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DEVELOPMENT & PRODUCTION OF A 3.5mm HAV

3.5mm HAV AS mBTT SHUNT IN NHP MODEL

HAV mBTT Shunt
Systemic Anastomosis
Pulmonary Anastomosis
Implant (Day 0)

Day 10
Systemic Anastomosis
Pulmonary Anastomosis

Month 6
Systemic Anastomosis
Pulmonary Anastomosis

HAV mBTT Shunt

HAV REPOPULATION

Month 6
H&E

SSkrm

HAV
L

cSMA / vWF

HAV
L

5µm
Evaluation of Tissue Engineered Human Acellular Vessels as a Blalock-Taussig-Thomas Shunt in a Juvenile Primate Model

Kevin M. Nash, PhD¹, Brian A. Boe, MD², Sergio A. Carrillo, MD³, Andrew Harrison, AS, ARRT², Ryuma Iwaki, MD⁴, John Kelly, MD²,⁴, Robert D. Kirkton, PhD¹, Ramkumar Krishnamurthy, PhD⁵, Jeffrey H. Lawson, MD, PhD¹,⁶, Yuichi Matsuzaki, MD, PhD⁴, Heather L. Prichard, PhD¹, Kejal Shah, MD⁴, Toshiharu Shinoka, MD, PhD²,³,⁴, Christopher K. Breuer, MD⁴,⁷,⁸

Affiliations: ¹Humacyte, Inc. Durham, North Carolina; ²The Heart Center, Nationwide Children's Hospital, Columbus, Ohio; ³Department of Cardiothoracic Surgery, Nationwide Children's Hospital, Columbus, Ohio; ⁴Center for Regenerative Medicine, The Abigail Wexner Research Institute at Nationwide Children's Hospital, Columbus, Ohio; ⁵Department of Radiology, Nationwide Children's Hospital, Columbus, Ohio; ⁶Department of Surgery, Duke University, Durham, North Carolina. ⁷Department of Surgery, The Ohio State University Wexner Medical Center, Columbus, Ohio; ⁸Department of Surgery, Nationwide Children's Hospital, Columbus, Ohio; Electronic address: christopher.breuer@nationwidechildrens.org

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Glossary of Abbreviations: αSMA (α-smooth muscle actin), CCHD (cyanotic congenital heart disease), ECM (extracellular matrix), ePTFE (expanded polytetrafluoroethylene), FLASH (fast low angle shot), HAV (human acellular vessel), H&E (hematoxylin & eosin), IH (intimal hyperplasia), IHC (immunohistochemistry), mBTT (modified Blalock-Taussig-Thomas), MRI (magnetic resonance imaging), NHP (non-human primate), PA (pulmonary artery), PGA (polyglycolic acid), STS (Society of Thoracic Surgeons), vWF (von Willebrand factor).

Central Message:

Preclinical evaluation of a 3.5mm Human Acellular Vessel (HAV) as a mBTTs in a non-human primate model demonstrated patency through 6 months and host cell repopulation of the engineered vessel.

Central Picture Legend. Implantation and angiography imaging of a 3.5 mm HAV as a mBTTs in a NHP for 6 months.

Perspective Statement:

Clinical use of ePTFE mBTTs as palliative treatment in CCHD has associated risks for thrombosis and infection. The 6mm HAV is in clinical trials for adult vascular indications and has demonstrated the capacity to repopulate with host cells. The successful implantation and evaluation of a 3.5mm HAV mBTTs in this nonhuman primate preclinical model suggests its potential for use in CCHD.

Abstract
Objective(s): Palliative treatment of Cyanotic congenital heart disease (CCHD) utilizes systemic-to-pulmonary conduits, often a modified Blalock-Taussig-Thomas shunt (mBTTs). Expanded polytetrafluoroethylene (ePTFE) mBTTs have associated risks for thrombosis and infection. The Human Acellular Vessel (HAV) (Humacyte Inc., Durham, NC) is a 6mm decellularized tissue engineered blood vessel currently in clinical trials in adults for vascular trauma, peripheral artery disease, and end-stage renal disease requiring hemodialysis. In addition to restoring blood flow, the engineered HAV demonstrates the capacity for host cellular remodeling into native-like vasculature. Here we report preclinical evaluation of a small diameter (3.5mm) HAV as a mBTTs in a non-human primate model.

