Macrophage Notch Ligand Delta-Like 4 Promotes Vein Graft Lesion Development

Implications for the Treatment of Vein Graft Failure


Objective—Despite its large clinical impact, the underlying mechanisms for vein graft failure remain obscure and no effective therapeutic solutions are available. We tested the hypothesis that Notch signaling promotes vein graft disease.

Approach and Results—We used 2 biotherapeutics for Delta-like ligand 4 (Dll4), a Notch ligand: (1) blocking antibody and (2) macrophage- or endothelial cell (EC)–targeted small-interfering RNA. Dll4 antibody administration for 28 days inhibited vein graft lesion development in low-density lipoprotein (LDL) receptor-deficient (Ldlr−/−) mice, and suppressed macrophage accumulation and macrophage expression of proinflammatory M1 genes. Dll4 antibody treatment for 7 days after grafting also reduced macrophage burden at day 28. Dll4 silencing via macrophage-targeted lipid nanoparticles reduced lesion development and macrophage accumulation, whereas EC-targeted Dll4 small-interfering RNA produced no effects. Gain-of-function and loss-of-function studies suggested in vitro that Dll4 induces proinflammatory molecules in macrophages. Macrophage Dll4 also stimulated smooth muscle cell proliferation and migration and suppressed their differentiation.

Conclusions—These results suggest that macrophage Dll4 promotes lesion development in vein grafts via macrophage activation and crossstalk between macrophages and smooth muscle cells, supporting the Dll4–Notch axis as a novel therapeutic target. (Arterioscler Thromb Vasc Biol. 2015;35:2343-2353. DOI: 10.1161/ATVBAHA.115.305516.)

Key Words: endothelial cells  inflammation  lipid  macrophage  nanoparticles

Vein graft failure is a global health burden with no effective medical solutions.1 Because of the pandemic of atherosclerotic peripheral artery disease and the growing prevalence of underlying metabolic disorders,2 the incidence of vein graft failure is rising. Although many mechanisms for arterial diseases have been established, the pathogenesis of vein graft failure remains incompletely understood. Autologous saphenous vein grafts are widely used for peripheral artery disease because they remain patent longer than artificial conduits.3 Approximately 50% of lower extremity saphenous vein grafts, however, become occluded or narrowed within a year.4 When peripheral artery disease grafts fail, the only available therapeutic options are devastating limb amputation or invasive and expensive angioplasty or surgical revascularization. Coronary artery saphenous vein graft also fail at high rates.5 Although current therapies such as statins can reduce the onset of complications of arterial diseases (eg, myocardial infarction),6 no effective medical solutions are available for vein graft failure.

See cover image

The Notch pathway, involving ligands (Delta-like ligand [Dll]1, Dll3, Dll4, Jagged1, and Jagged2) and receptors (Notch1–4), contributes to biological processes during development and to disease mechanisms in adults.7,8 Direct cell-to-cell contract via the binding of a ligand to a Notch receptor, both of which are expressed on the cell surface, triggers downstream responses.9 We previously demonstrated that Dll4-mediated Notch signaling promotes macrophage activation.10,11 Clinical and preclinical evidence has established the causal role of macrophages in arterial atherosclerosis.12,13 Failing vein
grants also tend to contain macrophages, but their role in the disease progression remains unclear. To test the hypothesis that macrophage Notch signaling contributes to the pathogenesis of vein graft disease, this study used 2 clinically relevant biotherapeutics: (1) Dll4 blocking antibody and (2) Dll4 small-interfering RNA (siRNA) encapsulated in macrophage- or endothelial cell (EC)–targeted lipid nanoparticles (LNPs).

