INTRODUCTION

Solid organ transplantation (SOT) is the gold standard for treatment of end-stage organ failure. However, patient and allograft survival hinges on the life-long use of pharmacological immunosuppression associated with significant side effects, such as increased risk of infection, malignancies, and also drug toxicities. In reconstructive transplantation, the use of vascularized composite allografts (VCA) has been enthusiastically employed for reconstruction of complex tissue defects.

Methods: In this study, we introduce a novel murine model for en bloc chest wall, heart, and thymus transplantation and thereby the use of complex tissue allografts for reconstruction of both chest wall defects and also end-stage organ failure. Additionally, this model allows us to study the features of combined vascularized bone marrow (VBM), thymus, and heart transplantation on allograft survival and function. Heterotopic chest wall, thymus, and heart transplants were performed in untreated syngeneic and allogeneic combinations and in allogeneic combinations treated with costimulation blockade (CTLA4-Ig and MR-1).

Results: Indefinite (ie, 150 d, N = 3) graft survival was observed in syngeneic controls. In untreated recipients of allogeneic grafts, the skin component was rejected after 10 (±1) days, whereas rejection of the heart occurred after 13 (± 1) days (N = 3). Costimulation blockade treatment prolonged survival of the heart and chest wall component (130 d, N = 3) as well as the VBM niche as evidenced by donor-specific chimerism (average: 2.35±1.44%), whereas interestingly, the skin component was rejected after 13 (±1) days.

Conclusion: Thus, this novel microsurgical model of VCA combined with solid organ transplantation is technically feasible and results in split tolerance when treated with costimulatory blockade. (Plast Reconstr Surg Glob Open 2017;6:e1595; doi: 10.1097/GOX.0000000000001595; Published online 28 December 2017.)

Background: Congenital and acquired chest wall deformities represent a significant challenge to functional reconstruction and may impact feasibility of heart transplantation for patients with end-stage organ failure. In the recent past, the concept of replacing like-with-like tissue by using vascularized composite allografts (VCA) has been enthusiastically employed for reconstruction of complex tissue defects.

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bone marrow, which have been shown to prolong graft survival while decreasing the immunosuppression necessary for preventing chronic allograft rejection. In that regard, certain VCAs may present unique immunological features and advantages as they inherently contain donor-derived vascularized bone marrow (VBM), a continuous source of bone marrow progenitor cells. Combining SOT with VCA could therefore have a beneficial effect on tolerance induction and immunosuppression minimization in SOT. Furthermore, thymus-dependent mechanisms are also involved in the induction and maintenance of donor-specific tolerance and may be further exploited in the scenario of a thymus containing allograft.

Combining VCA with heart transplantation may help address challenges in heart-transplanted patients in need for another transplant because preceding corrective surgeries often result in complex regional anatomy. Specifically in the pediatric setting, chest wall deformities and scar tissue encroaching on mediastinal volume may limit the possible size of a donor heart. Gottlieb et al. have designed a concept for an en bloc chest wall, thymus, and heart VCA in the cadaveric setting. Although this shows the feasibility of clinical application of such a procedure, we herein present the first mouse model for a heterotopic en bloc chest wall, thymus, and heart transplantation. Using this murine model system, we investigate immunological features inherent to a bone marrow and thymus containing VCA combined with SOT.

MATERIALS AND METHODS

Animals
Male 8 to 10 week old Balb/C (H-2d), C57BL/6 (B6; H-2b) mice were purchased specific pathogen free from The Jackson Laboratories. Mice were housed at Johns Hopkins University, Baltimore, Md., in individually ventilated caging, 1–3 mice per cage, with autoclave sterilized caging, 1–3 mice per cage, with autoclave sterilized caging and on Teklad corncob bedding and received Teklad 2018 diet and bottled water ad libitum. All procedures were approved by the Johns Hopkins Animal Care and Use Committee (ACUC) under protocol #MO13M490.

