EphB4 monomer inhibits chronic graft vasculopathy in an aortic transplant model

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ABSTRACT

T cells and macrophages play an important role in the formation of allograft vasculopathy, which is the predominant form of chronic rejection in cardiac transplants. Arteries express Ephrin-B2 as a marker of arterial identity, whereas circulating monocytes express the cognate receptor EphB4, which facilitates monocyte adhesion to the endothelial surface. Adherent monocytes transmigrate and differentiate into macrophages that activate T cells and are a main source of tissue damage during rejection. We hypothesized that inhibition of Ephrin-B2-EphB4 binding would decrease immune cell accumulation within a transplanted graft and prevent allograft vasculopathy. We used EphB4 monomer to inhibit Ephrin-B2-EphB4 binding in a rat infrarenal aortic transplant model. Rats treated with EphB4 monomer had fewer macrophages and T cells in the aortic allografts at 28 days, as well as significantly less neointima formation. These data show that the Ephin-B2-EphB4 axis may be an important target for prevention or treatment of allograft vasculopathy. (JVS—Vascular Science 2023;4:100109.)

Keywords: Ephrin-B2; Eph-B4; Allograft; Allograft vasculopathy; Neointimal hyperplasia

Allograft vasculopathy is usually due to neointimal hyperplasia leading to decreased blood flow to an allograft with subsequent organ ischemia. This phenomenon is present in ≤50% of heart transplant recipients at 10 years and is the predominant form of chronic rejection.[1] Importantly, macrophages and T cells play an essential role in the development of chronic graft vasculopathy.[2,3] Therefore, interventions that target macrophages and T cells may be an important therapeutic target to prevent allograft vasculopathy.

The Eph receptors are the largest family of tyrosine kinase receptors and interact with cell membrane-bound Ephrin ligands; although Ephrin-A ligands are membrane anchored, the Ephrin-B ligands are transmembrane and thus capable of inducing bidirectional signal transduction.[4] There are five different EphB receptors that bind to three different Ephrin-B ligands.[5] Interaction between the Ephrin-B ligands and EphB receptors has several functions during development including arteriovenous specification; in adults, Ephrin-B2-EphB4 interaction seems to be an important signaling pathway regulating the immune response.

Juxtacrine cell signaling via Eph receptors and their Ephrin ligands regulates inflammation; endothelial EphrinB2 interacts with monocyte EphB4 receptors to promote monocyte adhesion and transmigration through the endothelium.[6] Ephrin-B2 also activates T cells, leading to maturation and cell proliferation.[7,8] Therefore, targeting Ephrin-B2-EphB4 interaction may be a potential treatment or prevention strategy for allograft vasculopathy.

EphB monomer is a new and highly specific therapy to inhibit the immune response; by binding Ephrin ligands, the monomer prevents both forward and reverse signaling.[9,10] For example, EphB4 monomer decreased inflammation and damage to the colon in an inflammatory bowel disease model via reduction of T cell accumulation in the mesenteric lymph nodes.[10] We hypothesized that the Ephrin-Eph axis is a novel regulator of immune activation that leads to chronic graft vasculopathy; inhibition of this axis may diminish immune cells within the graft to prevent rejection. We tested this hypothesis using EphB4 monomer to prevent Ephrin-Eph signaling in a rat aortic transplant model.

METHODS

Animal model. All animal experiments were performed in compliance with federal guidelines and with approval from the institutional Animal Care and Use Committee of Yale University. Lewis rats were between 36 and

