Endothelial Gab1 deficiency aggravates splenomegaly in portal hypertension independent of angiogenesis

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Jiang B, Deng Q, Huo Y, Li W, Shibuya M, Luo J. Endothelial Gab1 deficiency aggravates splenomegaly in portal hypertension independent of angiogenesis. Am J Physiol Gastrointest Liver Physiol 308: G416–G426, 2015. First published December 11, 2014; doi:10.1152/ajpgi.00292.2014.—Certain pathological changes, including angiogenesis, actively contribute to the pathogenesis of splenomegaly in portal hypertension (PH), although the detailed molecular and cellular mechanisms remain elusive. In this study, we demonstrated that endothelial Grb-2-associated binder 1 (Gab1) plays a negative role in PH-associated splenomegaly independent of angiogenesis. PH, which was induced by partial portal vein ligation, significantly reduced Gab1 expression in endothelial cells in a time-dependent manner. Compared with controls, endothelium-specific Gab1 knockout (EGKO) mice exhibited a significant increase in spleen size while their portal pressures remained similar. Pathological analysis indicated that EGKO mice developed more severe hyperactive white pulp and fibrosis in the enlarged spleen but less angiogenesis in both the spleen and mesenteric tissues. Mechanistic studies showed that the phosphorylation of endothelial nitric oxide synthase (eNOS) in EGKO mice was significantly lower than in controls. In addition, the dysregulation of fibrosis and inflammation-related transcription factors [e.g., Krüppel-like factor (KLF) 2 and KLF5] and the upregulation of cytokine genes (e.g., TNF-α and IL-6) were observed in EGKO mice. We thus propose that endothelial Gab1 mediates multiple pathways in inhibition of the pathogenesis of splenomegaly in PH via prevention of endothelial dysfunction and overproduction of proinflammatory/profibrotic cytokines.

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Portal hypertension (PH), characterized by elevation of the hepatic venous pressure gradient >5 mmHg (3), is a frequent and dreadful complication of chronic liver diseases (12). Bleeding esophageal varices, ascites, encephalopathy, and splenomegaly are well-known clinical complications of PH (62). Splenomegaly has its prevalence in cirrhotic patients from 36 to 92% in different series, contributes to morbidity, is detrimental to quality of life (41), and may also limit the treatment options in some liver diseases (11). Thus, spleen enlargement during follow-up has been used as a simple, objective, and noninvasive parameter to predict the complications of PH (7).

So far, the mechanisms underlying PH-related splenomegaly have been poorly characterized. Traditionally, splenomegaly was thought to be passive venous congestion as a result of the increased resistance to splenic blood outflow and the rise in portal venous pressure (42, 44). However, such passive congestion could not be the only cause of the enlarged spleen in PH, since a relatively weak correlation was found between spleen size and portal pressure (PP) or the degree of varices (24, 60). Increasing evidence has shown that splenomegaly arises as a consequence of the interplay of several factors, including not only spleen congestion, but also enhanced inflammation, fibrogenesis, and angiogenesis (7, 8, 42). Consistently, increased expression of genes encoding proangiogenic, proinflammatory, and profibrotic cytokines was observed in the enlarged spleen (48, 60). For example, angiogenic factors such as vascular endothelial growth factor and platelet-derived growth factor are highly expressed both in the PH spleen (42, 60) and mesenteric tissues (20, 43). Importantly, studies using angiogenesis inhibitors have shown beneficial effects on PP and splenomegaly (20, 56, 57). These studies have revealed a critical role of angiogenesis in PH and its complications.