Methods: 3.5mm HAVs were implanted as right subclavian artery to pulmonary artery mBTTs in non-immunosuppressed juvenile rhesus macaques (n=5). HAV patency, structure, and blood flow were assessed by post-operative imaging from 1 week to 6 months. Histology of HAVs and surrounding tissues was performed.

Results: Surgical procedures were well tolerated, with satisfactory anastomoses, showing feasibility of using the 3.5mm HAV as a mBTTs. All macaques had some immunological reactivity to the human extracellular matrix, as expected in this xenogeneic model. HAV mBTTs remained patent for up to 6 months in animals exhibiting mild immunoreactivity. Two macaques displaying more severe immunoreactivity to the human HAV material developed midgraft dilatation without bleeding or rupture. HAV repopulation by host cells expressing smooth muscle and endothelial markers was observed in all animals.
Conclusions: These findings may support use of 3.5mm HAVs as mBTTs in CCHD and potentially other pediatric vascular indications.

Keywords: Congenital Heart Disease; Tissue Engineered Vascular Grafts; Blalock-Taussig-Thomas Shunt
Introduction

Cyanotic congenital heart disease (CCHD) results in decreased blood oxygenation, causing cyanosis in neonates. CCHD is associated with a high risk of developmental delay, arrhythmia, heart failure, cardiac arrest, and stroke. Several meta-analyses estimate a CCHD prevalence of ~1.5 per 1,000 live births (5,859 affected children per year in the US).\(^1\)\(^-\)\(^3\) Palliative surgical intervention is often required to alleviate symptoms of CCHD before proceeding to definitive repair of structural cardiac and vascular defects.

The first surgical treatment for CCHD was described by Drs. Alfred Blalock and Helen Taussig\(^4\) following pioneering preclinical work by Vivien Thomas.\(^5\) The modified Blalock-Taussig-Thomas shunt (mBTTs) utilizes a synthetic vascular conduit, typically 3 – 4 mm diameter expanded polytetrafluoroethylene (ePTFE), anastomosed from the subclavian or innominate artery to the pulmonary artery as first described by Klinner et al.\(^6\) and optimized by de Leval et al.\(^7\) This treatment redirects blood from the systemic to the pulmonary circulation, thereby increasing overall blood oxygenation. The mBTTs provides palliation in infants for 3 – 6 months, when they typically undergo definitive cardiac repair or staged single ventricle palliation. Occlusion is the leading mBTTs failure mechanism and elevates risk of mortality, with up to 21% of shunts more than 50% occluded at time of elective takedown.\(^8\) Shunt infection is also associated with longer hospital stays and greater in-hospital mortality in shunt recipients.\(^9\) The Human Acellular Vessel (HAV) (Humacyte Inc., Durham, NC) is a decellularized tubular, tissue-engineered blood vessel consisting of human extracellular matrix (ECM) proteins. The HAV is created by culturing human vascular cells within a biodegradable scaffold under carefully controlled biochemical and biomechanical conditions. The cultured vascular tissue is
then decellularized to yield a mechanically robust and clinically non-immunogenic conduit (Fig 1). The 6 mm HAV has been evaluated in eight Phase 2 and Phase 3 clinical trials, and has been implanted in over 500 patients with over 1,000 patient-years of clinical exposure.

To generate smaller engineered blood vessels that may be suitable for pediatric cardiac surgery, the HAV manufacturing platform was modified to produce small diameter (3.5 mm) HAVs. The 3.5 mm vessels are 23 cm in length and have mechanical characteristics and composition comparable to the 6 mm vessels currently in clinical development. Since the HAV material has shown to be more resistant to infection than ePTFE, the 3.5 mm HAV has potential as a mBTTs in CCHD. In this study, the 3.5 mm HAV was evaluated in a xenogeneic juvenile nonhuman primate (NHP) mBTTs model.

