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Role Of The Innate Immune Response In Tissue Engineered Vascular Graft Stenosis

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Role of the Innate Immune Response in Tissue Engineered Vascular Graft Stenosis

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Tissue Engineered Vascular Grafts (TEVGs) hold great promise in advancing the field of pediatric cardiac surgery and other applications in which a vascular conduit is required. The first human clinical trial of these grafts indicated that stenosis was the primary mode of graft failure. Here, we investigate the role of the immune response in stenosis through a CB17 murine inferior vena cava interposition model. We demonstrate that this mouse model undergoes a dramatic stenotic response, and that this is nearly completely abolished in a SCID/bg immunodeficient variant (average luminal diameter (LD) WT: 0.071+/-0.035mm, SCID/bg: 0.804+/-0.039mm; P<0.001). SCID-only mice, which lack T- cells and B-cells, undergo stenosis with luminal diameters approaching those of wild type mice. The *bg* mutation is characterized by defects in natural killer (NK) cell and platelet dysfunction, and treatment of wild-type mice with either anti-platelet therapy (aspirin/clopidogrel) or an anti- natural killer cell antibody (NK 1.1) results in patency that is half of that achieved in the SCID/bg mouse (WT: 0.071+/-0.035mm, SCID/bg: 0.804+/-0.039mm, SCID: 0.137+/-0.032mm, Asp/Pla: 0.452+/-0.130mm, NKab: 0.356+/-0.151mm; P<0.001). We additionally show that SCID/bg mice show a blunted immune response to scaffold implantation, wheras wild-type mice show a dramatic initial burst of activity as demonstrated by macrophage number, mRNA, and cytokine levels. Implicating the initial innate immune response to the scaffold as a critical contributing factor in graft stenosis may provide a strategy for prognosis and therapy of future TEVGs.

Acknowledgements

I would like to sincerely thank Dr. Breuer for his support in working on this exciting project. His oversight and his words of advice have been invaluable over the past year, both in matters of research, and in matters of discerning how to shape my future career in medicine. I cannot thank him enough for his support.

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Introduction

Congenital heart disease is a significant cause of mortality and morbidity among pediatric patients, affecting approximately 9 newborns per 1000 births. It represents the leading cause of infant death due to congenital defects, and more than 30% of infants that die of congenital abnormalities have a cardiac defect (1).

Single ventricle anomalies collectively comprise one of the largest groups of potentially fatal cardiac conditions. They include tricuspid atresia, pulmonary atresia, and hypoplastic left heart syndrome, all of which result in mixing of the pulmonary and systemic circulations with subsequent chronic hypoxia, volume overload of the single ventricle, and eventual heart failure. Without correction, they associated with a 70% mortality rate in the first year of life, with the highest risk of death occurring within the first postnatal week (2).

Advancement of the surgical therapy of these patients has drastically changed this course, with continued declines in mortality occurring even into modern times as further refinements are made. Deaths from single ventricle defects decreased by 65% in the interval 1979-1997 and occurred at progressively older ages (3).

The treatment of choice remains staged surgical reconstruction (4), (5), whereby, in a series of operations, the pulmonary circulation is separated from the systemic

circulation, normalizing the volume delivered to the ventricle and preventing heart failure as well as improving systemic oxygenation. The deoxygenated blood is then passively circulated through the pulmonary circulation. This latter step is most commonly achieved through a modified Fontan operation, where a conduit is placed that connects the vena cava to the pulmonary artery. This modified Fontan procedure, the extra cardiac total cavopulmonary connection (EC TCPC), is considered the standard of care for patients with single ventricle anomalies.

Polytetrafluoroethylene (PTFE or Gore-Tex®) conduits are currently the most widely used vascular grafts for EC TCPC (5). Long term data regarding the performance of these grafts is not yet widely available, but data regarding the long term performance of valved and unvalved conduits used in similar operations suggests that there may be complications in the future. Late problems encountered in these latter grafts include conduit degeneration with progressive obstruction, increased susceptibility to infection, and increased risk for thromboembolic complications (6). PTFE grafts also lack growth potential, necessitating re-operation when patients outgrow them. Re-operations are associated with significant morbidity and mortality (6).

Tissue engineered vascular grafts (TEVG) hold great promise for surgical applications such as these in which a vascular conduit is required. The ideal TEVG, comprised of living, autologous tissue, would address the problems associated with the use of synthetic biomaterials. Since there should be no foreign materials present in the mature

TEVG, the risk of microbial adherence and infection would be reduced. As with native vessels, the anti-thrombogenic endothelial cell intima would greatly decrease the risk of thromboembolic complications. Perhaps most significantly, these grafts should possess the capability for growth and remodeling, effectively negating the necessity of re-operation.

In previous studies, using the classic tissue engineered paradigm, we implanted biodegradable scaffolds seeded with vascular cells as pulmonary artery grafts, and subsequently as IVC interposition grafts in ovine and canine models, to simulate the high flow, low pressure conditions of the EC TCPC conduit (7), (8). Over time, we have demonstrated the in-vivo degradation of the scaffold and the generation of a mature, laminated vascular structure that closely approximates native vessels. The neovessels formed in this fashion had an endothelial-lined intima, a media containing smooth muscle cells (SMCs), and an outer advential layer. Additionally, they showed evidence of growth and development, increasing in size proportional to the surrounding tissue (9). There was no evidence of failure associated with aneurismal dilation or thrombosis.

The mechanisms underlying neovessel formation were incompletely understood, and it was assumed that the neotissue was formed as a result of repopulation of the scaffold from the seeded cells. Culturing vascular cells for autologous cell seeding proved to be a cumbersome process, requiring initial vein biopsy, as well as an extended culture period during which there was risk for contamination (10). Based upon reports that showed

that bone marrow mononuclear cells (BM MNCs) stimulated the endothelialization of a synthetic prosthesis (11), and that bone marrow derived endothelial progenitors were incorporated into areas of injury-induced neovascularization (12),(13), we theorized that BM MNCs might prove to be an easily accessible source for autologous cell seeding. This was borne out in our canine and ovine models, where BM MNC were harvested from the sternum, seeded upon polymer scaffolds, and implanted into the animals on the same day (10), (14), (15).

Based upon the success of these animal models, we performed a pilot clinical study of autologous BM MNC-seeded TEVG as conduits in the modified Fontan procedure (16), (17), (18), (19). A total of 25 patients were enrolled in the study with a follow up of 7 years. Serial MRI imaging confirmed the potential of the grafts for growth (18). There was no graft related mortality, and again, no complications associated with thrombosis or aneurysm. Four of the grafts, however, did develop complications from significant stenosis. These patients were treated successfully with angioplasty or angioplasty and stenting, since the neovessel conduits were amenable to coronary interventions that would have been impossible with synthetic grafts (19). To date, no graft has had to be replaced. This clinical trial demonstrated that tissue engineering has the potential to provide functional, growing grafts that avoid the pitfalls associated with synthetic conduits, though a significant subset of grafts are currently complicated by graft stenosis.

To better understand the mechanisms by which stenosis, and indeed neotissue formation, takes place, we have developed sub-1mm scaffolds for implantation in a murine model (20). Advances in characterization and manipulation of mouse genetics make the murine host a good model for the study of these processes on a molecular level. Immunodeficient mice, in particular, allow for the direct study of human cell seeded scaffolds without fear of graft rejection.

Using the SCID/bg mouse model, which lacks an adaptive immune response, we have recently studied the development of scaffolds seeded with human BM MNCs, and we have shown that the seeded cells do not give rise to neotissue directly, as has previously been thought (21). Rather, we have proposed that neotissue formation is a regenerative, immune-mediated process characterized by early degradation of BM MNCs, infiltration of the scaffold by monocytes, the secretion of cytokines such as VEGF, and the resultant recruitment of host endothelial and smooth muscle cells. In further studies which are in press, we have found that the host cells that populate the graft migrate laterally from adjacent native tissue (22). Additionally, we have found that the seeded BM MNCs function by a transient paracrine effect, modulating the immune response and accelerating the kinetics of neotissue formation (21). This is borne out by previous studies, where BM MNC-seeded grafts exhibited faster endothelialization, smooth muscle recruitment and collagen deposition than unseeded controls (15), (9).

