

Decellularization reduces the immune response to aortic valve allografts in the rat

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Objectives: Cryopreserved valve allografts used in congenital cardiac surgery are associated with a significant cellular and humoral immune response. This might be reduced by removal of antigenic cellular elements (decellularization). The aim of this study was to determine the immunologic effect of decellularization in a rat allograft valve model.

Methods: Brown Norway and Lewis rat aortic valves were decellularized with a series of hypotonic and hypertonic buffers, protease inhibitors, gentle detergents (Triton X-100), and phosphate-buffered saline. Valves were implanted into Lewis rats in syngeneic and allogeneic combinations. Cellular (CD3 and CD8) infiltrates were assessed with morphometric analysis, and the humoral response was assessed with flow cytometry.

Results: Morphometric analysis identified a significant reduction in CD3⁺ cell infiltrates (cells per square millimeter of leaflet tissue) in decellularized allografts compared with that seen in nondecellularized allografts at 1 (79 ± 29 vs 3310 ± 223 , $P < .001$), 2 (26 ± 11 vs 109 ± 20 , $P = .004$), and 4 weeks (283 ± 122 vs 984 ± 145 , $P < .001$). Anti-CD8 staining confirmed the majority of infiltrates were cytotoxic T cells. Flow cytometric mean channel fluorescence intensity identified a negative shift (abrogated antibody formation) for decellularized allografts compared with nondecellularized allografts at 2 (19 ± 1 vs 27 ± 3 , $P = .033$), 4 (35 ± 2 vs 133 ± 29 , $P = .001$), and 16 weeks (28 ± 2 vs 166 ± 54 , $P = .017$).

Conclusions: Decellularization significantly reduces the cellular and humoral immune response to allograft tissue. This could prolong the durability of valve allografts and might prevent immunologic sensitization of allograft recipients.

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Allograft tissue is used extensively in congenital cardiac surgery as valved conduits and patches to replace or reconstruct diminutive and malformed structures. Although the cryopreserved allograft valve has acceptable results in adults, with freedom from reoperation approaching 69% at 15 years,¹ failure is much more rapid in children. Freedom from reoperation has been reported to be as low as 69% at 48 months in children less than 2 years of age.² It was previously believed that this tissue was immunoprivileged. However, increasing evidence from laboratory^{3,4} and clinical^{5,6} investigations has clearly demonstrated that this tissue is immunogenic and stimulates an intense cell-mediated immune response. This immune response has been implicated in the failure of these tissues. Equally important is the more recent observation by our group^{6a} and others⁷ that these tissues also stimulate intense humoral responses, with panel reactive antibodies (PRA) approaching 100%. Although the evidence linking this humoral response to allograft failure is less clear, there is concern that these preformed antibodies might complicate future cardiac transplantation should it be required.⁸ An increased PRA level has been associated with early development of high-grade cellular rejection and

increased annual rejection frequency,^{9,10} increased graft vasculopathy,¹¹ and decreased survival.^{8,12,13}

It was previously believed that preserving cellular viability was essential for long-term valve durability.¹⁴ Consequently, methods have been developed to prepare allograft tissue with the intention of maintaining cellular viability. However, more recent investigations have correlated enhanced allograft viability with increased immunogenicity and increased rates of failure.¹⁵ There is increasing evidence that the source of the alloreactive immune response is the cellular elements of the allograft.¹⁶ These findings, in part, have prompted the recent interest in tissue engineering of valves, either creating valves from synthetic matrices or through decellularization techniques to remove the immunogenic cellular elements of the allograft tissue. It is hypothesized that creation of an acellular matrix will abrogate the alloreactive immune response, improving valve durability and possibly preventing antibody formation. The purpose of this study was to investigate the cellular and humoral immune response to decellularized allograft tissue in a rat allograft model.

Materials and Methods

Experimental Animals

Inbred male Brown Norway (RT1.An) and Lewis (RT1.Al) rats (weight, 175–250 g) were purchased from Charles River (Quebec) and housed in the institutional animal care facility with food and water ad libitum for 1 week before experimentation in accordance with the guidelines of the Canadian Council of Animal Care.¹⁷

Surgical Technique

Aortic valve allografts were implanted into recipient animals according to a well-established infrarenal implantation model.^{3,18} Briefly, this technique entails removing the aortic valve with approximately 5 mm of ascending aorta from a donor rat and transplanting it into the infrarenal abdominal aorta of the recipient rat by using end-to-end anastomoses (10-0 nylon, Sharpoint) during general anesthesia (isoflurane).