**Methods**

**3.5 mm HAV Production & Characterization**

Small diameter (3.5 mm) bioengineered HAVs were generated by modifying the previously described 6 mm x 42 cm HAV platform and production methods. In summary, banked primary human vascular cells isolated from a single cadaveric donor were expanded and then seeded onto rapidly degradable polyglycolic acid (PGA) tubular scaffolds having a 3.5 mm inner diameter. The seeded scaffolds were exposed to pulsatile cyclic distension within sterile bioreactors for 8 weeks of tissue culture. The cellular engineered human vessel is then rendered acellular through a decellularization process as previously described. HAVs were characterized prior to implantation by measuring suture retention strength, burst pressure, wall thickness and
Measurement of suture retention strength was performed in accordance with ISO 7198:2016 using an automated system.

**Animal Model and Surgical Implantation**

A juvenile Old World primate model was chosen to: a) provide size and phylogenetic similarity to infant humans; and b) attempt to minimize the potential for xenogeneic reactivity of the NHP to the human extracellular matrix of the HAV during long-term implantation. All procedures were approved by the Institutional Animal Care and Use Committee of Nationwide Children’s Hospital (Columbus, OH; protocol AR18-00149, 3/7/2019). Healthy male juvenile rhesus macaques (Macaca mulatta, n = 8, age 41 - 52 months) of Chinese origin were acquired from Orient BioResource (Alice, TX). Three NHPs were used for development of the surgical model prior to evaluation of the HAV mBTTs in n = 5 NHPs. All nonhuman primates (NHPs) received humane care in compliance with the NIH Guide for the Care and Use of Laboratory Animals.

All operations and angiography procedures were performed in sterile fashion under general anesthesia.

Non-immunosuppressed NHPs were implanted with 3.5 mm HAVs as right-sided mBTTs between the right subclavian, carotid, or innominate systemic artery (SA, proximal) and pulmonary artery (PA, distal) via an open thoracotomy using standard surgical techniques. Heparin (100 U/kg) and phenylephrine were administered prior to clamping the target arteries. Proximal and distal anastomoses were created in an end-to-side manner with a running 7-0 Prolene suture. Native blood flow through the PA was maintained (i.e. pulmonary flow was supplied from both the PA and the mBTTs after completion of the operation). Radiopaque markers were sutured adjacent to the mBTTs anastomoses to mark HAV placement for in vivo
imaging and post-mortem evaluation. Acute patency of the implanted mBTTs was confirmed post-operatively by doppler echocardiography. Acute, intra-operative thrombosis of the HAV mBTTs in animal NHP2 occurred near the PA anastomosis. Patency was restored by placement of a bare metal stent (Integrity Coronary Stent, Medtronic; 9 mm length, expanded to 3.5 mm) across this region, followed by completion of the procedure.

Pre- and post-operatively, NHPs were administered oral aspirin (3.5 mg/kg) daily throughout the study, and subcutaneous enoxaparin (10 mg) was administered for 30 days post-operatively. Throughout the study, NHPs were monitored by echocardiography and observed for clinical signs of congestive heart failure. Of the n = 8 total animals in this study, the initial n = 3 were used in the development of the surgical model and the subsequent n = 5 were survived for the planned 3- to 6-month evaluation.

Angiography

Fluoroscopic angiography of HAV mBTTs was performed 1-2 weeks post-operatively and prior to explant. The right femoral artery and femoral vein were accessed with separate angiographic catheters advanced to the aortic root and PA for systemic and pulmonary vascular imaging, respectively. HAV mBTTs and surrounding vasculature were visualized by iodinated contrast injections and captured by fluoroscopy (Infinix-I system, Canon Medical Systems, USA).

Rotational angiography was performed with simultaneous systemic and pulmonary injections while the C-arm rotated in a continuous movement around the region of interest. Measurements of graft and vessel diameters were performed using the Canon Infinix-I system. 3D reconstructions were generated using Vitrea software (Canon Medical Systems, USA).
Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) was performed at 1, 3, and 6 months as previously described. MR images were captured on a Prisma 3T MRI (Siemens Medical Systems, Erlangen, Germany). Animals were sedated with continuous propofol infusion, intubated, and mechanically ventilated. Primates were administered ferumoxytol (5 mL/kg, IV) as a contrast agent. Black blood fast spin echo MRI and contrast enhanced 3D MR angiography (MRA) were obtained for anatomic imaging with HAV diameter measured at proximal, medial, and distal regions. MR 2D and 4D flow sequences were obtained to assess flow velocities and bulk flow through the graft. (Suppl Fig 1).

