Title: Endothelial phosphoinositide 3-kinase-β inactivation confers protection from immune-mediated vascular injury

Running Title: PI3Kβ inactivation confers vascular protection

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Abbreviations

EC	endothelial cell
ECβKO	endothelial cell PI3K beta knockout
Cleaved caspase 3	aCasp3
GPCR	G protein-coupled receptor
HAEC	human aortic endothelial cells
HUVEC	human umbilical vein endothelial cells
PI3K	phosphoinositide 3-kinase
TNF	tumor necrosis factor
VSMC	vascular smooth muscle cell

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Abstract

Heart transplant and recipient survival is limited by immune cell-mediated injury of the graft vasculature. We examined the role of the phosphoinositide 3-kinase-β (PI3Kβ) isoform in endothelial cells (EC) during coronary vascular immune injury and repair in mice. In minor HY-antigen mismatched allogeneic heart grafts, a robust immune response was mounted to each wild-type, PI3Kβ inhibitor-treated, or endothelial-selective PI3Kβ knockout (ECβKO) grafts transplanted to wild-type recipients. However, microvascular EC loss and progressive occlusive vasculopathy only developed in the control, but not PI3Kβ -inactivated hearts. We observed a delay in inflammatory cell infiltration of the ECβKO grafts, particularly in the coronary arteries. Surprisingly, this was accompanied by impaired display of pro-inflammatory chemokine and adhesion molecules by the ECβKO ECs. *In vitro*, TNFα-stimulated endothelial ICAM1 and VCAM1 expression was blocked by PI3Kβ inhibition or RNAi. Selective PI3Kβ inhibition also blocked TNFα-stimulated degradation of IKBα, and nuclear translocation of NF-kB p65 in EC. These data identify PI3Kβ as a therapeutic target to reduce vascular inflammation and injury.

1. INTRODUCTION

Solid-organ transplant and heart transplant recipient survival is limited by chronic immune responses directed against the graft vasculature (1-3). In heart transplants, immune-mediated vascular injury affects both coronary arteries and the heart microvasculature (4, 5). Loss of the microvasculature and arterial injury is mitigated by dynamic vascular repair (6, 7), but the maladaptive repair response elicited in coronary arteries results in progressive occlusion of the arterial lumen and progresses despite modern immune suppression regimes. These therapies target mononuclear leukocyte proliferation and function, hence, there is a need to develop complementary clinical strategies that address the vascular response to inflammation and injury.

We previously demonstrated that expression of local vascular pro-reparative genes, such as the endothelial-derived peptide apelin, are induced by allo-immune vascular injury to mitigate structural damage of the coronary arteries (7). The apelin receptor is expressed by vascular smooth muscle and endothelial cells (ECs). In ECs the apelin receptor is linked, among others, to the pro-reparative phosphoinositide 3-kinase (PI3K) signal transduction pathway via the p110β catalytic isoform (8, 9). However, it is not clear how apelinergic signals are translated by vascular cells to mitigate injury.

Endothelial PI3Kβ activity links G protein coupled cell surface receptors (GPCRs) to mTOR and other effectors such as AKT (10, 11). The class I PI3K family comprises four catalytic subunits (p110α, β, γ and δ), each associated with a regulatory subunit (12). The

p110α and p110β catalytic isoforms are broadly expressed across cell types, while p110γ and p110δ are mainly found in leukocytes (13). The p110α isoform is the dominant form coupled to receptor tyrosine kinases such as the Vascular Endothelial Growth Factor receptor-2 in EC (14), whereas p110β and p110γ are coupled to pro-angiogenic endothelial GPCRs (14).

In this report, we studied the effect of vascular endothelial PI3Kβ inactivation on immune cell-mediated vascular injury. The experiments reveal a previously unknown requirement for endothelial PI3Kβ activity to regulate EC expression of pro-inflammatory chemokines and adhesion molecules. Inactivation protects against vascular injury and subsequent transplant vasculopathy.

2. MATERIALS AND METHODS

2.1 Reagents

Primary antibodies are shown in Supplemental Table 1. Class II tetramers I-A^b mouse H-Y peptide NAGFNSNRANSSRSS, I-A^b human CLIP 87-101 peptide PVSKMRMATPLLMQA, and class I tetramers H-$2D^b$ mouse H-Y (Uty) 246-254 peptide WMHHNMDLI were synthesized by the NIH tetramer core facility (Emory University, Atlanta, GA; http://tetramer.yerkes.emory.edu). Purchased pre-designed or custom primer sets (Integrated DNA Technologies) are shown in Supplemental Table 2 & 3. The selective PI3Kβ inhibitor, GSK2636771 (Selleckchem; cat # S8002) was dissolved in 0.5% methylcellulose with 0.2% Tween 80 (Sigma Aldrich; cat # M7140 & P1754) and

administered to mice at 30 mg/kg/day by daily gavage starting at week 2 through week 6 post-transplantation (15). The PI3Kβ inhibitor TGX221 (cat # 663619-89-4) used *in vitro* was from Cayman Chemical.

