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Deconstructing the Tissue Engineered Vascular Graft: Evaluating Scaffold Pre-Wetting, Conditioned Media Incubation, and Determining the Optimal Mononuclear Cell Source

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Abstract

Stenosis limits widespread use of tissue-engineered vascular grafts (TEVGs), and bone marrow mononuclear cell (BM-MNC) seeding attenuates this complication. Yet seeding is a multistep process, and the singular effects of each component are unknown. We investigated which components of the clinical seeding protocol confer graft patency and sought to identify the optimal MNC source. Scaffolds composed of polyglycolic acid and ϵ -caprolactone/ ν -lactic acid underwent conditioned media (CM) incubation ($n = 25$) and syngeneic BM-MNC ($n = 9$) or peripheral blood (PB)-MNC ($n = 20$) seeding. TEVGs were implanted for 2 weeks in the mouse IVC. CM incubation and PB-MNC seeding did not increase graft patency compared to control scaffolds prewet with PBS ($n = 10$), while BM-MNC seeding reduced stenosis by suppressing inflammation and smooth muscle cell, myofibroblast, and pericyte proliferation. IL-1 β , IL-6, and TNF α were elevated in the seeded BM-MNC supernatant. Further, BM-MNC seeding reduced platelet activation in a dose-dependent manner, possibly contributing to TEVG patency.

Graphical abstract

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Notes

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Keywords

tissue engineered vascular graft; biodegradable scaffold; stenosis; bone marrow; peripheral blood; mononuclear cell; seeding; IL-1 β ; TNF α ; IL-6; platelet; mouse model

I. INTRODUCTION

Surgical palliation of congenital heart disease often necessitates the use of Gore-Tex or Dacron vascular grafts, which are prone to infection, thrombosis, and stenosis.^{1–5} In addition, these conduits fail to grow with the child, often requiring serial operations to revise or upsize the graft, which each increase the risk of morbidity and mortality.⁶ The development of a tissue engineered vascular graft (TEVG) holds great promise for pediatric patients born with congenital cardiac anomalies.⁷ After implantation, the scaffold degrades, allowing the TEVG to remodel into a completely autologous neo-vessel that resembles native vasculature in form and function and possesses the capacity to grow with its host. We are currently in the midst of the first FDA-approved clinical trial evaluating the use of a biodegradable scaffold seeded with autologous bone marrow mononuclear cells (BM-MNCs) for use as a TEVG within the pediatric population. Initial experience suggests that while our graft possesses growth capacity and functional efficacy, rates of stenosis mimic currently utilized synthetic conduits.^{8,9} Therefore, current efforts are aimed at the rational design and validation of an improved TEVG.¹⁰

The clinical protocol for the preparation of the TEVG is outlined in Scheme 1 and has been previously described in detail.^{11,12} Briefly, a porous scaffold fabricated from a PGA felt sealed with a copolymer solution of ϵ -caprolactone and ι -lactic acid (PCL/LA) is situated on a perforated size-matched mandrel. The assembly is placed in a graduated cylinder into which phosphate buffered saline (PBS, 1 $\times$) is added until the scaffold is completely submerged. Negative pressure is induced via vacuum tubing, and all PBS is drawn through the scaffold. An enriched fraction of BM-MNCs obtained via density gradient centrifugation of autologous whole bone marrow aspirate is then added to the cylinder containing the prewet graft. The BM-MNC suspension is similarly drawn through the scaffold under a vacuum to complete seeding. Finally, the seeded graft is removed from the mandrel and placed in a container of autologous plasma collected after Ficoll separation (top layer) where it is incubated at 37 °C with 5% CO₂ until its delivery to the operating room and subsequent implantation (~2 h).

We developed a murine model of TEVG implantation,^{13,14} which is utilized to investigate the cellular and molecular mechanisms of neotissue formation and the natural history of graft evolution.¹⁵ Recent data from our lab demonstrated that the dose of seeded BM-MNCs is inversely related to the incidence of TEVG stenosis.¹⁶ In this study, we sought to further optimize our TEVG by evaluating the individual contribution of each component of the clinical seeding protocol to graft performance. In addition, a persistent clinical question revolves around substituting peripheral blood mononuclear cells (PB-MNCs) for graft seeding, as their harvest would pose less risk for pediatric patients and evidence supports their use in other cellular therapies. Thus, we also sought to compare the efficacy of PB-MNC versus BM-MNC seeding in preventing TEVG stenosis in this study.

