilution (**C**) and against GalKO PAECs at a 1/4 dilution (**D**), representing both the anti-αGal and non-αGal IgG Ab (C) versus only the anti–non-αGal IgG Ab (D).

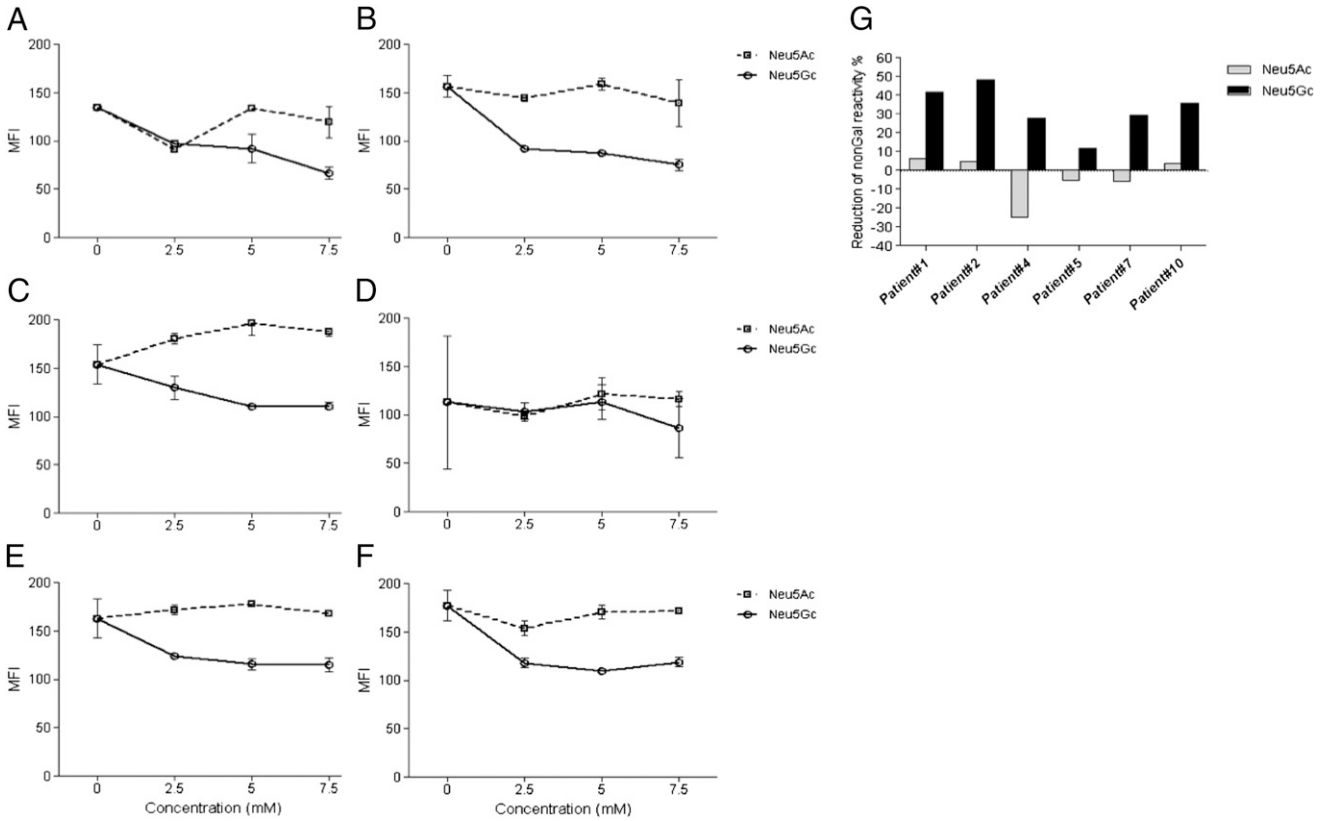


FIGURE 2. Blocking of IgG binding to GalKO PAEC by Neu5Gc. Anti–non-αGal IgG reactivity of serum with or without Neu5Ac (○, dashed line) or Neu5Gc (□, plain line) respectively at 0, 2.5, 5, and 7.5 mM: sera from patient 1 (**A**), patient 2 (**B**), patient 4 (**C**), patient 5 (**D**), patient 7 (**E**) and patient 10 (**F**) diluted in FACS buffer. (**G**) The percentage of reduction of anti–non-αGal IgG reactivity after serum absorption with Neu5Ac (gray bars) or Neu5Gc (black bars) in the six patients is shown (nos. 1, 2, 4, 5, 7, and 10).