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42 days old and Sprague-Dawley rats were between 250 and 350 g (Charles River Laboratories, Wilmington, MA), with five or six rats per group. Aortic transplantation was performed as previously described.[11] Briefly, rats were anesthetized with vaporized isoflurane. A laparotomy incision was made and a 1-cm segment of infrarenal aorta was harvested from either a Lewis rat (allograft) or Sprague-Dawley rat (isograft). These segments were flushed with ice-cold normal saline and stored in ice-cold normal saline while the recipient Sprague-Dawley rat was prepared. The recipient rat also had a laparotomy incision and the infrarenal aorta was exposed and controlled with vascular clamps. A 1-cm segment of aorta was excised, and the donor aorta was transplanted in an end-to-end fashion using 10-0 nylon suture. The laparotomy incision was closed with 4-0 nylon suture. Rats then had subdermal injections with either EphB4 monomer (20 μg/kg; Sino Biological Inc., Beijing, China) or IgG control (Sino Biological Inc.). EphB4 monomer dosing was based on a previous study at which this was an effective dose.[10] These injections were continued daily for a total of 28 days. Daily weights were measured using an Ohaus CS-2000 Portable Digital Scale (Ohaus, Parsippany, NJ).

Doppler ultrasound examination (Vevo770 High-Resolution Imaging System; Fujifilm Visual Sonics Inc., Toronto, Canada) using probe RMV704 (40 MHz) was used for in vivo measurements of flow velocities and aortic diameter and performed before surgery for baseline values and then serially every 7 days throughout the study. Mean wall shear stress was calculated using the Poiseuille parabolic model $S = 8 \mu V/D$, where S is the shear stress (dyne/cm^2), μ is blood viscosity (estimated to be 0.035 Poise), V is velocity (cm/s), and D is diameter (cm).[12]

Histology. Animals were euthanized at day 28 and perfused with normal saline followed by 10% formalin via the left ventricle. The graft was then harvested and embedded in paraffin. We used 5-μm sections from the center of the graft. Elastin Van Gieson staining was used to measure intima-media thickness. Digital images of the sections were captured with a microscope (BX40; Q Color 5; Olympus America, Center Valley, PA) and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

Immunofluorescence. We de-paraffinized 5-μm sections from the center of the graft using xylene and rehydrated them in graded series of alcohols. Sections were heated in citric acid buffer (pH 6.0) at 100°C for 10 minutes for antigen retrieval. The sections were then blocked with 2% bovine serum albumin for 1 hour at room temperature and then incubated overnight at 4°C with either CD68 (ab31630; Abcam) or CD3 (ab11089). Sections were then treated with secondary antibodies at

room temperature for 1 hour using Alexa Fluor 488-conjugated IgG (Life Technologies, Eugene, OR). Sections were stained with Slow Fade Gold Antifade Mount with DAPI (Life Technologies) and a coverslip was applied. Images were captured on an EVOS FL auto Imaging microscope and cells counted per image.

Statistical analyses. Data were analyzed using Prism 9 software (GraphPad Software, Inc., La Jolla, CA). Data are shown as mean ± standard error of the mean. The nonparametric Mann-Whitney *U* test was used for two-group comparisons owing to small sample sizes. *P* values of ≤.05 were considered significant.

RESULTS

Safety of EphB4 monomer. Systemic daily administration of EphB4 monomer (20 μg/kg) or control IgG (20 μg/kg) over 28 days was not associated with weight loss; there were no significant difference in weight in either the allograft or isograft groups (Fig 1, *A*). Similarly, serial ultrasound measurements of graft diameter and blood flow velocity (Fig 1, *B* and *C*), as well as calculated shear stress (Fig 1, *D*), showed no significant differences between allografts or isografts that received either EphB4 monomer or IgG. These data are consistent with lack of systemic effects or changes in hemodynamics, that is, the safety, of EphB4 monomer.

EphB4 monomer decreased inflammatory cell infiltration. Both the innate immune response, characterized by macrophages, and the adaptive immune response, characterized by T cells, play important roles in acute and chronic rejection,[13] such as present in allograft vasculopathy within transplanted organs.[14] To assess the potential function of the Ephrin-Eph axis as part of the innate immune response, we determined the effect of EphB4 monomer on CD68$^+$ cells within the aortic grafts. As expected, allografts were more immunogenic compared with isografts, with greater numbers of CD68$^+$ cells present throughout aortic wall in the allografts (Fig 2, *A*). There was no difference in the number of CD68$^+$ cells that were present within the isograft between the groups treated with EphB4 monomer or IgG (Fig 2, *B*). However, there were significantly decreased numbers of CD68$^+$ cells in allografts among rats treated with EphB4 monomer compared with those treated with IgG (Fig 2, *C*). These data are consistent with EphB4 monomer decreasing the innate immune response in allografts.

