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Brief Report **Vascular biology**

Glaucoma is a leading cause of blindness, afflicting more than 60 million people worldwide. Increased intraocular pressure (IOP) due to impaired aqueous humor drainage is a major risk factor for the development of glaucoma. Here, we demonstrated that genetic disruption of the angiopoietin/TIE2 (ANGPT/TIE2) signaling pathway results in high IOP, buphthalmos, and classic features of glaucoma, including retinal ganglion degeneration and vision loss. Eyes from mice with induced deletion of *Angpt1* and *Angpt2* (A1A2Flox^{WB} mice) lacked drainage pathways in the corneal limbus, including Schlemm's canal and lymphatic capillaries, which share expression of the PROX1, VEGFR3, and FOXC family of transcription factors. VEGFR3 and FOXCs have been linked to lymphatic disorders in patients, and FOXC1 has been linked to glaucoma. In contrast to blood endothelium, in which ANGPT2 is an antagonist of ANGPT1, we have shown that both ligands cooperate to regulate TIE2 in the lymphatic network of the eye. While A1A2Flox^{WB} mice developed high IOP and glaucoma, expression of ANGPT1 or ANGPT2 alone was sufficient for ocular drainage. Furthermore, we demonstrated that loss of FOXC2 from lymphatics results in TIE2 downregulation, suggesting a mechanism for ocular defects in patients with FOXC mutations. These data reveal a pathogenetic and molecular basis for glaucoma and demonstrate the importance of angiopoietin ligand cooperation in the lymphatic endothelium.

A lymphatic defect causes ocular hypertension and glaucoma in mice

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Introduction

The angiopoietin/TIE2 (ANGPT/TIE2) signaling pathway is a major regulator of vascular development, and altered expression of ANGPT ligands or activity of the TIE2 receptor is linked to a variety of vascular diseases and adverse outcomes in patients (1–4). However, while the blood vascular role of the pathway has been extensively studied, the function of angiopoietins in lymphatic endothelium is uncertain. In vascular endothelium, ANGPT2 is reported to function as a competitive antagonist of ANGPT1/TIE2 signaling, inhibiting ANGPT1-mediated phosphorylation of TIE2 (5). ANGPT2 and the orphan receptor TIE1 are known to be involved in lymphatic development (6), but until now, the roles of TIE2 and its canonical ligand ANGPT1 have not been described.

Surprisingly, while *Angpt1*-KO mice die between E9.5 and E12.5 due to major cardiovascular defects, conditional deletion of *Angpt1* after E13.5 produces no overt vascular phenotypes in adult mice (3). To determine whether there is unrecognized cooperation between ANGPT1 and ANGPT2, which provides compensation in mice lacking ANGPT1, we generated a conditional *Angpt2* allele and produced mice lacking both major angiopoietin ligands (A1A2Flox^{WB}). Strikingly, simultaneous deletion of both ligands at mid-gestation phenocopies deletion of *Tie2*, demonstrating cooperativity between ANGPT1 and ANGPT2. Whole-body deletion of the *Tie2* receptor or both *Angpt1* and *Angpt2* at E12.5 leads to gross subcutaneous edema in embryos with patterning defects in dermal lymphatic vessels (S.E. Quaggin, unpublished observations). While *Angpt2*-KO mice exhibit lymphatic valve defects and mesenteric lymphatic abnormalities resulting in chylous ascites (2), they did not develop the embryonic edema observed in A1A2Flox^{WB} or *Tie2*–conditional KO (cKO) mice, suggesting a compensatory role for ANGPT1 in lymphatic development.

Results and Discussion

To investigate the combined roles of ANGPT1 and ANGPT2 in adult mice, we deleted both ligands at E16.5. A1A2Flox$^{WB\Delta E16.5}$ mice (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI77162DS1) were born in Mendelian numbers and were indistinguishable from controls during the first 3 weeks of life. However, eyes of the mutant animals began to protrude 21–28 days after birth. Buphthalmos worsened with age, and by 8 weeks, mice had difficulty closing their eyelids (Figure 1, A–D). Gross examination revealed corneal enlargement and increased anterior chamber depth (Figure 1, F, G, I, and J). Eyes of TIE2COIN$^{WB\text{-}INV\Delta P0}$ mice (Supplemental Figure 2) exhibited