In addition to being essential cellular components of angiogenesis, endothelial cells (ECs) are critical for maintaining tissue homeostasis and inhibiting vascular inflammation in many pathological conditions, such as chronic liver disease and its complications, including PH. Endothelial nitric oxide synthase (eNOS) is an important enzyme in regulation of endothelial functions (26, 51), and deficient expression and decreased activity of eNOS are associated with augmented fibrosis/inflammation (37, 61) and splenomegaly (60). eNOS-derived NO was reported to suppresses the production of various cytokines in a number of cells, including ECs, during various airway inflammations and to inhibit fibrosis via inactivating fibroblast cells (53) and stellate cells (17, 35). These studies indicated that eNOS is a crucial negative regulator in tissue inflammation and fibrosis. It is known that eNOS can be activated by multiple kinases [e.g., protein kinase A (PKA), extracellular signal-regulated kinases (Erk), and protein kinase B (Akt)] (6, 9, 13, 18), but the exact upstream signaling cascades of eNOS activation in PH-induced splenomegaly remain to be defined. On the other hand, some members of Krüppel-like factor (KLF) family, such as KLF2 (45, 58) and KLF5 (16, 34, 47), have been shown to maintain vascular homeostasis via modulating inflammation and fibrosis-related genes, although upstream signaling regulators need to be defined.

Grb-2-associated binder 1 (Gab1), a scaffolding adaptor, belongs to a family of signaling proteins consisting of Gab1, Gab2, and Gab3 (23, 25, 46). Upon stimulation by growth factors, Gab1 undergoes tyrosine phosphorylation and association with phosphatidylinositol 3-kinase (PI3K) and the tyrosine phosphatase Shp2 (46), leading to activation of the Akt and Erk pathways. Homozygous disruption of the Gab1 gene results in embryonic lethality with multiple defects in the
placenta and heart, along with abnormal liver growth (28, 49).

Recently, using endothelium-specific Gab1 knockout (EGKO) mice, several groups, including ours, have demonstrated that Gab1 plays an important role in postnatal ischemic angiogenesis via mediating the PI3K/Akt, Shp2/Erk, and PKA-eNOS signaling pathways (40, 50, 63). Interestingly, especially the Gab1-SHP2 complex regulates the expression of KLF2 and early growth response 1 (Egr1) (27, 50), key transcriptional factors for endothelial homeostasis and anti-inflammation (5, 33). On the other hand, shear stress is able to activate eNOS via Gab1-PKA/Akt pathways (18, 31), suggesting a potential role of Gab1 in the maintenance of normal endothelial function and vascular homeostasis. These studies suggest that endothelial Gab1 signaling plays pivotal roles in both the promotion of angiogenesis and the prevention of endothelial dysfunction.

Because angiogenesis, inflammation, and endothelial dysfunction are important pathological events in PH-related splenomegaly, we set out to determine whether Gab1 plays a

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**Table 1. Gene-specific primers used in this study for RT-PCR**

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
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<tbody>
<tr>
<td>Collagen 1α</td>
<td>CTTAGAGTGGCCACACTTG</td>
<td>TCCGGTGTGACTGTCGAGAG</td>
</tr>
<tr>
<td>Collagen 1α2</td>
<td>GCTTCTGTGGAGAGAGGC</td>
<td>GCTCTGGCATCAGCTCGAGAG</td>
</tr>
<tr>
<td>Collagen 3α1</td>
<td>CGTGAATGTCTGAGTATG</td>
<td>CTAAGCTGAGGTCGAGAG</td>
</tr>
<tr>
<td>TNF-α</td>
<td>CTTGAGCACGACTGCTGAG</td>
<td>GGAGTTAGAACAGATGACACC</td>
</tr>
<tr>
<td>IL-1β</td>
<td>GAAATGCCCACCTTTGAGAGTG</td>
<td>TGGATGCTCTGATCAGGACAG</td>
</tr>
<tr>
<td>IL-17</td>
<td>AACCTTTCACCAGTACCCC</td>
<td>CAGCTTCTCCTCAGATT</td>
</tr>
<tr>
<td>IL-6</td>
<td>CGTGGAGAAGTGTTGAAA</td>
<td>GGACCTGCGCTGCTCCT</td>
</tr>
<tr>
<td>KLF5</td>
<td>CTTCCCAACCTGGGTCTCTCC</td>
<td>GCACCTGAGGCTGCTCTCG</td>
</tr>
<tr>
<td>KLF2</td>
<td>AGCTATCTGAGCTTGTCTTT</td>
<td>GCACCTGAGGCTGCTCTCG</td>
</tr>
<tr>
<td>EGR1</td>
<td>AAGCGACATGGGAGACAGG</td>
<td>GCACCTGAGGCTGCTCTCG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ACAGTCCACGGCATCAGT</td>
<td>CTGCTTCCACCTCCTTTG</td>
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KLF, Krüppel-like factor; EGR1, early growth response 1.