There are limitations to the use of this animal model which merit consideration. These

grafts are of a much smaller caliber than the grafts used in human applications, and are thus subject to different hemodynamic forces in-vivo, which may affect neotissue and stenosis formation. Animal models also have inherent species and strain specific characteristics that affect the applicability of their results to humans, or even to each other. In comparing the patency rates of autologous endothelial cell seeded synthetic grafts in dogs and lambs, for example, one study noted that the lambs had a much higher rate of thrombosis, with 89% of the grafts becoming occluded. The canine recipients, however, had a thrombosis rate of only 17% (23).

In a similar fashion, our studies in SCID/bg mice have only yielded largely patent vascular grafts. Scaffolds seeded with human BM MNCs, human aortic smooth muscle cells, as well as ovine arteriole decellularized xenografts (24), (20), (25), (21), have all been implanted into SCID/bg mice, and were tolerated well. Recent studies which are in press, by contrast, have shown that the immunocompetent C57Bl6 mouse strain exhibits a similar rate of stenosis to humans (20%) when implanted with syngenic mouse BM MNC- seeded grafts (26). The rate of stenosis increases dramatically to 80% when the graft is unseeded.

As stated above, our experiments in BM MNC seeded grafts have shown that the process of TEVG formation is an immune modulated process, and the differences in the occurrence of stenosis between the immunodeficient SCID/bg mouse and the immunocompetent C57Bl6 models is striking. We hypothesized that the formation of

stenosis was as a result of an exuberant inflammatory response, with excessive inflammation triggering excessive smooth muscle cell and extracellular matrix deposition. Seeding with BM MNC may serve to modulate this process and thus reduce graft stenosis. The two mouse models used, however, represent two different strains, with SCID/bg mice being mutants of an albino CB17 strain, while the C57Bl6 are normally pigmented variants. As previously noted, strain-specific differences in results cannot be neglected. In an attempt to better characterize the effect of immunodeficiency on rates of TEVG stenosis, here we compare grafts implanted in SCID/bg mice with grafts implanted into the wild-type CB17 background, and attempt to characterize the differences in immune response between the two. Unseeded grafts are used to maximize the inflammatory response, and thus accent the differences due to diminished immunity.

Hypothesis and Specific Aims

The formation of TEVG stenosis is as a result of an exuberant inflammatory response, and blunting this response in an immunodeficient mouse model, or by specific depletion of immune cell lines will abolish graft stenosis.

Specific Aims

1. To determine whether the immunodeficient SCID/bg mouse experiences lower rates of graft stenosis, and whether the cell line defects in T and B cells (SCID mutation) or NK cells and platelets (Bg mutation) are responsible for this effect

2. To determine whether a decreased hyperplastic response is reflected by a decreased inflammatory response, as demonstrated by macrophage number and cytokine levels

Methods

Immunohistochemistry, immunofluorescence and mouse anesthesia were performed by Dane Mejias. Scaffold fabrication and treatment of mice with aspirin, clopidogrel and NK 1.1 antibody were performed by Dane Mejias and Narutoshi Hibino. Microsurgical implantation of scaffolds was performed by Tai Yi and Narutoshi Hibino. qPCR and RT-PCR were performed by Narutoshi Hibino.

Scaffold Fabrication

Scaffolds were constructed from a nonwoven polyglycolic acid (PGA) mesh (Concordia Fibers) and a co-polymer sealant solution of poly-L-lactide and –ε-caprolactone (P(CL/LA)) using previously described methods (20)

TEVG Implantation

TEVG implantations were performed using microsurgical technique. The scaffolds were inserted into the infrarenal IVC of 3-4 month old, CB-17 backgound of wild type, SICD/bg, and SCID female mice (TECOIC). A total of 72 animals were implanted with TEVG. All animal experiments were done in accordance with the institutional guidelines for the use and care of animals, and the institutional review board approved the experimental procedures described.

Histology

Explanted grafts were pressure fixed in 10% formalin overnight and then embedded in paraffin or glycolmethacrylate using previously published methods (20),(21). H&E (Sigma, St Louis, MO) staining was conducted to evaluate graft morphometry. Graft luminal diameter were measured using Image J software (Image Processing and Analysis in Java; National Institutes of Health, Bethesda, MD).

Immunohistochemistry:

Primary antibodies included rat-anti-mouse F4/80 (AbD Serotec), mouse-anti-human Smooth Muscle Actin (SMA) (Dako), rabbit-anti-human vWF (Dako), and mouse-anti-mouse NK 1.1 (Abcam). Antibody binding was detected using appropriate biotinylated secondary antibodies, followed by binding of streptavidin-HRP and color development with 3,3-diaminobenzidine (Vector). Nuclei were then counterstained with hematoxylin. For immunofluorescence detection, a goat-anti-rabbit IgG-Alexa Fluor 568 (Invitrogen) or a goat-anti-mouse IgG-Alexa Fluor 488 (Invitrogen) was used with subsequent 4',6-diamidino-2-phenylindole nuclear counterstaining.

Ultrasonography:

Serial ultrasonography (Vevo® Visualsonics 770) was utilized for graft surveillance of the TEVG. Prior to ultrasonography, mice were anesthetized with 1.5% inhaled isoflurane. Graft luminal diameter was determined sonographically at the indicated time points after post-implantation.

Micro-computed tomography angiography (microCTA):

In vivo patency and morphology of the TEVG were evaluated using microCTA, as previously described (Jason 2008).

Computer Assisted Image Analysis (Quantitative IHC):

Mouse macrophages, identified by positive F4/80 expression, were measured for each explanted scaffold. Two separate sections of each explant were counterstained with hematoxalin and imaged at 400X magnification. The number of nuclei was then counted in five regions of each section and averaged.

qRT-PCR:

RNA Isolation and qRT-PCR to Characterize Macrophage Phenotype: Explanted tissue grafts were frozen in Tissue-Tek OCT (Sakura) and each sectioned into forty 10um sections using Cryocut 1800 (Leica). Excess Tissue-Tek OCT was removed by centrifugation in water. RNA extraction was performed with an RNeasy Mini Kit (QIAGEN VWR, Stockholm, Sweden), according to the manufacturer's protocol. RNA concentration of each sample was determined by Quant-iT™ RiboGreen® RNA Reagent and Kit (Invitrogen Co., Carlsbad, California, USA), according to the manufacturer's protocol. Real-time reverse transcriptase polymerase chain reaction was carried out with a 50 µL total volume containing 1 µg RNA, 5 µL 10xTaq-Man RT Buffer, 11 µL MgCl2 (25 mM), 10 µL dNTP mixture (10 mM), 2.5 µL Random Hexamer (50 mM), 1 µL RNase inhibitor (20 U/ µL), and 1.25 µL reverse transcriptase (50 U/ µL) (Applied Biosystems). RNAse-free water was added to bring total sample volume to 50 µL . The thermal cycling parameters were incubation at 25°C for 10 min, reverse transcription at 48°C for 30 min, and inactivation at 95°C for 5 min. Predesigned and validated gene-specific TaqMan Gene Expression Assays from Applied Biosystem (Foster City, CA, USA) were used in

duplicate for quantitative real-time PCR according to the manufacturer's protocol. Primers for the following genes were used: CCL3 (Mm00441258_m1), iNOS (Mm00441258_m1), TNF-alpha (Mm00443258_m1), CX3CR1 (Mm00438354_m1), Fizz1 (Mm00445110_g1), and MMP9 (Mm00600157_g1). Values were normalized to expression of HPRT (Mm00441258_m1).

Platelet Inhibition

Wild-type mice were treated with aspirin and clopidogrel for 10 weeks following TEVG implantation. Aspirin (30 mg/L) was placed in their drinking water, which was replaced with fresh water every other day. Considering that each animal drinks in average 3 to 4 mL of water per day, this would be equal to 90 to 120 μg aspirin per day for a mouse of 30-g of weight. This low quantity was administered to obtain an amount similar to that prescribed in clinical practice, i.e.,3–4 mg/kg/day (50), (51). Clopidogrel (Plavix) was obtained from the local hospital pharmacy, and 75-mg tablets were dissolved in 0.9% saline under sterile conditions. This solution was then diluted appropriately in 20 mg/kg clopidogrel. Daily treatment with clopidogrel was started immediately after transplantation. Because dissolved clopidogrel is unstable, the clopidogrel solution was freshly prepared every day and injected intraperitoneally immediately after preparation. (52) These mice were sacrificed at the end of the 10weeks treatment period, and the implanted scaffolds fixed for histologic examination as above.