2.2 Mice and heart transplantation

Animal experiments were carried out according to the Canadian Council on Animal Care guidelines, and were approved by the Animal Care and Use Committee at the University of Alberta. Wild-type male and female recipient C57BL/6 mice were purchased from Jackson Laboratories (cat # 000664). The PI3Kβ -deficient (Tie2-Cre^{ERT2}/ *Pik3cb*$^{flx/flx}$) and wild-type (*Pik3cb*$^{flx/flx}$) male littermate donor mice were bred on a C57BL/6 background at the University of Alberta as previously described (16). Donor mice were treated with tamoxifen, to delete *Pik3cb* in ECs, 2 weeks before heart harvest for heterotopic transplantation. Wild-type or mutant *Pik3cb* expression was confirmed by end-point PCR of lung (Suppl Fig 1).

2.3 Histological processing, staining, and image analysis

Details of tissue sample processing for histological analysis are provided in the Expanded Materials and Methods supplement.

2.4 qRT-PCR

The mouse heart apex, or micro-dissected coronary artery specimens were homogenized using a TissueLyser 2 (Qiagen), then RNA was extracted using the

RNAeasy micro-Kit (Qiagen), and used to synthesize cDNA with a QuantiTect Reverse Transcription Kit (Qiagen). qRT-PCR was done using an Eppendorf Mastercycler RealPlex2 thermocycler. Samples were normalized to the internal control (mouse or human *Hprt1*) and then to normal mouse heart samples or untreated ECs *in vitro*. Fold changes were calculated based on the log2−ΔΔCT method. Additional details are provided in the Expanded Materials and Methods supplement.

2.5 Mouse heart allograft cell isolation and flow cytometry

Leukocytes were isolated from the heart allografts as previously described (17). Additional details are provided in the Expanded Materials and Methods supplement.

2.6 Cell culture and RNA interference

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as previously described under approval from the Human Research Ethics Board of the University of Alberta (18). Additional details are provided in the Expanded Materials and Methods supplement.

2.7 Western blotting

Details are provided in the Expanded Materials and Methods supplement.

2.8 Statistical analysis

Datasets were tested for normality by the D'Agostino test using Prism 7 software (Graphpad). Data is presented as mean ± the standard error of mean (SEM). Using

Prism 7 software, statistical analysis was conducted by 1-way or 2-way ANOVA as appropriate followed by the Bonferroni post-hoc test. Pairwise comparisons were done by unpaired two-sided Student t-test, or in the case of the smaller datasets, the two-sided Mann Whitney test. A P value less than 0.05 was considered significant.

3. RESULTS

3.1 Endothelial cell PI3 kinase-β inactivation protects allografts from vasculopathy

Vascular injury and maladaptive repair limits the survival of heart and other vascularized solid organ allografts. We undertook investigation of the PI3K pathway, linked to the pro-reparative apelin receptor by the catalytic p110β isoform, to determine if EC PI3Kβ isoform activity is required to mitigate obliterative vasculopathy.

We exploited male to female heart transplantation in mice, known to elicit an indolent immune response and vascular disease akin to transplant vasculopathy in humans (19). In the first series of experiments, recipient mice were treated with the PI3Kβ isoform-selective oral inhibitor, GSK2636771, starting at 2 weeks after transplantation. The hearts were retrieved at 6 weeks post-transplantation. We found hearts harvested from recipients treated with the carrier developed marked expansion of the coronary arterial intima, largely occluding the arterial lumen (Fig 1 a, b). In striking contrast, arterial intimal expansion in hearts from GSK2636771-treated mice showed little change compared to non-transplanted donor hearts. Further, we observed the microvessel

density of the myocardial compartment in the vehicle-treated mice was reduced more than 50%, reflecting reperfusion and allo-immune injury directed against microvascular endothelium. GSK2636771-treatment protected the microvasculature from loss. This surprising result suggests that PI3Kβ inhibition in immune or parenchymal cells of the allograft attenuates vascular disease.