BP-PS (Fig. 3B). Furthermore, we examined several representative samples by inhibition with increasing concentrations of either Neu5Gc or with the human sialic acid *N*-acetylneuraminic acid (Neu5Ac), a control monosaccharide that differs by only one oxygen atom from Neu5Gc. Neu5Gc/Neu5Ac specificity is also relevant to the in vivo state where many circulating proteins in the serum are glycosylated and most carry Neu5Ac-glycoconjugates (rarely Neu5Gc). Therefore, in vivo, anti-Neu5Gc Abs must be able to pass this pool of high concentration of Neu5Ac. No inhibition was observed with Neu5Ac. In contrast, we saw inhibition with Neu5Gc that was maximal at 5 mM (Fig. 3C). Subsequently, Neu5Gc specificity was tested in all samples in the presence of 5 mM Neu5Gc or Neu5Ac, revealing specific inhibition of anti-Neu5Gc IgG binding with Neu5Gc, but not with Neu5Ac (Fig. 3D). These results indicate greater anti-Neu5Gc response in BP-PS (Fig. 3C). To address this result in more detail, we quantified anti-Neu5Gc Ab response in all these samples, relative to an IgG standard curve (Fig. 3E). These results collectively indicate that BP-PSs can have high and persistent levels of anti-Neu5Gc IgG, which likely contributes to the high IgG binding to GalKO PAECs (Fig. 1B).

Analysis of anti-Neu5Gc IgG profile on a sialoglycan microarray

Neu5Gc can be attached to a wide variety of naturally occurring glycans, resulting in the generation of a wide variety of epitopes. To reveal a more detailed Neu5Gc-Ag binding profile, anti-Neu5Gc IgGs were characterized further in BP-PSs and in BP-CTRLs using a sialoglycan microarray (46, 56, 60). The arrays were printed with matched pairs of Neu5Gc- or Neu5Ac-glycans, and serum IgG binding was analyzed (Fig. 4). Consistent with the above ELISA assay, BP-PSs showed significantly higher IgG binding to Neu5Gc-glycans compared with the BP-CTRLs, and both groups did not show significant binding to Neu5Ac glycans (Fig. 4A, 4B). To delineate the binding preferences of anti-Neu5Gc IgG in BP-PS recipients further, we performed receiver operating characteristic (ROC) analyses for each of the Neu5Gc-glycans (Fig. 4C). Overall, this array analysis suggests that both *N*-linked (Neu5Gcα2-6LacNAc/Lac) and *O*-linked (Neu5Gcα2-3Core1) glycans contribute to anti-Neu5Gc immune response in BP-PSs (Fig. 4C).

Discussion

Commercially available vital porcine skin has been shown to be effective in treating patients with severe burns, as the grafts are viable for up to 5 d after transfer to the wound and facilitate swift healing (8). This treatment has been practiced less frequently since 2005, mainly because of the potential risk of zoonosis (50). In this study, we examined transmission of PERV, the major safety issue in xenotransplantation, and long-term status of anti-pig Abs in BP-PSs; this could produce unique information on expected long-term humoral response to a xenotransplantation in the clinic.