Natural Killer Cell Inhibition

Wild type mice were treated with 200ul of NK 1.1 ab (ebioscience) at 2 days and 24 hours prior to TEVG implantation, then weekly thereafter for 10 weeks. This dose of NK antibody were used to sustain depletion of NK cells from the spleen without reduction of cellular or humoral immunity. As above, these mice were sacrificed and the grafts harvested for histologic analysis.

Results

We have previously demonstrated the feasibility of creating sub-1 mm biodegradable tubular scaffolds that are functional as vascular grafts in our previous study (20). Here, we investigated the natural course of the grafts using this same model. TEVG grafts implanted into SCID/bg mice were followed over 24 weeks. Histologic examination of the vessels at 1 week, 3 weeks, 6 weeks, 10 weeks and 24 weeks (N=4 for each time point) demonstrated progressive infiltration of the structure by macrophages, degradation of the polymer scaffold, and the formation of a laminated neovessel (fig1a). Up to 3 weeks after implantation, the wall thickness increased and luminal diameter decreased due to the cell infiltration into the graft. Thereafter, the degradation of scaffold led to decreased wall thickness and increased luminal diameter, and both values closely approximated native IVC at 24 weeks (fig1c). Immunofluorescence staining demonstrated vWF positive endothelial cell and alpha SMA positive cell layer in the inner layer of graft at 10 weeks following implantation (fig1b). This staining confirmed the presence of a confluent endothelialized intima and a medial smooth muscle layer at the 10 week time point, suggesting that this was an appropriate end point to estimate neotissue formation.

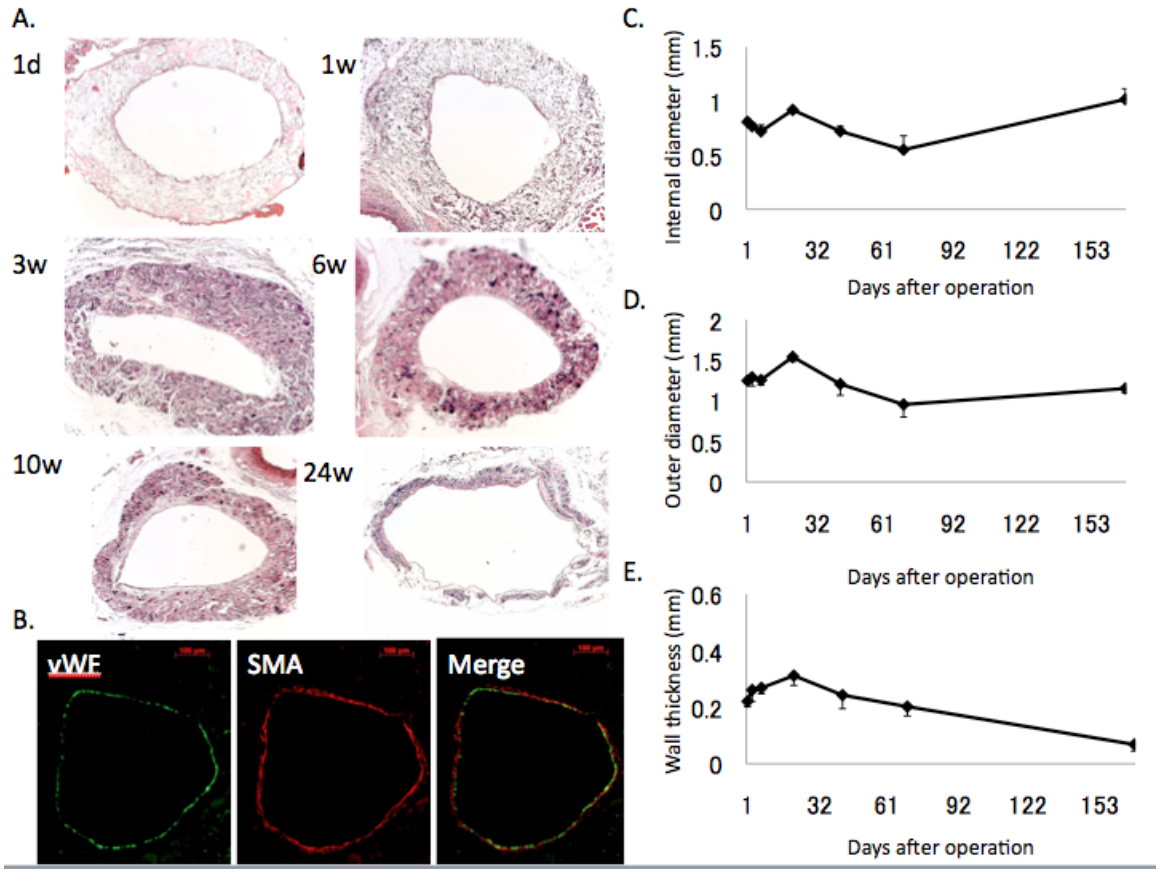

Fig 1. Characterizing neovessel formation in SCID/bg mice. A. H&E staining demonstrating infiltration of the scaffold wall with macrophages, and the serial degradation of the scaffold and neovessel formation (Bar=200um). B. Immunofluorescence of mature vessels showing positive staining for endothelial and smooth muscle cells. C, D, E Luminal diameter and wall thickness changes in the neovessels over time.

To investigate the effect of host immune function on TEVG neotissue formation, we implanted 8 unseeded scaffolds into CB17 SCID/bg mice and 8 unseeded scaffolds into wild type CB17 controls. Ultrasonography demonstrated stenosis of the grafts implanted in wild type mice after 2 weeks whereas the grafts of SCID/bg group remained patent up to 10 weeks. Histologic examination at 10 weeks confirmed the development of stenosed grafts in the wild type mice, with severe intimal thickening. All

of the SCID/bg grafts, by contrast, remained patent and demonstrated significantly higher diameter compared with wild type (average luminal diameter (LD) WT: 0.071+/-0.035mm, SCID/bg: 0.804+/-0.039mm; P<0.001).

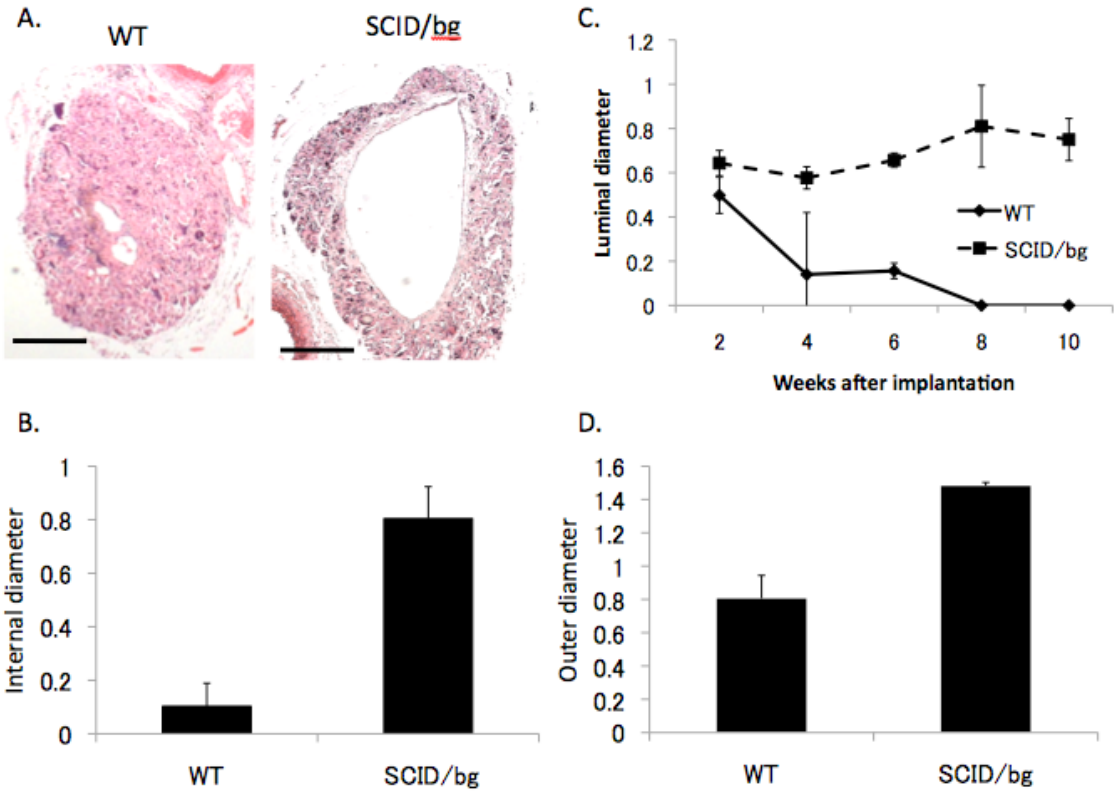

Fig 2. SCID/bg mice show markedly increased patency rates vs wild type controls A. H&E stain of TEVG cross sections from wild type and SCID/bg CB17 mice (Bar=200um). C. Luminal diameters of SCID/bg implanted mice are higher than WT mice at 2, 4, 6, 8 and 10 weeks post implantation. WT scaffolds show uniform occlusion at 8 weeks post-implantation. B. and D. SCID/bg mice show increased luminal and vessel diameters after 10 weeks vs WT controls.