To determine the role of heart endothelial PI3Kβ in this vascular protection from the effects of immune injury, we transplanted hearts from inducible Tie2-Cre$^{ERT2+/-}$/ *Pik3cb*$^{f/f}$ (ECβKO) donor mice, pretreated with tamoxifen 2 weeks before the donor heart was harvested for transplant. The effect of endothelial PI3Kβ inactivation in ECs was compared with tamoxifen-treated CRE$^{ERT2-/-}$/ *Pik3cb*$^{f/f}$ littermates. The result of recombination was monitored by RT-PCR of donor lung endothelium, acquired at the time of donor heart transplantation (Suppl Fig 1). Hearts were recovered at 2 or 6 weeks post-transplant. Transplantation of syngeneic hearts of wild-type littermates to male recipients elicited little arteriopathy (Suppl Fig 2). However, we observed a progressive increase in the neo-intima expansion in the allogeneic littermate control heart transplants, with a reduction in the microvessel density in the myocardium (Fig 1c, d). In contrast, donor endothelial *Pik3cb* knockout protected the graft artery since intimal occlusion was similar to the syngeneic transplants. EC *Pik3cb* loss also protected the microvasculature. This finding establishes that loss of graft EC PI3Kβ activity, independent of PI3Kβ expression in the recipient immune system, is necessary to protect against vasculopathy.

The vascular endothelium is a target for allo-immune injury, hence we directly evaluated the effect of PI3Kβ inactivation on injury to the graft endothelium. As shown in Fig 2a (and Suppl Fig 3), discontinuities or gaps in the arterial endothelium were quantified from tissue sections immuno-stained for CD31. Isogeneic male-to-male heart transplants had no endothelial gaps detected at 2 weeks post-transplant. However, a progressive increase in the focal loss of endothelial cell coverage was observed in the littermate control male to female hearts at 2 and 6 weeks post-transplant. In contrast, in ECβKO hearts the area of focal gaps in the endothelium were markedly reduced at 2 weeks post-transplant, and did not measurably increase at the later timepoint. These data are supported by evaluation of activated caspase 3 immunostaining of the CD31-positive coronary arterial endothelium in 1 μm confocal tissue sections. Cleaved caspase 3 (aCasp3) staining of the endothelium, cells in the expanded intima, and medial vascular smooth muscle cells was evident in littermate control hearts (Fig 2b, c; Suppl Fig 4). A substantial reduction in the $CD31^+$ EC co-stained with aCasp3 was evident in the ECβKO coronary arteries. Notably, the aCasp3 staining of vascular smooth muscle cells (VSMC) was also markedly reduced in ECβKO hearts (Suppl Fig 4), indicating that PI3Kβ loss in ECs protected VSMC in the arterial media. Taken together these data show that the ECβKO graft coronary arteries displayed less immune cell-mediated injury compared to the wild-type littermate controls.

Next, we evaluated vascular repair in the microdissected coronary artery and myocardial compartments to determine if PI3Kβ loss enhanced repair. Previous work identified the upregulation of reparative endothelial tip cell genes in mouse and human

transplant coronary vessels, and indicated that apelin-dependent repair is rate-limiting in progressive vasculopathy (7). Although the EC tip cell gene *Apln* was upregulated *vs* expression in the baseline donor heart, expression was not increased in the ECβKO *vs* wild-type hearts either in the arterial (e.g. *Apln* 8 ± 1 *vs* 32 ± 9 fold change; p= 0.01) or myocardial microvascular (e.g. *Apln* 9 ± 3 *vs* 15 ± 5; p=NS) compartments at 2 weeks post-transplant. These data indicate that enhanced repair is not a dominant feature of the protection afforded by EC PI3Kβ inactivation.

3.2 Characterization of graft-infiltrating immune cells

T cell and monocyte infiltration and subsequent injury of the graft vasculature initiates vasculopathy(19). We therefore examined the effect of endothelial PI3Kβ loss on $CD8^+$, $CD4^+$ lymphocyte, and Mac-2^+ activated macrophage infiltration of the artery and myocardium. In the ECβKO myocardium at 2 weeks post-transplant, when early vascular injury is evident, we observed a modest reduction in the density of lymphocytes and macrophages, while at 6 weeks post-transplant $CD8^+$, but not $CD4^+$ lymphocyte infiltration was similar to the wild-type littermate hearts (Fig 3a, b; Suppl Fig 5). In parallel, M2 macrophage infiltration was decreased in ECβKO hearts (Suppl Fig 6). In contrast, in the artery, the reduction in leukocyte infiltration was more pronounced, and where evident, leukocytes localized to the adventitia (Fig 3c). At 6 weeks post-transplant the leukocytes in the expanded arterial intima of the control hearts were too numerous to count, whereas the infiltrate was little changed from 2 weeks post-transplant in ECβKO arteries (Fig 3a).

