Bioengineered human and allogeneic pulmonary valve conduits chronically implanted orthotopically in baboons: Hemodynamic performance and immunologic consequences

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Objective: This study assesses in a baboon model the hemodynamics and human leukocyte antigen immunogenicity of chronically implanted bioengineered (decellularized with collagen conditioning treatments) human and baboon heart valve scaffolds.

Methods: Fourteen baboons underwent pulmonary valve replacement, 8 with decellularized and conditioned (bioengineered) pulmonary valves derived from allogeneic (N = 3) or xenogeneic (human) (N = 5) hearts; for comparison, 6 baboons received clinically relevant reference cryopreserved or porcine valved conduits. Panel-reactive serum antibodies (human leukocyte antigen class I and II), complement fixing antibodies (C1q binding), and C-reactive protein titers were measured serially until elective sacrifice at 10 or 26 weeks. Serial transthoracic echocardiograms measured valve function and geometry. Differences were analyzed with Kruskal–Wallis and Wilcoxon rank-sum tests.

Results: All animals survived and thrived, exhibiting excellent immediate implanted valve function by transthoracic echocardiograms. Over time, reference valves developed a smaller effective orifice area index (median, 0.84 cm²/m²; range, 1.22 cm²/m²), whereas all bioengineered valves remained normal (effective orifice area index median, 2.45 cm²/m²; range, 1.35 cm²/m²; *P* = .005). None of the bioengineered valves developed elevated peak transvalvular gradients: 5.5 (6.0) mm Hg versus 12.5 (23.0) mm Hg (*P* = .003). Cryopreserved valves provoked the most intense antibody responses. Two of 5 human bioengineered and 2 of 3 baboon bioengineered valves did not provoke any class I antibodies. Bioengineered human (but not baboon) scaffolds provoked class II antibodies. C1q⁺ antibodies developed in 4 recipients.

Conclusions: Valve dysfunction correlated with markers for more intense inflammatory provocation. The tested bioengineering methods reduced antigenicity of both human and baboon valves. Bioengineered replacement valves from both species were hemodynamically equivalent to native valves. (J Thorac Cardiovasc Surg 2013;145:1098-107)

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There is increasing interest in subhuman primates for biomedical research.¹ However, they are rarely used for evaluating bioengineered cardiovascular constructs. The genus *Papio* (baboons) is potentially valuable for comparative tissue-engineering research given significant similarities with humans in cardiac anatomy, physiology, valve dimensions, resident antigens, and innate and acquired immune systems. Yet there are few reported baboon chronic survival cardiac surgical replacement valve performance studies.^{2,3} A baboon orthotopic valve replacement model could be a significant preclinical tool for bridging cardiovascular tissue engineering to “real world” clinical applications. *Papio hamadryas Anubis*, an Old World monkey species, is genetically more similar to *Homo sapiens* compared with other nonhominoid primates and far more so than hoofed food stock *ungulate* mammals (Figure E1). The latter are often used both as valve test models (eg, sheep) and as source material (eg, porcine) for bioprosthetic valves. International standards and regulatory guidelines for implantable cardiovascular devices typically require large animal preclinical in vivo validation studies.⁴ For evaluating

Abbreviations and Acronyms

EOA	= effective orifice area
H&E	= hematoxylin and eosin
HLA	= human leukocyte antigen
PRA	= panel-reactive antibody
TEE	= transesophageal echocardiogram

bioprosthetic heart valves, the classic juvenile sheep model is typically chosen because it is robust and sensitive for predicting valve structural failure due to dystrophic calcification.⁵ However, all *ungulates* express powerful non-human leukocyte antigen (HLA) xeno-epitopes, such as alpha-galactosyl, to which all *catarrhines* (humans, apes, and Old World Monkeys) possess natural antibodies. Conversely, *ungulates* cannot be used to directly test human-derived uncrosslinked tissue because of profound xenotransplant rejection.⁶ After removal of cells, it is not known whether heart valve extracellular matrix scaffolds remain functionally antigenic and proinflammatory across *catarrhine* species. Thus, for both mechanistic research and possible regulatory utility, these studies were undertaken to evaluate the suitability of the baboon as a subhuman primate model for testing clinical prototype bioengineered heart valves.

The current clinical standard for pediatric valved conduit reconstructions is the cryopreserved, cadaver-derived allograft “biologic” pulmonary valve, historically termed valve “homografts.” As traditionally prepared, donor cells are retained through surgical implantation, yet these valves do not grow, and the donor cell population disappears; durability is limited especially in infants and children, typically failing because of dystrophic calcification driven by chronic inflammation.⁷ Cryopreserved human homografts have been conclusively shown to contain HLA antigens capable of provoking antibodies in recipients.⁸ To reduce inflammatory responses, various tissue decellularization treatments of allogeneic tissues have been devised, and some show promise.^{9–11} Unlike allografts, the clinical results with decellularized porcine valve xenografts have been poor with accelerated inflammatory destruction.^{12,13} This research uses valve scaffolds bioengineered (decellularized and conditioned) and designed to be optimal, minimally proinflammatory, and attractive biologically to cells in vitro and in vivo.^{14,15} As tested in the current study, these are not tissue-engineered heart valves. Tissue-engineered heart valves are a subset of bioengineered in which the scaffolds are seeded with cells in a bioreactor before implantation. The bioengineered valve scaffolds tested in these studies are acellular but otherwise inherently similar to native valves in design, hemodynamic performance, matrix chemistry, and material properties.^{10,16,17} As such, they could function as the platform or scaffold for a tissue-

engineered valve, and because a scaffold without cells at the time of surgical implantation is the “worst case” scenario for a tissue-engineered construct in which seeded cells have failed to repopulate, it is the appropriate starting point for the development of new evaluative tools for preclinical assessment of tissue-engineered heart valves.