Grafts were implanted as either nondecellularized (fresh) or decellularized grafts in both syngeneic (Lewis to Lewis) or allogeneic (Brown Norway to Lewis) combinations ($n = 6/\text{time}/\text{group}$). Nondecellularized grafts were harvested from donor animals and implanted without delay into recipient animals; decellularized grafts were treated as described below before implantation.

Decellularization

Tissues were decellularized by using methods similar to those previously described by Courtman and colleagues.¹⁹ Aortic valves were harvested from donor animals, rinsed with saline solution, and stored in Tris buffer (pH 8.0, 50 mmol/L, on ice) for transport. The valves were stored in CMRL solution (90 mL, Gibco), fetal bovine serum (FBS; 10 mL, Sigma), and penicillin-streptomycin solution (penstrep; 0.5 mL, Sigma) for 24 hours at 4°C. Samples were placed in hypotonic Tris buffer (10 mmol/L, pH 8.0) containing phenylmethylsulfonyl fluoride (0.1 mmol/L) and ethyl-

enediamine tetraacetic acid (5 mmol/L) for 48 hours at 4°C. Next, samples were placed in 0.5% octylphenoxy polyethoxyethanol (Triton X-100, Sigma) in a hypertonic Tris-buffered solution (50 mmol/L, pH 8.0; phenylmethylsulfonyl fluoride, 0.1 mmol/L; ethylenediamine tetraacetic acid, 5 mmol/L; KCl, 1.5 mol/L) for 48 hours at 4°C. Samples were then rinsed with Sorensen's phosphate buffer (pH 7.3) and placed in Sorensen's buffer containing DNase (25 $\mu\text{g}/\text{mL}$), RNase (10 $\mu\text{g}/\text{mL}$), and MgCl_2 (10 mmol/L) for 5 hours at 37°C. Samples were then transferred to Tris buffer (50 mmol/L, pH 9.0; Triton X-100 0.5%) for 48 hours at 4°C. Finally, all samples were washed with phosphate-buffered saline at 4°C for 72 hours, changing the solution every 24 hours. All stages were performed with constant stirring. Valves were stored in Hanks balanced salt solution plus penstrep at 4°C until the time of implantation.

The efficacy of decellularization was confirmed with staining for overall morphology (hematoxylin and eosin), endothelial cells (anti-CD31; sc1506, Santa Cruz Biotechnology), and interstitial cells (anti-vimentin; MCA 862, Serotec) by the methods described below.

Immunohistochemistry

Tissue was harvested for histology at 1, 2, and 4 weeks. Samples were formalin fixed (10%), paraffin embedded, and serially sectioned (5 μm) for histologic and immunohistochemical examination, ensuring valve leaflets were visualized in all sections. Immunohistochemistry involved standard staining techniques with biotinylated secondary antibodies, a peroxidase avidin-biotin complex, and 3,3'-diaminobenzidine as the chromogen. Primary monoclonal antibodies for T cells (anti-CD3; sc1127, Santa Cruz Biotechnology) and cytotoxic T cells (anti-CD8; sc7970, Santa Cruz Biotechnology) were used.

Tissue Analysis

Samples were examined with a light microscope, and images were captured with a digital camera (QImaging). Morphometric analysis was performed to measure cell counts per area (Simple PCI, Compix). Leaflet cell density (number of labeled cells per square millimeter) was obtained from sampling of the entire leaflet area from representative tissue cross-sections from each animal. Cell counts were obtained from the average cell density of 3 representative areas sampled at the 12, 4, and 8 o'clock positions from 2 representative tissue cross-sections from each animal.

Flow Cytometric Cross-Match

Serum harvest. At the time of death at 2, 4, and 16 weeks, rats were exsanguinated, and blood was collected in 10-mL phlebotomy tubes ($n = 6/\text{group}/\text{time point}$). Blood was allowed to clot, and the tubes were centrifuged for 5 minutes at 1500 rpm. Serum was transferred to 5-mL polypropylene tubes and stored at -86°C until needed.

Cell isolation. Spleens were harvested from Brown Norway rats and mechanically dissociated between sterile slides. Splenocytes were isolated through repeated washing with saline, centrifugation (1500 rpm \times 5 minutes), and red blood cell lysis (pH 7.3). Cells were resuspended in dimethyl sulfoxide and FBS and frozen at -86°C until needed.