We further examined the expression of *Ifng* and *Tnfa*, characteristic cytokines of allo-immune responses implicated in the development of vasculopathy. We found reduced *Ifng* expression in the ECβKO artery at the early 2 week timepoint (Fig 3d), but could resolve little change in *Ifng* or *Tnfa* between the ECβKO and littermate coronary arteries at 6 weeks post-transplant. In the myocardial compartment reduced *Ifng* and *Tnfa* expression in ECβKO hearts was evident at the later timepoint (Fig 3e). Moreover interferon-γ-dependent expression of CXCL11 in the artery and the myocardial compartment was unchanged by endothelial *Pik3cb* loss (Suppl Fig 7). These data indicate that leukocyte infiltration was delayed in the myocardium and markedly reduced in the artery of ECβKO *vs* wild-type hearts, but the vascular cells were nevertheless exposed to complex pro-inflammatory stimuli.

Perturbation of immune cell trafficking to the allograft has been associated with altered populations of lymphocytes, reported to be biased to regulatory T cell enrichment, that might mitigate direct alloreactive T cell activities against graft cells (20). We therefore extracted lymphocytes infiltrating the myocardium for more detailed phenotyping by flow cytometry. We exploited H-2^b-HY and I-A^b-HY tetramers to identify allo-reactive lymphocytes within cell populations in the spleen and myocardial infiltrate. We confirmed that an increased frequency of allo-reactive $CD8^+$ and $CD4^+$ T cells were detected in the spleen from grafted mice, consistent with an established allo-immune response at 2 weeks post-transplant (Fig 4a). Heart-infiltrating lymphocytes were enriched in the fraction stained with each of the class I and class II tetramers, but no difference in total or $CD44^{hi}$ memory allo-reactive lymphocytes was observed between

ECβKO and wild-type hearts (Fig 4b, c). Further, a similar fraction $CD8^+$ T cells expressed the cytolytic markers, granzyme B and perforin, between the groups (Fig 4d). We observed a low fraction of $FoxP3^+$ $CD4^+$ T cells, but no enrichment in the ECβKO hearts (Fig 4e). Moreover, we did not find a difference in expression of exhaustion markers, TIM3 and LAG3, among lymphocytes from ECβKO *vs* wild-type hearts (Fig 4f, g). Taken together, these data indicate that lymphocyte egress from blood to the artery and the myocardium was reduced, however the alloreactive lymphocytes maintained similar functional capacity in ECβKO *vs* wild-type hearts.

3.3 PI3 kinase-β deficient endothelial cells lack fractalkine and ICAM 1 expression

To investigate defective recruitment of mononuclear inflammatory cells to the arterial intima, we examined expression of CX3CL1, a chemokine that participates in graft rejection and is selectively expressed by arterial ECs (21, 22). CX3CL1 was absent from coronary endothelium of the naïve donor heart. However, CX3CL1 was highly expressed by arterial, but few microvascular, EC at 2 weeks after transplantation of wild-type hearts (Fig 5a, b). In striking contrast, CX3CL1 expression was absent from ECβKO endothelium post-transplant. Next, we studied *CX3CL1* expression in human aortic EC (HAEC) *in vitro*. As previously reported, CX3CL1 was optimally induced with dual interferon-γ plus TNFα stimulation. However, treatment of HAEC with TGX221 to selectively inhibit PI3Kβ activity, completely blocked *CX3CL1* expression (Fig 5c, d). TGX221 treatment completely blocked HAEC IFN-γ plus TNFα-induced CX3CL1 expression by qRT-PCR, and expression of EC adhesion molecules VCAM1 and

ICAM1, but not expression of the interferon-γ -induced genes, CD74 or CXCL10 (Fig 5e).

We studied the effect of endothelial PI3Kβ inactivation on EC expression of pro-inflammatory adhesion molecules. ICAM1 immunostaining of the mouse heart allografts 2 weeks post-transplant demonstrated widespread ICAM1 expression by microvascular EC in wild-type, but not ECβKO hearts (Fig 5f, g). ICAM1 staining of infiltrating leukocytes was visible in the myocardium of both groups.

3.4 PI3 kinase-β inactivation blocks TNFα-stimulated NF-kB activation in endothelial cells

In vitro, HUVEC were treated with TGX221 to inhibit PI3Kβ activity, then stimulated with TNFα and examined by western blot to evaluate adhesion molecule expression (Fig 6a, b, e, f). We observed TNFα treatment induced phosphorylation of AKT, and the AKT substrate eNOS, indicating upstream PI3K activity, in a TGX221-sensitive manner (Fig 6a). TGX221 did not inhibit VEGF-stimulated PI3Kα activity in EC (Suppl Fig 8). Thus, TNFα stimulation elicits PI3K activity via the p110β isoform in EC. Further, TGX221 blocked TNFα-stimulated ICAM1 induction (Fig 6a, b). To confirm the observation, HUVEC were treated with siRNA to knockdown *PIK3CB* expression (Fig 6c, g). TNFα-stimulated expression of ICAM1 was abolished in *PIK3CB*-deficient EC (Fig 6c, d). Next, the effect of EC PI3Kβ inactivation on EC expression of VCAM1 was examined. TNFα-stimulated induction of VCAM1 was blocked by either TGX221 or *PIK3CB* knockdown, similar to the effect on ICAM1 (Fig 6e-h). Together, we observe endothelial

inactivation of PI3Kβ *in vivo* in the mouse heart, and *in vitro* in human EC, prevents expression of pro-inflammatory chemokine and adhesion molecules used to recruit mononuclear cells to sites of inflammation.