We next assessed the potential function of the Ephrin-Eph axis on the adaptive immune response and determined the effect of EphB4 monomer on CD3$^+$ cells within the aortic grafts. As expected, there were more CD3$^+$ cells in the allografts compared with the isografts (Fig 3, *A*). There was no difference in the number of CD3$^+$ cells that were present within the isograft between
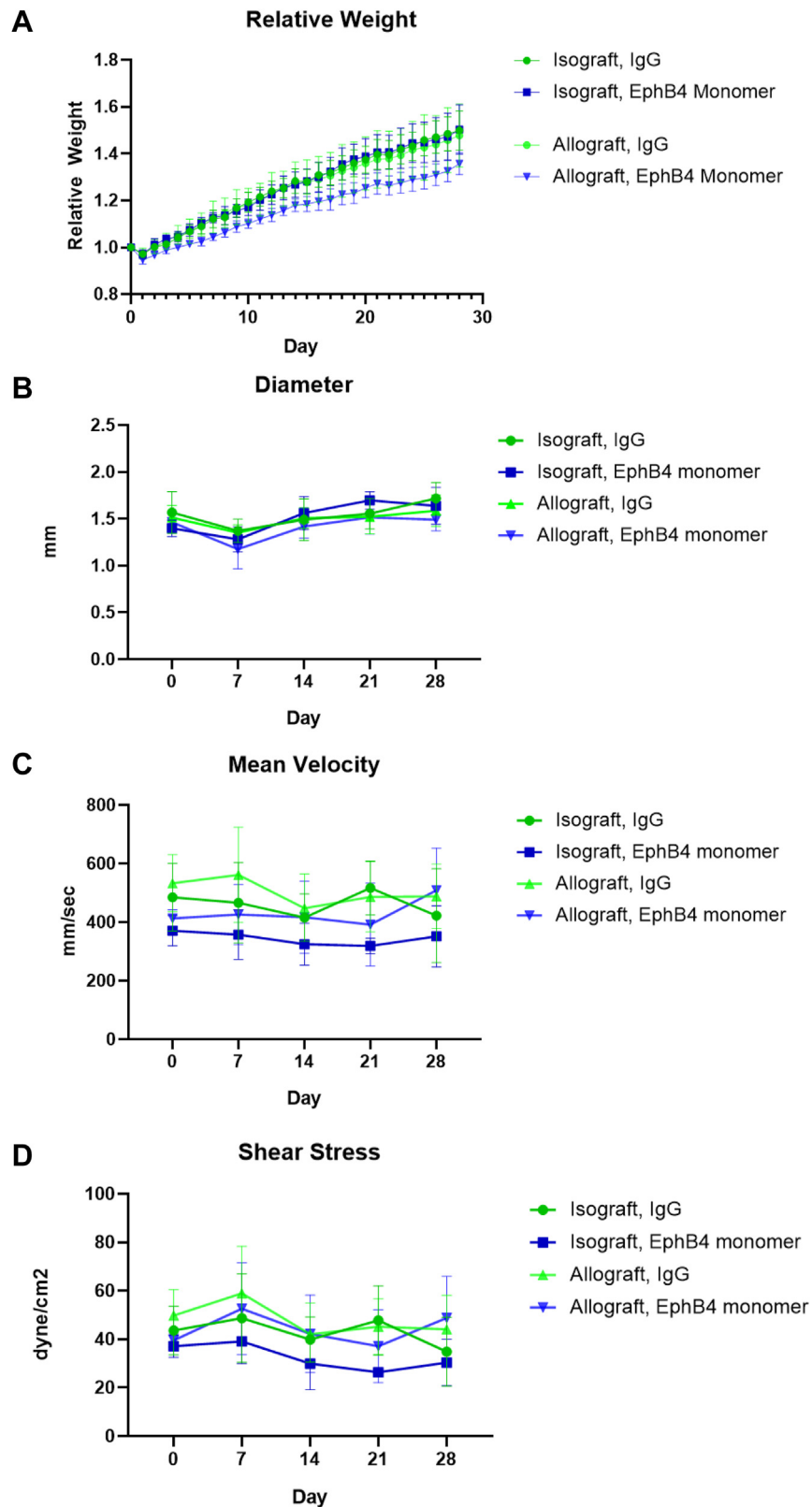