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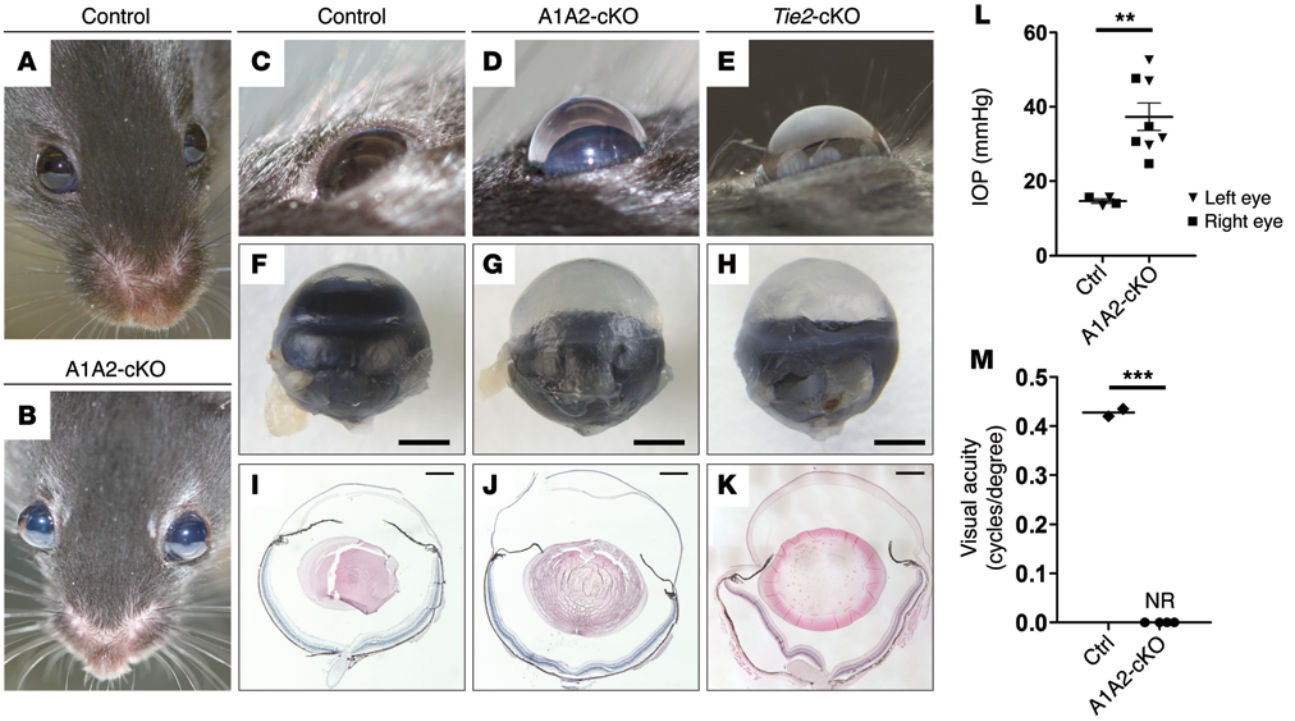

Figure 1. A1A2Flox$^{WB\Delta E16.5}$- and TIE2COIN$^{WB\text{-}INV\Delta P0}$-cKO mice develop buphthalmos. While control eyes (**A**, **C**, **F**, and **I**) appeared normal, 8- to 9-week-old A1A2- (**B**, **D**, **G**, and **J**) and *Tie2*-cKO (**E**, **H**, and **K**) mice exhibited anterior chamber enlargement due to increased IOP (**L**). Optomotor response tests (**M**) showed impaired vision in mutant animals. Scale bars: 1 mm (**F**, **G**, and **H**) and 500 μm (**I**, **J**, and **K**). $^{**}P < 0.01$ and $^{***}P < 0.001$ by Student's 2-tailed *t* test. Error bars indicate SEM. No response (NR) indicates an optomotor response of less than 0.042 cycles per degree.

an identical phenotype, confirming that the effect was mediated through the canonical ANGPT receptor TIE2 (Figure 1, E, H, and K). We measured the intraocular pressure (IOP) at 10 weeks using a rebound tonometer (7). While control animals were within the normal range of 12 to 16 mmHg, the IOP of mutant littermates was significantly elevated, ranging from 24 to 52 mmHg (Figure 1L). Using an optomotor response test (8), we found that A1A2Flox$^{WB\Delta E16.5}$ mice had severely impaired vision, with visual acuity of less than 0.042 cycles per degree (Figure 1M). Histological analysis revealed optic nerve excavation (Figure 2, A and G) and other features of glaucomatous eye disease.