The transcription factor NF-kB integrates a variety of pro-inflammatory cues to elicit chemokine and adhesion molecule expression by vascular endothelial cells, among these CX3CL1, ICAM1, and VCAM1. We tested the effect of endothelial PI3Kβ inhibition on TNFα-stimulated NF-kB activation. Western blot of TNFα-stimulated coronary ECs showed a time-dependent degradation of IkBα, the final regulatory component that prevents the release of sequestered NF-kB from the cytosol (Fig 7a, b). Treatment with TGX221 blocked TNFα-stimulated IkBα degradation. To develop an additional line of evidence, we examined the translocation of NF-kB p65 from the cytosol to the nucleus. TNFα stimulation of EC monolayers for 30 min resulted in nuclear accumulation of NF-kB p65, visualized by immunofluorescent confocal microscopy of mouse (Fig 7c, d) and human (Suppl Fig 9) EC. Treatment with TGX221 blocked p65 translocation. These data indicate that TNFα recruitment of PI3Kβ-dependent signaling is required for TNFα-stimulated NF-kB activity, and provides a mechanistic explanation for the dependence of TNFα-stimulated proinflammatory CX3CL1, ICAM1, and VCAM1 induction on PI3Kβ activity.

Discussion

Immune injury and maladaptive repair of the arterial vascular endothelium limits graft and recipient survival after heart and other solid-organ transplantation despite modern immune suppression treatment (2). Here we identify PI3Kβ blockade, and a repurposed pharmacologic compound (GSK2636771) currently in clinical trial for salvage cancer chemotherapy (ClinicalTrials.gov Identifier NCT02465060 (23)), as an approach to reduce vascular inflammation and limit injury.

The principal finding of this study is that TNFα-stimulation of inflammatory responses in vascular endothelial cells requires PI3Kβ activity. We show that TNFα uses the PI3Kβ isoform to elicit mTORC2-driven phosphorylation of AKT, and downstream AKT activation of eNOS in cultured EC. Further, NF-kB-dependent induction of the endothelial chemokine, CX3CL1, stimulated by IFN-γ plus TNFα, or the adhesion molecules ICAM1 and VCAM1, elicited by TNFα stimulation alone, was blocked by pharmacological or genetic PI3Kβ inactivation. The induction of genes dependent on IFN-γ alone was not affected by selective PI3Kβ inactivation. Blockade of adhesion molecule and CX3CL1 expression was evident in both human and mouse ECs, indicating that the requirement for PI3Kβ activity is conserved across species.

In vivo murine heart transplant rejection directed against the minor HY histocompatibility antigen expressed by the male coronary arterial and microvascular endothelium, in the absence of confounding immune suppression, elicits chronic vascular inflammation. Oral administration of the GSK2636771 PI3Kβ inhibitor, initiated after the allo-immune response was established, markedly reduced arterial immune cell accumulation,

arrested progression of obliterative arterial vasculopathy, and preserved the coronary microvasculature against immune injury. Selective knockout of the p110β catalytic isoform in the graft endothelium, leaving the recipient bone marrow-derived cells unperturbed, phenocopied the effect of the inhibitor to protect the graft vasculature from injury, indicating the effect is independent of PI3Kβ activity in the recipient's immune cells. Examination of the graft-infiltrating lymphocytes confirmed the presence of $CD4^+$ and $CD8^+$ lymphocytes in the graft myocardium, albeit with delayed kinetics in ECβKO hearts. EC PI3Kβ loss was not associated with expression of markers of T cell exhaustion, or enrichment of regulatory T cells as has been reported in chronic tumor inflammation (24). $CD8^+$ and $CD4^+$ T cells were functional, reflected by TNFα, interferon-γ, and interferon-γ-dependent chemokine CXCL11 expression in the graft myocardium and arterial compartments. Nevertheless, immunostains showed markedly reduced *in vivo* expression of CX3CL1 and ICAM1 on EC, consistent with the findings *in vitro*.