MATERIALS AND METHODS

Donors, Recipients, and Surgery

Fourteen healthy postpubescent male baboons weighing 28 to 34 kg were selected as recipients. Donors were unrelated animals 2 to 4 kg larger than the recipients. Anesthesia and cardiopulmonary bypass methods were refined as previously reported by us.¹⁸ All recipients had excision of native valves and main pulmonary artery segments, and then test valve conduits were inserted with standard homograft surgical techniques using end-to-end anastomoses. Five were replaced with human bioengineered pulmonary valves for 10 weeks (N = 3) or 26 weeks (N = 2); 3 received bioengineered baboon pulmonary valves for a duration of 10 weeks. Three types of clinically analogous alternatives (N = 6) were used as reference valves: cryopreserved pulmonary valves from humans (N = 2), baboons (N = 2), and stentless porcine (N = 2) bioprostheses (size 21 Medtronic Freestyle, Medtronic, Inc, Minneapolis, Minn). Studies were performed with Institutional Animal Care and Use Committee approval (Southwest National Primate Research Center), in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011).

Preparation of Bioengineered Valves

Baboon pulmonary valves were harvested and cryopreserved with clinical methods.⁷ Seven cryopreserved human valves at the end of storage time limits were obtained from a clinical tissue bank (LifeNet Health, Virginia Beach, Va). Human and baboon bioengineered valves were prepared by subsequent decellularization using a previously reported laboratory developed, double-solvent, multidetergent, enzyme-assisted, reciprocating osmotic cell fracture method optimized for pulmonary valves.¹⁰ Before implantation, all decellularized valves were conditioned in solutions designed to acidify tissue pH, rehydrate the collagen helix moisture envelope, align and compact collagen fibrils, and restore soluble proteins to the matrix.¹⁶ These decellularized-conditioned valves are termed “bioengineered.”

Transesophageal and Dobutamine Stress Echocardiography

Valve performance was evaluated by transesophageal echocardiogram (TEE) and dobutamine stress echocardiography with geometric dimensions and functional parameters measured and calculated as recommended for evaluation of prosthetic heart valves by the American Society of Echocardiography.¹⁹ A 5.0/3.7 MHz omni-plane TEE probe (Philips Medical Systems, Andover, Mass) was positioned after induction of general anesthesia. Images were captured in real time on an HP SONOS 2500 platform (Philips Medical Systems) and recorded for quantitative offline analyses. Baseline measurements were performed before cardiopulmonary bypass and after sternotomy closure during stable hemodynamics. Dobutamine was administered intravenously as a continuous infusion (Sigma Spectrum Volumetric Infusion pump; Sigma International, Inc, Medina, NY), with measurements repeated at dosages of 2 and 4 $\mu\text{g/kg/min}$. Terminal measurements were similarly performed. Leaflet thicknesses were measured in triplicate by 2-dimensional and M-mode echocardiography.

Serial Panel-Reactive Antibodies, Complement Fixation, C-Reactive Protein Titers

Immediate pre- and postoperative and serial weekly postoperative serum samples were assayed qualitatively for the development of

TABLE 1. Echocardiography measurements of test and reference valves

Valve implant type	Examination	N	Valve diameter (2D echocardiography) cm	Maximum geometric valve area* cm ²
Bioengineered valves				
<i>Papio</i>	Day 0	3	1.58 (0.29)	1.96 (0.75)
Bioengineered	10 wk	3	1.76 (0.19)	2.43 (0.50)
Human	Day 0	5	2.11 (0.64)	3.50 (2.11)
Bioengineered	10 wk	5	2.00 (0.51)	3.14 (1.49)
	26 wk	2	1.97 (0.13)	3.04 (0.40)
Reference valves				
<i>Papio</i>	Day 0	2	1.68 (0.15)	2.21 (0.39)
Cryopreserved	10 wk	2	1.52 (0.08)	1.82 (0.19)
Human	Day 0	2	2.01 (0.18)	3.18 (0.57)
Cryopreserved	10 wk	2	1.75 (0.30)	2.42 (0.82)
Porcine	Day 0	2	1.65 (0.40)	2.17 (1.04)
Glutaraldehyde	10 wk	2	1.97 (0.06)	3.05 (0.19)
All bioengineered‡	10 wk	8	1.76 (0.54)	2.43 (1.56)
All reference valves‡	10 wk	6	1.75 (0.52)	2.42 (1.42)

Data reported as median (range). Porcine glutaraldehyde, *Papio* cryopreserved, and human cryopreserved valves are reference valves, whereas *Papio* 10-week, human 10-week, and human 6-month implant valves are bioengineered valves. 2D, 2-dimensional; EOA, effective orifice area; EOA_I, effective orifice area index; BSA, body surface area.

*Maximum geometric test valve areas (theoretic) are calculated by measuring the internal annulus diameters at the time of implantation and explantation with Hegar dilators.