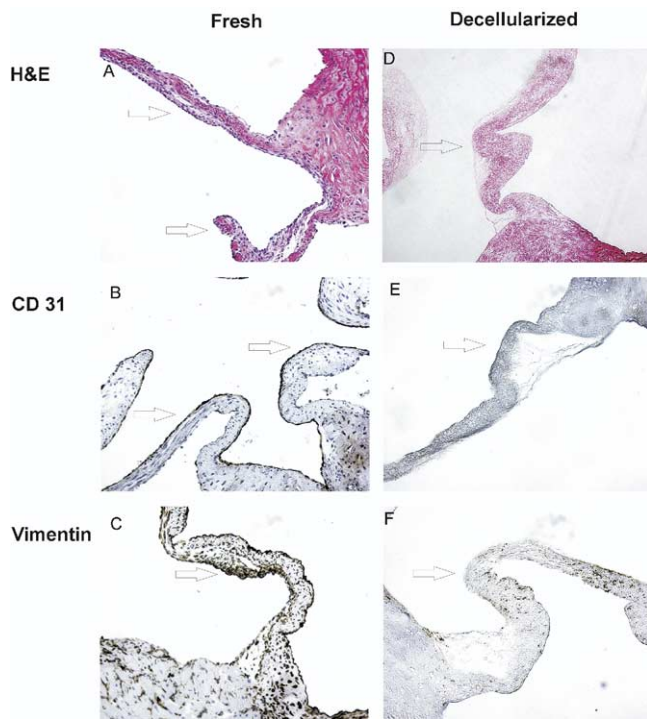


Figure 1. Comparison of decellularization techniques at 200 \times original magnification. **Arrows** indicate aortic valve leaflets. Hematoxylin and eosin (*H&E*) staining (overall morphology) and immunohistochemistry for endothelial cells (anti-CD31) and interstitial cells (anti-vimentin) are shown. **Fresh** (nondecellularized): note the normal laminar architecture and extensive cellularity with hematoxylin and eosin staining. Immunohistochemistry reveals the intact endothelial layer and extensive interstitial cells. **Decellularized**: preserved extracellular matrix, complete removal of the endothelial layer, and absence of intact cellular elements. Some residual interstitial cellular fragments are noted with anti-vimentin staining.

Cell preparation. Cells were thawed and reconstituted in saline. Cell number was counted in a hemocytometer with Trypan blue. Cells (10^6) were washed with saline, centrifuged (1500 rpm \times 5 minutes), combined with 100 μ L of serum from recipient rats (1:50 dilution in FACS buffer [1% FBS in phosphate-buffered saline]) and incubated at 37 $^{\circ}$ C for 30 minutes. Cells were washed twice with FACS buffer, centrifuged, and incubated with 100 μ L of secondary antibody (FITC-conjugated F[ab']₂ fragment goat anti-rat IgG, Fc γ fragment specific; Jackson ImmunoResearch Laboratories Inc, Cat. No. 112-096-008, 1:200 dilution in FACS buffer) at 4 $^{\circ}$ C for 30 minutes in the dark. Cells were washed with FACS buffer, centrifuged, resuspended in FACS buffer, and analyzed by means of flow cytometry (BD FACS-Calibur) for mean channel fluorescence intensity, which represents antibody binding to target cells. A rightward shift indicated the presence of serum antibodies.

Statistical Analysis

Continuous data are expressed as means \pm standard error of the mean. Analysis of variance testing was used to compare multiple