Fig 1. Graft type and treatment does not affect weight or blood flow. **(A)** Relative weight of animals compared to baseline weight. **(B)** Diameter of grafts measured serially with ultrasound over time. **(C)** Mean velocity of blood flow through the graft. **(D)** Mean calculated shear stress over time. (n = 5-6 rats per group.)
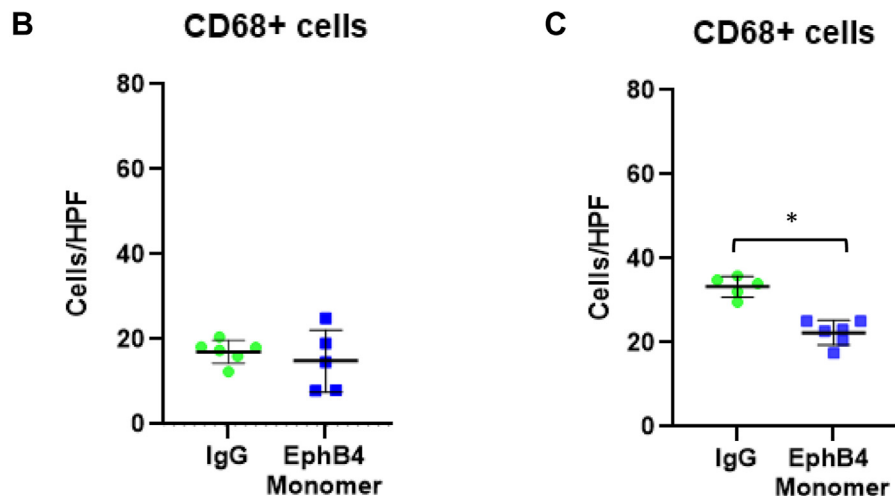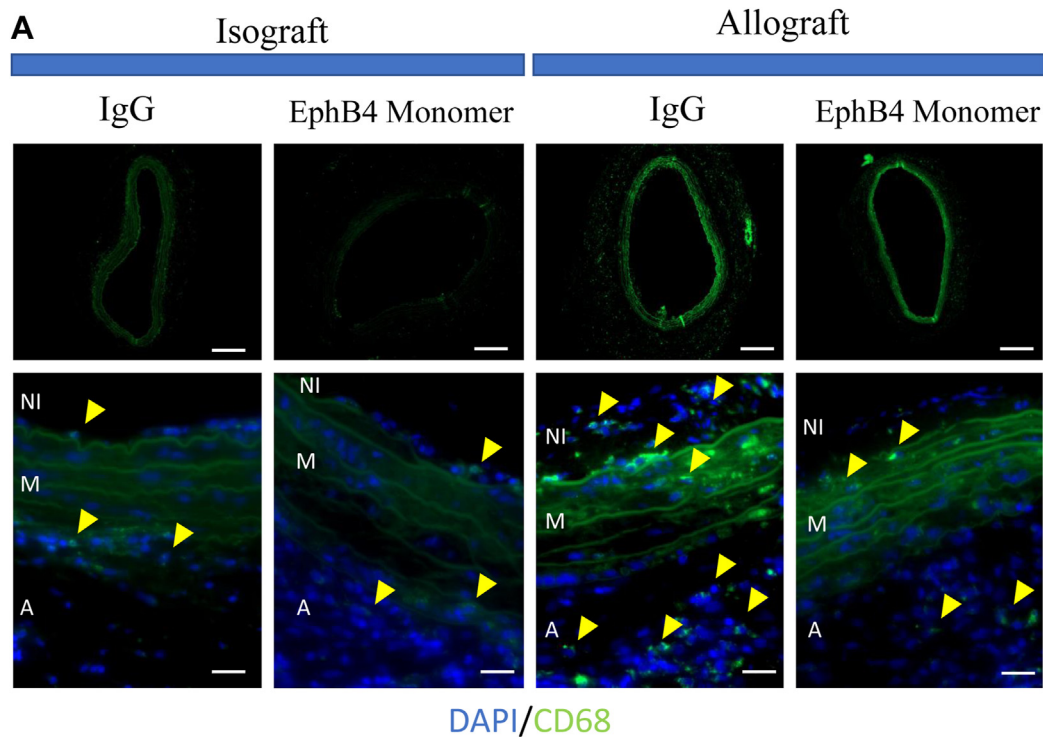
A