†BSA calculated by the Haycock formula. ‡All bioengineered versus all reference valves at 10 weeks: §*P* = .0003, ||*P* = .003, ¶*P* = .005, #*P* = .002.

panel-reactive antibodies (PRAs) using LABScreen mixed class I and II HLA bead assay (One Lambda, Inc, Canoga Park, Calif) run on a Luminex 200 System (Luminex, Inc, Austin, Tex) after serum purification (HiTrap; Amersham Biosciences, Buckinghamshire, England). All sera were also assayed with a second assay, LABScreen PRA (One Lambda, Inc), to confirm positivity and calculate the clinically familiar percentage PRA developing to class I and II antigens. This %PRA assay contains bead sites to 55 class I and 32 class II phenotypes and allows for some discrimination of responsible antigen specificities. Serum C-reactive protein titers were measured by enzyme-linked immunosorbent assay at day 0 and days 7, 35, and 70 postoperatively (Monkey CRP; Life Diagnostics, West Chester, Pa). Class I complement-fixing immunoglobulin-G antibodies were measured with a solid-state assay (C1q Screen, One Lambda, Inc).

Explant Pathology Methods

At necropsy, the heart lung block was removed and severity of pericardial adhesions was scored (absent, mild, moderate, severe). Test valves were excised with cuffs of native tissue beyond both anastomoses for explant pathology evaluations. Explanted valves were fixed with HistoChoice MB (Amresco, Solon, Ohio) and photographed without magnification (Sony Cybershot, San Diego, Calif) and at macroscopic 6.3× (SteREO Discovery V12; Carl Zeiss, Thornwood, NY) to document visual observations and imaged with radiographic methods for calcium mineral formation (Faxitron-LR, Lincolnshire, Ill). Longitudinal sections were cut through each cusp, from the free edge to the base, including the corresponding sinus and arterial wall. Paraffin-embedded sections were used for routine hematoxylin and eosin (H&E) stains (American Histo Laboratory, Gaithersburg, Md). For immunohistochemistry, after antigen retrieval (10 minutes, 90°C; Antigen Unmasking Solution, Vector Laboratories, Burlingame, Calif) deparaffinized sections of each valve were incubated overnight with a 1:25 dilution of CD79 (B-cell; mouse monoclonal; Dako, Glostrup, Denmark) followed by application of a secondary antibody (alkaline phosphatase-conjugated; VECTASTAIN ABC-AP Kits, Vector Laboratories). Slides were examined via light and fluorescence microscopy with digital camera and imaging software (Axio Imager.Z1; Axio Vision, Carl Zeiss). Multiple

low-magnification (2.5×) H&E images from each entire valved conduit were blindly reviewed (S.L.H.), and the inflammatory response was scored on the basis of the maximum percentage of tissue area per low-power field infiltrated with inflammatory cells: minimal (0%-10%), mild (10%-25%), moderate (25%-50%), or marked (50%-100%).

Statistical Methods

Kolmogorov-Smirnov test for normality indicated these data sets were not normally distributed, so nonparametric tests were used and values reported as median (range). Differences between groups were analyzed for continuous variables with the Kruskal-Wallis test and for ordinal variables by the Wilcoxon rank-sum test. SAS 9.2 (SAS Institute, Inc, Cary, NC) and SPSS version 17 (SPSS, Inc, Chicago, Ill) were used. Bonferroni correction was applied for multiple comparisons.

RESULTS

Clinical Outcomes

All baboons were successfully weaned from cardiopulmonary bypass and survived and thrived. One recipient easily tolerated emergency open surgical removal of an intercurrent gastric bezoar (hair).

Two-Dimensional and Doppler Echocardiographic Assessment of Valve Performance

Compared with bioengineered valves, reference valves developed higher peak (20.0 [29.0] mm Hg vs 5.5 [6.0] mm Hg, *P* = .0003) and mean (12.5 [23.0] mm Hg vs 3.5 [4.0] mm Hg, *P* = .003) pressure gradients by 10 weeks postimplant (Table 1). None of the bioengineered valves developed elevated resting valve gradients (Figure 1). The majority (5 of 6) of the reference valves

Table 1. Continued

Peak gradient (Doppler) mm Hg	Mean gradient (Doppler) mm Hg	EOA (Doppler) cm ²	EOAI to BSA (Doppler) cm ² /m ²	Cardiac output mL/min	BSA† m ²
4.0 (6.0)	3.0 (4.0)	1.96 (0.93)	2.40 (0.93)	3251 (2994)	0.79 (0.16)
5.0 (3.0)	2.0 (2.0)	2.28 (0.92)	3.09 (1.03)	4143 (11)	0.76 (0.11)
3.0 (1.0)	2.0 (0.0)	2.05 (0.36)	2.65 (0.39)	3398 (1878)	0.78 (0.09)
8.0 (6.0)	3.0 (4.0)	1.73 (1.14)	2.32 (1.28)	4705 (3797)	0.78 (0.15)
4.0 (4.0)	2.0 (2.0)	2.5 (0.06)	3.15 (0.07)	3708 (1344)	0.80 (0.04)
6.0 (0.0)	3.5 (1.0)	2.12 (0.43)	2.64 (0.06)	5040 (2955)	0.80 (0.15)
23.0 (18.0)	14.5 (11.0)	0.68 (0.41)	0.84 (0.35)	2755 (2)	0.79 (0.16)
4.5 (1.0)	2.0 (0.0)	2.33 (0.47)	2.75 (0.17)	3980 (2379)	0.84 (0.14)
12.0 (8.0)	7.5 (5.0)	1.35 (0.41)	1.57 (0.43)	3940 (2179)	0.85 (0.03)
5.0 (4.0)	3.0 (2.0)	2.19 (1.15)	2.59 (1.34)	4270 (383)	0.84 (0.01)
35.5 (23.0)	21.5 (13.0)	0.52 (0.06)	0.61 (0.08)	2776 (825)	0.85 (0.01)
5.5 (6.0)§	3.5 (4.0)	1.77 (1.19)¶	2.45 (1.35)#	4148 (3797)	0.78 (0.16)
30.0 (39.0)§	12.5 (23.0)	0.72 (1.08)¶	0.84 (1.22)#	2813 (2666)	0.85 (0.16)