Histology

Following euthanasia, a full necropsy was performed, and harvested tissues were fixed in 10% neutral buffered formalin. Representative sections of non-HAV tissues (heart, spleen, liver, kidney, brain, lung, lymph nodes, and thymus) were sent to StageBio (Mason, OH) for histopathology evaluation following standard operating procedures. The HAV mBTts were pressure-fixed, explanted en bloc, and then processed for paraffin embedding followed by histological sectioning (5 μm thickness) and staining. Hematoxylin & Eosin (H&E, StatLab reagents) and Picrosirius Red (ab150681, Abcam) staining were performed using standard techniques. Immunohistochemistry (IHC) with fluorescence microscopy was performed as previously described. Explanted tissue sections and tissue slides (American MasterTech) were immunostained for alpha smooth muscle actin (αSMA, Dako M0851), von Willebrand Factor (vWF, Abcam 179451), CD3 (Dako A0451), CD20 (Abcam ab9475), CD11b (Abcam 52478), and CD68 (R&D Systems MAB2040). Fluorescent secondary antibodies included anti-mouse
IgG Alexa Fluor 488 (Thermo Fisher A-11001) and anti-rabbit IgG Alexa Fluor 594 (Thermo Fisher A-11012). Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Thermo Fisher EN62248). Brightfield H&E images were taken using an Olympus BX41 microscope with an Olympus DP25 camera and cellSens software. Polarized light (Picrosirius Red) and fluorescence microscopy were performed using a Nikon TE2000U microscope with a Photometrics CoolSNAP HQ2 camera. Images were acquired and processed using μManager and Fiji software.

Results

Development and Characterization of 3.5 mm HAV

Generation of HAVs at different diameters and lengths has been previously reported. The manufacturing platform (Fig. 1) was minimally modified for the production of small diameter tissue engineered blood vessels, by reduction of silicone tube outer diameter and PGA mesh inner diameter from 6 mm to 3.5 mm. Bioreactors were designed to allow culture of 3.5 mm HAVs that were 23 or 42 cm long. The 3.5 mm HAVs produced were comparable to 6 mm HAVs by suture retention and had similar burst pressure strength. Characteristics of both HAVs were similar to those of native vessels (Fig 1.). Decellularization was confirmed by staining with H&E to confirm the absence of visible nuclei, in addition to assays which verified minimal concentrations of residual β-actin protein, MHC I protein, and an absence of DNA bands greater than 200bp by gel electrophoresis.

Evaluation of 3.5mm HAV in an NHP mBTTs Model
3.5 mm HAV mBTTs (2.5 cm – 4.0 cm in length) were implanted via a thoracotomy approach in healthy juvenile rhesus macaques (3 - 4 years, 5.1 – 6.3 kg) with the proximal anastomosis to the innominate, right subclavian, or right carotid artery; and the distal anastomosis to the right PA. Target PA and SA diameters in these juvenile rhesus macaques (3.4 – 6.2 mm and 2.3 – 3.0 mm) were similar to those of human infants (approx. 4.1 mm and 2.9 mm, respectively).23

Model development animals (n=3) were used to optimize the complicated surgical implantation procedure and post-operative care, and were followed up to 10 days post-operative. During the development of the model, the HAV-to-PA anastomosis was found to be challenging due to the thin wall of the juvenile macaque PA.24 A longitudinal arteriotomy was initially used for the PA anastomosis, similar to that used when anastomosing more rigid ePTFE mBTTs,23 but this caused flattening of the HAV distal anastomosis upon tightening of the sutures leading to intra-operative thrombosis. Subsequent implantations optimized the HAV-to-PA anastomotic approach by creating a circular arteriotomy matching the diameter of the HAV.