Fig 2. EphB4 monomer reduces CD68$^+$ cell infiltration in allografts. **(A)** Representative immunofluorescence of isografts and allografts (day 28) treated with either control IgG or EphB4 monomer; top row, 40× (scale bar, 250 μm); bottom row, 400× (scale bar, 25 μm). **(B)** Mean number of CD68$^+$ cells per high-power field (*HPF*) in isografts; *P* = .66 (Mann-Whitney *U* test; n = 5-6 rats per group). **(C)** Mean number of CD68$^+$ cells per HPF in allografts; *P* ≤ .01 (Mann-Whitney *U* test; n = 5-6 rats per group).

the groups treated with EphB4 monomer or IgG (Fig 3, *B*). However, there were significantly decreased numbers of CD3$^+$ cells in allografts among rats treated with EphB4 monomer compared with those treated with IgG (Fig 3, *C*). These data are consistent with EphB4 monomer reducing the adaptive immune response in allografts, that is the Ephrin-Eph signaling axis may regulate inflammation and/or the immune response after transplant.

EphB4 monomer decreased neointimal thickness. Because the EphB4 monomer regulates immune cell infiltration into allografts (Figs 2 and 3), we next determined the effect of EphB4 monomer on neointimal thickening, an accepted model of allograft
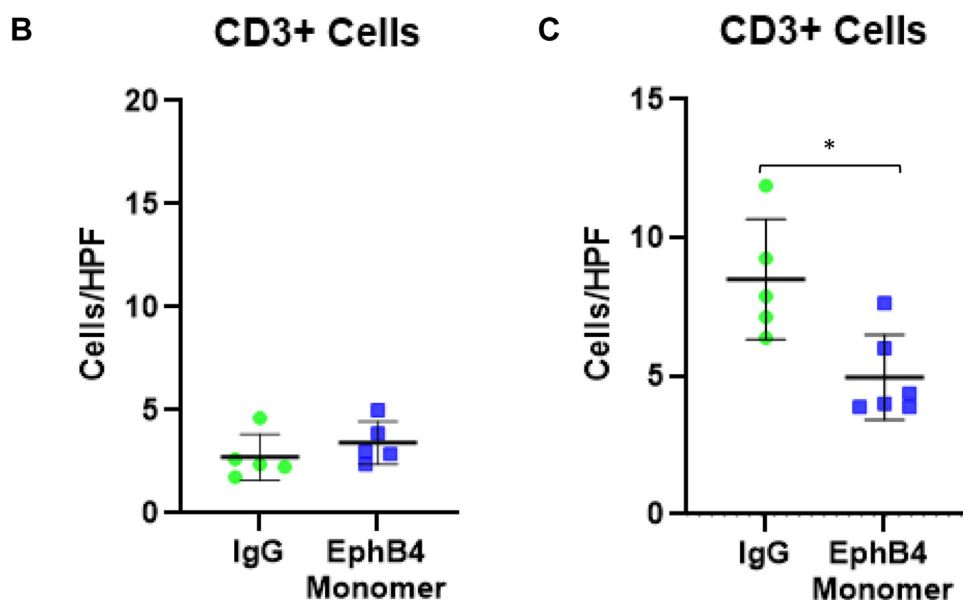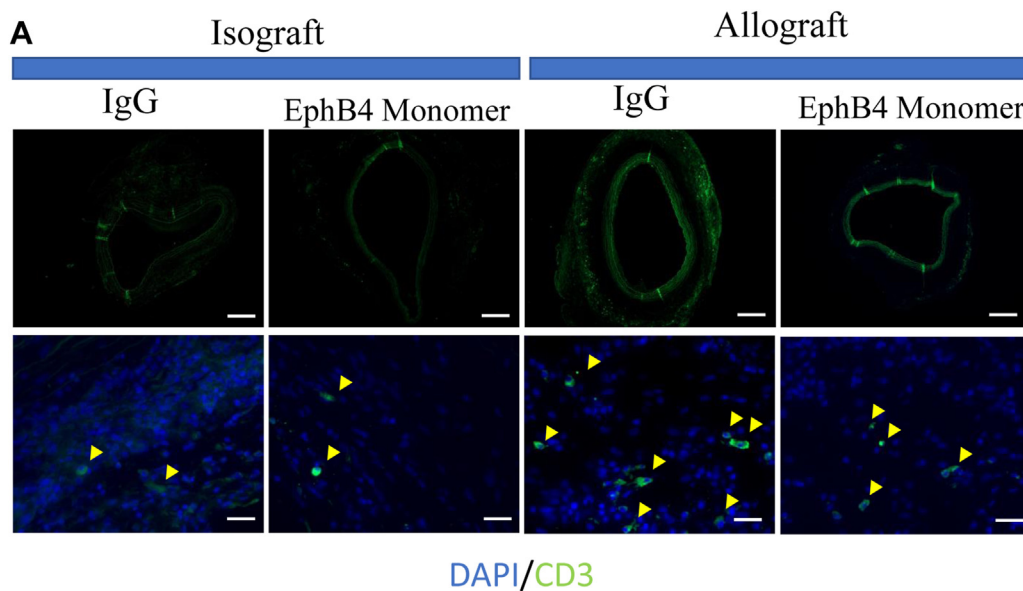
Fig 3. EphB4 monomer reduces CD3$^+$ cell infiltration in allografts. **(A)** Representative immunofluorescence of isografts and allografts (day 28) treated with either control IgG or EphB4 monomer; top row, 40× (scale bar, 250 μm); bottom row, 400× (scale bar, 25 μm). **(B)** Mean number of CD3$^+$ cells per high-power field (*HPF*) in isografts (*P* = .17, Mann-Whitney *U* test ; n = 5-6 rats per group). **(C)** Mean number of CD3$^+$ cells per HPF in allografts; *P* = .02 (Mann-Whitney *U* test; n = 5-6 rats per group).

vasculopathy.[14] As expected, little neointima was present in isografts (Fig 4, *A*), and there was no difference in the thickness of neointima among rats treated with EphB4 monomer compared with those treated with IgG (Fig 4, *B-D*). However, there was significantly less neointima in allografts among rats treated with EphB4 monomer compared with those treated with IgG (Fig 4, *E-G*). These data are consistent with the Ephrin-

Eph signaling axis regulating neointimal thickness after transplantation and suggest a potential translational role of this axis in regulation of graft vasculopathy.