developed cusp thickening (≥ 1.5 mm) and restriction in cusp excursion (Table 2). In contrast, mild cusp thickening was seen in only 1 bioengineered valve (animal number 10), and all had normal cusp mobility. Bioengineered valves maintained stable normal effective orifice areas (EOAs) to 10 and 26 weeks. Reference valves developed markedly decreased EOA compared with the bioengineered valve group ($0.72 [1.08]$ cm² vs $1.77 [1.19]$ cm²,

$P = .005$). Bioengineered cusp dysfunctional regurgitation was typically trace to mild, but was moderate for 1 C1q⁺ recipient receiving a human-derived bioengineered valve (animal number 10).

Dobutamine Stress Echocardiography

Dobutamine stress testing demonstrated marked differences in EOA and EOA index between reference and

FIGURE 1. Dobutamine echocardiography results after chronic implantation of human and baboon bioengineered and control valves. Source species and process preparation for each group are noted. Porcine valves (light blue) were glutaraldehyde crosslinked stentless valves (Medtronic Freestyle; Medtronic Inc, Minneapolis, Minn). Transvalvular gradients were uniformly lower for all bioengineered valves (red) at rest and during dobutamine challenge compared with the clinical reference valves (green) (A). The human bioengineered valves (orange) functionally had low gradients and normal indexed EOA at maximum dobutamine stimulation (B). * $P \leq .005$. PV, Pulmonary valve; BEV, bioengineered valve; RVCO, right ventricular cardiac output.

TABLE 2. Serum human leukocyte antigen antibody serial assays and corresponding valve cusp function by transesophageal echocardiography

Valve type	No.	LABScreen mixed HLA assay* (One Lambda Inc, Canoga Park, Calif)				LABScreen % PRA assay†			
		Class I		Class II		Class I		Class II	
		Result	Time	Result	Time	Result	Time	Result	Time
Porcine	1	+	wk 3	–	N/A	0%	N/A	0%	N/A
glutaraldehyde	2	–	N/A	+	wk 10	0%	N/A	0%	N/A
<i>Papio</i> cryopreserved	3	++	wk 3	++	wk 3	85%	wk 3	98%	wk 3
	4	+	wk 10	++	wk 4	95%	wk 10	70%	wk 6
Human cryopreserved	5	++	wk 2	++	wk 4	98%	wk 2	98%	wk 5
	6	+	wk 4	++	wk 3	60%	wk 6	100%	wk 4
Bioengineered valves									
<i>Papio</i> 10-wk implant	7	–	N/A	–	N/A	–	–	–	N/A
	8	–	N/A	–	N/A	–	N/A	–	N/A
	9	+	wk 6	–	N/A	65%	wk 6	–	N/A
Human 10-wk implant	10	+	wk 9	++	wk 2	76%	wk 9	100%	wk 4
	11	+	wk 1	+	wk 8	96%	wk 1	14%	wk 8
	12	+	wk 6	+	wk 5	75%	wk 6	20%	wk 5
Human 6-mo implant	13	–	N/A	+	wk 6	0%	N/A	100%	wk 7
	14	–	N/A	+	wk 9	0%	N/A	100%	wk 9

Porcine glutaraldehyde, *Papio* cryopreserved, and human cryopreserved valves are reference valves, whereas *Papio* 10-week, human 10-week, and human 6-month implant valves decellularized and conditioned are termed “bioengineered valves.” HLA, Human leukocyte antigen; PRA, panel-reactive antibody; N/A, not available. *Results of the LABScreen mixed assay are scored as positive (+; median fluorescence intensity > 1000 units) or strongly positive (++; peak median fluorescence intensity > 7000 units) for the postoperative week at which the serum first measured as antibody positive. †Results of the LABScreen % PRA assay are reported as the maximum PRA titer (%) for the postoperative week after the % PRA first exceeded 10%. Time points where the % PRAs were negative for the study duration are reported as N/A. ‡Results are the likely antigens responsible for HLA-I or II sensitization as identified by reporting bead epitope sites. Epitopes can be shared by multiple antigens, so antibody specificities can arise against HLA antigens other than those actually provoking the response. §Highly reactive indicates an inability to assign specificities due to intense reactivity. ||Cusps were designated as thickened when the thickness measured by TEE was ≥ 1.5 mm; mildly thickened = cusp thickness ≥ 1.2 mm. Cusp regurgitation scores represent function at pre-death examination: trace (I+), mild (2+), and moderate (3+). ¶There was complete agreement on the measurement of cusp thickness by TEE and explant qualitative observations except for animal number 3 (compare with Table E1).