The SCID/bg phenotype is due to defects in T and B cell function (SCID mutation) and natural killer and platelet dysfunction (bg mutation). To ascertain whether defects in these cell lines could be independently responsible for decreased

intimal hyperplasia, we implanted scaffolds into mice that bore only the SCID mutation (SCID) (N=8), as well as into wild type CB17 mice that were treated with either an NK-cell depleting antibody (NKab) (N= 8), or with platelet-inhibiting aspirin and clopidogrel (Asp/Pla) (N=6). Ultrasonography demonstrated a difference in luminal diameter at 2 weeks following implantation. The SCID mice developed graft stenosis at a rate equivalent to WT mice, while each of the treated groups exhibited luminal diameters that were halfway between SCID/bg mice and the WT group. (WT: 0.071+/-0.035mm, SCID/bg: 0.804+/-0.039mm, SCID: 0.137+/-0.032mm, Asp/Pla: 0.452+/-0.130mm, NKab: 0.356+/-0.151mm; P<0.001)

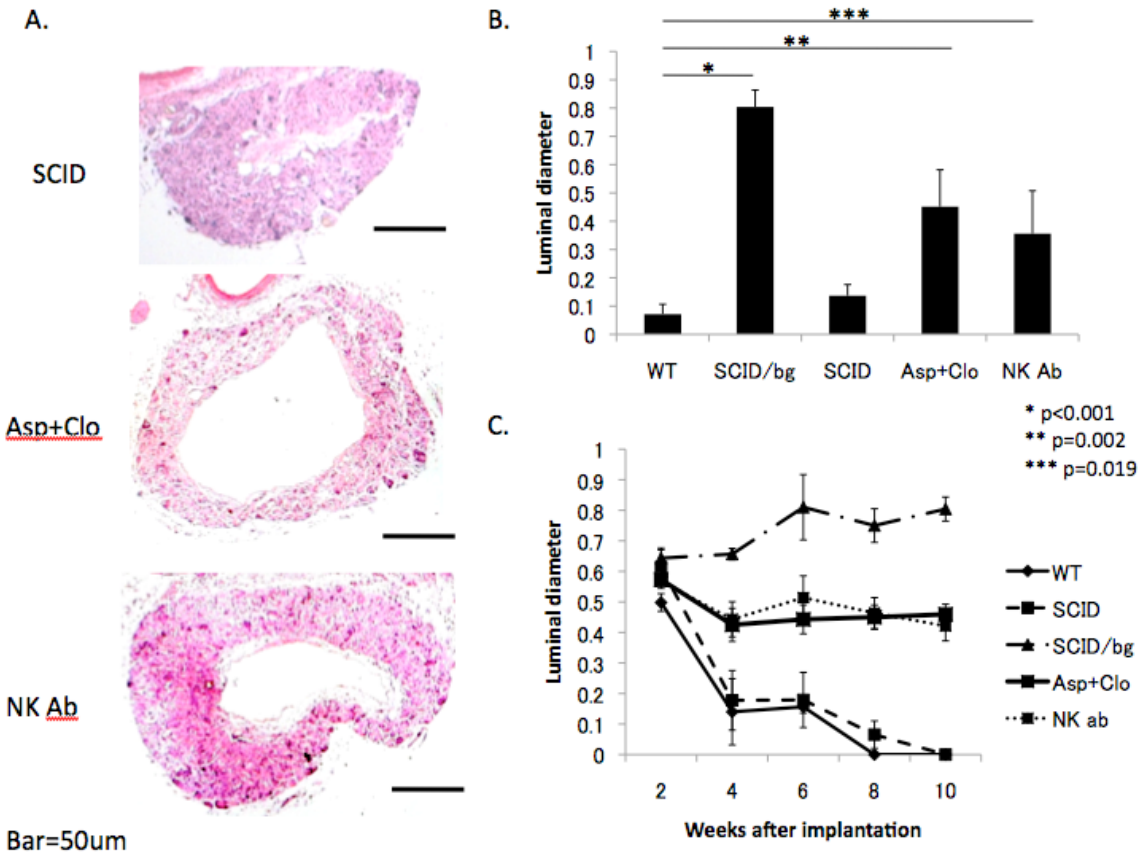

Fig 3. A. H&E staining showing complete occlusion of a TEVG implanted into a SCID mouse, and patent grafts implanted into mice treated with aspirin and clopidogrel or an

anti-NK antibody (Bar=200um). B. and C. SCID-only mice display stenosis that mirrors the wild-type response. Mice treated with aspirin/clopidogrel or NK 1.1 antibody achieve patency rates that are approximately half of that achieved in the SCID/bg mouse.

To further investigate the role of Natural Killer cells in intimal hyperplasia, we performed immuohistochemical analysis of the stenosed wild type grafts as well as the patent SCID/bg grafts using the anti-NK antibody NK 1.1. The stenosed grafts stained positive for Natural Killer cells, and in grafts that showed evidence of hyperplasia without complete stenosis, NK 1.1- positive cells were observed in the hyperplastic region. Scaffolds were subsequently implanted into pfp gene deficient mice which exhibit severe cytotoxic dysfunction of NK cells. As the background of this strain is C57/BL6, we explanted scaffolds at 2 weeks that is the optimal end point to examine the patency of graft based on our previous study (26). There was no significant difference in luminal diameter, however, between these mice and the wild type controls. This result suggested that not cytotoxic function but cytokines secreted from NK cell could be an important factor causing graft stenosis.

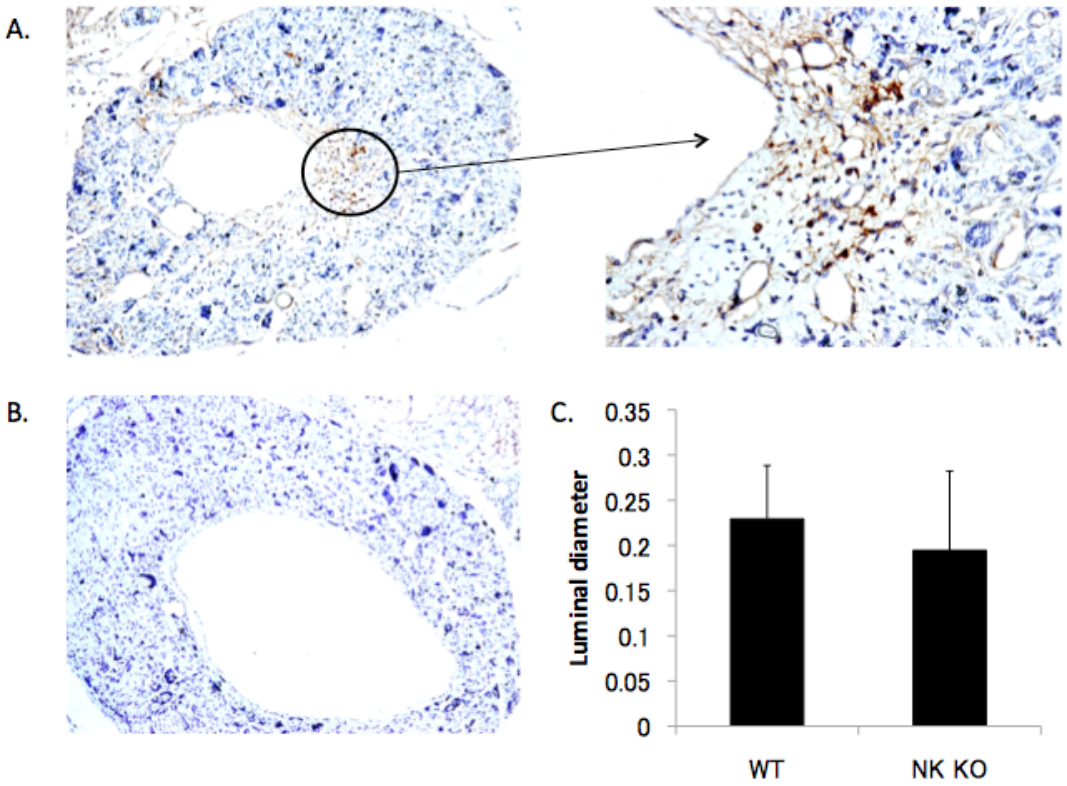

Fig 4. A. IHC staining with NK 1.1 antibody showing that NK cells are present at sites of neointimal hyperplasia. B. SCID/bg mice show no positive staining with NK 1.1. C. NK-KO mouse show no significant differences in luminal diameter when compared to wild-type controls.