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**Fig. 1. Endothelial Grb-2-associated binder 1 (Gab1) expression is induced by partial portal vein ligation (PPVL) in a time-dependent manner.**

A: portal pressure measurements after PPVL in control (CTR) mice (n = 5, a P < 0.001 vs. sham). B: top, immunoblots for Gab1, Gab2, and GAPDH after sham operation or PPVL for the indicated times in wild-type mouse mesenteric tissues. Bottom, quantification of Gab1 expression normalized to GAPDH (n = 5). C: representative images of mouse mesenteric veins after sham operation and PPVL for the indicated times, which were stained for Gab1 (brown staining, arrows) and counterstained with hematoxylin. Scale bar, 5 μm.
role in this process. Using a PH model induced by partial portal vein ligation (PPVL), a well-characterized model in studies of the pathophysiology of PH (2, 22), we found that endothelial Gab1 signaling inhibited splenomegaly in PH independent of angiogenesis.

MATERIALS AND METHODS

Reagents. Anti-Gab1, GAPDH, Akt, and Erk2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Gab2 antibody was from Millipore (Bedford, MA). Anti-phosphorylated Akt (Ser473), phosphorylated Erk1/2 (Thr42/44), and phosphorylated PKA substrate antibodies were from Cell Signaling Technology (Danvers, MA). Anti-CD45R, phosphorylated eNOS (Ser1177), and eNOS antibodies were from BD Biosciences (San Jose, CA). Anti-CD31 antibody was from Dianova (Hamburg, Germany). Rabbit antiserum against vascular endothelial growth factor receptor 2 (VEGFR2) was as previously described (40). The Sirius red staining kit for detection of fibrosis was from Leagene Biotech (Beijing, China). All other reagents were from Sigma-Aldrich (St. Louis, MO).

Animals. Mice with endothelium-specific knockout of Gab1 were generated as previously described (40). Experiments were performed on 6- to 9-wk-old age-matched males. Animal procedures were carried out according to the guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care International and approved by the Animal Care and Use Committee of Peking University.

Induction of PH by PPVL. PH was induced by PPVL under sterile conditions (54, 57). Briefly, mice were anesthetized with ketamine (80 mg/kg body wt ip). After a midline abdominal incision, the portal vein was separated from the surrounding tissue. A calibrated constriction was made using a single ligature (silk gut 6-0) tied around the portal vein along with a blunt-ended 27-gauge needle, and then the needle was withdrawn. In sham-operated mice, the portal vein was isolated by 10.220.32.246 on June 25, 2017 http://ajpgi.physiology.org/ Downloaded from

Fig. 2. Endothelial Gab1 deficiency aggravates spleen size enlargement after PPVL. A: representative images of mouse mesenteric veins after sham operation or PPVL stained for Gab1 (brown staining, arrows) and counterstained with hematoxylin. Scale bar, 5 μm. B: portal pressure measurements after PPVL in CTR and endothelium-specific Gab1 knockout (EGKO) mice (n = 5, *P < 0.001 vs. sham-CTR; bP < 0.001 vs. sham-EGKO). C: left, photographs of spleens from CTR and EGKO mice after sham operation or PPVL. Right, quantification of spleen-to-body weight ratio (n = 10, *P < 0.01 vs. sham-CTR; bP < 0.001 vs. sham-EGKO; cP < 0.05 vs. PPVL-CTR). D: left, representative images of spleen stained for Gab1 (brown, arrows). Please note that some Gab1-positive areas in control mice exhibited microvascular structures. Scale bar, 5 μm. Right, quantification of Gab1-positive area (n = 5, *P < 0.05 vs. sham-CTR; bP < 0.001 vs. sham-CTR; cP < 0.05 vs. Sham-EGKO; dP < 0.01 vs. PPVL-CTR).
but not ligated. After the operation, the abdomen was closed, and the animals were allowed to recover under a heat lamp. Unless specially noted, all studies were performed 7 days after operation, since at this time the PH syndrome is fully established (4, 19).