bioengineered valves (Figure 1). Peak and mean transvalvular pressure gradients were consistently lower for all bioengineered valves. At maximum dobutamine stimulation, the reference valves had peak and mean pressure gradients of 44.5 (76.0) mm Hg and 29.5 (49.0) mm Hg, respectively. In contrast, the human bioengineered valves had functionally low valve gradients (peak, 12.0 [12.0] mm Hg; mean, 7.0 [8.0] mm Hg) despite cardiac indexes of 5 L/min/m² or greater at the maximum dobutamine stimulation ($P \leq .005$ for all comparisons). For all bioengineered valves, transvalvular gradients during stress echocardiography were comparable to those across native valves in each animal, suggesting the bioengineered valves were functionally equivalent to native valves. The geometric valve annulus diameters (Table 1) by direct TEE measurement after 10 weeks for bioengineered (1.76 [0.54] cm) and reference (1.75 [0.52] cm) valves were the same ($P > .05$). Thus, the reductions in EOA measured in reference valves were likely not due to anatomic shrinkage but rather to increasing cuspal stiffness, restricted cusp opening, and loss of conduit wall compliance.

Mixed Class I and II Human Leukocyte Antibodies Screening Assay

The mixed screening assay accurately predicted whether class I or II PRA titers would exceed 10% when measured by quantitative LABScreen PRA % assay (Table 2). Both allogeneic and xenogeneic cryo reference valves provoked class I and II antibodies, typically as “strongly positive,” beginning relatively early (weeks 1–6) after implantation. In contrast, 2 of 5 human and 2 of 3 baboon bioengineered valves did not provoke class I antibodies. None of the bioengineered baboon valves provoked class II antibodies, whereas all human tissues did.

Class I and Class II % Panel-Reactive Antibody Assay

All cryopreserved donor valves provoked class I and II titers exceeding 60% by week 10 (Table 2) regardless of species of origin. Baboon bioengineered valves were negative for both class I and II elevations except for 1 animal (number 9) that developed 75% class I (and C1q⁺) by week 6, but which declined by week 10% to 47%. Two human bioengineered valves remained class I negative (<10%) to 6 months,

Table 2. Continued

Candidate HLA culprit specificities ^{‡,§}			Echocardiography (cusp) ^{,¶}		
Class I	Class II	C1q complement	Thickness	Mobility	Regurgitation
None	None	–	Thick	Restricted	0
None	None	–	Thick	Restricted	1+
A32, A29, A74, B18, B42, B27, B37, B38, B39, B47, B57	DQ7, DQ8, DR11, DR13, DR14	–	Thick	Restricted	0
A1, A10, A23, A24, A25, A3, A32, B48, B13, B62, B44, B45, B60	DR1, DR10, D9, DR4, DQ7, DQ5, DQ8, DQ9	+(62%)	Thick	Restricted	2+
Highly reactive	DR7, DQ6	+(100%)	Thin	Restricted	3+
A2, A68, A69, B58	Highly reactive	–	Thick	Normal	1+
None	None	–	Thin	Normal	2+
None	None	–	Thin	Normal	2+
A25, A26, A33, A34, A32, A74, A23, A24, A66	None	+(58%)	Thin	Normal	2+
A24, B7, B8, B81, B48, B27, B42, B54, B60, B64, B65	DR4	+(25%)	Mildly thickened	Normal	3+
A25, A26, B48, B60	DR7 Dr9	–	Thin	Normal	2+
A25, A29, A66, B81, B56, B54, B55, B57, B7, B42, B51, B53, B35	DR7, DR9	–	Thin	Normal	2+
None	DR7, DR8, DR9, DR17	–	Thin	Normal	2+
None	DR11, DR12, DR13, DR14, DR17, DR8	–	thin	normal	2+

but all eventually provoked class II antibodies. PRA time courses were variable (Figure 2): Bioengineered valves that elicited positive PRA titers typically did so by 4 weeks and then returned to zero by 10 weeks, whereas cryopreserved valves (even allogeneic) tended to evoke more persistent elevations. Valves maintaining superior hemodynamics typically had absent or shorter elevations of PRA.

Complement Fixing Antibody, C-Reactive Protein Assays

C1q binding class I antibody titers were positive in 4 animals receiving 4 different valve types (Table 2). Elevated CRP titers consistently occurred only perioperatively (P = .005) (Figure E2).

Explant Pathology

Both human and baboon bioengineered valves appeared to have virtually normal cusps qualitatively except for 1 human valve in a strongly class II⁺ and C1q⁺ recipient (number 10) that was described as “slightly thickened.” By echocardiography, this valve’s leaflets were measured as “mildly thickened” (Table 2). Only 1 valve (number

3, cryopreserved) had discordant leaflet scoring between echocardiography and explant observations (compare Tables E1 and 2), although this particular valve did exhibit gross findings of inflammatory degradation with sinus wall thickening (Table 2). In contrast, all but 1 reference valve exhibited cusp thickening quantitatively by echocardiography (Table E1). Frank calcifications were not observed by radiographic examination in any of the explanted valves. Inflammatory histologic scores demonstrated some variability yet tended to correlate with the PRA titers (Tables E1 and 2). With the exception of a single baboon valve, all cryopreserved valves elicited a marked inflammatory infiltrative response (mimicking clinical experience), whereas only 2 human bioengineered valves did. Immunohistochemical staining confirmed the presence of B-cells in these inflammatory infiltrates (Figure E3).