DISCUSSION

We show that EphB4 monomer is associated with reduced number of infiltrating macrophages and T cells in an aortic graft (Figs 2 and 3); in addition, fewer
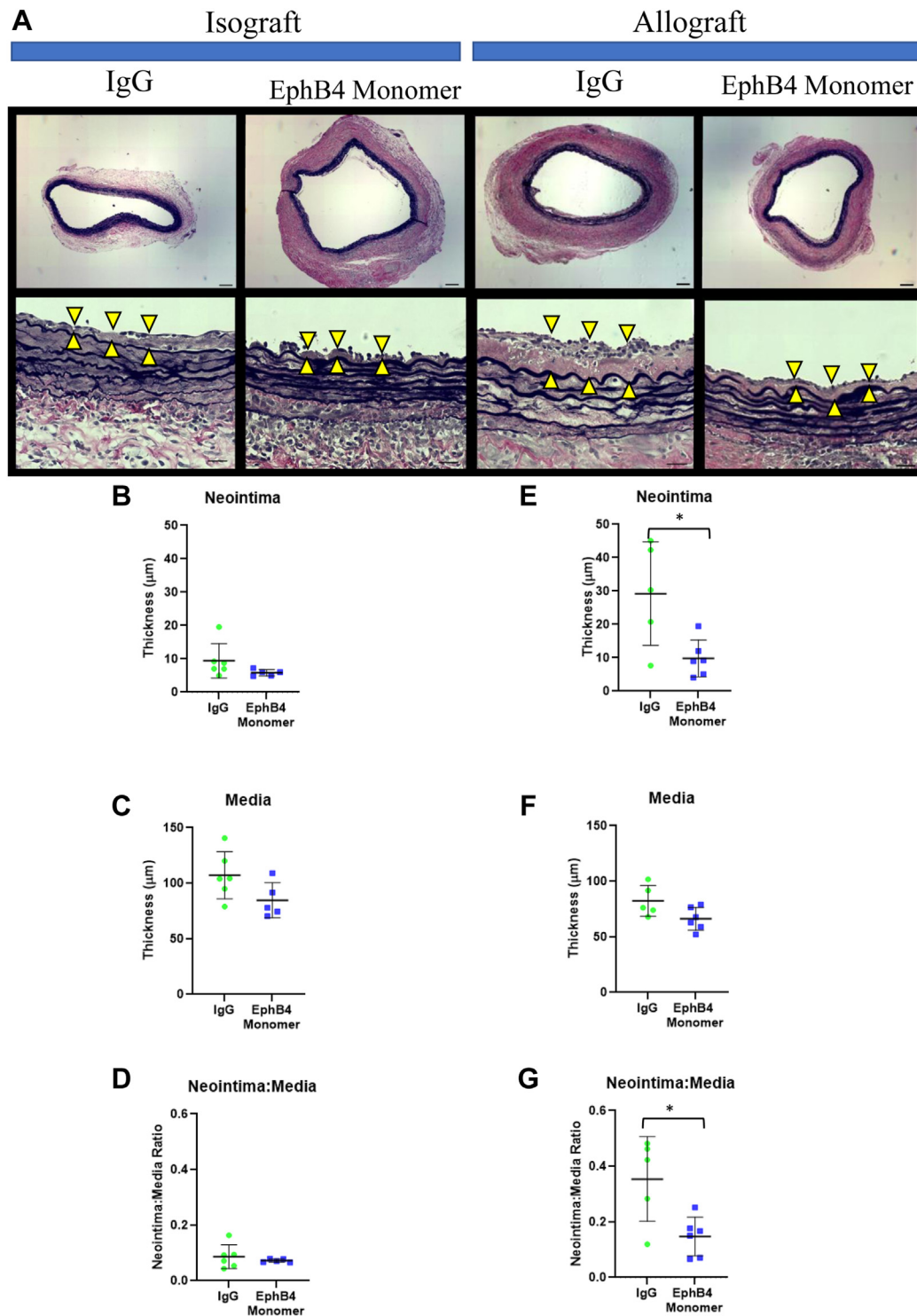
Fig 4. EphB4 monomer reduces neointimal thickness in allografts. **(A)** Representative EVG staining of isografts and allografts (day 28) treated with either control IgG or EphB4 monomer; top row, 40× (scale bar, 250 μm); bottom row, 400× (scale, bar 25 μm). **(B)** Mean neointimal thickness in isografts ($P = .08$, Mann-Whitney U test; n = 5-6 rats per group). **(C)** Mean medial thickness in isografts ($P = .08$, Mann-Whitney U test; n = 5-6 rats per group). **(D)** Ratio of neointima:media thickness in isografts ($P = .66$, Mann-Whitney U test; n = 5-6 rats per group). **(E)** Mean neointimal thickness in allografts ($P = .05$, Mann-Whitney U test; n = 5-6 rats per group). **(F)** Mean medial thickness in allografts ($P = .12$, Mann-Whitney U test; n = 5-6 rats per group). **(G)** Ratio of neointima:media thickness in allografts ($P = .05$, Mann-Whitney U test; n = 5-6 rats per group).