The dramatic protection against arterial disease likely reflects more stringent requirements for chemokine and adhesion molecule presentation by the vascular endothelium to recruit leukocytes under high shear stress conditions. The reduction in immune cell recruitment is reflected by the marked reduction in arterial EC and smooth muscle injury. Conversely, the low shear condition of the capillary and post-capillary coronary vessels allowed lymphocyte and monocyte transendothelial migration. Similar parenchymal lymphocyte infiltration has been demonstrated in transplanted ICAM1-deficient donor hearts, consistent with redundant adhesion ligand use by leukocytes

undergoing transendothelial migration (25). Moreover, CX3CL1-deficiency alone does not change heart transplant inflammation and survival, unless immune suppression is added (21). However, simultaneous genetic perturbation of the expression of several pro-inflammatory molecules by EC cumulatively reduces inflammation in other models (26). The effect of PI3Kβ inactivation has not been tested in fully MHC-mismatched grafts.

The effect of endothelial PI3Kβ loss of function on the vascular response to inflammation is complex, since PI3Kβ activity contributes to pro-angiogenic repair responses, and to the defense of the repairing vasculature from immune cell infiltration. For example, apelin-stimulated nitric oxide synthase activity in the endothelium is coupled to PI3Kβ, attenuates leukocyte recruitment, and is required to protect the vasculature against aggressive immune injury (7). Here we find TNFα-stimulated eNOS activation is similarly dependent on PI3Kβ. Thus, PI3Kβ blockade-mediated inhibition of TNFα-stimulated chemokine and adhesion molecule display dominates blunted TNFα-stimulated eNOS and pro-reparative activities in the inflamed vasculature.

Selective perturbations of the graft microenvironment have been shown to confer immune tolerance to an allograft. For example, disruption co-stimulation molecules (27), or macrophage activation (28) may induce long-term allograft survival associated with impaired lymphocyte effector function and enrichment of regulatory T lymphocytes. Similarly, in anti-tumor immunity, suppression of T cell activation via PD-L1 or exhaustion of the infiltrating lymphocytes from chronic stimulation may occur (24).

Disruption of selective adhesion molecules have generally not afforded long-term protection of graft rejection (25, 29-31), but systemic ICAM-1/ LFA-1 blockade limits recipient allo-reactive lymphocyte expansion(32, 33). In our experiments, we found robust enrichment of the graft with $CD44^{hi}$ allo-specific lymphocytes showing cytotoxic perforin and granzyme molecules and interferon-γ expression indicative of competent effector function. The lymphocytes were not enriched in $FoxP3^{+}$ Treg cells or cells bearing exhaustion markers. We speculate that T cell-mediated cytotoxicity against the microvascular EC may be blunted by defective interaction with EC displaying low amounts of adhesion molecule ligands, enabling net preservation of the microvasculature.

The finding that selective PI3Kβ inactivation blocks the effect of TNFα-dependent pro-inflammatory responses in EC was unexpected. NF-kB-dependent gene transcription drives expression of CX3CL1 and endothelial adhesion molecules to promote leukocyte recruitment in response to inflammatory cues and disturbed flow at sites of atherosclerosis (34, 35). However, earlier study of the effect of PI3K inhibition using pan-isoform first-generation compounds did not identify a role of PI3K in EC NF-kB activation or pro-inflammatory adhesion molecule expression in response to TNFα (36, 37). In agreement with this finding, subsequent work has defined the core molecular detail of NF-kB pathway activation downstream of diverse proinflammatory receptors, such as the TNFα superfamily, IL-1, and Toll-like receptors in a variety of cell types acting independent of PI3K activity (38). However, co-stimulation-driven PI3K activity in T lymphocytes modulates NF-kB-dependent gene expression (39). In cancer cells,

oncogenic PI3Kα or AKT gain-of-function mutants have been demonstrated to drive NF-kB activity (40-42).

Recent interest in clinical PI3Kβ inhibition, primarily for treatment of PTEN-deficient cancers that upregulate PI3K activity using the p110β isoform, has prompted detailed investigation of the roles of individual PI3K isoforms and revealed complexity in the regulation of the parallel pathways. In cancer cells, for example, resistance to PI3Kα inhibition appears dependent on PI3Kβ (15, 43). These studies identify complex cellular responses to isolated PI3K isoform inhibition, including use of alternate PI3K isoforms and release of constitutive inhibitory pathways that normally constrain downstream mTOR and AKT activities (15, 43, 44). Taken together, these findings suggest that PI3Kβ inactivation is likely to have an indirect effect on TNFα-induced NF-kB pathway activation in EC.

Transplant vasculopathy remains problematic despite aggressive modern immune suppression regimes. This data suggests that a treatment approach with a re-purposed oral PI3Kβ inhibitor may be useful to mitigate injury and complement immune suppression.