**Longitudinal Evaluation of HAV mBTTs**

After completion of the 3 model development implantations, we undertook 5 additional HAV implantations into the experimental animals (Table 1). All animals remained healthy throughout the study and there were no adverse events attributed to the implanted HAV. Implanted HAV mBTTs were evaluated by angiography and MRI through 3 - 6 months, similar to the median duration of mBTTs palliation in infants with CCHD.25 All HAV mBTTs were patent by angiography 1-2 weeks post-implant, and at all time points prior to termination (Fig 2). Midgraft dilatations were noted in 2 of the 5 experimental animals: NHP1 at 3 months and NHP3 at 6
months post-implant. The corresponding regions in these grafts were further investigated by histology, as discussed later.

By non-invasive MRI flow imaging (Fig 3), blood flow through the mBTTs systemic-to-pulmonary circuit was $911 \pm 167 \text{ mL/min (n = 3)}$ and $473 \pm 205 \text{ mL/min (n = 5)}$ at 1- and 3-months, respectively (Fig 3A, 3E). Clinically, blood flow through mBTTs in human infants ranges from $256 – 2400 \text{ mL \cdot min}^{-1} \cdot \text{m}^{-2}$ ($81.9 – 768 \text{ mL/min for 5.5 kg [0.32 m}^2 \text{ body surface area]}$), making these flow rates comparable to those observed clinically.\textsuperscript{26} The ascending aortic blood flow rate in the juvenile rhesus was approximately $1430 \text{ mL/min (n=1)}$. Luminal diameters of mBTTs at the proximal, midgraft, and distal regions (Fig 3B-D) measured by MRI correlated well with measurements by fluoroscopy at termination (Additional data in Supplemental Figure 3). Some stenosis (luminal diameter: $2.28 \pm 0.63 \text{ mm}$) was observed within all HAV mBTTs through 3 months (n = 5/5), and moderate stenosis ($1.40 \pm 0.42 \text{ mm}$) was observed in two of the three mBTTs evaluated at month 6. Narrowing in all mBTTs predominantly occurred near the PA anastomosis (Fig 3F).

**Explant Histology**

HAV mBTTs were explanted at month 3 (NHP1), month 4 (NHP2) and month 6 (NHP3-5) and evaluated by histology. Sections stained with H&E showed remodeling of the 3.5 mm diameter HAV with host cells similar to that observed in previous preclinical and clinical studies of the 6 mm diameter HAV.\textsuperscript{10,17} Infiltration of host cells was observed as early as 10 days post-implantation (Fig. 4A; model development NHP) and progressed to near complete recellularization of the HAV wall, as well as creation of surrounding neoadventitial tissue, in the 3, 4, and 6 month explant samples (Fig. 4B-D). In addition, stent placement at implant within
the mBTTs in NHP2 did not adversely impact host cell infiltration or remodeling of the HAV (Suppl Fig 2). Pannus ingrowth was noted in the distal regions of all grafts near the PA anastomosis, which correlated with graft stenosis.

Immunostaining revealed that most cells repopulating the wall of HAVs expressed αSMA, a vascular smooth muscle marker (Fig. 4E-H). Additionally, many cells lining the HAV lumen expressed vWF, an endothelial cell marker, as early as 3 months post-implantation (Fig 4 I-L) suggesting the formation of luminal endothelium. Picrosirius red stained tissue sections visualized under polarized light showed dense collagen I (orange-red) fibers with some collagen III (green-yellow) fibers throughout HAV mBTTs explants at all time points (Fig 4 M-P). Average wall thickness measurements of explanted HAVs by histology (964 ± 360 μm) are similar to the initial 3.5 mm HAV wall thickness (729 ± 144 μm) and did not appear to trend with implant duration.

HAV mBTTs explanted from these non-immunosuppressed rhesus macaques revealed varying levels of immune cell infiltration, as demonstrated by small clusters of T-cells (CD3+) and B-cells (CD20+) surrounding grafts from NHP2, NHP4, and NHP5 (Fig 5 A-I). In NHP1 and NHP3, there were substantial populations of T- and B-cells, monocytes (CD11b+) and macrophages (CD68+) that had infiltrated the walls of the two HAV mBTTs. These were the two animals that developed dilatation of the implants over time (Fig 5 P-U). The most concentrated areas of immune and inflammatory cells were found within the midgraft dilatations, suggesting a correlation between host immune reactivity and dilatation of the HAV.