II. EXPERIMENTAL SECTION

II.A. Materials and Methods

II.A.1. Animal Care and Ethics Statement—All animals received humane care in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (2010). The Institutional Animal Care and Use Committee of the Research Institute at Nationwide Children's Hospital approved and monitored the use and care of all mice described in this report. Syngeneic 8–12 week old female C57BL/6 (wild type) mice ($n = 83$) were purchased from Jackson Laboratories (Bar Harbor, ME) and were either graft recipients ($n = 64$) or peripheral blood and/or bone marrow donors ($n = 19$).

II.A.2. Graft Fabrication—TEVG scaffolds (3.0 mm length, 0.819 mm inner diameter) were fabricated from nonwoven polyglycolic acid (PGA) felt sealed with a 50:50 copolymer solution of ϵ -caprolactone and α -lactic acid (PCL/LA) as previously described.¹⁷ Scaffolds were manufactured by Gunze, Ltd., sterilized with ethylene oxide gas, and stored at -20°C until implantation.

II.A.3. Bone Marrow and Peripheral Blood Mononuclear Cell Isolation—Peripheral blood and bone marrow donor mice were administered an overdose cocktail of ketamine (300 mg/kg) and xylazine (30 mg/kg). Approximately 1.0–1.5 mL of peripheral blood was collected in a 3 mL syringe preloaded with 100–200 μL of citrate-dextrose solution (C3821, Sigma). Bone marrow was collected by disarticulation of the hind limbs, removal of the femoral and tibial heads, and repeated flushing with 5.0 mL of RPMI 1640 (Sigma) + 1% penicillin/streptomycin (P/S, Sigma) as previously described. The pooled bone marrow was filtered (100 μm cell strainer, Fisher) to remove bone spicules and macroaggregates. Peripheral blood and bone marrow aspirates were diluted to 10 mL with PBS (1 $\times$, Sigma), and then carefully layered atop Ficoll Histopaque solution (1083, Sigma) in a 1:1 (vol/vol) ratio. After density gradient centrifugation, the mononuclear cell layer (buffy coat) was collected, twice washed with PBS, and resuspended at a concentration of 2.0×10^8 cells/mL (1.0×10^6 cells/ $5 \mu\text{L}$) in RPMI-1640 + 1% P/S. Cell concentrations were identified via Trypan Blue exclusion using a Countess automated cell counter (Invitrogen) and were determined as the mean of two separate cell counts. Differential cell counts from smears of each suspension were performed by a pathologist (MB) to identify differences in MNC subpopulations.

II.A.4. Graft Preparation

II.A.4.1. PBS Control: Control scaffolds ($n = 10$) were prepared by applying 5 μL of PBS + 1% P/S to the scaffold lumen for 5 min. Excess PBS was aspirated prior to implantation. This condition was utilized to mimic the PBS prewetting component of the clinical protocol for graft seeding, which occurs in under 5 min directly prior to cell seeding.

II.A.4.2. Mononuclear Cell Seeding: Scaffolds were prewet with PBS as described above, then 5 μL (1.0×10^6 cells) of prepared mononuclear cell suspension was introduced into the scaffold lumen ($n = 9$ BM-MNC, $n = 20$ PB-MNC). Cells were incubated for 10 min, and then a 22 G needle was threaded through the seeded graft before immersion in 1 mL of RPMI 1640 + 1% P/S in a 24-well plate. Seeded grafts were incubated overnight at 37 °C with 5% CO₂ prior to implantation as previously described.^{13,18}

II.A.4.3. Conditioned Media Incubation: Scaffolds were seeded with 1.0×10^6 bone marrow mononuclear cells as described above. After incubation, the media from each were aspirated and filtered using a 20 μm syringe filter to remove any cellular debris. A 22 G needle was threaded through untreated scaffolds ($n = 25$), which were then incubated in the filtered conditioned media overnight at 37 °C with 5% CO₂ prior to implantation.