Data availability: All data supporting the findings of this study are available within the paper and from the corresponding author on request.

Author contributions: AGTM, JXL, GYO and AGM conceived the project. AGTM, JXL, LFZ, and KT generated the data. LW, BV, GYO, and ZK provided technical support. AGTM, JXL, CCA, and AGM interpreted the data. AGTM and AGM wrote the manuscript. GYO, CCA, LW, BV and RBM provided review, critical appraisal and editing of the final manuscript.

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Figure legends

Figure 1 PI3Kβ inactivation mitigates vasculopathy in transplanted hearts. (A) Transplanted hearts from vehicle (n = 9) or GSK2636771-treated mice (n = 9) were recovered 6 weeks after transplantation, stained with the van Gieson elastin stain (top panels) or immuno-stained for endothelial CD31 (PECAM; bottom panels). Fibrocellular expansion of the intima, bounded by the internal elastic lamina and the lumen, in a coronary artery from a vehicle-treated heart is marked (arrow; upper panels). Little neointima expansion is evident in coronary arteries from a normal heart, or a transplanted heart from a GSK2636771-treated mouse. Rarefaction of myocardial microvessels is evident in a vehicle-treated *vs* normal or GSK2636771-treated heart (lower panels). (B) Quantification of the intima occlusion (left), and myocardial $CD31^+$ microvessel density (right) of the groups in (A) as described in Methods. (C) Transplanted ECβKO or wild-type hearts (n=4-10) were recovered at 2 or 6 weeks post-transplant and stained as in (A). (D) Quantification of the intima occlusion (left), and myocardial microvessel density (right) of the groups in (C). HPF = high-power field. Scale bars: 50 μm. Mean ± SEM; *P < 0.05, **P < 0.01, NS non-significant by 1-way ANOVA with Bonferroni's post hoc test.

Figure 2 PI3Kβ inactivation protects heart allografts from endothelial injury. (A) Quantification of gaps in the arterial endothelium in cross-section as described in Methods, from ECβKO or wild-type hearts recovered post-transplant. (B) Confocal photomicrographs (1 μm optical section) of intramyocardial coronary arteries in cross-section from the transplanted hearts. $CD31^+$ EC (left panels), activated caspase 3 (aCasp3, red; center panels), and the merged channels (right panels) are shown. (C) Quantification of the fraction of $CD31^+$ EC co-stained with aCasp3 in the groups (n=4-10 mice/ group) in (B). Scale bars: 50 μm. Mean ± SEM; **P < 0.01, by 1-way ANOVA with Bonferroni's post hoc test.

Figure 3 Immune cell infiltration of heart allografts. (A) Photomicrographs of the $CD8^+$ lymphocytes (top panels), and Mac-2^+ monocyte/ macrophages (bottom panels) infiltrating hearts from ECβKO or wild-type donors (n=4-10 mice/ group) recovered at 2 or 6 weeks post-transplant. (B) Quantification of the number of myocardial-infiltrating $CD8^+$ (left) and Mac-2^+ cells (right) in (A). (C) Quantification of the $CD8^+$ (left) and Mac-2^+ (right) cells infiltrating the arterial compartment in hearts recovered 2 weeks post-transplant (n=8-10 mice/ group). Mean ± SEM; **P < 0.01 by Student's t-test. Pro-inflammatory *Infg* or *Tnfa* cytokine gene expression determined by qRT-PCR among micro-dissected coronary arteries (D) or myocardium (E) samples from ECβKO or wild-type donor transplanted hearts (n=4-10 mice/ group). HPF = high-power field. Scale bars: 50 μm. Mean ± SEM; *P < 0.05, **P < 0.01, NS, non-significant by 1-way ANOVA with Bonferroni's post hoc test.

Figure 4 Characterization of male HY-alloreactive lymphocytes in heart allografts. Splenic or graft-infiltrating lymphocytes were isolated from non-transplanted female, or ECβKO or wild-type donor heart recipient mice (n=4-7 mice/ group) recovered 2 weeks post-transplant, followed by staining with HY-peptide/ Major Histocompatibility Class (MHC) I or II tetramers to identify allo-reactive cells, counter-immunostained as indicated, then detected by flow cytometry as described in Methods. (A) Quantification of the abundance of MHC I (left) or MHC II (right) tetramer-stained lymphocytes to detect $CD8^+$ and $CD4^+$ alloreactive T lymphocytes, respectively, in the spleen of heart allograft recipient mice. (B) Quantification of the abundance of tetramer-stained lymphocytes recovered from the hearts at 2 weeks post-transplant. (C) Quantification of the $CD44^+$ memory T lymphocytes among allo-reactive lymphocytes in heart allografts. (D) Quantification of the cytotoxic granzyme B^+ (left) or $perforin^+$ (right) fraction of the HY-MHC class I $tetramer^+$ cells. (E) Quantification of the $FoxP3^+$ Treg fraction of the HY-MHC class II $tetramer^+$ cells. Quantification of the lymphocyte exhaustion markers (F) $Tim3^+$ and (G) $LAG3^+$ populations among allo-reactive lymphocytes in heart allografts. Mean ± SEM; NS = non-significant by unpaired Student's t-test.