We observed reductions in the thickness of retinal cell layers in mutant eyes, including the ganglion and nuclear layers, as well as loss of the nerve fiber layer (Figure 2, B, C, H, I, and O, and Supplemental Figures 3 and 4). Unlike human glaucoma, which is rarely associated with photoreceptor loss, A1A2Flox$^{WB\Delta E16.5}$ mice showed outer nuclear layer thinning that worsened toward the retinal periphery. This outer retina damage was similar to that described in laser-induced models of mouse glaucoma, suggesting the possibility of pressure-related or ischemic effects (9). While photoreceptor loss may be partially responsible for the vision loss we observed, this degree of atrophy was not sufficient to explain the dramatic decrease in visual acuity seen in A1A2Flox$^{WB\Delta E16.5}$ mice. Instead, we hypothesize that the defect was due to loss of retinal ganglion cells and interneurons — which would confirm that A1A2Flox^{WB} mice represent a new model of murine glaucoma.

To determine the cause of high IOP and glaucoma in A1A2Flox$^{WB\Delta E16.5}$ mice, we studied the aqueous humor drainage system of the eye. While the trabecular meshwork and ciliary body were indistinguishable between KO and control mice, Schlemm's canal was absent in 8 of 8 A1A2Flox$^{WB\Delta E16.5}$ eyes examined (Figure 2, D and J). Schlemm's canal was present in all control littermates. Although Schlemm's canal is a major route of aqueous humor drainage, defects in Schlemm's canal formation have not been reported to raise IOP in mice. Transgenic mice haploinsufficient for the transcription factor FOXC1 have been reported to exhibit a small or absent Schlemm's canal, yet did not develop high IOP (10). Previous studies of aqueous humor dynamics in mice have suggested that only 20% of total fluid drainage is carried out via Schlemm's canal, suggesting that alternate drainage routes, including uveoscleral and lymphatic routes, may be able to provide compensation for defects in Schlemm's canal (11).

To better understand alternate drainage pathways of the anterior chamber, we studied lymphatic and vascular capillaries in the corneal limbus. We examined vessels by confocal microscopy, which revealed a complete absence of LYVE1-positive lymphatic endothelium in the limbus of A1A2Flox$^{WB\Delta E16.5}$ mice (Figure 2, E and K). CD31-positive blood vasculature was present, but exhibited disturbed patterning with some capillary loops extending into the cornea. It is unclear whether this aberrant vascular morphology is a direct effect of *Angpt1* and *Angpt2* deletion, or a response to loss of lymphatic drainage and/or stretching of the cornea. Mice lacking ANGPT1 or ANGPT2 alone develop lymphatic vasculature in the corneal limbus (Figure 2, M and N), suggesting the presence of inter-ligand compensation in the lymphatic endothelium.