Although SCID/bg mice do not typically exhibit a pronounced defect in the innate immune response, we nonetheless investigated the difference in macrophage recruitment response between the SCID/bg mice (N=8) and WT CB17 (N=8) mice through semi-quantitative assessment of the degree of macrophage infiltration. The SCID/bg mice showed significantly less macrophages per high-powered field (WT: 113+/-12 /HPF, SCID/bg: 66+/-18/HPF; p=0.006). This result is consistent with the result of PCR analysis of scaffold at 3 days, which showed significantly less F4/80 mRNA production in SCID/bg grafts compared with WT (fig 5).

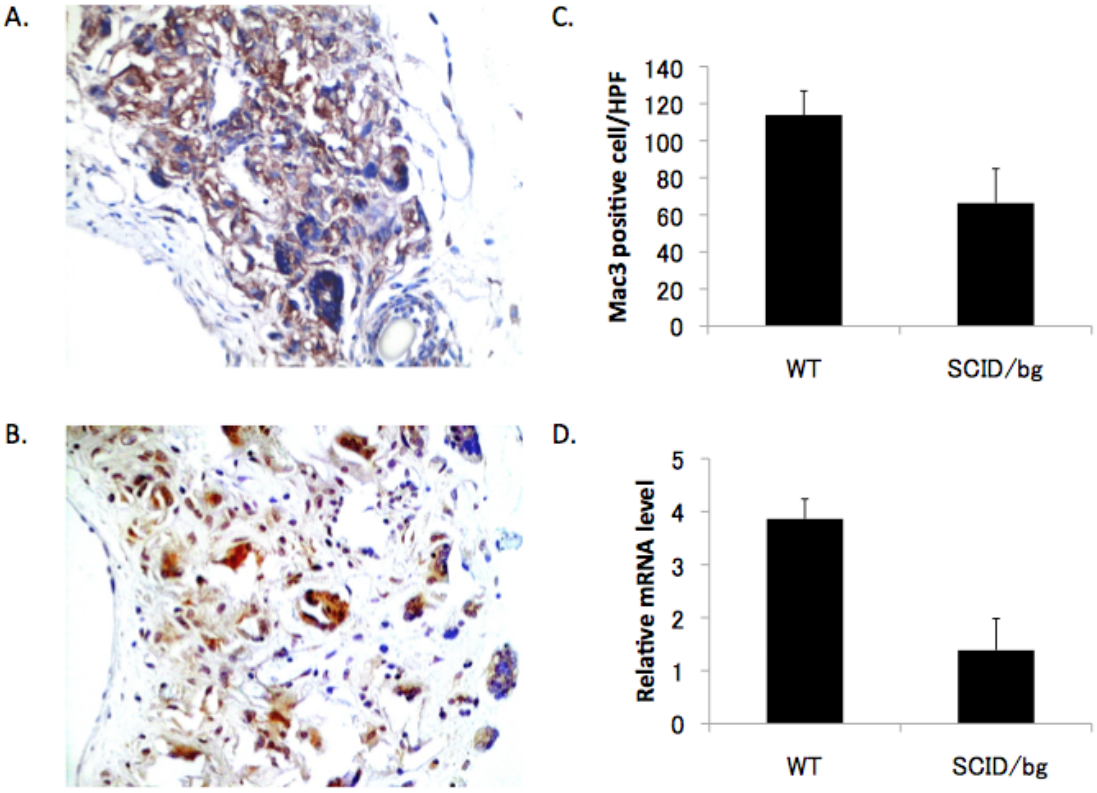

Fig 5. A. Immunohistochemistry of wild-type CB17 mice demonstrating more robust macrophage infiltration than in SCID/bg mice, B. C. SCID/bg mice show fewer macrophages than wild-type mice upon semi-quantitative analysis. D. RT-PCR demonstrating less macrophage cellular activity in SCID-bg mice

The decreased macrophage activity in the SCID/bg grafts suggested that there is less inflammatory cytokine production, and evidence of a blunted immune response to the implanted scaffold. To further explore this, we performed PCR for CCL3 (a macrophage recruitment and activation protein), iNOS (an anti-inflammatory cytokine) and TNF-alpha (an inflammatory cytokine) in SCID/bg and WT grafts at both an early 3 day and a later 4 week time point. For the 3 day time point, a sharp initial production of all of these cytokines was demonstrated in the WT mice, but not the SCID/bg mice. These levels all decreased by the 4 week time point in the WT mice, while the SCID/bg mice

showed minimal declines. We also examined the levels of CXCL1 and Fizz, which represent makers of the later reparative macrophage response as well as MMP9, an extracellular matrix degrading enzyme which is thought to play a role in tissue remodeling. Levels of these proteins could be expected to be higher in a hyperplastic response, with an exaggerated remodeling response resulting in the creation of a hyperplastic neointima. By contrast, our results demonstrated a slightly higher expression of CX3CL1 in the SCID/bg mice, and comparable levels of Fizz and MMP9 in both groups.

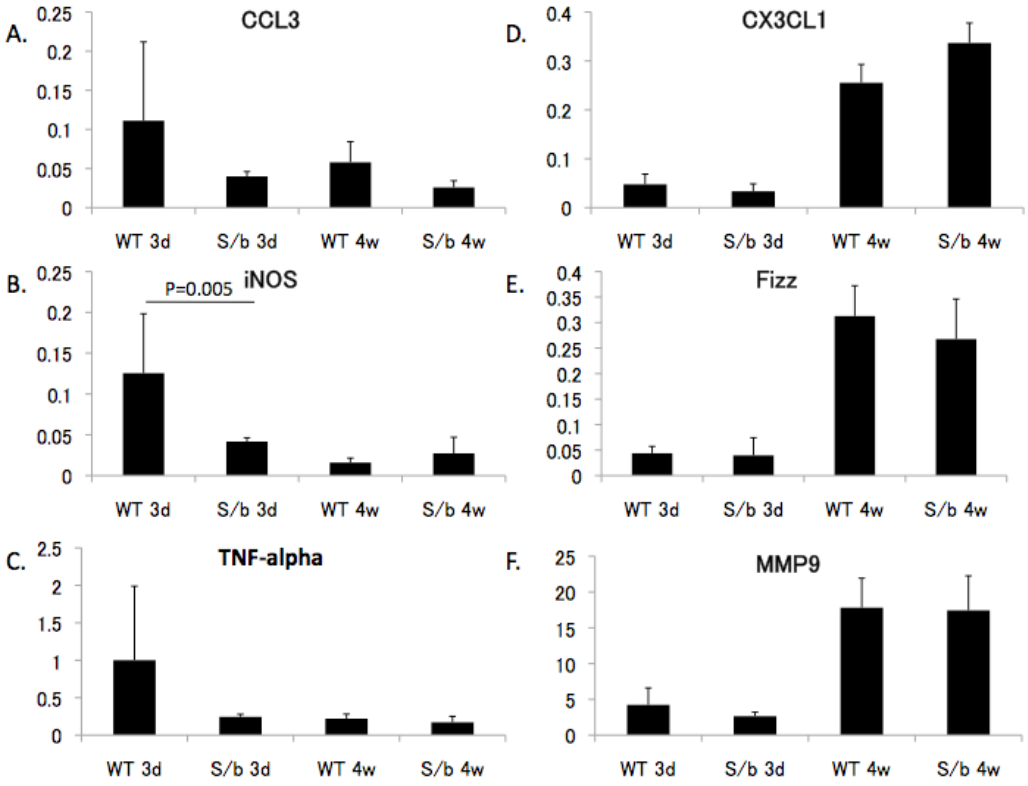

Fig 6. A-C. Wild-type mice show a greater initial inflammatory response after graft implantation, which drops rapidly by the 4th week. SCID/bg mice show only slight decreases in cytokine activity after 4 weeks. D-F. Markers of the later macrophage response and MMP9. CX3CL1 is slightly higher in SCID/bg mice after 4 weeks wheras Fizz is slightly higher in WT mice. MMP9 levels are equivalent in both types.