Figure 5 EC-specific PI3Kβ inactivation blocks pro-inflammatory molecule expression *in vivo*. (A) Expression of CX3CL1 by intramyocardial coronary artery EC from ECβKO or wild-type donor hearts at 2 weeks post-transplant. Confocal photomicrographs (1 μm optical section) of endothelial CD31 (green, left panels), CX3CL1 (red, center panels),

and the merged images (right panels). Scale bars: 50 μm. (B) Quantification of the fraction of $CD31^+$ EC co-stained with CX3CL1 in the groups (n=8-10 mice/ group) in (A). Mean ± SEM; **P < 0.01 by Student's t-test. (C) Primary human aortic ECs stimulated with both TNFα (10 ng/ml) + IFNγ (50 ηg/ml) ± TGX221 (100 nM). Cell lysates were collected at 48 h then immunoblotted for CX3CL1. (D) Quantification of CX3CL1 expression relative to β actin (n=4 independent experiments). (E) Human aortic ECs were treated as in (C) and expression was quantified by qRT-PCR (n=3 independent experiments). Mean ± SEM; *P < 0.05, **P < 0.01, NS, non-significant by 1-way ANOVA with Bonferroni's post hoc test. (F) Expression of ICAM1 expression by myocardial microvessel EC from ECβKO or wild-type donor hearts at 2 weeks post-transplant. Confocal photomicrographs of endothelial CD31 (green, left panels), ICAM1 (red, center panels), and the merged images (right panels). (Scale bars: 50 μm). (G) Quantification of the fraction of $CD31^+$ EC co-stained with ICAM1 in the groups (n=8-10 mice/ group) in (F). Mean ± SEM; **P < 0.01 by Student's t-test.

Figure 6 EC PI3Kβ inactivation blocks TNFα-stimulated adhesion molecule expression *in vitro*. (A) HUVECs were stimulated with TNFα (10 ng/ml) ± TGX221 (100 nM). Cell lysates were collected at 12 h then immunoblotted for ICAM1, and phospho-AKT^{S473}. (B) Quantification of ICAM1, phospho-AKT^{S473}, and phospho-$eNOS^{S1177}$ expression (n=4 independent experiments). (C) HUVECs were transfected with *PIK3CB* or scrambled siRNA as described in Methods, then stimulated with TNFα as in (A). Cell lysates were immunoblotted for ICAM1. (D) Quantification of ICAM1 expression (n=4 independent experiments). (E) HUVECs were stimulated with TNFα (10 ng/ml) ±

TGX221 (100 nM). Cell lysates were collected at 24 h then immunoblotted for VCAM1, and phospho-AKT^{S473}. (F) Quantification of VCAM1 expression (n=4 independent experiments). (G) HUVECs were transfected with *PIK3CB* or scrambled siRNA, then stimulated with TNFα as in (E). Cell lysates were immunoblotted for VCAM1. (H) Quantification of VCAM1 expression (n=4 independent experiments). Mean ± SEM; *P < 0.05, **P < 0.01, NS, non-significant by 1-way ANOVA with Bonferroni's post hoc test. HUVEC, human umbilical vein endothelial cells.

Figure 7 EC PI3Kβ inactivation blocks the canonical NF-kB pathway. (A) Mouse microvascular heart ECs were stimulated with TNFα (10 ng/ml) ± TGX221 (100 nM). Cell lysates were collected at the indicated times, then immunoblotted for IKBα. (B) Quantification of IKBα abundance (n=4 independent experiments). TGX221 treatment protected against IKBα degradation (P<0.01 by 2-way ANOVA). Mean ± SEM; *P < 0.05, **P < 0.01, NS, non-significant by ANOVA. (C) MHEC were stimulated with TNFα (10 ng/ml) ± TGX221 (100 nM), then imaged at 30 min. Confocal photomicrographs of endothelial NF-kB p65 (red, left panels), DAPI (blue, center panels), and the merged images (right panels). Scale bars: 50 μm. (D). Co-localization of NF-kB p65 with the DAPI^{+} nucleus was quantified as mean fluorescent intensity of NF-kB p65. Mean ± SEM; **P<0.01 by 1-way ANOVA with Bonferroni's post hoc test.