Experimental Design
To assess feasibility of the surgical model, en bloc chest wall, thymus, and heart transplants were first performed in syngeneic mice. Subsequently, fully H2-mismatched transplants were performed from Balb/c (donor) into C57BL6 (recipient) mice. Graft survival was assessed daily by visual inspection of skin and palpation of the heart-beat. Full rejection was defined as Grade 3 skin rejection with epidermolysis and focal necrosis and cessation of a palpable heart beat. Analysis of donor-derived chimerism was used to evaluate the viability of the donor bone marrow and chest wall component and also to measure donor bone marrow cell engraftment.

Surgical Technique
The surgery was performed using a technique described by Oh et al. (Fig. 1). After transplantation, skin survival was monitored daily. Equally, heart grafts were monitored by daily palpation.

Imaging
To obtain representative information on both graft position and graft component viability, micro-CT scans (Perkin Elmer IVIS Spectrum/CT optical imaging device, PerkinElmer, Waltham, WA) and MR-imaging (Bruker 11.7T system, Brunker, Belgium) were performed.

Flow Cytometry Analysis of Mixed Chimerism and T Cell Receptor (TCR) Vβ TCR Families
Four-color flow cytometry was performed to distinguish donor from host mononuclear leukocytes on a FACS Calibur flow cytometer (BD Biosciences) and subsequently analyzed with FlowJo software. The percentage of donor cells circulating in host peripheral blood was calculated as described previously. Mouse peripheral blood was incubated after red blood cell (RBC) lysis with fluorescein isothiocyanate (FITC)-conjugated mAb directed against H-2d (SF1-1.1), PerCP-Cy5.5-conjugated antimouse CD3e (145-2C11), PE-conjugated B220 (RA3-6B2), and Alexa Flour 647-conjugated CD11b (M1/70) were used (BD Pharmingen, BioLegend and ebioscience) and non-Ag-specific FcγR-related binding blocker (2.4G2).

Histopathology
Euthanasia was performed by CO2 inhalation and then exsanguination and blood collection by cardiotomy. Mice were perfused with heparinized saline, followed by 10% neutral buffered formalin, via the left ventricle of the host heart. The grafts were dissected en bloc to permit assessment of all graft components. Fixed tissues were processed routinely to paraffin in graded alcohols, sectioned at 5 u, and stained with hematoxylin and eosin (H&E). Specimens were assessed by a veterinary pathologist.

Statistical Analysis
Results are expressed as means. Survival curves were generated with Prism Software package (GraphPad Software, GraphPad, San Diego, Calif.), and Kaplan–Meier analysis was used to determine significance of differences in graft survival between groups.

RESULTS

Graft Survival
Syngeneic B6 grafts demonstrated long-term survival that exceeded 150 days (Figs. 2A, C), attesting to the technical feasibility of the model. In untreated allogeneic recipients, the skin component was rejected after an average of 10±1 days, whereas rejection of the solid organ graft occurred after 13±1 days (Fig. 2A). Clinically, this rejec-
tion profile is similar to previous results of hind limb allograft rejection in 4 distinct rejection stages—beginning with skin loss. In allogeneic recipients under costimulation blockade treatment, skin rejection was observed after 13 ± 1 days. However, we observed long-term survival of all other allograft components, exceeding 130 days (Fig. 2B).

Pathology
In the syngeneic graft recipient after >130 days, the graft tissues were viable clinically and perfused successfully. The graft heart features were similar to the host heart, thymus morphology was similar to the host, and marrow cellularity and hematopoiesis were similar to the host (Fig. 3). In the allogeneic graft recipient at >130 days, the graft tissues were viable clinically and perfused successfully. The graft heart showed inflammation and adhesions to adjacent tissues, graft thymus was identified, and there was hematopoiesis in graft bone marrow (Fig. 3).

Chimerism
Using flow cytometry analysis, donor-specific multilineage mixed chimerism was observed in peripheral blood mononuclear cells of long-term survivors (average: 2.35±1.44%, range: 1.0–4.36%) (3G) indicating a viable donor bone marrow compartment after costimulation blockade–based treatment. Although levels of donor-type leukocytes diminished with time in some animals, their presence persisted in all animals for the duration of the analysis.