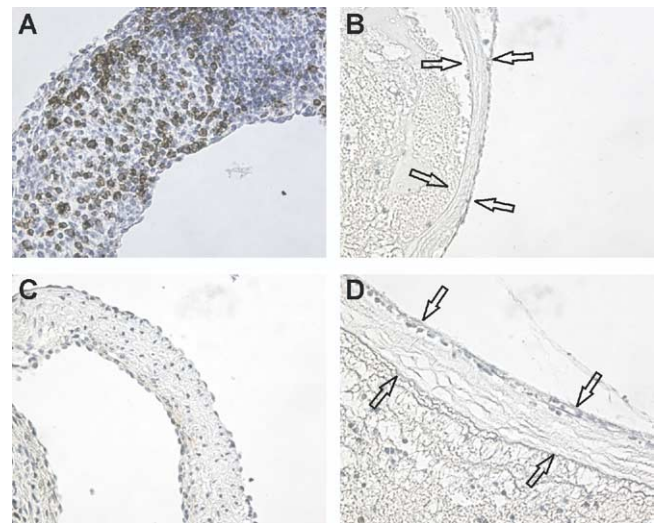


Figure 2. Representative immunohistochemistry for T cell infiltrates (CD3) 1 week after transplantation in allogeneic non-decellularized (A), allogeneic decellularized (B), syngeneic nondecellularized (C), and syngeneic decellularized (D) samples. Untreated allogeneic valve: early and intense T cell infiltrate and significant thickening of the valve leaflets. This was not present in the syngeneic model. Decellularized specimens: in both the allogeneic and syngeneic models, there is a dramatic attenuation of the immune response, as noted by normal leaflet thickness and the absence of T cells. **Arrows** indicate the location of valve leaflets adjacent to the thrombosis in the sinus of Valsalva. (Original magnification 400 \times .)

groups, with Scheffé post hoc analysis used to compare individual groups. The funding organizations assumed no role in the collection of data, its analysis and interpretation, or in the right to approve or disapprove publication of the finished manuscript.

Results

Samples of decellularized tissues are shown in [Figure 1](#). These images confirm the efficacy of the decellularization process in removing the majority of cellular elements. In the fresh (nondecellularized) tissue there was extensive cellularity, an intact endothelial layer, and a laminar architecture. In the decellularized tissue there was an absence of intact cells, complete removal of the endothelial layer, and relative preservation of the extracellular matrix. Some residual cellular fragments were detected by means of vimentin staining.

Representative sections of tissue explanted at 1 week are shown in [Figure 2](#). Most notable in these images are the intense T cell infiltrates and leaflet thickening in the allogeneic nondecellularized tissues compared with that seen in the decellularized tissues. This difference in T cell infiltrates was quantified by means of morphometric analysis and summarized in [Figure 3, A](#). Scheffé post hoc analysis confirmed a significant decrease in CD3⁺ T cell infiltrates for