DISCUSSION

The key findings of this study include the following: (1) Bioengineering using valve decellularization and conditioning reduces antigenicity and prolongs normal

functional valve performance especially well for baboon (allogeneic) but also for human (xenogeneic) sourced valve scaffolds; (2) the intensity of immune and inflammatory responses is correlated with valve functional durability; (3) prolonged and higher HLA titers seem to predict accelerated functional degradation; (4) C1q⁺ complement fixing antibodies were provoked by 4 different test valves, all developed 2+ to 3+ valve regurgitation; (5) technical implant surgery in baboons is analogous to human clinical surgery; and (6) development of HLA antibodies in baboons can be quantified with commercially available solid-state assays used in transplantation surgery. Our rationale for selecting the baboon was similarity to humans in ontogeny, phylogeny, immunology (eg, 4 immunoglobulin-G subclasses, complement fixation, macrophage, and T- and B-cell functions), absence of α -Gal epitope, similar semilunar valve microstructure, semi-upright posture, lack of susceptibility to simian herpes B virus, and robust tolerance of cardiopulmonary bypass.¹⁸ Our data suggest that bioengineered cross-species human to baboon valve scaffold transplants are different immunologically (eg, provoking class II⁺ antibodies) than allotransplants, but functionally less provocative than cryopreserved valves of either species.⁹

Valve Functional Performance Correlates With Markers of Human Leukocyte Antigen Mismatch

The baboon valve diameters as measured at implant scaled allometrically to normalized (body surface area) pediatric valve annulus diameters with values similar to those of humans (Figure 3). The EOA index increased by the time of explant for bioengineered valves (*Papio* $\uparrow$ 29%, human $\uparrow$ 19%) but decreased for cryopreserved (*Papio* $\downarrow$ 68%, human $\downarrow$ 57%) and porcine ($\downarrow$ 76%). The differences in resting pressure gradients across the clinical reference valves versus the bioengineered valves are likely underreported because the cardiac outputs at 10 weeks were generally higher in the animals with bioengineered valves. There was no echocardiographic evidence of impending bioengineered valve stenotic failure because transvalvular gradients did not increase over time, nor did calculated valve EOAs decrease significantly at 10 or 26 weeks postimplant. The dobutamine-driven elevated cardiac outputs only provoked physiologic flow gradients comparable to expected values for human native valves. In contrast, echocardiographic evidence of critical dysfunction with decreasing EOA was manifest in all 3 types of reference valves even by 10 weeks, suggesting that the baboon is a severe test species and robust model for such assessments. Dysfunction by TEE correlated with measurements of more intense PRA responses. Only 1 bioengineered valve (number 10) demonstrated cuspal thickening. This recipient had a high class II⁺ response and was class

I C1q⁺. The 2 valves that developed leaflet dysfunction with greater than 2+ regurgitation (numbers 5 and 10) were both associated with higher PRA titers and C1q⁺ antibodies. To our knowledge, the correlation of the appearance of C1q complement fixing antibodies and transplanted biological valve dysfunction has not been reported. Intuitively, C1q⁺ antibodies may be implicated in more consequential antibody-mediated clinical tissue transplant rejection and may thus be an observation perhaps worthy of clinical evaluation in homograft valve recipients exhibiting accelerated valved conduit dysfunction. Thus, various indicators of the severity of provoked immunogenic inflammatory responses seemed to correlate with the severity of valve dysfunction, which decellularization and conditioning seemed to mitigate. These observations corroborate current mechanistic theories emphasizing the immune and inflammatory pathogenesis of valve degradation.²⁰

Baboon and human HLA molecules have been compared and are approximately 90% identical, with cross-species differences concentrating in positions typically demonstrating polymorphisms in human alleles that serve to activate T cells.²¹ Cryopreserved valves provoked strong HLA I and II responses regardless of donor species. Of note, in the baboon model, as in human transplantation, suspect culprit antigen specificities tended to group with known shared epitopes (eg, B8 with B64 and B65, DQ7 with DQ8, and B7 with B42).²² The duration of the PRA elevations was more sustained for cryo-implants, but when occurring in recipients of bioengineered valves, the transient elevations were as brief as 5 weeks duration, highlighting the need for well-timed blood draws to identify sensitization (Figure 2). This has significant implications for the interpretations of PRA titers in older reports and assay timing in future clinical studies.

Study Limitations

The 10-week duration of these studies was too short to adequately assess in situ autologous in vivo recellularization but was intentionally chosen to best capture early immune-inflammatory responses to correlate with echocardiography function and PRA titers. Only 2 recipients with human bioengineered scaffolds were kept to 6 months; both had persistent excellent valve functionality without MHCI⁺ or C1q⁺ antibodies, suggesting that when antigenicity is minimized, longer-term hemodynamic performance studies are feasible. Why antigenicity can be variably retained at all in decellularized valves (albeit typically mild) is not clear from these studies. All mammalian semilunar valves have microscopic interdigitations of ventricular muscle deep within the fibrous annulus where some fragmentary cell remnants could remain despite 97% to 99% removal of DNA and other indicators of decellularization effectiveness. Epitopes