We did not find any evidence for PERV transmission up to 408 mo after xenotransplantation, the longest term analyzed to date. These results support previous negative results in patients who had received various types of xenotransplantation treatment (13–19). These results support the use of closely monitored xenotransplantation clinical trials, especially those that do not require the use of systemic immunosuppression, such as alginate embedded pancreatic islet cell transplantation (1, 61). However, our analysis of the humoral response in BP-PS revealed that PS treatment can induce persistent anti-pig cell IgG responses (including the

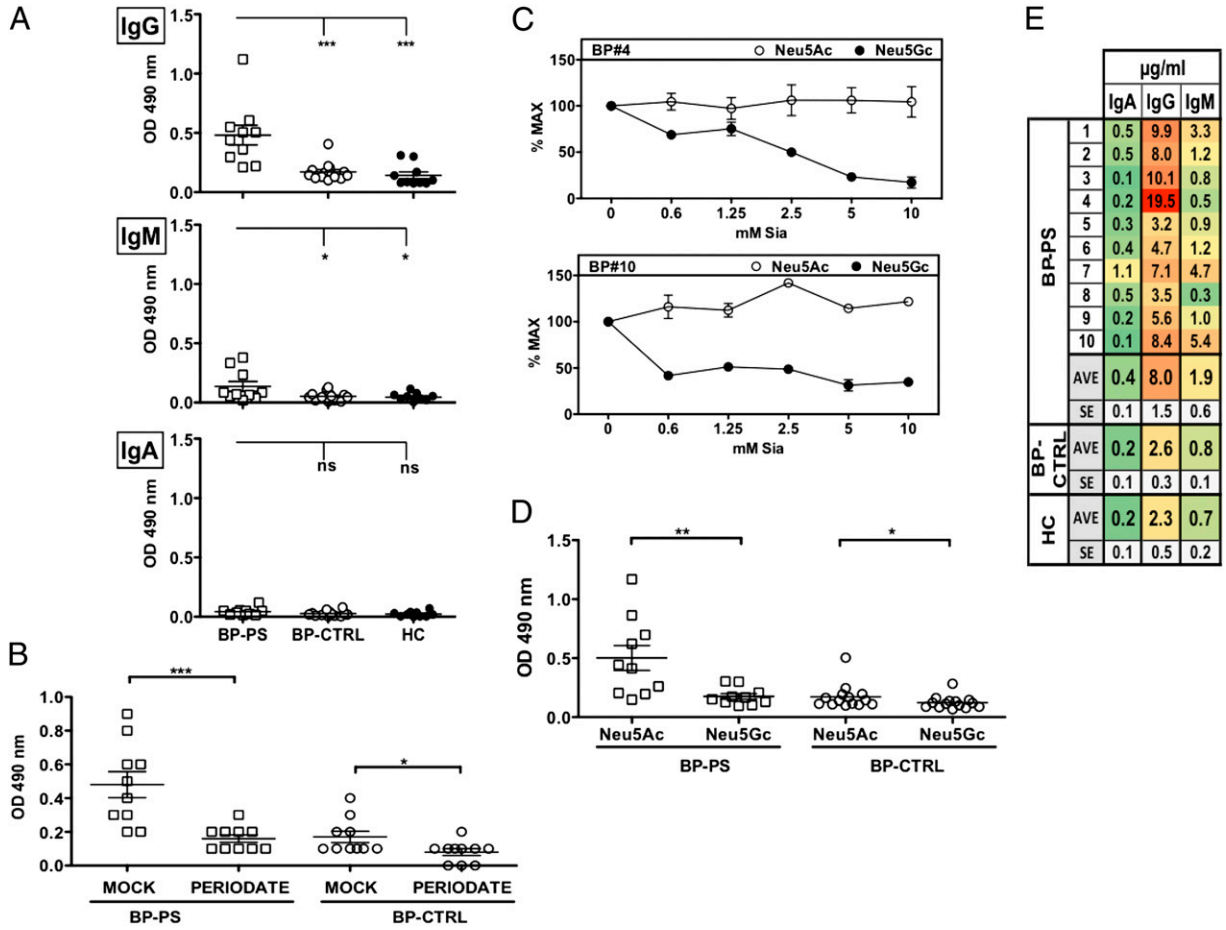
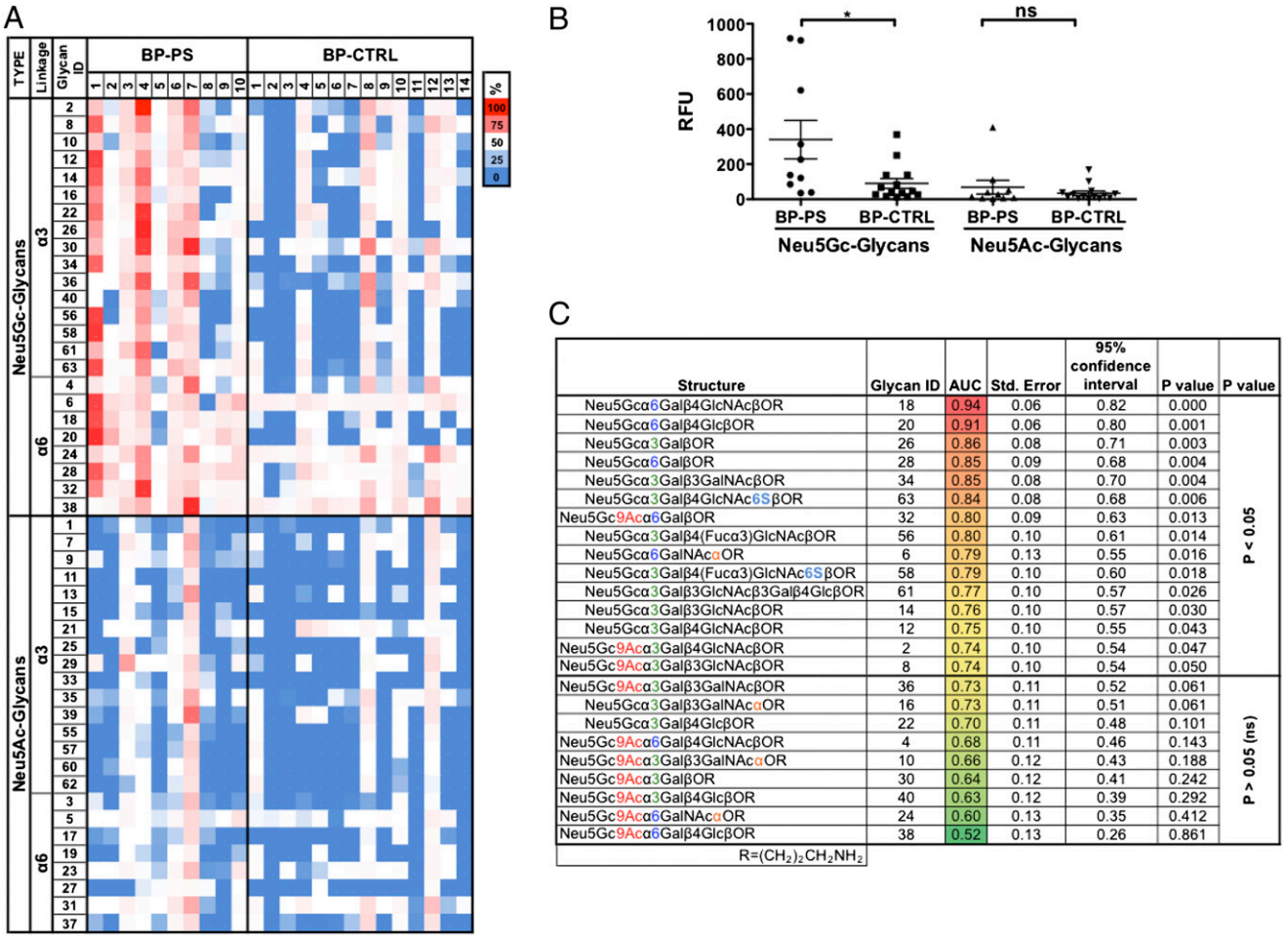