This type of adaptive immune response to an implanted HAV, accompanied by dilatation, has not been observed in baboons, nor in any human explant samples analyzed to date. We speculate...
that the dilatation may have been induced by adaptive immunity with elaboration of proteases which degraded the structure of the HAV. Interestingly, evaluation of peripheral tissues from NHP1 and NHP3 also revealed evidence of organism-wide adaptive immunity, with reactive lymph nodes and observable immune response in the lung, spleen, and thymus (Suppl Fig 3). In contrast, examinations of peripheral tissues from NHP2, NHP4, and NHP5 were unremarkable, and did not show signs of systemic immune reaction. Since the only two instances of HAV dilatation were seen in animals that also generated a local and peripheral immune response, we hypothesize that the dilatation was caused by host reactivity, as opposed to primary mechanical weakness of the implanted 3.5 mm HAVs.

Discussion

HAVs, engineered from allogeneic smooth muscle cells and then decellularized, have been studied in clinical trials for >9 years. The 6 mm HAV has been evaluated in arteriovenous access for hemodialysis, in the treatment of vascular injury, and in peripheral artery disease. However, a smaller conduit is necessary to address clinical needs in pediatric cardiac and vascular surgery, in adult coronary artery surgery, and in distal revascularization of the limbs. The goal of this study was to evaluate function of a 3.5 mm HAV in a preclinical model of CCHD.

The HAV has shown resistance to infection, which is one of the leading causes of mBTTs failure. The HAV is also extensively repopulated and remodeled by host cells, which suggests that the HAV may have the potential to grow along with a growing pediatric patient. The potential for growth of implanted tissue engineered vascular grafts in pediatric patients has been previously shown, and such growth-capable materials could reduce or eliminate the need for repeated operations to revise synthetic conduits in pediatric heart patients.
To evaluate the 3.5 mm HAV as a mBTTs conduit, a non-immunosuppressed juvenile NHP surgical model was developed. Healthy animals were implanted with a 2.5 – 4.0 cm length of 3.5 mm HAV from the SA to PA and evaluated over 3 – 6 months. Native blood flow through the PA, which would be reduced in CCHD infants but was not in this healthy animal model, did not appear to compete with flow through the mBTTs. Rather, blood flow persisted through the HAV from the systemic to the pulmonary circulation throughout the experiments. Blood flow rate through the HAV mBTTs, as measured by MRI, was similar to that observed in humans. The blood flow rate decreased from 1 month to 3 months, which may have occurred due to narrowing of the HAV as observed in 3- and 6-month explant tissue. A conservative thrombosis prophylaxis approach was taken (post-op enoxaparin) following intra-operative occlusion of HAV mBTTs in model development NHPs; however, optimization of the distal anastomosis technique in the study animals may have played a larger role in the patency observed.

To date, only one prior study of mBTTs in NHPs has been published. In this study, 4 mm diameter ePTFE grafts were implanted as aorta-to-pulmonary mBTTs in 5 animals weighing between 3.6 and 9 kg. Three months after implantation, two of the five grafts occluded due to thrombosis, resulting in a 40% occlusion rate. The authors also noted that most grafts were narrowed by ~1 mm, primarily at the anastomotic regions with more buildup of tissue at the distal region than the proximal. These data are a useful comparator to the 3.5 mm HAV mBTTs data here and show comparable performance between the conduits despite a significant xenogeneic response to the HAV material in two NHPs.

In contrast to the prior reported outcomes with ePTFE in NHPs, all five of the implanted HAV mBTTs remained patent for 3 – 6 months. Distal stenosis ≥ 50% was not observed in any of the
HAV mBTTs at 3 months but was identified in two out of the three HAVs at 6 months. This outcome is similar to that observed clinically by Wells and colleagues, wherein 21% of mBTTs were occluded more than 50% at elective take-down.\(^8\) The 3.5 mm HAV therefore seems to perform comparably to historical clinical mBTTs data, despite evidence of an intermittent xenogeneic reaction to the HAV human extracellular matrix material in these non-immunosuppressed animals.