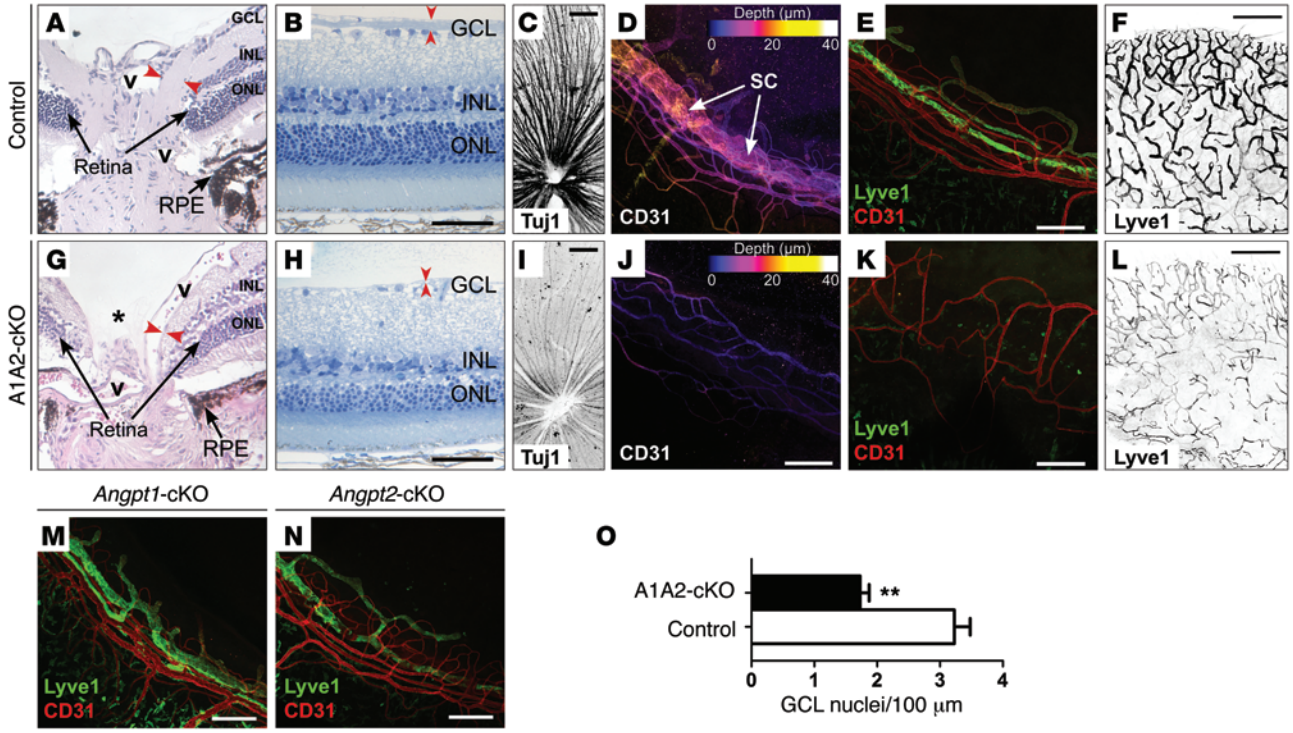

Figure 2. A1A2Flox$^{WB\Delta E16.5}$-cKO mice develop glaucoma due to defects in ocular drainage. Compared with controls (**A**), the optic nerve head appeared abnormal in A1A2Flox$^{WB\Delta E16.5}$ mice (**G**), with thinning of the nerve fiber layer (red arrowheads) and optic nerve excavation (asterisk). Semi-thin sections showed thinning of the nerve fiber, ganglion, and nuclear cell layers in the central retina (**B** and **H**). Loss of nerve fibers was confirmed by Tuj1 staining (**C** and **I**). Unlike their littermate controls (**D**), Schlemm's canal was absent in A1A2-cKO mice (**J**). Anterior chamber drainage was further diminished by a loss of LYVE1-positive lymphatic capillaries in the corneal limbus (**E** and **K**). Lymphatic vasculature was present in nonocular tissues, but exhibited disturbed patterning as shown here in the dermis of the ear (**F** and **L**). Unlike double-KOs, mice lacking *Angpt1* or *Angpt2* individually developed lymphatics in the corneal limbus (**M** and **N**). Compared with controls, A1A2-cKO mice had fewer nuclei in the retinal ganglion cell layer (**O**). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium; V, blood vessel; SC, Schlemm's canal. Red arrowheads indicate thickness of the nerve fiber layer. Scale bars: 200 μm and 1 mm (**F** and **L**). $^{**}P < 0.01$.