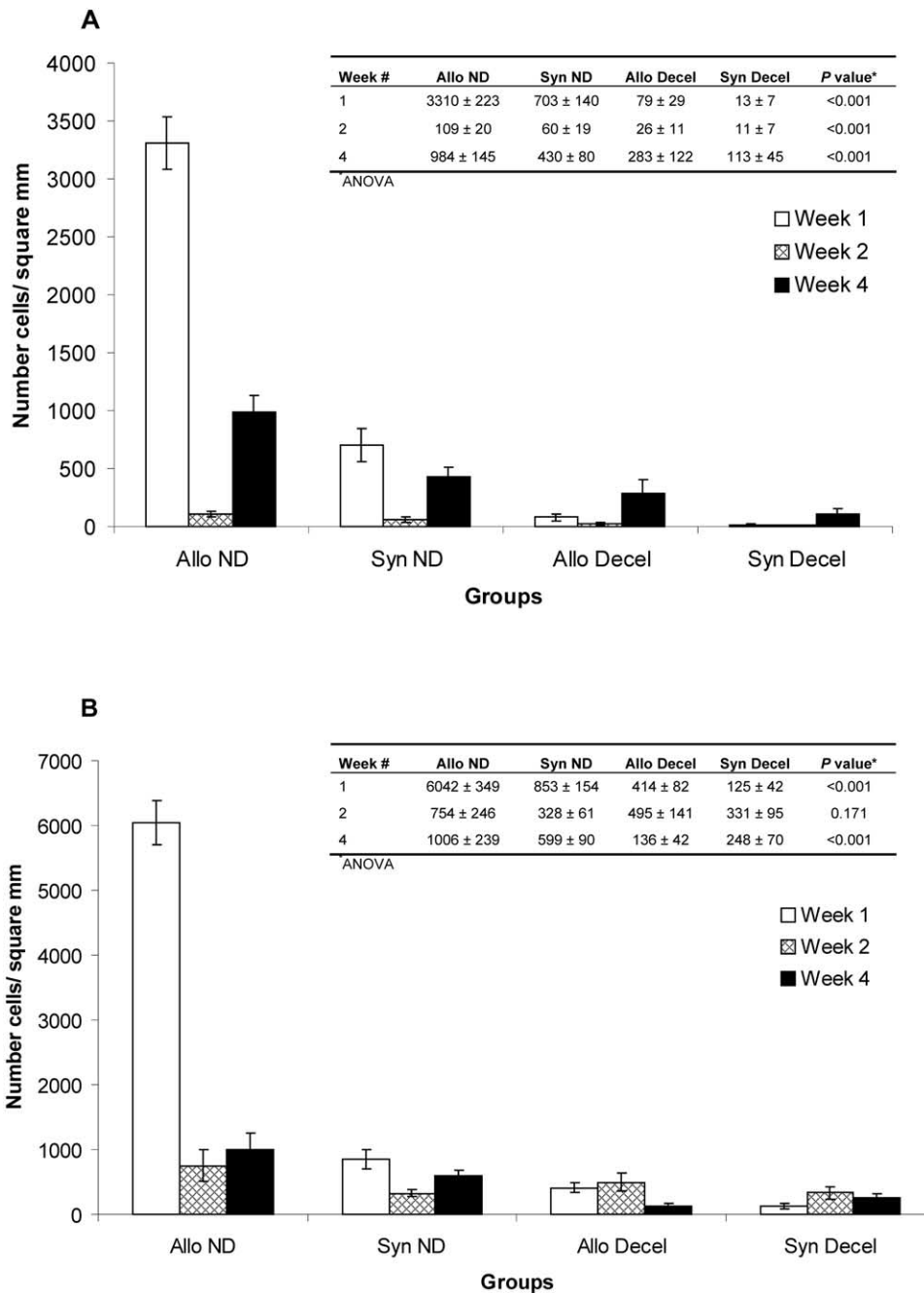


Figure 3. Decellularization reduces CD3⁺ (A) and CD8⁺ (B) mean cell counts in leaflets of aortic valve grafts at 1, 2, and 4 weeks after transplantation. *Allo ND*, Allogeneic nondecellularized; *Syn ND*, syngeneic nondecellularized; *Allo Decel*, allogeneic decellularized; *Syn Decel*, syngeneic decellularized.

allogeneic decellularized tissue compared with that for allogeneic nondecellularized tissue at 1 (79 ± 29 vs 3310 ± 223 , $P < .001$), 2 (26 ± 11 vs 109 ± 20 , $P = .004$), and 4 weeks (283 ± 122 vs 984 ± 145 , $P < .001$). There was a modest increase in CD3⁺ T cell infiltrates at 1 week in the syngeneic nondecellularized group, which differed slightly

from that seen in the allogeneic decellularized tissues (703 ± 140 vs 79 ± 29 , respectively; $P = .013$). Otherwise, the T cell infiltrates of the decellularized tissue were similar to those of the control nondecellularized and decellularized syngeneic grafts. Decellularized grafts, however, were very thrombogenic, as evidenced by thrombosis of the sinus of