Discussion

The results of this study highlight the critical role that the immune system plays in TEVG stenosis. We have recently proposed a mechanism for the natural history of neotissue formation within bone marrow mononuclear cell (BM MNC) seeded scaffolds in SCID/bg mice, and surmised that it is an immune-mediated process (21). The process begins with the infiltration of the scaffold by host macrophages, which degrade the polymer scaffold while secreting angiogenic cytokines and growth factors, such as VEGF. This results in the recruitment of smooth muscle cells and endothelial cells, with eventual neovessel formation. BM-MNC secreted MCP-1, a monocyte chemoattractant protein, speeds up the kinetics of both macrophage recruitement and neovessel formation (21). Here, we assert that an overly robust burst of inflammation may be at the core of neovessel stenosis.

This model, of a macrophage inflammatory response, followed by cell recruitment and remodeling, mirrors exactly the model that has been proposed for the formation of intimal hyperplasia, which is a well known pathologic process that causes arterio-venous graft failure in hemodialysis grafts (27), (28), and in coronary artery restenosis following angioplasty and stenting (29). The primary inciting factor that precipitates intimal hyperplasia appears to be vessel wall/endothelial damage as a result of surgical or interventional trauma, and in the case of hemodialysis AV grafts, graft bioincompatibility (30). These upstream events provoke a cascade of events comprising

an acute inflammatory phase with macrophage infiltration, a proliferation phase with smooth muscle cell recruitment, and a remodeling phase (31). Presumably in the case of TEVGs, bioincompatibility, as well as the surgical anastamosis of the scaffold to the vasculature, would provide an ample stimulus for activation of the immune system, and provoke a similar sequence of events.

In previous studies, the magnitude of the inflammation that occurs following vessel injury appears to be linked to the subsequent intimal hyperplastic response. Sustained high levels of systemic inflammation markers such as CRP predict increased risk of human coronary artery stenosis following stenting (32). The number of monocytes recruited to the vessel wall has also been directly correlated with the volume of neointima formation (33). Studies have shown that MCP-1 is an essential chemokine in this process, and that blockade of MCP-1 abrogates both macrophage recruitment and intimal hyperplasia (34), (35), (36).

Inflammation has thus clearly been implicated in vascular hyperplasia in response to vessel injury. In the context of TEVG formation we propose, however, that inflammation is not purely pathogenic—and is in fact critical for neotissue generation—but that an overly exuberant inflammatory response causes excessive recruitment of SMCs, and resulting intimal thickening. We have currently submitted for review a study that further supports this idea. Pharmacologic inhibition of macrophages results in grafts that show

no stenosis, but also show a lack of neotissue formation (26). A balanced immune response is required for optimum TEVG development.

SCID/bg mice, by virtue of their genetic derangements, appear to have this near-optimum internal mileu. Though we hypothesized that an immunodeficient mouse would enjoy greater TEVG patency rates than an immunocompetent control, the contrast between the SCID/bg and wild-type mice is startlingly pronounced (Fig 1.). Wild type mice develop uniformly stenosed grafts, and the immunodeficient mutant develops uniformly patent, though fully cellularized and mature neovessels (Fig 1., Fig 2.). Additionally, the exuberant stenosis exhibited in wild type CB17 mice is beyond that observed in humans, or indeed in other animal models (18), (26). The exaggerated response of this mouse species, and that of its immunodeficient variant, lie on the extreme ends of the spectrum of intimal hyperplasia in TEVGs. They provide a unique window through which the mechanisms that underlie this process can be viewed.

In investigating what characteristics make the SCID/bg mouse resistant to graft stenosis we have attempted to separate the component cell line defects present. To this end, we implanted scaffolds in SCID- only mice that lack an adaptive immune response due to defective T and B cells. We have also implanted mice treated with anti-platelet agents aspirin and clopidogrel, and mice treated with an NK-neutralizing antibody, to mimic the platelet and natural killer cell defects present in the bg mutation.

In this study, T and B cells have appeared to play no role in abrogating TEVG intimal hyperplasia. This is in line with studies done in cyclosporine- suppressed rabbits (37), where suppression of humoral immunity had no effect, though at odds with studies done in athymic and T-cell depleted rats (38), (39). The latter two studies, however, have shown opposite results. The mixed evidence of the role played by the adaptive immune system may be due to species-specific differences, as well as differences in the method of diminishing the adaptive immune response. Nonetheless, in our study SCID mice demonstrated uniform occlusion of the graft, with no difference between these and wild-type CB17 mice.

The inhibition of platelets and natural killer cells, by contrast, appeared to individually reduce stenosis rates to half of the rates expected of a SCID/bg mouse (Fig 3), implying that the beige mutation underlies the suppression of graft stenosis. The beige mutation is characterized mainly as a deficiency of granular cells, with decreased anti-microbial efficacy of granulocytes (40), severe NK cell deficiencies (41), and defective platelet function (42), (43). It is thought to be analogous to Chediak- Higashi syndrome in humans. Though the molecular underpinnings of this mutation remain to be elucidated, it appears to be a deficiency of the innate immune response. A defect of the innate, not adaptive, immune system thus appears to underlie the decreased stenosis seen in SCID/bg mice.

Additionally, previous studies have implicated platelet adherence to damaged endothelium, and subsequent macrophage recruitment, as being a critical factor in the formation of a hyperplastic intima. Platelet P-Selectin expression is necessary for triggering monocyte arrest and adhesion, and P selectin -/- knockout mice demonstrate markedly decreased macrophage infiltration, as well as a subsequent decrease in intimal hyperplasia (44), (45). Direct blockade of P-selectin binding by sialyl-Lewis^X substrate replicates this response (46). Here, inhibition of platelets by pharmacologic means has decreased the degree of stenosis in TEVGs (Fig 3.). That SCID/bg mice also show decreased macrophage recruitment (Fig 5.) would be commensurate with this. Aspirin, as an NSAID drug, also possesses anti-inflammatory properties that might confound these results, but the standard dose used is thought to be below the threshold at which the anti-inflammatory effects become apparent. We have also tried to substantiate the importance of platelets by direct anti-thrombocyte antibody depletion following scaffold implantation, but this proved to impair the hemostatic capabilities of the mice beyond the limit to which they could survive the implantation procedure. The decrease in stenosis as a result of administration of readily available anti-platelet medications, however, does have implications for therapy of TEVG recipients.

Evidence in our study regarding the role of natural killer cells in graft stenosis is mixed. Antibody treatment of mice with NK 1.1 appeared to have a beneficial effect on graft patency (Fig 3.), but scaffolds implanted into NK KO mice showed no difference from their wild-type counterparts (Fig 4.). In the literature, NK cells have been demonstrated

to promote arteriogenesis in a murine hindlimb ischemia model (47), while $Cd1d^{-/-}$ mice, which have faulty lipid antigen presentation to NK cells, have shown a 60% decrease in intimal hyperplasia in a carotid artery injury model (48). In this study, it is certainly possible that the NK 1.1 antibody exerted its effect through NK inhibition as expected, and that the NK KO mouse possesses other genetic derangements that negated the effect of defective NK cells. It is however, equally possible that the antibody exerted its effect through an indirect means. Reconciling this discrepancy brings into focus one of the limitations of the immunodeficient mouse models used in this study—namely that while the gene mutations do have a significant effect on immune cell lines, there may be unforeseen consequences on other biochemical pathways.

Whatever the individual molecular derangements of the SCID/bg mouse, a clear contrast in the initial innate inflammatory response to graft can be drawn between these mice and their wild type counterparts. In broad strokes, this is represented by both a decrease in overall macrophage number by quantitative histology, as well as by a lower level of macrophage mRNA production (Fig 5.).