While rhesus macaques are useful models for xenotransplantation research,\(^{37}\) implantation of human-derived tissues into rhesus macaques is not well-studied. The HAV is decellularized to remove cellular antigens with the goal of making it non-immunogenic for human implantation. However, chronic implantation in a non-immunosuppressed, non-human model may elicit a xenogeneic response to the human extracellular matrix. Since the 6 mm HAV had previously been implanted successfully into baboons for 6 months,\(^{10}\) we hypothesized that the smaller but phylogenetically related rhesus macaque would tolerate the 3.5 mm HAV as a mBTT for a similar duration. However, histological evaluation showed that all mBTTs explants, even those having no dilatation, had regions of T- and B-cell infiltration which were not observed in baboon or human clinical HAV explants to date (Fig 5). Additionally, explants from the animals with dilated HAVs exhibited systemic evidence of immune reactivity, implying adaptive response to the implanted human matrix. Because the degree of observed systemic immune response was correlated with vessel dilatation, it is likely that the adaptive immune response preceded and precipitated the formation of pseudoaneurysms in NHP1 and NHP3.

Recellularization of mBTTs were observed as early as 10 days post-implant (Fig 4A, model development NHP), with \(\alpha\text{SMA}^+\) cells migrating into the HAV primarily from the abluminal
surface. Extensive re-population by αSMA+ cells and partial luminal coverage of vWF+ endothelial cells were noted in explants harvested at 3 – 6 months. This is similar to observations from clinical explants of HAVs, wherein repopulation by αSMA+ cells by 16 weeks precedes expression of the mature smooth muscle cell marker, calponin 1 (CNN1), and nearly complete endothelial coverage of the HAV lumen by 44 weeks.17 Thus, recellularization of the HAV in the NHP thoracic cavity seems to mimic what is observed in human implants in more peripheral implantation sites, though further studies are needed to support this finding.

Here we demonstrated the development of a small diameter HAV which was successfully evaluated in an NHP mBTTs surgical model and remained patent in non-immunosuppressed NHPs for up to 6 months (figure 6). Since the HAV is repopulated with host cells, an implanted HAV in a juvenile patient may be capable of growing with the patient, potentially reducing or eliminating the need for multiple surgical corrections as the patient ages. However, since these animal recipients had very little somatic growth during these studies, we were not able to assess growth of the HAV here. Further studies are needed to evaluate the HAV’s long-term growth potential within juvenile recipients.
References


Table 1. Outcomes of 3.5mm HAV mBTT Shunts in Juvenile NHPs

<table>
<thead>
<tr>
<th>Animal</th>
<th>Weight (kg)</th>
<th>HAV mBTTs Length (cm)</th>
<th>Implant Duration</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHP1</td>
<td>6.0</td>
<td>2.5</td>
<td>3 Months</td>
<td>Midgraft dilatation, HAV patent&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
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<td>NHP3</td>
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<td>3.0</td>
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<td>6 Months</td>
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<tr>
<td>NHP5</td>
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<td>3.0</td>
<td>6 Months</td>
<td>Planned takedown, HAV patent</td>
</tr>
</tbody>
</table>

<sup>a</sup> Early termination
Figure Legends

**Figure 1.** 3.5mm HAV development schematic and characteristics. HAVs are manufactured by seeding human vascular cells onto a polymer mesh scaffold within a bioreactor bag, culturing the cells to produce extracellular matrix as the polymer mesh degrades, and decellularizing the resulting tissue to remove cellular material. The resulting 3.5 mm and 6 mm inner diameter HAVs remain in a sterile bioreactor bag until opening in the operating room. Bioengineered HAVs have mechanical properties that are similar to human native blood vessels. Data for native human blood vessel characterization from Konig et al.\textsuperscript{10}, L’Heureux et al.\textsuperscript{11}, and Canham et al.\textsuperscript{12}

**Figure 2.** Surgical implantation and evaluation of 3.5mm HAV mBTT Shunts in juvenile Rhesus Macaques for 3-6 Months. Representative photographs of a 3.5mm HAV mBTT Shunt at implant (A) and at 6 months (D) in NHP4. The HAV mBTT shunt was evaluated at 1-2 Weeks (B, C) and 6 months (E, F) by fluoroscopy and 3D rotational angiography, respectively. The HAV mBTT shunt in NHP4 remained patent for 6 months and was found to be well incorporated into the host tissue at explant.