FIGURE 3. Characterizing the anti-Neu5Gc Ab response. (**A**) Anti-Neu5Gc response was tested in BP-PS, BP-CTRL, and HCs at 1:100 diluted serum by the enzyme immunoassay (EIA) method, revealing higher anti-Neu5Gc IgM or IgG (but not IgA) in BP-PS compared with BP-CTRL or HCs (two independent experiments; one-way ANOVA, ns $p = 0.1502$; $*p = 0.0177$; $***p < 0.0001$). (**B**) Binding is specific to sialic acids (Sia) because pretreatment of coated glycans with mild-periodate (that removes Sia side chain), but not control mock pretreatment, eliminates binding (representative of two experiments). Paired *t* test, $*p = 0.0187$, $***p = 0.0004$). (**C**) EIA IgG binding (of two sera with high anti-Neu5Gc reactivity) is inhibited by increasing concentrations of Neu5Gc, but not with Neu5Ac (two independent experiments showing mean and SEM). (**D**) IgG binding is specific to Neu5Gc because serum EIA binding is inhibited by 5 mM Neu5Gc but not 5 mM Neu5Ac (representative of two experiments). Paired *t* test, $*p = 0.0213$, $**p = 0.0083$). (**E**) Serum anti-Neu5Gc Abs (detected by EIA) were quantified using standard curves of human Ig (for each Ig standard curve, all sera samples were tested in duplicates on the same plate; two independent experiments showing mean and SEM). This analysis confirmed that BP-PS have 3–4-fold higher anti-Neu5Gc Abs (IgG: 8 ± 1.5 µg/ml; IgM: 1.9 ± 0.6 µg/ml) compared with BP-CTRL (IgG: 2.6 ± 0.3 µg/ml; IgM: 0.8 ± 0.1 µg/ml) or HC (IgG: 2.3 ± 0.7 µg/ml; IgM: 0.7 ± 0.2 µg/ml).

common anti-αGal), a major component of which is the anti-Neu5Gc IgG, which can be detected up to 34 y after PS removal from the burn injuries.

Our study shows that BP-PSs possess increased xenoreactivity long after the treatment despite the short exposure to PS. Because humans have lost the functional gene *CMAH*, encoding a hydroxylase that converts the acetylated form of neuraminic acid (Neu5Ac) to its glycolylated form (Neu5Gc) (62, 63), Neu5Gc epitopes are recognized as foreign by the human immune system, similar to the αGal epitopes (37, 39–41, 43). Using a recently developed method designed to detect overall anti-Neu5Gc reactivity to multiple Neu5Gc-glycans in a single assay (55), we showed that the sera of BP-PS had a significant increase in anti-Neu5Gc IgG and IgM compared with BP-CTRL even years after the burn PS dressing. The specificity of Ab binding for Neu5Gc was confirmed by several means, including competitive inhibitor experiments. Moreover, using a sialoglycan microarray displaying the various underlying molecular scaffolds of Neu5Gc, we showed that the distribution pattern of the Abs for these various determinants was strongly affected by the PS treatment (Fig. 4C). In BP-PS, anti-Neu5Gc IgG showed a strong preference to Neu5Gcα2-6LacNAc/Lac (Fig. 4C). We also detected elevated anti-Neu5Gc IgG to Neu5Gcα2-3 linked to Core-1 *O*-glycan that was rare in BP-CTRL. Furthermore, using specific competitive inhibition of anti-Neu5Gc Abs in the GalKO PAEC cell binding assay, we show that a substantial IgG fraction of anti–non-αGal Abs (ranging 10–50%; average of 30%) in the tested sera were anti-Neu5Gc. This result highlights the possibility that induction of anti-Neu5Gc Abs represents a major late immune response in clinical xenotransplantation, although Abs against other uncharacterized pig cell Ags were likely to be induced in BP-PS as well.