II.A.5. Graft Implantation—TEVG scaffolds were implanted as abdominal inferior vena cava (IVC) interposition grafts following previously described microsurgical techniques.¹⁴ This model of IVC grafting is used to mimic the high flow, low-pressure environment found in the Fontan circulation, as this operation would not be technically feasible in the mouse. Briefly, mice were administered a preanesthetic analgesic dose of ketoprofen (5 mg/kg) and anesthetized with a cocktail of ketamine (100 mg/kg) and xylazine (10 mg/kg). The abdomen was shaved, prepped, draped, and opened via a midline laparotomy. A self-retaining retractor was placed and the intestines eviscerated and wrapped in moist gauze. A 5.0 mm segment of the infra-renal inferior vena cava (IVC) was bluntly dissected, and vascular control was obtained. The IVC was cross-clamped and divided, and TEVG scaffolds were implanted using running 10–0 nylon sutures to complete the end-to-end anastomoses. Cross-clamps were removed, hemostasis obtained, and patency of the anastomoses evaluated. The intestines were returned to the abdomen, which was closed in 2 layers with 6–0 prolene suture.

II.A.6. Graft Explantation—Two weeks after implantation, graft recipients were administered an overdose cocktail of ketamine (300 mg/kg) and xylazine (30 mg/kg). The abdomen was opened by midline laparotomy and the graft was isolated. Pneumothorax was induced via sternotomy. The right atrium was incised, and the systemic circulation was perfused via left ventricle puncture with PBS followed by 10% neutral buffered formalin (NBF) using a 25 G needle and 10 mL syringe. Perfusion-fixed grafts were subsequently explanted and fixed overnight in 10% NBF at 4 °C before dehydration and paraffin embedding. Slides for each sample were prepared from 4- μm -thick serial sections.

II.A.7. Tissue Analysis

II.A.7.1. Histology and Immunohistochemistry: For histologic analysis, at least one slide from each graft explant was stained with hematoxylin and eosin (H&E) following standard techniques. Immunohistochemistry identified vascular smooth muscle cells, myofibroblasts, and pericytes as α -smooth muscle actin (α -SMA) positive and macrophages as F4/80 positive. Slides were deparaffinized, rehydrated, blocked for endogenous peroxidase activity (0.3% H₂O₂ in MeOH), and antigens were retrieved using the citrate buffer method (90 °C, pH 6.0). Slides were then blocked for nonspecific binding (Background Sniper, BioCare Medical) and incubated overnight at 4 °C with mouse antihuman α -SMA (1:500, Dako) and rat antimouse F4/80 (1:1000, AbD Serotec). Antibody binding was detected with biotinylated goat antimouse or goat antirat IgG (1:200–300, Vector) followed by incubation with streptavidin–horse radish peroxidase and chromogenic development with 3,3'-diaminobenzidine. Nuclei were counterstained with Gill's hematoxylin (Vector) and slides were dehydrated and coverslipped. Photomicrographs were captured on a Zeiss AxioObserver.Z1 inverted microscope with a Zeiss AxioCam 105 digital camera, at either 5× or 63× (oil immersion) magnifications.

II.A.7.2. Computer Aided Image Analysis: ImageJ (NIH, Bethesda, MD) was used to measure the TEVG lumen diameter from H&E photomicrographs. A patent graft was defined as a graft with a lumen diameter greater than 0.41 mm (50% the lumen diameter at implantation). Immunohistochemical stains were quantified by conversion of the photomicrograph to the hue, saturation, and lightness (HSL) color space followed by pixel specific thresholding to isolate positively stained cells. Reported area fractions correspond to the number of pixels satisfying the threshold requirements relative to the total number of pixels in the region of interest analyzed. Four high-powered field (HPF, 63×) images of one representative section from at least five animals in each group were analyzed for every stain.