**Figure 3.** Longitudinal MRI and angiographic measurements of HAV mBTT shunts implanted in juvenile NHPs. (A) Representative 4D-Flow MR imaging of blood velocities through the systemic circulation, the HAV mBTT shunt, and into the pulmonary artery at 6 Months. (B-D) Sagittal slices at 1-, 3-, and 6-Months post-implant with inner diameters for proximal, midgraft, and distal regions. (E) Peak blood flow velocities within HAV mBTT shunts at months 1 (n=4)
and 3 (n=5). (F) Longitudinal individual measurements for proximal, midgraft, and distal HAV mBTT shunt regions. Dotted line indicates size of HAV at implant (3.5mm).

**Figure 4.** Histological evaluation of host remodeling of HAV mBTT Shunts in NHPs over 6 Months. HAV explants were stained by H&E (A-D), for αSMA (E-L) and vWF (E-L), and by picrosirius red (M-P) at day 10 (left column), month 3, month 4, and month 6. Infiltration of host αSMA+ cells was observed as early as 10 days post-implant, and endothelial cells expressing vWF on the lumen were observed as early as month 3.

**Figure 5.** Histological evaluation of host immune response. Midgraft sections from 3.5 mm HAV mBTT shunt explants stained by IHC for CD20 (B-cell), CD3 (T-cell), CD68 (macrophage), and CD11b (monocyte) markers. NHPs with midgraft dilatations (NHP1 and NHP3 [J-O]) exhibited markedly higher levels of host immune infiltration within the mBTT shunt wall than NHPs with no dilatations (NHP2, NHP4, NHP5 [A-I]). Host immune cells in explants from NHP1 and NHP3 were predominantly localized around the regions of dilatation.

**Figure 6.** Graphical Abstract.
Supplemental Video 1. Representative MRI 4D Flow Cine of an HAV mBTTs at 6 Months (NHP 4).

Supplemental Figure 1. Stented HAV mBTTs explant H&E (A) and trichrome (B) histology (NHP 2).

Supplemental Figure 2. Representative histopathology of immunoreactivity in peripheral tissues of implanted animal with mBTTs midgraft dilatation (NHP 1, 3 Months).

Supplemental Figure 3. Individual NHP longitudinal HAV mBTTs diameter by graft region (A-C). Representative MR imaging and cross-sectional mBTTs luminal diameter measurement (D-G).
<table>
<thead>
<tr>
<th>Test Material</th>
<th>Burst Pressure (mm Hg)</th>
<th>Suture Strength (g)</th>
<th>Wall Thickness (µm)</th>
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<tbody>
<tr>
<td>Humacyte 3.5 mm HAVs</td>
<td>4788 ± 432 (n=78)</td>
<td>213 ± 35 (n=233)</td>
<td>478 ± 87 (n=202)</td>
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<td>Humacyte 6 mm HAVs</td>
<td>3727 ± 392 (n=1,007)</td>
<td>251 ± 41 (n=1,010)</td>
<td>451 ± 84 (n=966)</td>
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<td>Human Saphenous Vein</td>
<td>1599 ± 877 (n=7)</td>
<td>196 ± 2 (n=7)</td>
<td>180 to 650 (n=11)</td>
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<tr>
<td>Human Internal Mammary Artery</td>
<td>3196 ± 1264 (n=16)</td>
<td>138 ± 50 (n=6)</td>
<td>180 to 430 (n=23)</td>
</tr>
</tbody>
</table>
DEVELOPMENT & PRODUCTION OF A 3.5mm HAV

3.5mm HAV AS mBTT SHUNT IN NHP MODEL

- HAV mBTT Shunt
- Systemic Anastomosis
- Pulmonary Anastomosis
- Implant (Day 0)
- Day 10
- Month 6
- HAV mBTT Shunt
- Systemic Anastomosis
- Pulmonary Anastomosis
- Explant (Month 6)

HAV REPOPULATION

- HAV
- H&E
- cSMA / vWF