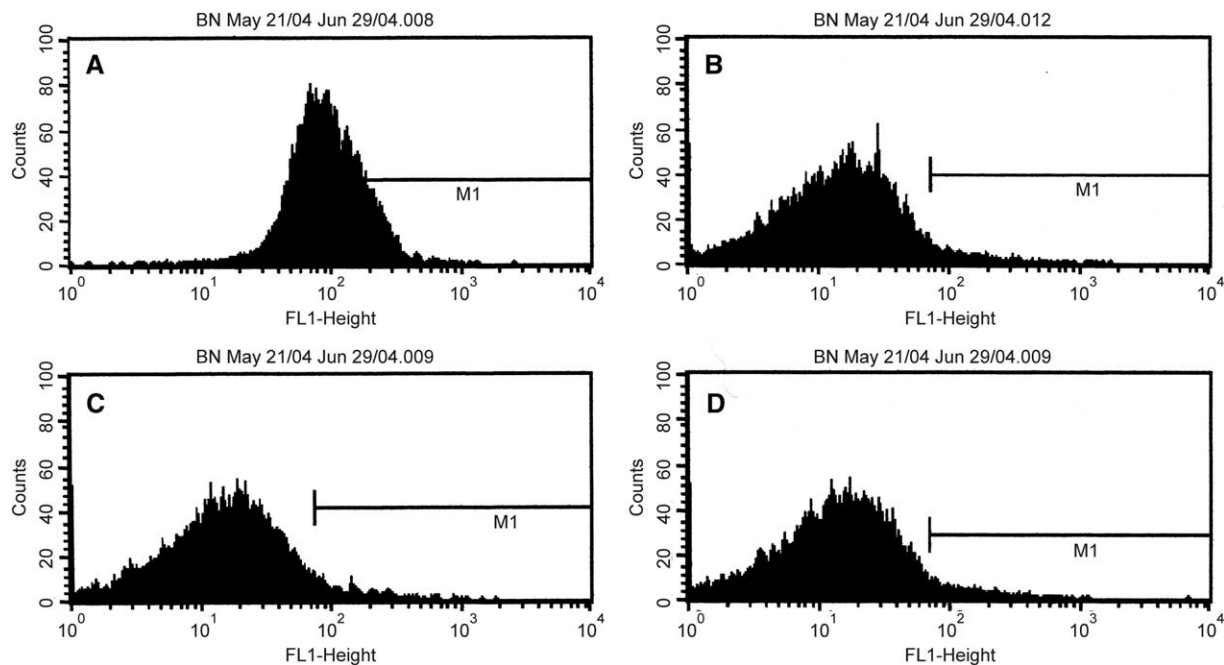


Figure 4. Representative histograms for flow cytometric analysis at 16 weeks for allogeneic nondecellularized (A), allogeneic decellularized (B), syngeneic nondecellularized (C), and syngeneic decellularized (D) samples. Allogeneic decellularized tissue was associated with a significant leftward shift indicative of reduced antibody production compared with the nondecellularized allogeneic tissue. Allogeneic nondecellularized tissue had curves that were nearly identical to those of syngeneic tissues.

Valsalva (Figure 2, B and D). At 16 weeks, the grafts were completely thrombosed, with complete loss of valve leaflets precluding meaningful analysis. Staining for cytotoxic CD8⁺ T cells (Figure 3, B) confirmed that the majority of the infiltrates were cytotoxic T cells. There was a similar decrease in CD8⁺ T cell infiltrates in the decellularized allograft tissue at all time points.

Flow cytometric cross-match identified a significant reduction in antibody production to levels that were similar to those of syngeneic nondecellularized grafts. Representative histograms at 16 weeks are provided in Figure 4. The allogeneic decellularized grafts were characterized by a leftward shift of the histogram compared with that seen in allogeneic nondecellularized grafts, indicating the failure to generate antibodies to decellularized allogeneic grafts. Similar findings were noted at 2 and 4 weeks, with the former being less pronounced. Flow cytometric mean channel fluorescence intensity was used to quantify these results, and values are summarized in Figure 5. Scheffé post hoc analysis confirmed the difference between decellularized allografts and nondecellularized allografts at 2 (19 ± 1 vs 27 ± 3 , $P = .033$), 4 (35 ± 2 vs 133 ± 29 , $P = .001$), and 16 weeks (28 ± 2 vs 166 ± 54 , $P = .017$).

Discussion

Allograft tissue is used extensively in cardiac surgery. In adults allograft valves are used for reconstruction of the right ventricular outflow tract in the pulmonary autograft (Ross) procedure, and they provide an important option for reconstruction of the aortic root, especially in cases of endocarditis. Allograft tissue plays an even more important role in congenital cardiac surgery, in which it is used both as valved conduits and as patches to reconstruct congenital abnormalities. Despite the advantages of absence of thrombogenicity, resistance to infection, and excellent hemodynamics, allograft valves fail with a predictable time course, which is particularly rapid in young children.^{1,2} Allograft tissues contain living cells, at least initially.⁵ Thus, it is not surprising that studies have demonstrated that these tissues stimulate a profound cell-mediated immune response with diffuse T cell infiltrates within 1 week of implantation in an animal model.^{3,4} The progressive failure of the allograft valve has been attributed to this alloreactive immune response. More recently, evidence has been accumulating that allograft tissue used in congenital cardiac surgery also stimulates a profound humoral response.^{6a,7} The role of this humoral response in allograft failure is less clear. However, more concerning is the effect that the presence of preformed