**PP measurement.** All experiments were performed on overnight-fasted mice. Under anesthesia with ketamine (80 mg/kg body wt ip), a saline-filled PE-10 catheter was inserted in the portal vein toward the liver. The catheter in turn was inserted into PE-50 tubing and connected to a highly sensitive pressure transducer (Powerlab; AD Instruments, Spechbach, Germany). The pressure was continuously monitored to obtain stable and reliable average data. After death, the spleen was removed and weighed to calculate the spleen-to-body ratio.

**Western blot analysis.** Snap-frozen tissues were homogenized in lysis buffer containing 50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM MgCl₂, 1 mM EGTA, 5 mM EDTA, 0.27 g/ml Na₃P₂O₇, 5 µg/ml aprotinin, 1 µg/ml prostatin A, 1 µg/ml antipain, 10 µg/ml leupeptin, 1 mg/ml phenylmethylsulfonyl fluoride, 2 mM β-glycerol phosphate, 10 mM NaF, and 2 mM Na₃VO₄ (40). The lysates were separated by 10% SDS-PAGE, following by a standard Western blot analysis.

**Histological analysis.** Mouse mesentery and spleen tissues were fixed in 4% phosphate-buffered paraformaldehyde, dehydrated in graded ethanols, and embedded in paraffin using standard procedures. Six-micrometer sections were stained with hematoxylin and eosin (H&E) or incubated with antibodies against Gab1, CD45R (a B lymphocyte-specific marker), and CD31 (an EC-specific marker) or with immunoglobulin G (IgG) as a negative control. Bound antibodies were visualized using diaminobenzidine with hematoxylin for nuclear counterstaining. For semiquantitative analysis of spleen fibrosis, sec-
tions were stained with 0.1% Sirius red and 1% brilliant green. Next, six to eight fields from each slide were randomly selected, photographed by using a microscope equipped with a digital camera (Olympus, Tokyo, Japan), and analyzed using Image-Pro Plus software.

Quantitative real-time PCR. Total RNAs were extracted from mouse spleen using TRIzol reagent (Sigma) and reverse-transcribed to cDNAs using a Superscript first-strand synthesis kit (Invitrogen, Gaithersburg, MD) according to the manufacturer’s instructions. Quantitative real-time (RT) PCR was carried out using SYBR green as the fluorescent dye. Fluorescence data were collected and analyzed using Mastercycler ep realplex2 (Eppendorf, Hamburg, Germany). The primers used for amplification were shown in Table 1.

Statistical analysis. Data are shown as means ± SD. Statistical comparisons were made using Student’s t-test or two-way ANOVA. A probability value <0.05 was considered statistically significant.

RESULTS

Endothelial Gab1 expression is increased by PH in a time-dependent manner. To test if Gab1 is involved in PH, we assessed its expression level in mice with PH induced by PPVL. The successful induction of PH was confirmed by PP measurement (Fig. 1A). The Western blot data revealed that PPVL dramatically increased Gab1 expression in mesenteric veins in a time-dependent manner, whereas the expression of Gab2, another widely expressed Gab family protein, remained unchanged (Fig. 1B). To identify the cells in which Gab1 expression was induced by PH, we carried out immunohistochemistry (IHC) in mesenteric veins using Gab1 antibody. Although basal Gab1 expression was relatively high in endothelium, it was upregulated in both ECs and smooth muscle cells (Fig. 1C). This result showed that Gab1, but not Gab2, was specifically upregulated in PH, indicating a potential pathophysiological role of the Gab1 gene.

Endothelial Gab1 deficiency aggravates spleen size enlargement after PPVL. To determine the role of endothelial Gab1 in the pathogenesis of PH, we used EGKO mice, which were established in our laboratory and had been well characterized in terms of the efficiency and specificity of Gab1 deletion (40). Age-matched EGKO and control mice were subjected to PPVL. IHC analysis showed that EGKO mice exhibited deficient expression of Gab1 both under basal and PPVL conditions, demonstrating efficient Gab1 deletion (Fig. 2A). Next, their PPs were measured. The basal PPs were comparable in control and EGKO mice; whereas the mean PP value in EGKO mice was slightly higher than in controls, the difference was not statistically significant (Fig. 2B). As expected, 7 days after PPVL control mice displayed significant splenomegaly with an increased spleen-to-body (g/g) weight ratio (0.51 ± 0.069) (Fig. 2C), which is consistent with the published data (22, 52). Interestingly, however, EGKO mice developed more pro-