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Increased Expression of Dll4 in Macrophages in Human and Mouse Vein Grafts

In control human saphenous veins before grafting, little if any intimal cells were immunoreactive for Dll4, whereas the thickened intima of failed human saphenous vein graft contained many cells expressing Dll4 (Figures 1A; Figure I in the online-only Data Supplement). In the failed grafts, some CD68-positive intimal macrophages were immunoreactive to Dll4 antibody (Figure IB in the online-only Data Supplement). In high-cholesterol/high-fat–fed Ldlr−/− mice, IVC implanted into the carotid artery developed more advanced lesions than in wild-type mice. The neointima of vein grafts in the carotid artery developed more advanced lesions than in wild-in hypercholesterolemic ApoE3*Leiden mice by the Paul Quax Supplement), supporting previous reports on a similar model intraplaque hemorrhage (Figure II in the online-only Data Supplement). Ligand binding promotes the cleavage of Notch receptors and release of the intracellular domain. The amount of Notch1 intracellular domain (NICD), as identified by the antibody that recognizes the neoepitope, thus indicates the levels of Notch-signaling activation. NICD accumulated primarily in intimal macrophages of vein grafts 28 days after implantation, whereas few if any SMC and EC were stained positively (Figure III in the online-only Data Supplement). Dll4 and NICD were almost undetectable in the native IVC (Figure IVA in the online-only Data Supplement). But the amounts of immunoreactive Dll4 and NICD in the intima of mouse vein grafts increased in parallel over time (Figure IVA and IVC in the online-only Data Supplement), indicating acceleration of Notch-signaling activation during the lesion development. Furthermore, the amounts of Dll4 and NICD correlated positively with the wall area (Figure IVC in the online-only Data Supplement). These results suggest that Dll4–Notch signaling is accelerated during the development of vein graft lesions.

Blockade of Dll4 Reduces Lesion Formation and Inflammation in Vein Grafts

Blocking antibody for Dll4 was administered to Ldlr−/− mice twice a week for 28 days. Reduced amounts of NICD after antibody administration indicate that Dll4 mediates Notch activation in vein graft lesions (Figure 1D). Dll4 blockade produced

![Image 1A: Delta-like ligand 4 (Dll4) expression increases in human and mouse vein grafts.](http://atvb.ahajournals.org/)

![Image 1B: Dll4 mRNA in mouse vein grafts.](http://atvb.ahajournals.org/)

![Image 1C: Immunostain of Dll4 and Mac-3](http://atvb.ahajournals.org/)

![Image 1D: NICD/Intima](http://atvb.ahajournals.org/)
no effects on serum levels of total cholesterol (801.0±51.5 versus 867.0±15.7 mg/dL), triglycerides (216.2±19.3 versus 249.3±36.1 mg/dL), and body weight (29.6±0.7 versus 30.5±1.1 g). We previously verified that administration of the same antibody for 3 months did not affect blood pressure, food consumption, and physical activity in \( Ldlr^{−/−} \) mice.\(^{11}\)

Histological assessment demonstrates that 28 days of Dll4 antibody treatment decreased the area and thickness of the intima of vein grafts in \( Ldlr^{−/−} \) mice but produced no significant changes in the lumen diameter, media/adventitia thickness, or vessel diameter (Figure 2A). Noninvasive ultrasonography is routinely used to monitor the patency of

![Figure 2](http://atvb.ahajournals.org/)

**Figure 2.** Antibody blockade of Delta-like ligand 4 (Dll4) for 28 days inhibits lesion formation in vein grafts. **A**, Vein graft harvested 28 days after implantation from control IgG or Dll4 antibody-treated animals and results of morphometric analyses. Scale bar, 400 \( \mu \text{m} \); \( n=10 \) and 9. **B**, Ultrasonographic images of vein grafts treated with control IgG (left) or anti-Dll4 antibody (right) 28 days after implantation. The white dotted line indicates vessel wall area. **C**, Lumen and vessel wall area (\( \text{mm}^2 \)) and vessel wall volume (\( \text{mm}^3 \)) of ultrasonographic images in control IgG or Dll4 antibody treated vein grafts (\( n=5 \) and 3). **D**, Immunostaining of Mac-3, \( \alpha \)-SMA, and proliferating cell nuclear antigen (PCNA) at day 28 and quantitative data shown as percentages of staining positive area (Mac-3, \( \alpha \)-SMA) and PCNA-positive nucleus in the intima. Scale bar, 200 \( \mu \text{m} \); \( n=4 \) to 7.
vein grafts in patients. Clinically relevant ultrasound imaging visualized and quantified the decreased wall area and volume of vein grafts, but no significant changes in the lumen area, in mice treated with Dll4 antibody (Figure 2B and 2C), which is consistent with the microscopic data. Dll4 blockade also reduced the accumulation of macrophages (Mac3-positive cells), SMC (α-SMA), and proliferating cell nuclear antigens in the intima (Figure 2D). Proliferation of macrophages and SMC may contribute to the pathogenesis of vascular diseases. Dll4 blockade inhibited macrophage phagocytic activity and MMP activity in parallel (Figure 6B). Notably, Dll4 antibody therapy inhibited collagen degradation by macrophage-derived proteolytic activity, indicating that Dll4 suppression diminishes the proinflammatory microenvironment in vein grafts.