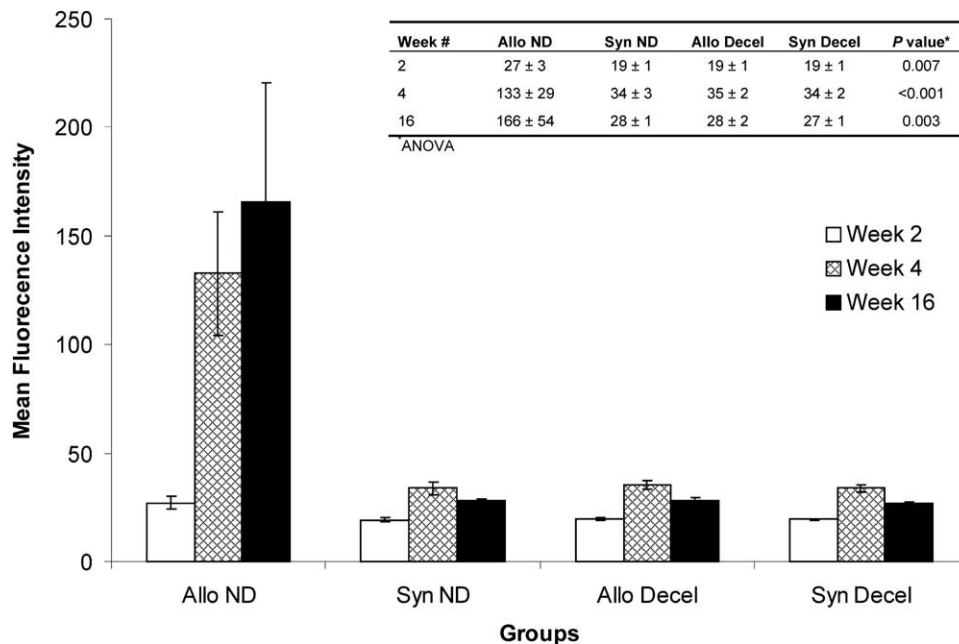


Figure 5. Decellularization reduces the humoral response to decellularized allograft valves, as identified by means of flow cytometric analysis at 2, 4, and 16 weeks. *Allo ND*, Allogeneic nondecellularized; *Syn ND*, syngeneic nondecellularized; *Allo Decel*, allogeneic decellularized; *Syn Decel*, syngeneic decellularized.

anti-HLA antibodies will have on future cardiac transplantation,⁸ a possibility in many children with congenital heart disease undergoing palliative procedures, such as the Norwood operation for hypoplastic left heart syndrome.

There are 2 options to attenuate this alloreactive response: alter the host (eg, immunosuppression) or alter the valve. Although shown to be beneficial in animal models, currently available immunosuppression is not acceptable for many valve candidates, especially young children, because of toxicity. Altering the valve to reduce its immunogenicity is another approach. As previously mentioned, it is believed that the cellular elements are the antigenic stimulus for the alloreactive immune response, and thus decellularization has been proposed to reduce the antigenicity of these tissues. Numerous protocols have been proposed to decellularize tissues.¹⁹⁻²² A similar number of studies have been performed to assess the effectiveness of removing cellular elements and to assess the biomechanical properties of decellularized tissues. However, there is a relative paucity of detailed studies investigating the immunologic consequences of decellularization of these tissues. Thus, the current study used a modification of a previously described decellularization technique¹⁹ and a well-established allograft heart valve implantation model^{3,18} to investigate the cellular and humoral immune response to decellularized allograft tissues.

In the present study we clearly demonstrated that decellularization of aortic valve allografts reduces the aforemen-

tioned cellular immune response. Similar to previous reports, allogeneic nondecellularized grafts were associated with significant CD3⁺ and CD8⁺ T cell infiltrates in aortic valve leaflets by 1 week after transplantation, rapidly decreasing in the following weeks.³ Decellularized grafts, on the other hand, were associated with significant reductions in T cell infiltrates, which were similar in density to those of syngeneic control grafts. The explanation for the anomalous increase in CD3⁺ T cell infiltrates in the nondecellularized grafts at 4 weeks is not entirely clear. This finding was not noted in the CD8⁺ T cells counts. Thus, it leads us to believe that the increased CD3⁺ T cell counts at 4 weeks might represent infiltration of CD3⁺ cells other than CD8⁺ T cells, perhaps CD4⁺ T cells. These findings are consistent with previous work by our group and others. Legare and colleagues³ demonstrated a nadir of infiltrates at 2 weeks in allogeneic aortic valve transplants. Similarly, Green and associates⁴ demonstrated a nadir of infiltrates in the intima and media of aortic valve allografts at 14 days, which rebounded somewhat at 28 days. Moreover, Green and associates demonstrated an increased proportion of CD4⁺ T cells and a decreased proportion of CD8⁺ T cells at 28 days compared with that at 7 and 14 days.