![Fig. 4](https://example.com)
nounced splenomegaly (Fig. 2C). We then examined the change of Gab1 expression in spleen of mice after PPVL. Gab1 was expressed at a relatively low level in sham/control mice, but it was hardly detected in sham/EGKO mice (Fig. 2D). This is consistent with our preliminary finding that Gab1 was mostly expressed in ECs in spleens of control mice (data not shown). After PPVL, Gab1 expression was enhanced in spleens of control mice, which appeared to be more abundant in the structure of vascular cells, significantly higher than that in EGKO mice (Fig. 2D). The above data demonstrated a protective role of endothelial Gab1 against the development of splenomegaly in PH.

**Endothelial Gab1 deficiency results in hyperactive white pulp after PPVL.** To get insight into the pathogenesis of splenomegaly in PH, we focused attention on the histological changes in the enlarged spleen. Consistent with a previous study (42), the percentage of white pulp (WP) area in spleen sections was significantly increased in PH mice (Fig. 3A). Endothelial Gab1 deficiency further increased the splenic WP area in PPVL mice (Fig. 3A). Consistently, B lymphocytes, which were identified by IHC for CD45R, were more abundant in the WP area of EGKO mice than controls (Fig. 3B). In addition, the spleen of EGKO mice had enhanced immunological function post-PPVL compared with controls, since the expression of lymphocyte-derived IgG-class antibody was clearly higher (Fig. 3C). These findings suggested that endothelial Gab1 has an inhibitory effect on WP activation in PH-related splenomegaly.

**Endothelial Gab1 deficiency aggravates splenic fibrogenesis after PPVL.** Chronic splenomegaly is usually associated with increased fibrogenesis (7, 8). To assess this, we carried out Sirius red staining, a well-accepted histochemical stain for fibrosis, as well as complementary quantitative RT-PCR to detect the presence of fibrosis. The area of Sirius red-stained collagen fibers was expanded in PH mice (Fig. 4A), and the splenic fibrosis in EGKO mice was significantly higher than controls (Fig. 4A). Consistently, we found that the mRNA expression of fibrogenic genes (collagens 1α1, 1α2, and 3α1) was strongly induced at least threefold by PH, and the induction was greater in EGKO mice than in controls (Fig. 4B). Collectively, these results strongly suggested an inhibitory role of endothelial Gab1 in PH-induced splenic fibrogenesis.

**Endothelial Gab1 deficiency impairs splenic and mesenteric angiogenesis after PPVL.** Because angiogenesis is also an essential process in PH-related splenomegaly (7, 8), we evaluated angiogenesis by H&E and IHC for CD31 (an EC-specific marker). Angiogenesis was remarkably induced by PPVL both in spleen (Fig. 5A) and in mesenteric tissues (Fig. 6A) in control mice, consistent with previous studies (20, 42), but the phenomenon was attenuated in EGKO mice (Figs. 5A and 6A). Based on all these data, we drew the conclusion that endothelial Gab1 deficiency promotes splenomegaly following PPVL independent of angiogenesis.

**Endothelial Gab1 deficiency attenuates eNOS activation after PPVL.** To explore the molecular mechanism for splenomegaly caused by endothelial Gab1 deficiency, we studied Gab1-related signaling pathways. Previously, we showed that Gab1 was important for the VEGFR2 signaling pathway (40). Besides, VEGFR2 has been shown to play a role in PH (43). We thus checked the change of VEGFR2 expression after PPVL. VEGFR2 expression was lower in EGKO mice than the controls both in spleen and mesenteric tissues (Figs. 5B and 6B).
6B), consistent with the previous finding. As noted above, eNOS is a negative regulator of vascular inflammation and tissue fibrosis. Previously, we and others have demonstrated that Gab1 is essential for the activation of eNOS (18, 40). Therefore, we assessed the involvement of these pathways in PH-associated splenomegaly and found that EGKO and control mice displayed similar levels of basal phosphorylation of eNOS, PKA, Erk, and Akt (Fig. 7). In contrast, after PPVL, the levels of eNOS phosphorylation in both spleen (Fig. 7A) and mesenteric tissues (Fig. 7B) in EGKO mice were significantly lower than those in controls. Similarly, the phosphorylation levels of PKA, Erk, and Akt were significantly decreased in EGKO mice, albeit with some variations in spleen and mesenteric tissues. In summary, endothelial Gab1 deficiency impaired the activation of VEGFR2-eNOS pathways, which may contribute to the exaggerated pathological changes of inflam-
mation and fibrosis in aggravated splenomegaly in EGKO mice.