Corneal neovascularization and glaucoma have been described in patients with Axenfeld–Rieger syndrome due to mutations in *FOXC1*, and *Foxc1* haploinsufficient mice exhibit defects in the anterior chamber and Schlemm's canal, suggesting a link to this molecular pathway (10). In mice, a similar phenotype has been described for the related transcription factor FOXC2 (10, 12). Intriguingly, the downstream targets of FOXC1/2 responsible for these phenotypes have not been elucidated. Given the similar expression pattern of FOXC1/2 with TIE2 and ANGPT2 in lymphatics, as well as the overlapping phenotypes (13, 14), we hypothesized that FOXC1 or FOXC2 are responsible for regulating expression of TIE2 or angiopoietin ligands in the lymphatic endothelium. De Val and colleagues have reported the presence of a FOX:ETS transcriptional enhancer sequence in the *Tie2* promoter region and demonstrated FOXC2-mediated enhancer activation using an in vitro reporter system, further suggesting a link between these pathways (15). We investigated the possibility of FOXC-mediated TIE2 regulation using complimentary in vivo and in vitro systems. siRNA targeting of FOXC2 in human dermal lymphatic cells caused a marked reduction in TIE2 protein expression compared with targeting of FOXC1 or scrambled siRNAs (Figure 3A). This result was replicated in vivo using a *Foxc2* transgenic mouse line. Lymphatic endothelial cells were isolated from lymphatic-specific *Foxc2*-KO (*Foxc2$^{fl/fl}$ Prox1-CreERT2*; ref. 16) embryos at E15.5 using FACS. mRNA was isolated, and real-time PCR revealed a 74.5% reduction ($n = 6$ animals per group, $P = 0.015$) in *Tie2* mRNA expression relative to that in controls (Figure 3B). These results suggest a mechanism for lymphatic phenotypes in patients with *Foxc2* mutations or in *Foxc2*-KO mice and provide additional evidence of a connection between FOXC and ANGPT/TIE2 molecular pathways.

Surprisingly, LYVE1-positive lymphatic vessels were present in nonocular tissues of A1A2Flox$^{WB\Delta E16.5}$ mice, although patterning in some organs was abnormal. In the dermis of the ear, lymphatic vessels appeared sparse with variable vessel diameter and abnormal branching similar to those described in *Angpt2*-null mice (Figure 2, F and L, and ref. 2). It is possible that specialized lymphatic vessels of the anterior chamber are more dependent on ANGPT/TIE2 signaling than other lymphatic endothelia, suggesting that, like blood vascular endothelia, lymphatic capillaries are heterogeneous, with unique functions and regulatory mechanisms. Indeed, Schlemm's canal has been described as a hybrid vessel, with features of both blood and lymphatic endothelium, expressing blood endothelial markers CD34 and endomucin and a subset of lymphatic markers, including PROX1 and VEGFR3, but not podoplanin (17).

The cardiovascular phenotypes reported in *Angpt1*- and *Tie2*-KO models highlight the importance of these molecules in cardiac development and angiogenesis. In older animals, our data suggest

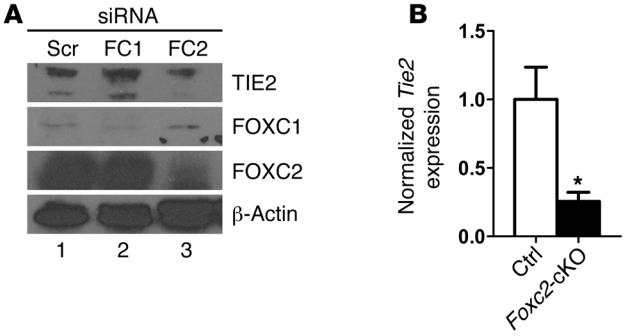

Figure 3. FOXC2 regulates TIE2 expression in the lymphatic endothelium. (**A**) Human dermal lymphatic endothelial cells were cultured in the presence of specific siRNAs targeting FOXC1, FOXC2, or a scrambled siRNA. Compared with scrambled siRNA (lane 1) or FOXC1 siRNA (lane 2), siRNA targeting FOXC2 (lane 3) caused a reduction in TIE2 protein expression. (**B**) *Tie2* mRNA expression was measured by real-time PCR in lymphatic endothelial cells isolated from E15.5 *Foxc2*$^{fl/fl}$ (Ctrl) or *Foxc2*$^{fl/fl}$ *Prox1-CreERT2* (*Foxc2*-cKO) mouse embryos. n = 6 control and 6 *Foxc2*-cKO embryos. Error bars indicate SEM. *$P < 0.05$.

that ANGPT/TIE2 signaling is less critical in quiescent blood vasculature, but continues to play a major role in the lymphatic endothelium. At the outset, we hypothesized that A1A2Flox^{WB} mice would develop severe vascular defects, revealing a compensatory role for ANGPT2 in the blood vasculature of mice lacking ANGPT1. Instead, we observed that the lymphatic defects reported in *Angpt2*-null mice were enhanced by the additional loss of ANGPT1, demonstrating cooperation between ligands in the lymphatic endothelium.