Unfortunately, the decellularization exposed the extracellular matrix, and thus the graft was highly thrombogenic. The model used was a nonworking model, with the valve leaflets remaining open throughout the cardiac cycle. In contrast to a working model, there is no backflow in the

sinuses of Valsalva during diastole, leading to stasis and thrombosis. Whether this thrombosis would occur in a working model is unknown. The effect of this thrombosis on cellular infiltrates is not entirely clear. However, because the allogeneic decellularized grafts had a paucity of T cell infiltrates similar to that seen in syngeneic nondecellularized control grafts, it could be cautiously assumed that the effect is minimal. Moreover, the humoral response to the allografts closely mimics the cellular data presented.

Flow cytometry demonstrated that decellularization similarly reduces the humoral response to allograft tissue. Decellularization was associated with a profound reduction of antibodies to the level of syngeneic tissues. These animal studies are consistent with recent work in human subjects using cryopreserved decellularized allograft tissue. Elkins and coworkers²³ recently reported that the humoral immune response to allograft heart valves (measured by PRA) was absent in 52 (91%) of 57 patients at 1 month and was absent in 43 (88%) of 49 at 3 months in allograft valves treated with the SynerGraft process for antigen reduction. Short-term valve function was reported as satisfactory. These findings are particularly relevant given the current research demonstrating that allograft tissue might be sensitizing infants undergoing congenital cardiac surgery,^{6a,7} infants who might eventually require cardiac transplantation. Although the tissues in the current study were valved conduits, it could be expected that decellularization of allograft patches, such as those used in the Norwood procedure, would have a similarly reduced humoral immune response.

The results of this study confirm that decellularization reduces the immunogenicity of allograft tissue. This finding might in turn be associated with improved allograft durability. Moreover, the finding of a reduced humoral response might prevent the sensitization that is currently seen with allograft tissue in children, complicating subsequent transplantation. However, the ultimate widespread use of such tissue will be limited by donor availability. Others have investigated the use of decellularized xenograft tissue, but this has met with failure,²⁴ presumably because of interspecies extracellular matrix immunogenicity.²⁵ Use of synthetic matrices seeded with autologous cells has shown promise,²⁶ but they might be limited by the need to harvest, expand, and seed autologous cells, a process that might take 14 days or greater. At present, it thus appears that decellularization of allograft tissue might be the most feasible of the 3 aforementioned options.

One of the major limitations to this study is use of a nonfunctional model that results in sinus of Valsalva thrombosis. One might consider using functional models to overcome such difficulties, including larger animal models of pulmonary artery implants or a recently described functional rat model with aortic insufficiency.²⁷ The current issue of thrombosis makes it difficult to speculate on the

effect the reduced cellular infiltrates will have on long-term durability. However, because the cellular infiltrates have been correlated with reduced valve durability, it could be expected that durability would be improved. In addition, longer-term studies are required to investigate the capacity for the matrix to repopulate with host endothelial and interstitial cells. Because it could be expected that many of these decellularized allografts would be stored with cryopreservation, the effect of cryopreservation on an acellular matrix requires careful assessment. However, in this experiment we wanted to limit the number of variables that would confound our interpretation of the effects of decellularization. In previous work with the same rodent model, our group had demonstrated that cryopreservation is associated with a lack of protection from allorecognition, increased valve leaflet injury, and increased cellular infiltrates, even in a syngeneic model.²⁸ Finally, work is required to ensure the integrity of the decellularized graft and the individual components of the extracellular matrix (eg, glycosaminoglycans), which might be essential for repopulation of the graft by host cells.

In summary, we have demonstrated that decellularization of aortic valve allografts is associated with a significant reduction in cellular and humoral immune responses to levels seen with nonimmunogenic syngeneic tissue. These findings suggest that the acellular matrix might provide a suitable environment for repopulation by host cells (eg, fibroblasts), which would provide the extracellular matrix with a regenerative capacity typical of native valves, thus improving allograft durability. Moreover, the reduced humoral response might prevent sensitization of recipients of allograft tissue. This would have enormous implications for the survival and quality of life of all valve recipients, especially children. Ongoing studies in a larger animal model of orthotopic implantation will provide information on the thrombogenicity, functional integrity, and capacity for repopulation by host cells.

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