High anti-Neu5Gc Ab responses (up to 30-fold higher than in controls) in BP-PS were observed for a strikingly long time (up to 34 y; average of 9.5 y) after the PS treatment. This result occurred even though the PS was applied only temporarily (average for 3.9 wk and up to 8 wk for the maximum application time, with applications and changes up to a maximum of 18 times). It is likely that despite short-term exposure to pig tissues in BP-PS, pig Ags were presented in the strong and persisting inflammatory context of a severe burn (64). The inflammation may have provided a



C

Structure	Glycan ID	AUC	Std. Error	95% confidence interval	P value	P value
Neu5Gcα6Galβ4GlcNAcβOR	18	0.94	0.06	0.82	0.000	
Neu5Gcα6Galβ4GlcβOR	20	0.91	0.06	0.80	0.001	
Neu5Gcα3GalβOR	26	0.86	0.08	0.71	0.003	
Neu5Gcα6GalβOR	28	0.85	0.09	0.68	0.004	
Neu5Gcα3Galβ3GalNAcβOR	34	0.85	0.08	0.70	0.004	
Neu5Gcα3Galβ4GlcNAc6SβOR	63	0.84	0.08	0.68	0.006	
Neu5Gc9Acα6GalβOR	32	0.80	0.09	0.63	0.013	
Neu5Gcα3Galβ4(Fucα3)GlcNAcβOR	56	0.80	0.10	0.61	0.014	
Neu5Gcα6GalNAcαOR	6	0.79	0.13	0.55	0.016	
Neu5Gcα3Galβ4(Fucα3)GlcNAc6SβOR	58	0.79	0.10	0.60	0.018	
Neu5Gcα3Galβ3GlcNAcβ3Galβ4GlcβOR	61	0.77	0.10	0.57	0.026	
Neu5Gcα3Galβ3GlcNAcβOR	14	0.76	0.10	0.57	0.030	
Neu5Gcα3Galβ4GlcNAcβOR	12	0.75	0.10	0.55	0.043	
Neu5Gc9Acα3Galβ4GlcNAcβOR	2	0.74	0.10	0.54	0.047	
Neu5Gc9Acα3Galβ3GlcNAcβOR	8	0.74	0.10	0.54	0.050	
Neu5Gc9Acα3Galβ3GalNAcβOR	36	0.73	0.11	0.52	0.061	
Neu5Gcα3Galβ3GalNAcαOR	16	0.73	0.11	0.51	0.061	
Neu5Gcα3Galβ4GlcβOR	22	0.70	0.11	0.48	0.101	
Neu5Gc9Acα6Galβ4GlcNAcβOR	4	0.68	0.11	0.46	0.143	
Neu5Gc9Acα3Galβ3GalNAcαOR	10	0.66	0.12	0.43	0.188	
Neu5Gc9Acα3GalβOR	30	0.64	0.12	0.41	0.242	
Neu5Gc9Acα3Galβ4GlcβOR	40	0.63	0.12	0.39	0.292	
Neu5Gc9Acα6GalNAcαOR	24	0.60	0.13	0.35	0.412	
Neu5Gc9Acα6Galβ4GlcβOR	38	0.52	0.13	0.26	0.861	
$R=(CH_2)_2CH_2NH_2$						

P < 0.05

P > 0.05 (ns)

FIGURE 4. Analysis of serum binding to sialoglycan microarray. (**A**) Binding pattern of BP-PSs versus BP-CTRLs (at 1:250 serum dilution) on sialoglycan microarray, including various matched pairs of Neu5Ac-glycans and Neu5Gc-glycans (array 1 full glycan list is detailed in Ref. 56). Digital array data are presented in Supplemental Table I. (**B**) Statistical analysis of serum binding to the various glycans reveals that only binding to Neu5Gc-glycans is significantly higher in BP-PS compared with the BP-CTRL (Mann–Whitney *U* test; Neu5Gc-glycans, $p = 0.0207$; Neu5Ac-glycans, $p = 0.5775$). (**C**) ROC curve analysis on binding to Neu5Gc-glycans in BP-PS versus BP-CTRL. The area under the ROC curve (AUC) revealed a significantly ($p < 0.05$) elevated IgG response to some Neu5Gc-glycans in BP-PS compared with the BP-CTRL. In general, the least difference is observed for Abs to Neu5Gc-glycans that are modified with *O*-acetylation at position C-9 (Neu5Gc9Ac), suggesting that those Ags do not contribute to anti-Neu5Gc immune response to PS ($p < 0.05$, glycans 36, 4, 10, 30, 40, 24, and 38). However, there is a strong preference to Neu5Gcα2-6LacNAc/Lac over Neu5Gcα2-6GalNAc or Neu5Gcα2-3LacNAc (glycans 18, 20 > 6, 12) with no binding to Neu5Gcα2-3Lac (glycan 22). In addition, Neu5Gcα2-3Core1 is preferred over Neu5Gcα2-3Type1, Le^X or 6S-Le^X (glycans 34 > 14, 56, 58).