Commensurate with this, our more specific PCR analysis (Fig 6) demonstrated that the macrophage recruitment cytokine CCL3 was higher in the wild type than SCID/bg groups, as was the proinflammatory cytokine TNF-alpha. The anti-inflammatory iNOS was also higher in the wild type mice than in the SCID/bg group. As iNOS plays a role in the modulation of inflammation (49), this suggests that there is certainly greater

inflammatory activity in the wild-type mice, with both proinflammatory cytokines and their anti-inflammatory modulators being engaged. This discrepancy is only apparent at the 3 day time point; by 4 weeks, when stenosis has already become apparent (Fig 1.), all of these cytokines have fallen to levels that are comparable with the levels seen in SCID/bg mice, with only a modestly higher level of CCL3 and a modestly lower level of iNOS in the wild type mice vs. SCID/bg. Also of note is the near-constant profile of these cytokines in SCID/bg mice at 3 days and four weeks. Where wild type mice show an initial upswing of inflammatory activity after vessel implantation followed by a sharp decline, the SCID/bg mice show negligible changes of these cytokine levels during the four week time course.

Our findings with regard to "late" markers of inflammation—CX3CL1, Fizz, and MMP, which are thought to represent the later remodeling response—are less striking. Both wild type and SCID/bg mice show comparable responses (Fig 6.). It appears that it is not the remodeling phase but the inflammatory phase, or lack thereof, that is unique to the SCID/bg mice.

We surmise that it is the initial burst of inflammatory activity that correlates with stenotic grafts in the wild type CB17 mice, whether this is as a direct result of impaired platelet and natural killer activity or no. Our parallel study that shows a causative role of macrophage infiltration in neotissue formation and intimal hyperplasia is consistent with this. Further characterization of the molecular determinants of this initial response

could, at the least, provide a measurable predictor of graft patency. Furthermore, this has implications for the rational design of second generation TEVGs. We have already demonstrated the ability to construct cytokine-eluting scaffolds (21). Future graft designs may include, in a similar fashion, the ability to elute appropriately targeted anti-inflammatory, and possibly, anti-thrombotic agents that will increase the utility of TEVGs as vascular conduits for surgical applications.

References

1. Heart disease and stroke statistics. American Heart Association Report. 2008;1:20.
2. Samanek M. Children with congenital heart disease: Probability of natural survival. Pediatr Cardiol [Internet]. 1992 07/01;13(3):152-8.
3. Boneva RS, Botto LD, Moore CA, Yang Q, Correa A, Erickson JD. Mortality associated with congenital heart defects in the united states: Trends and racial disparities, 1979-1997. Circ [Internet]. 2001 05/15;103(19):2376-81.
4. Giannico S, Hammad F, Amodeo A, Michielon G, Drago F, Turchetta A, Di Donato R, Sanders SP. Clinical outcome of 193 extracardiac fontan patients: The first 15 years. J Am Coll Cardiol [Internet]. 2006 05/16;47(10):2065-73.
5. Petrossian E, Reddy VM, Collins KK, Culbertson CB, MacDonald MJ, Lamberti JJ, Reinhartz O, Mainwaring RD, Francis PD, Malhotra SP, Gremmels DB, Suleman S, Hanley FL. The extracardiac conduit fontan operation using minimal approach extracorporeal circulation: Early and midterm outcomes. J Thoracic Cardiovasc Surg [Internet]. 2006 11/01;132(5):1054-63.
6. Dearani JA, Danielson GK, Puga FJ, Schaff HV, Warnes CW, Driscoll DJ, Schleck CD, Ilstrup DM. Late follow-up of 1095 patients undergoing operation for complex congenital heart disease utilizing pulmonary ventricle to pulmonary artery conduits. Ann Thorac Surg [Internet]. 2003 02/01;75(2):399-1.
7. Shinoka T, Shum-Tim D, Ma PX, Tanel RE, Isogai N, Langer R, Vacanti JP, Mayer JE. Creation of viable pulmonary artery autografts through tissue engineering. J Thoracic Cardiovasc Surg [Internet]. 1998 03/01;115(3):536-.
8. Watanabe M, Shin'oka T, Tohyama S, Hibino N, Konuma T, Matsumura G, Kosaka Y, Ishida T, Imai Y, Yamakawa M, Ikada Y, Morita S. Tissue-engineered vascular autograft: Inferior vena cava replacement in a dog model. Tissue Eng [Internet]. 2001 08/01;7(4):429-39.
9. Brennan MP, Dardik A, Hibino N, Roh JD, Nelson GN, Papademitris X, Shinoka T, Breuer CK. Tissue-engineered vascular grafts demonstrate evidence of growth and development when implanted in a juvenile animal model. Ann Surg [Internet]. 2008 09/01;248(3):370-7.
10. Matsumura G, Miyagawa-Tomita S, Shin'oka T, Ikada Y, Kurosawa H. First evidence that bone marrow cells contribute to the construction of tissue-engineered vascular autografts in vivo. Circ [Internet]. 2003 10/07;108(14):1729-34.
11. Noishiki Y, Tomizawa Y, Yamane Y, Matsumoto A. Autocrine angiogenic vascular prosthesis with bone marrow transplantation. Nat Med [Internet]. 1996 01/01;2(1):90-3.
12. Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, Magner M, Isner JM, Asahara T. Ischemia- and cytokine-induced mobilization of bone marrow-derived

endothelial progenitor cells for neovascularization. Nat Med [Internet]. 1999 04/01;5(4):434-8.
13. Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, Kearne M, Magner M, Isner JM. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res [Internet]. 1999 08/06;85(3):221-8.
14. Matsumura G, Ishihara Y, Miyagawa-Tomita S, Ikada Y, Matsuda S, Kurosawa H, Shin'oka T. Evaluation of tissue-engineered vascular autografts. Tissue Eng [Internet]. 2006 11/01;12(11):3075-83.
15. Hibino N, Shin'oka T, Matsumura G, Ikada Y, Kurosawa H. The tissue-engineered vascular graft using bone marrow without culture. J Thoracic Cardiovasc Surg [Internet]. 2005 05/01;129(5):1064-70.
16. Matsumura G, Hibino N, Ikada Y, Kurosawa H, Shin'oka T. Successful application of tissue engineered vascular autografts: Clinical experience. Biomater [Internet]. 2003 06/01;24(13):2303-8.
17. Naito Y, Imai Y, Shin'oka T, Kashiwagi J, Aoki M, Watanabe M, Matsumura G, Kosaka Y, Konuma T, Hibino N, Murata A, Miyake T, Kurosawa H. Successful clinical application of tissue-engineered graft for extracardiac fontan operation. J Thoracic Cardiovasc Surg [Internet]. 2003 02/01;125(2):419-20.
18. Shin'oka T, Matsumura G, Hibino N, Naito Y, Watanabe M, Konuma T, Sakamoto T, Nagatsu M, Kurosawa H. Midterm clinical result of tissue-engineered vascular autografts seeded with autologous bone marrow cells. J Thoracic Cardiovasc Surg [Internet]. 2005 06/01;129(6):1330-8.
19. Hibino N, McGillicuddy E, Matsumura G, Ichihara Y, Naito Y, Breuer C, Shinoka T. Late-term results of tissue-engineered vascular grafts in humans. J Thoracic Cardiovasc Surg [Internet]. 2010 02/01;139(2):431-e2.
20. Roh JD, Nelson GN, Brennan MP, Mirensky TL, Yi T, Hazlett TF, Tellides G, Sinusas AJ, Pober JS, Saltzman WM, Kyriakides TR, Breuer CK. Small-diameter biodegradable scaffolds for functional vascular tissue engineering in the mouse model. Biomater [Internet]. 2008 04/01;29(10):1454-63.
21. Roh JD, Sawh-Martinez R, Brennan MP, Jay SM, Devine L, Rao DA, Yi T, Mirensky TL, Nalbandian A, Udelsman B, Hibino N, Shinoka T, Saltzman WM, Snyder E, Kyriakides TR, Pober JS, Breuer CK. Tissue-engineered vascular grafts transform into mature blood vessels via an inflammation-mediated process of vascular remodeling. Proc Natl Acad Sci [Internet]. 2010 03/09;107(10):4669-74.
22. Hibino N, Villalona G, Pietris N, Schoffner A, Roh J, Yi T, Wawrzyniec D, Mejias D, Sawh-Martinez R, Harrington J, Sinusas A, Krause D, Kyriakides T, Saltzman M, Pober J, Shinoka T, Breuer CK. Tissue Engineered Vascular Grafts form neovessels that arise from regeneration of the adjacent blood vessel. *In press*.
23. Ortenwall P, Bylock A, Kjellstrom BT, Risberg B. Seeding of ePTFE carotid interposition grafts in sheep and dogs: Species-dependent results. surg [Internet]. 1988 02/01;103(2):199-205.
24. Goyal A, Wang Y, Su H, Dobrucki LW, Brennan M, Fong P, Dardik A, Tellides G, Sinusas A, Pober JS, Saltzman WM, Breuer CK. Development of a model system for