II.A.8. In Vitro Experiments

II.A.8.1. Cytokine Secretion Assay: BM-MNC and PB-MNC seeded scaffolds ($n = 10$ /group) were incubated in 1 mL of RPMI 1640 + 1% P/S overnight at 37 °C with 5% CO₂. After incubation, 50 μ L aliquots of the MNC-conditioned media were used to quantify the cytokine concentrations secreted from each MNC population using a V-PLEX Plus Proinflammatory Panel (Meso Scale Discovery, Rockville, MD) following the manufacturer's recommended protocol.

II.A.8.2. Platelet Activation Assay

II.A.8.2.1

Platelet Isolation: Whole blood was harvested by cardiac puncture using a 25 G needle and 3 mL syringe preloaded with 100–200 μ L of citrate-dextrose solution (C3821, Sigma) as previously described.¹⁷ Throughout the platelet isolation process, pipetting was performed gently to minimize platelet activation. Blood was centrifuged (120g for 8 min) to separate red cells and leukocytes (pellet) from platelet rich plasma (PRP, supernatant). PRP was aspirated and centrifuged (120 g for 3 min) to further enrich the platelet fraction in the supernatant. Centrifugation (740g for 10 min) of the collected supernatant yielded a pellet

that was resuspended with 200 μL of PBS. Platelet concentration was quantified using an ABX Micros 60 hematology analyzer (Horiba) and determined as the mean of two separate measurements.

II.A.8.2.2

ATP Assay: To assess the effect of seeded MNCs on platelet activation, either PB-MNC or BM-MNC seeded grafts were prepared as described above using cell seeding doses of 0.1, 0.3, 1.0, and 3.0×10^6 cells/scaffold. Then, 5.0×10^5 platelets were added to wells of a black polystyrene 96-well assay microplate (Corning Costar), which contained either PB-MNC or BM-MNC seeded grafts. A total of 50 μL of PBS was added to each well in order to assess both thrombin-activated (0.1 U/mL) and resting conditions, and to keep grafts submerged in solution ($n = 4/\text{cell dose/MNC type}$). Plates were incubated at RT with gentle shaking for 1.0 h, and grafts were removed from the wells. The concentration of platelet-derived ATP in each well was determined by the addition of 50 μL of ChronoLume reagent (Chrono-Log Corp.) followed by luminescent detection using a LUMIstar Omega microplate luminometer (BMG Labtech) as previously described.¹⁹ Measured values were applied to a standard curve, and the concentration of platelet-derived ATP was determined.

II.A.9. Statistical Analysis—Numeric values are presented as mean $\pm$ standard error of the mean (SEM). Patency rates (dichotomous variables) were compared using a chi-square test. Lumen diameter, area fraction, and positive cell/HPF comparisons between the PBS, conditioned media, BM-MNC seeded, and PB-MNC seeded groups were performed via one-way ANOVA using Tukey's correction for multiple comparisons. Cytokine assay data were analyzed by performing multiple unpaired two-tailed t tests. The ATP assay was analyzed by two-way ANOVA followed by Tukey's correction for multiple comparisons. Results were considered statistically significant if $p < 0.05$. All data analyses were performed with GraphPad Prism 6 (GraphPad Software, Inc.).

III. RESULTS

III.A. Only BM-MNC Seeding Prevents TEVG Stenosis

The incidence of stenosis in BM-MNC seeded grafts was significantly lower than that of PBS, conditioned media, and PB-MNC seeded groups (Table 1). The incidence of stenosis was comparable between the PBS, conditioned media, and PB-MNC seeded groups (Table 1). Histomorphometric analysis supported these findings by revealing a significantly greater mean lumen diameter in the BM-MNC seeded group when compared to PBS, conditioned media, and PB-MNC seeded groups (Figure 1A; $p = 0.0291$, 0.0093 , and 0.0455 respectively); no significant difference in mean TEVG lumen diameter between the PBS, conditioned media, and PB-MNC seeded groups was found (Figure 1A). Semiquantitative immunohistochemistry for macrophages (F4/80⁺ cells, Figure 2A) and synthetic smooth muscle cells, myofibroblasts, and pericytes (αSMA^+ area fraction, Figure 3A) was performed to identify any differences in the progression of graft stenosis between the groups. Significantly more F4/80⁺ monocytes and macrophages were identified in sections from the PBS, conditioned media, and PB-MNC seeded groups when compared to the BM-MNC seeded group (Figure 2A, $p = 0.0001$, 0.0005 , and 0.0001 , respectively). In addition,