DISCUSSION
Animal models have provided valuable insights into transplant immunology research. Multiple murine transplant models for either organ transplantation or VCA are available. In contrast, models are needed for an investigation of combined SOT and VCA. The major advantage of the en bloc chest wall, thymus, and heart transplant murine model is that it includes both types of transplant and also a thymic component that has the potential to support T-cell development. Heterotopic heart transplantation to the neck, using a nonsuture cuff technique, is a well-established procedure in mice. To study VBM transplantation, Santiago et al. developed a heterotopic sternal transplantation model in rats. In 2013, Bozkurt et al. extended the heterotopic sternal transplantation model to include thymus and the entire osteomyocutaneous portion of the chest wall. Of note, Bozkurt et al. did not include a solid organ component into their allograft. In line with our results, Bozkurt et al. concluded that donor-derived thymus, transplanted with donor-derived bone marrow, is beneficial to induction and maintenance of chimerism in a heterotopic sternal transplant setting. Similarly, a superior outcome of heart allografts was shown in a swine model when the thymus was cotransplanted. The long-
term viability of hematopoietic allograft components and thus continuous hematopoietic output could provide the basis for strategies with the potential to reduce maintenance immunosuppression needed to sustain allograft survival. To date, no in vivo studies have reported combined solid organ, thymus, and vascularized bone marrow transplantation. Despite being technically challenging, the model presented in this study has applications for in vivo studies of immunological properties of combined SOT and VCA.

Evident from the robust allograft rejection in untreated recipients in our study, the presence of thymus and VBM alone does not prolong survival. Interestingly, CoB treatment extended heart allograft survival beyond 130 days, whereas rejection of the skin component occurred after an average of 13 days (Fig. 2B). This is reminiscent of split tolerance, the acceptance of one donor-derived tissue while rejecting another one from the same donor.20 One explanation for the failure of CoB to induce acceptance of the skin component may be the tissue’s higher immunogenicity when compared with musculoskeletal tissues—a result of the skin’s unique immunological defense functions. As the first barrier to pathogen invasion, the skin has a large population of Langerhans’ cells and other dendritic cells that allow for efficient antigen presentation and T-cell costimulation.21 Interestingly, the application of CoB in a comparable model of hind limb transplantation promotes long-term survival.

Fig. 2. Kaplan–Meier survival curve. Syngeneic (N = 5) versus allogeneic (N = 3) en bloc chest wall, thymus, and heart VCA (A). Although syngeneic grafts show long-term survival of both skin and heart components clinically and histologically, rejection of fully H2-mismatched grafts appear early with skin component rejection at an average of 10 days and heart component at an average of 13 days after transplantation. Survival of en bloc chest wall, thymus, and heart allograft components using costimulation blockade (N = 3). Skin component rejection occurs as early as 9 days after transplantation (average: 10 ± 1 d), whereas survival of the heart component and other tissue components is prolonged with costimulation blockade (B). Micro-computed tomography (CT) image of the chest wall recipient from a lateral–dorsal perspective showing graft inset location at the lateral aspect of the right neck ([C], left). Overlay of micro-CT and magnetic resonance imaging (MRI) showing both contours of the chest wall and the heart as a VCA ([C], right).
of all allograft components equally\(^2\) with only minor mononuclear cell infiltration present at post operative day (POD) 50. Nonetheless, epidermal mononuclear cell infiltration had progressed to be more pronounced by POD 70, highlighting increased immunogenicity of the skin component (data not shown). Tung et al.\(^2\) observed an accelerated rejection of the skin component in a murine model of limb allotransplantation. Although rejection of the allograft skin occurred at an increased pace in our study, additional factors, such as size of the target organ\(^2\) and the presence of ischemia-reperfusion injury,\(^2\) also have an influence on the rejection dynamic. Compared with Tung et al., our graft was smaller in size, and the technical complexity of the surgery leads to a longer ischemia time. In contrast to our results, previous studies were able to induce prolonged survival of the skin allograft component using the same treatment protocol. However, the Emory group used a different pairing of donor and recipient mouse strain,\(^2\) where costimulation blockade was more efficacious. When the same group published another article more recently,\(^2\) using a Balb/c to C57BL/6 combination, the time of alloskin survival was closer to the results presented here. In this study, histopathologic and flow cytometry–based analysis of the bone marrow compartment of the chest wall showed high cellularity and trilineage cell differentiation with higher levels of donor leukocytes after POD 50 (Fig. 3). The percentage of mixed hematopoietic chimerism is comparable to our hind limb allografts where multilineage mixed chimerism (in T, B, and myeloid cells) ranged between 1% and 5% after CoB only (data not shown). We found no difference in the number of T regulatory cells present between the groups (data not shown). In future studies, testing of myeloid-derived suppressor cells in the periphery could provide additional valuable insight into the underlying immune mechanisms. To advance the field of transplantation, novel treatment modalities are needed that prolong allograft survival, while minimizing or even abandoning the need for immunosuppression. The induction of mixed chimerism, even if only...
transiently,\textsuperscript{38,39} has established immunosuppression-free donor-specific tolerance. Additionally, tolerance induction may also prevent chronic rejection, a major cause of graft loss in SOT.\textsuperscript{20} However, the extensive preconditioning regimens necessary for bone marrow transplantation in combination with solid organ\textsuperscript{30} or VCA transplantation\textsuperscript{31,32} represent a major disadvantage for this approach. The potentially advantageous feature of VCAs containing VBM could overcome this limitation. As shown by our results, the unaltered bone marrow microenvironment of VBM may provide continuous hematopoietic output, rendering VBM superior to conventional cellular bone marrow transplantation for tolerance induction and immunomodulation.\textsuperscript{33-35} The presence of thymic tissue in addition to VBM could facilitate chimerism induction and maintenance. However, tolerance induction was not achieved in our model.