FIGURE 2. Examples of PRA time curves. A, Animal 3 received a cryopreserved allograft and developed both class I and II PRAs by week 3, sustained through 10 weeks. B, Animal 9 received a bioengineered allograft, did not elevate to class II, and was the only bioengineered allograft recipient in which class I increased at all. Titer returned to zero by 10 weeks. C, Animal 13 received a human bioengineered valve that provoked class II antibodies at week 7 but returned to baseline by weeks 26 and 27; no class I antibodies were detected. These serial measurements demonstrate the potential difficulties in interpretation of random isolated PRA titer measurements after tissue transplants. *PRA*, Panel-reactive antibody; *Pre*, preoperatively; *Post*, immediately after surgery.

could be associated with extracellular matrix (as seen with α -gal). Alternatively, mass spectrometry of detergent decellularized equine carotid arteries has revealed small residuals of more than 300 cell-associated proteins.²³ These could include epitopes accounting for some retention of xenotransplant immunogenicity despite the

absence of cell fragments. Our complete cardiac experience with baboons has provided some unique observations (Table E2) that may aid other investigators considering this species for cardiac preclinical studies.¹⁸ Cardiac surgery studies in baboons cost 500% more than sheep. As in this study, this limits feasible animal numbers per test group.

Tissue-Engineered Valves and Preclinical Testing With Baboons

The need for chronic animal models that are especially relevant to humans has been identified by multiple authorities as critical to “bench to bedside” translation of tissue-engineering strategies.^{1,2,24} A principal tenet in the field of tissue engineering is that the more a reestablished cellular population resembles normal tissue, in cell density, location, and phenotypes, the more likely it is that such tissues will effectively mimic native, particularly with the capacity for constructive and adaptive remodeling. One pathway to a “personal” heart valve would be to “tissue engineer” by repopulating a decellularized allogeneic scaffold with the putative recipient’s own cells. This could be achieved by 1 or more strategies: (1) preimplant direct bioreactor cell seeding (classic tissue engineering); (2) use of physical, chemical, and biological conditioning treatments of scaffold matrix to promote cell adhesion and in migration in vivo as an in situ postimplantation autologous process; and (3) introduction into the scaffold “homing” molecules (“breadcrumbs” strategy) that accelerate in vivo cell seeding.^{6,25} In this study, method 2 was used without ex vivo cell seeding. However, regardless of technique, the base scaffold must be minimally proinflammatory or the result may be scar tissue, not physiologic, healthy, reconstituted tissue structures. Previous research has demonstrated that autologous cell in migration, even without preseeding, effectively repopulates decellularized conduit vascular walls.¹⁰ Cuspal reendothelialization is improved with conditioning, but the matrix of all semilunar cusps of such valve scaffolds is not consistently (ie, all 3 cusps every time) fully repopulated with valve interstitial cells by just autologous postsurgical in vivo cell migration and proliferation.^{10,16} This is an important distinction because repopulated cells are needed to provide matrix remodeling protein synthesis to establish the capacity for growth and repair, which suggests that enhanced recellularization strategies could be useful.^{14,25} Development of viable personal heart valves using any combination of these strategies will be strengthened by assessment methods designed to emulate putative clinical paradigms as closely as possible. Both clinical hemodynamic failure modes for biological replacement valved conduits (insufficiency due to leaflet

FIGURE 3. Implanted valve sizes. Nomogram of BSA normalized human pediatric pulmonary valve annulus diameters derived from 14,128 normal pediatric transthoracic echocardiograms archived in the Children's Mercy Cardiac Database and on which the 14 implanted valve annulus diameters measured by TEE at baseline after implant surgery (day 0) are graphed (green dots). Note that all but 3 were above the 50% median; the smallest valve for the recipient size was a baboon bioengineered valve (number 8). An inadvertent "downsizing" bias would not explain the greater functional deterioration of the cryo-preserved valves. Numbers identify recipient animals for each valve as identified in Table 2. PV, Pulmonary valve.

dysfunction and stenosis with progressive gradients) were replicated in this model. Thus, the baboon could be useful for both mechanistic studies and as a bridge from traditional large animals to clinical trials of tissue-engineered cardiovascular constructs.

CONCLUSIONS

The bioengineering processes used seemed to reduce the antigenicity of semilunar valve scaffold tissues and prolong functional performance, especially within species, but also across genera from 2 primate families of high genomic congruence. Absent or evolving postoperative major histocompatibility complex antibody titers were assessed and seemed to correlate with functional measurements. This experience suggests that the baboon model may be useful in the future for chronic functional testing of clinical prototype human bioengineered or even "tissue-engineered" replacement valves for which human decellularized scaffolds could be recellularized with recipient (ie, baboon) cells to closely simulate clinical paradigms.

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FIGURE E1. Relative primate homology. Time domain is estimated with fossil [] or mtDNA evidence (). Primates first appeared as fossils dated to c. 95 million years ago. The depicted divergences represent generalized combinations of physical anthropological findings and computational algorithms based on genetic sequencing data. Of the Old World monkeys, the baboon has the most genomic homology to humans. Data from multiple sources, including Raaum RL, Sterner KN, Noviello CM, Stewart C-B, Disotell TR. Catarrhine primate divergence dates estimated from complete mitochondrial genomes: concordance with fossil and nuclear DNA evidence. *J Hum Evol.* 2005;48:237-57. Adapted with permission from material provided by Jeffrey Rogers, PhD, SNPRC. Ma, Million years ago.

FIGURE E2. C-reactive protein serum titers for each animal consistently peaked 1 week postoperatively, indicating classic proinflammatory effects of surgery and cardiopulmonary bypass. Pre, Preoperatively; Post, immediately after surgery.