vigorous stimulus resulting in the increased basal level of immunity against Neu5Gc-containing epitopes, followed by class switch to IgG as a long-term immune response. The immune response against some carbohydrate Ags (e.g., bacterial capsular polysaccharide) can elicit strong and long-lasting production of Abs if the BCR cross-linking by the repetitive antigenic motif is also contemporary to strong T cell or non–T cell help (65). Understanding the long-lasting and sometimes lifelong response following vaccination or virus infection has generated several models, including continuous stimulation (e.g., boost of vaccines, repeated infections, cross reactivity driven memory). However, convincing experiments suggest that the initial "imprinting" resulting from a vigorous BCR cross-linking and Th cell or TLR driven innate immunity help is determinant for establishing long-term memory B cells and plasma cells (66). Possibly, a high density of Neu5Gc or its protein/lipid scaffolds, or both, on xenogeneic cells synergize to major innate and cognate immunity helper signals contemporary to a severe burn itself and to the frequent surrounding bacterial complications. Furthermore, the coexistence of Neu5Gc Ags, Abs, and complement in a normal individual could promote long-lasting persistence of Neu5Gc on follicular dendritic cells in germinal centers of lymph nodes (67), all of which possibly contributing to the significant elevation of the Abs almost a decade after the burns.

The concomitant presence of the xeno-autoantigen Neu5Gc and related anti-Neu5Gc xeno-autoantibodies at significant titers in humans promoted several studies and hypotheses suggesting that long-term exposure to the resulting inflammation can facilitate inflammatory diseases (44–46). In a preclinical study, alginate-embedded pig islet implantation also generated a vigorous humoral anti-pig response (61), indicating that the alginate protection does not prevent immunization. However, nonhuman primates retain the intact *CMAH* gene (62). Thus, the human host response to Neu5Gc epitopes cannot be studied in preclinical xenotransplantation experiments using nonhuman primates. Instead, it is possible that patients currently treated with alginate-embedded (3) neonatal pig islets also develop persisting anti-Neu5Gc Abs, showing that this technique does not prevent their possible deleterious effects on recipients. Furthermore, our data raise the possibility that anti-Neu5Gc response also occurs in other xenotransplant regimens involving acellular pig

tissues, such as heart valves, or skin that is routinely used in the clinic. However, it is notable that the current study of BP-PS may have only limited implication to the use of acellular pig skin (which is expected to be less immunogenic than vital pig skin), vascularized xenografts, or injected porcine cells (47). Indeed, the analysis of the xenoantibody response in acellular pig skin recipients is of interest for future studies. Unlike this study, there is theoretically no risk of PERV transmission. In any case, pigs doubly null for αGT and CMAH may represent desirable or even required source animals for clinical xenotransplantation. It is also likely that there are additional Ags requiring some control measure, and such Ags are yet to be identified. Investigating that may require further study in patients' sera after exposure to xenografts, like the current study.

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Disclosures

A.V. is cofounder of and scientific advisor to Sialix, Inc., a startup biotech company interested in the practical relevance of anti-Neu5Gc Abs to human disease. H.Y. and X.C. are cofounders of Glycohub, Inc., a startup biotech company focused on the syntheses and applications of carbohydrates and glycoconjugates. The other authors have no financial conflicts of interest.

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