significantly more F4/80⁺ monocytes and macrophages were found in the PB-MNC seeded group when compared to the conditioned media group (Figure 2A, $p = 0.005$). No significant difference was identified when comparing the PBS and conditioned media or PBS and PB-MNC seeded groups. Following similar trends, α SMA⁺ area fraction was significantly lower in the BM-MNC seeded group when compared to the conditioned media and PB-MNC seeded groups (Figure 3A, $p = 0.005$ and 0.05 , respectively). Sections from the conditioned media group had a significantly greater α SMA⁺ area fraction when compared to the PBS group (Figure 3A, $p = 0.05$). No significant difference was found when comparing the conditioned media and PB-MNC seeded or PBS and BM-MNC seeded groups.

III.B. Hematology Analysis: BM-MNCs Have a Greater Monocyte:Lymphocyte Ratio than PB-MNCs

Differential cell counts of Jenner-Geimsa stained smears of BM-MNC and PB-MNC seeding suspensions identified differences in MNC subpopulations (images not shown). Totals of 189 BM-MNC and 100 PB-MNCs were counted from 8 to 10 HPF per group. The BM-MNC composition was 25% lymphocytes, 21% monocytes, 30% neutrophils, 6% eosinophils, 13% metamyelocytes, 4% promonocytes/blasts, and 6% erythroid precursors. The PB-MNC composition was 93% lymphocytes and 7% monocytes.

III.C. In Vitro Cytokine Analysis: Levels of Secreted IL-1 β , IL-6, and TNF α Are Greater from Seeded BM-MNC

The concentrations of 10 common immunomodulatory cytokines (IFN γ , IL-10, IL-12p70, IL-1 β , IL-2, IL-4, IL-5, IL-6, KC-GRO, and TNF α ; Figure 4A) in the cell culture media were compared after either BM-MNC or PB-MNC seeded graft incubation as described above. The concentrations of IL-1 β , IL-6, and TNF α from seeded BM-MNC were significantly greater than those from seeded PB-MNC ($p = 0.02640$, 0.00197 , and 0.00012 , respectively).

III.D. Paracrine Effect of Cell Seeding In Vitro: Seeded BM-MNCs Attenuate Platelet Activation

After seeding scaffolds with varying concentrations of BM-MNCs or PB-MNCs, their effect on thrombin-activated platelets was assessed *in vitro*. BM-MNC seeding reduced the concentration of measurable platelet-derived ATP in a dose-responsive manner, in stark distinction to PB-MNC seeded grafts where this effect was not observed (Figure 4B). Specifically, the concentration of platelet-derived ATP significantly decreased with increasing BM-MNC dose (0.1×10^6 vs 0.3×10^6 , $p = 0.005$; 0.3×10^6 vs 1.0×10^6 , $p = 0.0005$; 1.0×10^6 vs 3.0×10^6 , $p = 0.0001$), whereas the amount of platelet-derived ATP was unchanged with increasing PB-MNC dose. All wells with PB-MNC seeded grafts had significantly higher concentrations of platelet-derived ATP than those with BM-MNC seeded grafts at all cell doses ($p = 0.0001$) except for 0.1×10^6 cells.

IV. DISCUSSION

Congenital heart disease affects nearly 1% of all live births, and a significant portion of these patients require surgical intervention during their lifetime.¹ Many of these patients will

require a synthetic conduit or vascular patch during these procedures, something that places them at risk for future complications or need for reoperation.^{2,7,20} Tissue-engineering offers a potential solution to problems associated with infection or somatic overgrowth. The results of the initial clinical trial using TEVGs have identified that while these grafts can grow with the child, they are still subject to stenosis in up to 25% of patients.^{8,9} The work of our lab has focused on identifying both the mechanism behind stenosis and possible preventative measures.