**Endothelial Gab1 deficiency results in abnormal expression of fibrosis and inflammation-related genes after PPVL.** To further reveal the molecular mechanisms of enhanced inflammation and fibrosis in EGKO mice, we examined the expression of fibrosis and inflammation-related genes in the spleen after PPVL, using quantitative RT-PCR to examine the mRNA level of inflammatory cytokines and transcriptional factors. As shown in Fig. 8, the expression of KLF2 and EGR1, which have been shown to have inhibitory effects of vascular inflammation (5, 33), was significantly downregulated in the spleen of EGKO mice compared with those of control mice (Fig. 8). In contrast, KLF5 mRNA, which is known to be a proinflammatory and profibrotic transcription factor (34, 47), was significantly upregulated. Consistently, the mRNA levels of inflammatory cytokine genes, including TNF-α, IL-1β, IL-6, and IL-17, were significantly increased. Taken together, these findings suggest that endothelial Gab1 deficiency leads to the dysregulation of fibrosis and inflammation-related transcription factor genes and the upregulation of cytokine genes.

**DISCUSSION**

The major new finding in this study is that Gab1 in ECs plays an inhibitory role in PH-induced splenomegaly. Gab1 expression was significantly upregulated in ECs by PH, whereas endothelium-specific Gab1 deletion exaggerated spleen enlargement, accompanied by significant fibrosis and hyperactivation of the WP. In addition, the inhibitory effects of endothelial Gab1 signaling seem to be exerted via multiple pathways, including the activation of the eNOS pathway, modulation of the expression of fibrosis and inflammation-related transcription factor genes, and the downregulation of proinflammatory genes. It has been suggested that several pathological changes, including angiogenesis, are actively in-
Fig. 8. Endothelial Gab1 deficiency results in enhanced expression of inflammatory cytokines after PPVL. Expression of inflammatory cytokines (TNF-α, IL-6, IL-1β, and IL-17) and transcriptional factors [Krüppel-like factor (KLF) 5, KLF2, and early growth response 1 (EGR1)] in whole spleen after sham operation or PPVL for 3 days in EGKO mice vs. CTR. Data are shown as 5, a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p, q, r, s, t, u, v, w, x, y, z, A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P, Q, R, S, T, U, V, W, X, Y, Z.

Fig. 9. Schematic illustration of the role of Gab1 in regulation of portal hypertensive splenomegaly. Upon vascular injury, endothelial Gab1 transmits signaling from the activated receptors [e.g., VEGFR2, platelet-derived growth factor receptor 2 (PDGFR2)] to downstream pathways, which activate eNOS signaling, modulate the expression of fibrosis/inflammation-related transcription factor genes, and inhibit the expression of cytokine genes. Anti TFs, transcription factors with the function of anti-fibrosis and anti-inflammation, e.g., KLF2 and EGR1; Pro TFs, transcription factors with the function of stimulating fibrosis and inflammation, e.g., KLF5.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: B.J. and J.L. conception and design of research; B.J., Q.D., Y.H., and M.S. performed experiments; B.J., Q.D., and Y.H. analyzed data; B.J., Q.D., W.L., and J.L. interpreted results of experiments; B.J. and Q.D. prepared figures; B.J. drafted manuscript; J.L. edited and revised manuscript; J.L. approved final version of manuscript.

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ENDOTHELIAL Gab1 DEFICIENCY AGGRAVATES SPLENOMEGALY