FIGURE E3. Histopathology examples of inflammatory infiltrate grades. Histologic and immunohistochemical stains of pulmonary valves. A, Histologic section of cryopreserved human pulmonary valve explanted at 10 weeks (H&E, magnification 2.5×) exemplifying a microscopic field that would rate a mild inflammation score. Elsewhere in the specimen, areas of marked inflammation were observed; thus, the overall score for this valve explant was “marked” (animal number 5, Table 2). B, Immunohistochemical stain for B-cells (red, alkaline phosphatase, magnification 10×) within the conduit wall inflammatory infiltrate shown in A. C, Histologic section of cryopreserved *Papio* pulmonary valve explanted at 10 weeks (H&E, magnification 2.5×) exemplifying a score of “marked” inflammation (animal 4). D, Immunohistochemical stain for B-cells (red, alkaline phosphatase, magnification 10×) within the inflammatory infiltrate in the conduit of PRA⁺ recipient in C.

TABLE E1. Explant valve necropsy observations

Valve type	Animal ID no.	Pericardial adhesions	Valve conduit wall	Valve sinus wall	Valve cusps	Histologic inflammatory response score
Porcine glutaraldehyde*	1	Moderately severe	Stiff	Stiff, thick	Very stiff, thick	Marked
	2	Severe	Rigid	Stiff, thick	Stiff, thick	Marked
<i>Papio</i> cryopreserved	3	Moderate	Stiff, nodular suture line	Nodules, scarring	Thin	Mild
	4	Severe	Scarring at suture lines	Stiff, thick	Thick, contracted	Marked
Human cryopreserved	5	Moderately severe	No scar	No scar or calcium	Thin, normal	Marked
<i>Papio</i> 10-wk implant	6	Moderate	No scar	Stiff, thick	Stiff, thickened	Marked
	7	Mild	Normal	Normal	Thin, normal	Minimal–mild
	8	Mild	Normal	Normal	Thin, normal	Minimal–mild
Human 10-wk implant	9	Mild	Normal	Fibrin deposits	Thin, normal	Minimal–mild
	10	Moderate	Thickened	Inflammatory adventitia	Slightly thickened	Marked
	11	Mild	Normal	Normal	Thin, normal	Moderate
Human 6-mo implant	12	Moderate	Small nodule at STJ	Normal to slight thickening	Thin, pliable	Marked
	13	Mild	Normal	Normal	Thin, normal	Moderate
	14	Mild	Normal	Normal	Thin, normal	Minimal–mild

Porcine glutaraldehyde, *Papio* cryopreserved, and human cryopreserved valves are reference valves, whereas *Papio* 10-week, human 10-week, and human 6-month implant valves are bioengineered valves. ID, Identification; STJ, sinotubular junction. *Porcine glutaraldehyde valve inflammatory infiltrates were limited to the adventitia only.

TABLE E2. Observed differences between humans and baboons that may affect cardiovascular modeling

Observation	Human	Baboon	Consequence
Liver	Right sided	Transverse	Precludes retroflexed gastric TEE views or subxiphoid needle approaches.
Cardiac orientation	Normal = levocardia	Normal = levocardia	Base of apex alignment and orientation within the chest are similar to humans and not transversely situated as in 4-legged food stock animals.
Right ventricle wall thickness	“Normal”	Thinner	Less myocardial reserve; poorly tolerates distention, infundibular incisions, or acutely increased afterloads; may be useful for Fontan modeling.
Left ventricle muscle mass	“Normal”	Thicker with essentially concentric hypertrophy compared with human	Diastolic stiffness. Narrow left ventricular outflow tract; optimized cardioplegia strategies required.
Right coronary ostia	Typically smaller than left main	Miniscule	Smallest coronary perfusion cannulae (1 mm) cannot cannulate.
Splanchnic venous reserve	Limited	Large blood volume for autotransfusion via autonomic reflexes	Careful weaning from cardiopulmonary bypass with surgeon and TEE chamber volume monitoring to avoid acute cardiac distention. Avoid “light” anesthesia. After CPB, transfuse pump blood residual slowly.
Arterial wall thickness	Large lumen to wall thickness ratio	Very thick and stiff and hyper-vasoconstrictive	Peripheral arterial cannulation precluded; not likely a good species for transvascular catheter methods; enhanced fight/flight responses; older baboons develop hypertension and presbycardia.
Body mass	Evolves with age from upper body to lower body dominant	Upper body dominant	BSA calculations should be based on formulae that assume less contribution from lower body mass (eg, Haycock formula) when normalizing measurement to BSA (eg, indexing EOA of valves).
Demeanor	Age-dependent calm	Aggressive potentially bellicose	All interventions under sedation/anesthesia; perioperative management and husbandry by experts with proper caging, socialization procedures, management techniques, and so forth are required.

Regional Primate Centers are supported by National Institutes of Health Comparative Medicine Programs (formally in the National Center for Research Resources) and tasked with optimizing breeding, experimental modeling, availability, and medical knowledge for many Old and New World species for use in biomedical research; we found it preferable to travel as a team and operate onsite at the Texas Biomedical Research Institute, where subhuman primate veterinary medicine and husbandry are optimized. However, this adds to the additional limitation of expense. *TEE*, Transesophageal echocardiography; *CPB*, cardiopulmonary bypass; *BSA*, body surface area; *EOA*, effective orifice area.