Early work has shown that TEVG stenosis is caused by an overproliferation of smooth muscle cells, driven by excessive stimulation from infiltrating monocytes and macrophages.^{21–23} Cell seeding has been shown to reduce the rate of stenosis in both murine and ovine models.^{12,22,23} In fact, our lab recently demonstrated that this phenomenon is dose-responsive.¹¹ Currently, patients enrolled in the clinical trial undergo a harvest of 3–5 mL/kg of bone marrow, from which the mononuclear cell fraction is enriched and used for scaffold seeding. Our current understanding is that higher concentrations of seeded cells would further reduce the incidence of TEVG stenosis. However, increasing the amount of bone marrow harvested from this patient population may lead to new complications, including the potential for extended immunologic suppression. Current evidence suggests that it may take up to 19 days for the hematopoietic system to recover from larger volumes of bone marrow aspiration.²⁴ As critical as the immunologic suppression, there would be an increased risk of requiring transfusion after harvest due to either hemodynamic instability or anemia, especially in smaller patients.²⁵ While healthy donors undergoing larger volume aspiration may have only a small risk of serious complications,²⁶ the feasibility of this process may be limited in pediatric patients undergoing a major cardiac procedure. In addition, increasing the amount of marrow harvested would lead to increased anesthetic time. Thus, a research focus has been to determine the mechanism by which BM-MNCs prevent TEVG stenosis, aiming to optimize current techniques or identify potential alternatives or adjuncts to scaffold seeding.

Understanding the individual contribution of each component of the seeding protocol to graft performance is the first necessary step in defining the mechanism by which cell seeding prevents graft stenosis. In this report, we demonstrated that either scaffold prewetting with PBS or conditioned media incubation cannot individually prevent graft stenosis in our model. Scaffolds incubated in BM-MNC-conditioned media prior to implantation fared no better than PBS control grafts in terms of luminal narrowing and incidence of critical stenosis. Excessive numbers of F4/80⁺ monocyte and macrophage infiltration were identified in both the PBS and conditioned media groups, in contrast to the relatively low numbers of these inflammatory cells in the BM-MNC seeded group. Adverse remodeling indicative of intimal hyperplasia in the conditioned media group was further evidenced by significantly increased α SMA⁺ area fractions in graft sections when compared to the PBS control and the BM-MNC seeded group. These results indicate that utilization of unprocessed autologous BM-MNC-conditioned media as studied in this report as an alternative to BM-MNC seeding to prevent critical graft occlusion is not a viable strategy. It may be that further processing of the conditioned media is required, such as elution, enrichment, or alteration of a particular subset of critical factors, or that the hydrophobic scaffold polymers denatured proteins in solution and require modification prior to

incubation; however, in either scenario, the clinical utility of using conditioned media may be reduced.

After establishing that conditioned media incubation did not confer any resistance to graft stenosis in the absence of seeded mononuclear cells, we considered PB-MNCs as an alternative MNC source for cell seeding. Peripheral blood was identified as a promising and clinically relevant candidate due to its ready availability and its previous use in a variety of applications such as articular cartilage repair,²⁷ nerve regeneration,²⁸ as therapy for peripheral arterial disease^{29–32} and after myocardial infarction,³³ among others. PB-MNC-seeded TEVGs implanted for 2 weeks demonstrated significantly lower rates of patency than BM-MNC-seeded grafts, and the incidence of stenosis in this group was comparable to that of the PBS control and conditioned media groups. Monocyte and macrophage infiltration as well as smooth muscle cell/myofibroblast/pericyte area were both significantly elevated in the PB-MNC group, similar to the conditioned media incubated scaffolds and indicative of stenotic TEVGs reported in this model.^{17,21,34,35} These data refute the hypothesis that a Ficoll-enriched fraction of PB-MNCs are a viable alternative cell source for TEVG seeding in the mouse model described here. Heterogeneity in the mononuclear cell fraction from BM and PB has been well described; PB-MNCs are generally more mature than BM-MNC and PB-MNCs tend to contain greater frequencies of mature lymphocytes, whereas BM-MNCs contain greater numbers of CD34+ hematopoietic progenitor cells.^{30,36,37} We performed differential cell counts of MNC seeding suspensions to confirm these reports and found that the monocyte:lymphocyte ratio of seeded PB-MNCs was markedly lower than that of BM-MNC. As expected, the BM-MNC population contained progenitor cells such as metamyelocytes, promyelocytes, and myeloblasts, whereas these were not identified in the PB-MNC suspension. In light of this phenotypic heterogeneity, we sought to investigate any relevant functional differences between these two mononuclear cell populations in order to better understand how BM-MNC seeding prevents TEVG stenosis in the mouse.