infiltrating cells are associated with decreased graft neo-intima thickness (Fig 4). These data suggest that, in an aortic transplant model, which is an established model for chronic graft vasculopathy,[15-17] EphB4 monomer decreases the immune response to prevent neointimal thickness, suggesting its usefulness as a therapeutic strategy to prevent chronic graft vasculopathy.

Chronic rejection depends on multiple cell types, but both T cells and macrophages are critical.[2,13] T cells are essential regulators of macrophages; in a mouse model in which T cells were absent, there were no infiltrates of macrophages; reintroduction of T cells led to the presence of macrophages and neointimal formation in allografts.[13] Macrophages may be a critical effector of chronic graft vasculopathy; depletion of macrophages led to decreased allograft vasculopathy without change in the number of T cells in a mouse heterotopic heart transplant model.[2] These data suggest that both T cells and macrophages play roles in the formation of chronic graft vasculopathy; our data are consistent with these roles, because EphB4 monomer decreased the number macrophages and T cells in the allografts (Figs 2 and 3) that was associated with decreased neointimal thickening (Fig 4). There was no difference between the treatment groups of the isografts (Figs 2-4), which is likely due to isografts being less immunogenic and, therefore, not causing as large an inflammatory response as allografts.

Ephrin and Eph signaling are crucial in endothelial cell behavior during development, but also are active in the adult vasculature. Mouse models have shown that disruption of the EphB4-EphrinB2 signaling axis can cause pathologic cardiac remodeling.[18] Because EphB4 monomer inhibits EphB4-EphrinB2 signaling, we evaluated for potential hemodynamic changes that could suggest off target effects. Because the EphB4 monomer did not have any significant effects on graft blood flow (Fig 1), these data suggest a lack of significant off-target hemodynamic effects; in addition, the lack of significant weight change suggests that EphB4 is well-tolerated.

Our study has several limitations. First, we only assessed a single dose of EphB4 monomer (20 μg/kg); because previous data suggest a dose-dependent response, it is possible that a more optimal treatment dose exists.[10] Second, we only evaluated the effect of EphB4 on the graft and did not evaluate the effects on other organ systems or the circulating immune cells; thus, we cannot establish a mechanism of EphB4 monomer action. Similarly, we did not assess systemic markers of inflammation that could detect a decreased systemic inflammatory response. Third, there may be toxic or off-target effects that we did not identify in this limited study; similarly, we only evaluated a single time point; additional times could show additional hemodynamic effects of EphB4 monomer.

In summary, EphB4 monomer inhibits T cell and macrophage accumulation in aortic allografts, which is associated with decreased neointimal thickness. The Eph-B4 monomer may be a novel therapeutic strategy to prevent or treat chronic graft vasculopathy.

AUTHOR CONTRIBUTIONS

Conception and design: JL, LG, RT, AD
Analysis and interpretation: JL, LG, RT, AB, WZ, AD
Data collection: JL
Writing the article: JL
Critical revision of the article: JL, LG, RT, AB, WZ, AD
Final approval of the article: JL, LG, RT, AB, WZ, AD
Statistical analysis: JL
Obtained funding: AD
Overall responsibility: JL

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