While the overall mechanism responsible for human glaucoma remains obscure, the most important risk factor is elevated IOP (18). IOP is determined by the relative rates of aqueous humor drainage and formation, and the majority of glaucoma treatment has focused on lowering IOP by targeting these systems (19). Aqueous humor is thought to drain through 2 major pathways: the trabecular meshwork leading to Schlemm's canal and the uveoscleral pathway. In humans, studies have estimated that the uveoscleral pathway accounts for 46% to 54% of total outflow, with the remainder carried through Schlemm's canal (20). The contribution of lymphatic vessels to each pathway has not been reported, but studies in sheep have suggested that they play an important role in allowing fluid to escape from the anterior chamber (21). Since the increased IOP we observed in A1A2Flox$^{WB\Delta E16.5}$ mice was more severe than that of other models with an abnormal Schlemm's canal, we hypothesize that lymphatic vessels are essential for maintaining aqueous humor flow through the uveoscleral route.

In summary, we show that A1A2Flox$^{WB\Delta E16.5}$ mice lack both Schlemm's canal and the lymphatic capillaries of the corneal limbus, leading to a dramatic increase in IOP and glaucoma. These data raise the intriguing possibility that promotion of lymphangiogenesis with therapies such as VEGFC or ANGPT/TIE2 agonists might represent novel and much-needed therapies for glaucoma.

Methods

Complete methods, including antibody and primer information, are available in the Supplemental Methods.

Mice and breeding. To create doxycycline-inducible, whole-body (WB) *Angpt1*- and *Angpt2*-KO, we generated a novel *Angpt2*$^{fl/fl}$ allele and combined it with a previously described *ROSA-rtTA Tet-On-Cre Angpt1*$^{fl/fl}$–KO line (3). Pregnant dams were treated with doxycycline at E16.5 to generate A1A2Flox$^{WB\Delta E16.5}$ pups. To generate *Tie2* WB cKO mice, TIE2COIN (conditional by inversion; ref. 22) mice were crossed with *ROSA-rtTA Tet-On-Cre*–expressing mice. Nursing dams and neonates were treated with doxycycline at P0 to obtain TIE2COIN$^{WB\text{-}INV\Delta P0}$ offspring. *Prox1-CreERT2* mice were a gift of G. Oliver (St. Jude Children's Research Hospital, Memphis, Tennessee, USA).

Live-animal studies. IOP was measured in mice at 10 weeks of age using a Tonolab rebound tonometer (iCare Finland), as previously described (7). Visual acuity was estimated using an optomotor response test (8). The optomotor system used provides a maximum grating size of 0.042 cycles per degree, and mice were scored as having no response if they were unable to respond to this stimulus.

Histology. A1A2Flox$^{WB\Delta E16.5}$ mice and their littermate controls were 8 weeks of age before their tissue was harvested for histological studies. Eyes were perfusion fixed before embedding and sectioning following standard procedures. For immunofluorescence studies, eyes were dissected and immersion fixed. After fixation, retinas and optic nerves were removed and hemispheres stained as whole mounts for confocal microscopy.

FACS and quantitative PCR of lymphatic endothelial cells. Cells isolated from E15.5 control (*Foxc2*$^{fl/fl}$) and lymphatic-specific *Foxc2*-KO (*Foxc2*$^{fl/fl}$ *Prox1-CreERT2*) mouse embryos were stained for LYVE1 and CD31 and subjected to FACS as previously described (23). RNA was extracted using standard methods and cDNA synthesized for real-time PCR.

Lymphatic cell culture. Human dermal lymphatic endothelial cells were transfected with FOXC1, FOXC2, or control siRNAs and incubated for 48 hours. After incubation, cells were harvested and lysates prepared for Western blot analysis.

Statistics. P values were obtained using a 2-tailed Student's t test and are shown in the figures as *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

Study approval. All animal experiments were approved by the IACUC of the Center for Comparative Medicine at Northwestern University.

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