BM-MNC seeding is thought to regulate recruitment of host monocytes and macrophages which promote migration and proliferation of vascular endothelial and smooth muscle cells from the adjacent vessel;¹⁸ however, the exact mechanism of seeded cell activity has yet to be completely elucidated. We have recently demonstrated that excessive activation of infiltrating monocytes and macrophages in unseeded scaffolds leads to TEVG stenosis.^{35,38} Therefore, we hypothesized that the difference in patency between BM-MNC and PB-MNC seeded grafts could be due to cytokines and chemokines secreted from seeded cells that regulate the host response to TEVG implantation. *In vitro* evaluation of 10 different cytokines from the graft incubation supernatant directly prior to implantation revealed that seeded BM-MNCs secrete greater quantities of IL-1 β , IL-6, and TNF α than PB-MNCs, with differences in TNF α showing the greatest significance. TNF α is traditionally considered a pro-inflammatory cytokine, and our recent work suggests that it induces classical activation of infiltrating monocytes, leading to stenosis over a two-week time course.³⁸ While our *in vitro* results from this study may seem to contradict the hypothesis that pro-inflammatory signaling leads to graft stenosis, it is important to note that seeded BM-MNCs disappear within a few days after scaffold implantation,^{18,39} indicating that the cytokines and chemokines secreted from seeded cells are only relevant in the first ~72 h after implantation. The early effects of these secreted factors in the context of TEVG

patency is currently not understood, but the differences between the two MNC populations studied in this report identify targets for future work toward elucidating the early host response to TEVG implantation and how BM-MNC seeding regulates this interaction.

One process that could potentially be influenced in such an acute period is the development of graft thrombosis due to excessive platelet activation and aggregation; in fact, we have begun to evaluate the effects of antiplatelet therapy on TEVG stenosis in recent work.^{17,34} The inflammatory response to intravascular thrombus has been well characterized in models of deep vein thrombosis.^{40,41} After a thrombus forms within a vein, the number of infiltrating neutrophils and monocytes increases, and the vein wall thickens, often accompanied by hyperproliferation of smooth muscle cells and myofibroblasts resulting in progressive vessel occlusion. These responses are also observed in stenotic TEVGs at the 2-week time point identified in this report. We hypothesized that the excessive monocyte activation and macrophage infiltration found in stenotic TEVGs at 2 weeks could be triggered by early thrombus formation, and that one effect of cell seeding may be to abrogate this process.

We performed an *in vitro* experiment to determine whether seeded cells were capable of reducing platelet degranulation, a marker of platelet activation, by measuring ATP secretion from thrombin-activated platelets. Scaffolds seeded with BM-MNCs exerted a statistically significant dose-dependent inhibition of platelet derived ATP, whereas PB-MNC-seeded scaffolds did not. These results suggest that one means by which BM-MNC seeding prevents TEVG stenosis could be by potent inhibition of platelet activation in the acute period after implantation. The functional disparity between PB-MNCs and BM-MNCs could be attributed to our observed differences in cytokine expression; however further research is required to explore this potential mechanism. In addition, differences in the size and composition of intravascular thrombi between BM-MNC seeded and unseeded scaffolds will be characterized to substantiate or refute the hypothesis that differential thrombosis could be responsible for acute and/or long-term stenosis.