Nonetheless, these immunological findings may be relevant to the field of pediatric heart transplantation, as increasing long-term survival of a heart transplant is of utmost importance for this patient population because of the greater expected longevity and limited donor organ availability.\textsuperscript{56} Current immunosuppressive protocols seem inadequate to prevent chronic rejection and delayed appearance of cardiac allograft vasculopathy (CAV) in heart transplantation. In a recent report, the Pediatric Heart Transplant Study concluded that 48% of recipients will experience rejection during the first 5 years.\textsuperscript{37} The incidence of CAV in pediatric patients 10 and 15 years after transplantation was found to be 25% and 54%, respectively.\textsuperscript{58} Both, chronic rejection and CAV are thus major factors necessitating retransplantation,\textsuperscript{10} which is associated with a significant increase in mortality compared with primary transplantation.\textsuperscript{59} A mismatch in size between donor and recipient heart is another frequently encountered problem in pediatric heart transplantation. Twenty-five percent of pediatric heart transplants are performed with hearts from an adult donor (>18 y), owing to the severe donor shortage.\textsuperscript{40} Although proposing the integration of chest wall, thymus, and heart as a VCA may not address the shortage of suitable organs, the application of a more advanced reconstructive protocol may benefit selected patients with a size mismatch or those with the need for extensive chest reconstruction after multiple redo sternotomies. Most importantly, however, using a VCA containing VBM and thymus may contribute to the overall goal of reducing the need for long-term maintenance immunosuppression. As such a large transplant involves the transfer of a considerable amount of donor cells, the question of graft-versus-host disease needs to be addressed. In this study, there was no evidence of graft versus host disease (GVHD) despite the large load of donor immune cells transferred. In accordance with our findings, long-term survivors of a composite “thymoheart” transplant did not develop any signs of GVHD under cyclosporine A treatment.\textsuperscript{3}

This proof-of-concept study has a number of limitations. First, the use of anti-CD154 as costimulatory blockade is not directly translatable into the clinical setting. However, the used regimen represents an established approach to establishment of long-term survival in various murine transplant models and allowed a comparison with previous studies and historic data from our own group on hind limb transplantation. Second, albeit we observed consistent results within our small group sizes, an increase in experimental animals will certainly be necessary for future mechanistic studies.

In conclusion, results obtained in this study show evidence of VBM and thymus promoting allograft survival of all components, except the skin, in a fully mismatched murine model of chest wall, thymus, and heart transplantation.
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