preliminary evaluation of tissue-engineered vascular conduits. J ped Surg [Internet]. 2006 04/01;41(4):787-91.
25. Nelson GN, Mirensky T, Brennan MP, Roh JD, Yi T, Wang Y, Breuer CK. Functional small-diameter human tissue-engineered arterial grafts in an immunodeficient mouse model: Preliminary findings. Arch Surgery [Internet]. 2008 05/01;143(5):488-94.
26. Hibino N, Yi T, Duncan D, Rathore A, Dean E, Naito T, Dardik A, Kyriakides T, Madri J, Pober J, Shinoka T, Breuer CK. A Critical Role of Macrophages on Neovessel Formation and the Development of Stenosis in Tissue Engineered Vascular Grafts. *In press*.
27. Chang CJ, Ko PJ, Hsu LA, Ko YS, Ko YL, Chen CF, Huang CC, Hsu TS, Lee YS, Pang JH. Highly increased cell proliferation activity in the restenotic hemodialysis vascular access after percutaneous transluminal angioplasty: Implication in prevention of restenosis. Am J Kidney Dis [Internet]. 2004 01/01;43(1):74-84.
28. Roy-Chaudhury P, Kelly BS, Miller MA, Reaves A, Armstrong J, Nanayakkara N, Heffelfinger SC. Venous neointimal hyperplasia in polytetrafluoroethylene dialysis grafts. Kidney Int [Internet]. 2001 06/01;59(6):2325-34.
29. Kearney M, Pieczek A, Haley L, Losordo DW, Andres V, Schainfeld R, Rosenfield K, Isner JM. Histopathology of in-stent restenosis in patients with peripheral artery disease. Circ [Internet]. 1997 04/15;95(8):1998-2002.
30. Lee T, Roy-Chaudhury P. Advances and new frontiers in the pathophysiology of venous neointimal hyperplasia and dialysis access stenosis. Adv Chronic Kidney Dis [Internet]. 2009 09/01;16(5):329-38.
31. Patel SD, Waltham M, Wadoodi A, Burnand KG, Smith A. The role of endothelial cells and their progenitors in intimal hyperplasia. Ther Adv Cardiovasc Dis [Internet]. 2010 03/03;4(2):129-41.
32. Gaspardone A, Crea F, Versaci F, Tomai F, Pellegrino A, Chiariello L, GioffrÃ¨ PA. Predictive value of C-reactive protein after successful coronary-artery stenting in patients with stable angina. Am J Cardiol [Internet]. 1998 08/15;82(4):515-8.
33. Welt FG, Rogers C. Inflammation and restenosis in the stent era. Arterioscl Thromb Vasc Biol [Internet]. 2002 11/01;22(11):1769-76.
34. Furukawa Y, Matsumori A, Ohashi N, Shioi T, Ono K, Harada A, Matsushima K, Sasayama S. Anti-monocyte chemoattractant protein-1/monocyte chemotactic and activating factor antibody inhibits neointimal hyperplasia in injured rat carotid arteries. Circ Res [Internet]. 1999 02/19;84(3):306-14.
35. Roque M, Kim WJ, Gazdoin M, Malik A, Reis ED, Fallon JT, Badimon JJ, Charo IF, Taubman MB. CCR2 deficiency decreases intimal hyperplasia after arterial injury. Arterioscl Thromb Vasc Biol [Internet]. 2002 04/01;22(4):554-9.
36. Mori E, Komori K, Yamaoka T, Tanii M, Kataoka C, Takeshita A, Usui M, Egashira K, Sugimachi K. Essential role of monocyte chemoattractant protein-1 in development of restenotic changes (neointimal hyperplasia and constrictive remodeling) after balloon angioplasty in hypercholesterolemic rabbits. Circ [Internet]. 2002 06/18;105(24):2905-10.
37. Andersen HO, Hansen BF, Holm P, Stender S, Nordestgaard BG. Effect of cyclosporine on arterial balloon injury lesions in cholesterol-clamped rabbits: T

lymphocyte-mediated immune responses not involved in balloon injury-induced neointimal proliferation. Arterioscl Thromb Vasc Biol [Internet]. 1999 07/01;19(7):1687-94.
38. Ferns GA, Reidy MA, Ross R. Balloon catheter de-endothelialization of the nude rat carotid. response to injury in the absence of functional T lymphocytes. Am J Pathol [Internet]. 1991 04/01;138(4):1045-57.
39. Hansson GK, Holm J, Holm S, Fotev Z, Hedrich HJ, Fingerle J. T lymphocytes inhibit the vascular response to injury. Proc Natl Acad Sci [Internet]. 1991 12/01;88(23):10530-4.
40. Gallin JI, Bujak JS, Patten E, Wolff SM. Granulocyte function in the chediak-higashi syndrome of mice. Blood [Internet]. 1974 02/01;43(2):201-6.
41. The beige mutation in the mouse. I. A stem cell predetermined impairment in natural killer cell function. J Immunol [Internet]. 1979 11/01;123(5):2168-73.
42. Serotonin deficiency and prolonged bleeding in beige mice. Proc Soc Exp Biol Med [Internet]. 1976 01/01;151(1):32-9.
43. Novak EK, Hui SW, Swank RT. Platelet storage pool deficiency in mouse pigment mutations associated with seven distinct genetic loci. Blood [Internet]. 1984 03/01;63(3):536-44.
44. Manka D, Collins RG, Ley K, Beaudet AL, Sarembock IJ. Absence of p-selectin, but not intercellular adhesion molecule-1, attenuates neointimal growth after arterial injury in apolipoprotein e-deficient mice. Circ [Internet]. 2001 02/20;103(7):1000-5.
45. Smyth SS, Reis ED, Zhang W, Fallon JT, Gordon RE, Coller BS. Beta(3)-integrin-deficient mice but not P-selectin-deficient mice develop intimal hyperplasia after vascular injury: Correlation with leukocyte recruitment to adherent platelets 1 hour after injury. Circ [Internet]. 2001 05/22;103(20):2501-7. :
46. Barron MK, Lake RS, Buda AJ, Tenaglia AN. Intimal hyperplasia after balloon injury is attenuated by blocking selectins. Circ [Internet]. 1997 11/18;96(10):3587-92.
47. van Weel V, Toes RE, Seghers L, Deckers MM, de Vries MR, Eilers PH, Sipkens J, Schepers A, Eefting D, van Hinsbergh VW, van Bockel JH, Quax PH. Natural killer cells and CD4+ T-cells modulate collateral artery development. Arterioscl Thromb Vasc Biol [Internet]. 2007 11/01;27(11):2310-8.
48. Ström A, Wigren M, Hultgårdh-Nilsson A, Saxena A, Gomez MF, Cardell S, Fredrikson GN, Nilsson J. Involvement of the CD1d-natural killer T cell pathway in neointima formation after vascular injury. Circ Res [Internet]. 2007 10/12;101(8):e83-9.
49. Intimal hyperplasia in murine models. Curr Drug Targets [Internet]. 2008 03/01;9(3):251-60.
50. Cyrus T, Sung S, Zhao L, Funk C, Tang S, Praticó D. Effect of low dose aspirin on vascuar inflammation, plaque stability, and atherogenesis in low-density lipoprotein receptor-deficient mice. Circ 2002 Sep 4;106(10):1282-7.
51. Bulckaen H, Prévost G, Boulanger E, Robitaille G, Roquet V, Gaxatte C, Garçon G, Corman B, Gosset P, Shirali P, Creusy C, Puisieux F. Low-dose aspirin prevents age-related endothelial dysfunction in a mouse model of physiological aging. Am J Physiol Heart Circ Physiol 2008 Apr; 294(4):H1562-70.

52. Abele S, Weyand M, Wollin M, Hiemann NE, Harig F, Fischlein T, Ensminger SM. Clopidogrel reduces the develoment of transplant arteriosclerosis. J Thorac Cardiovasc Surg 2006 May;131(5):1161-6