The results presented in this report are limited by three primary factors. First, seeded BM-MNC-conditioned medium was used as a surrogate for the autologous plasma used clinically. In order to avoid the effects of plasma dilution due to the relatively small volume of bone marrow aspirated from each mouse and to standardize concentrations of potentially significant factors secreted from the seeded cells, we deem this approximation justified. Second, we did not evaluate the kinetics of graft occlusion with imaging techniques such as μ CT or ultrasonography. A thorough understanding of the time course of acute graft occlusion (on the scale of hours to days) would be beneficial in determining the incidence of graft occlusion due to thrombosis vs progressive stenosis. Third, the accuracy of phenotypic analysis of the MNC populations would be greatly enhanced by flow cytometric techniques. These valid limitations are the basis of ongoing experiments.

V. CONCLUSIONS

In this manuscript, we demonstrated that the patency achieved by seeding TEVG scaffolds with BM-MNCs cannot be solely attributed to other components of the clinical protocol for

graft preparation, such as PBS prewetting or plasma incubation. Next, we considered the effect of mononuclear cell source on TEVG patency and showed that only the BM-MNC preparation prevented TEVG stenosis when compared to grafts seeded with a Ficoll-enriched fraction of PB-MNCs. To characterize differences between these two populations of mononuclear cells, cytokine expression from graft seeding supernatants was quantified, and BM-MNCs were found to produce greater levels of IL-1 β , IL-6, and TNF α . Further, we investigated the effect of seeded cells on platelet activation and discovered a potent antiplatelet effect of seeded BM-MNCs. Though exploratory, our results reveal that these two populations are neither equivalent nor interchangeable in the context of TEVG seeding and clearly identify important questions for further mechanistic study.

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Figure 1.

Lumen diameter measurements (A) and representative H&E photomicrographs of patent (B–E) and occluded (F–I) TEVGs after 2-week implantation from the PBS (B, F), conditioned media (C, G), BM-MNC seeded (D, H), and PB-MNC seeded (E, I) groups. TEVGs with lumen diameters <50% of the original lumen diameter at implant (dashed line, 0.41 mm) were considered critically stenotic. The PBS, conditioned media, and PB-MNC seeded groups all had significantly smaller lumen diameters than the BM-MNC seeded group ($p = 0.0291$, 0.0093 , and 0.0455 , respectively). No other significant differences in lumen diameter between groups were identified. Images were acquired at 5 $\times$ magnification.

Figure 2.

Immunohistochemical evaluation of F4/80⁺ macrophages (A) and representative photomicrographs of patent (B–E) and occluded (F–I) grafts from the PBS (B, F), conditioned media (C, G), BM-MNC seeded (D, H), and PB-MNC seeded (E, I) groups. ***p* 0.005, ****p* 0.0005, *****p* 0.0001. Images were acquired at 63× magnification.

Figure 3.

Immunohistochemical evaluation of α SMA⁺ area fraction (A) and representative photomicrographs of patent (B–E) and occluded (F–I) grafts from the PBS (B, F), BM-MNC seeded (C, G) conditioned media (D, H), and PB-MNC seeded (E, I groups). * $p < 0.05$, ** $p < 0.005$. Images were acquired at 63 $\times$ magnification.

Figure 4.

In vitro cytokine analysis (A) from the media in which grafts were immersed after overnight incubation. Seeded BM-MNCs secreted significantly more IL-1 β , IL-6, and TNF α than seeded PB-MNCs. ATP secretion from thrombin activated platelets in the presence of TEVGs seeded with varying doses of either BM-MNCs or PB-MNCs demonstrates a dose-responsive decrease in measurable ATP from wells with BM-MNC seeded grafts, and this phenomenon was not identified with seeded PB-MNCs (B, ** p 0.005, *** p 0.0005, **** p 0.0001).

Scheme 1.
Clinical Protocol for TEVG Assembly

Table 1

TEVG Implant Groups Summary

implant group	<i>n</i>	survival rate	patent	occluded	patency rate (%)	χ^2 to BM-MNC seeded (<i>p</i>)
PBS	10	100%	2	8	20.0	0.0291
conditioned media	25	100%	6	19	24.0	0.0093
BM-MNC seeded	9	100%	8	1	88.9	
PB-MNC seeded	20	95%	6	14	30.0	0.0455