Dll4 Blockade Suppresses MMP Activity and Reduces Thin Collagen Fibers

In vivo molecular imaging further assessed the effects of Dll4 suppression on macrophage activation in vein grafts. We coinjected 2 imaging agents that elaborate near-infrared signals to visualize macrophage phagocytic activity (AminoSPARK, 750 nm) and MMP activity (MMPSense, 680 nm). Dll4 blockade inhibited macrophage phagocytic activity and MMP activity in parallel (Figure 4A). Notably, Dll4 antibody therapy reduced macrophage activation in vein grafts as early as 7 days after implantation, supporting our microscopic observation (Figure VII in the online-only Data Supplement). MMP produced by activated macrophages impairs collagen content and structures. Picrosirius red staining viewed under a circularly polarized microscope showed no significant difference in the content of total fibrillar collagen between 2 groups (Figure 4B). Collagen hue analysis, however, revealed that Dll4 blockade decreased thin collagen fiber (Figure 4B), indicating that collagen degradation by macrophage-derived proteolytic activity may have reduced.

Macrophage-Targeted Dll4 Silencing Inhibits Intimal Thickening and Macrophage Accumulation

To determine the relative contribution of macrophage Dll4 to the development of vein graft lesions, we used macrophage-targeted LNP (C12-200) to deliver Dll4 siRNA in...
vivo.25,26 In pilot experiments, a single injection of 0.5 mg/kg C12-200-siDll4 resulted in a 51% reduction of Dll4 mRNA in splenic macrophages in 72 hours (Figure 5A).

To validate the selectivity of Dll4 silencing to macrophages in vivo, we administered 0.5 mg/kg C12-200-siDll4 at 21 and 24 days after vein graft implantation. qPCR of intimal tissues containing endothelium or macrophage clusters isolated by laser capture microdissection showed inhibition of Dll4 expression in vein graft macrophages by >70%, but no effect in endothelium (Figure 5B).

C12-200-siDll4 was then injected at 0.5 mg/kg, twice a week, in Ldlr−/− mice. C12-200-siDll4 decreased intimal area and thickness as compared with control C12-200 containing nontargeting siRNA (Figure 5C). C12-200-siDll4 reduced macrophage accumulation in the intimal layer (Figure 5D), although it did not significantly increase thick collagen fibers (Figure 5E). These results indicate that Dll4 expressed by macrophages contributes to the lesion formation and macrophage burden in vein grafts.

Dll4 Regulates Expression of Proinflammatory Molecules in Macrophages

To explore mechanistic evidence for the causal role of Dll4 in macrophage activation, we performed gain-of-function and
loss-of-function experiments in mouse primary macrophages. Transient overexpression ofDll4 induced prototypical Notch target genes Hes1 and Hey1 (Figure 6A). Enforced expression ofDll4 induced proinflammatory molecules typical of M1 macrophages (eg, IL-1β, TNF-α; Figure 6B). In contrast,Dll4 blocking antibody exerted opposing effects (Figure 6C). Furthermore, Dll4 blockade inhibited the expression of proinflammatory genes iNOS and TNF-α induced by IFN-γ, a typical M1 stimulation (Figure 6D). These in vitro findings are consistent with in vivo data shown in Figure 3.

**Macrophage Dll4 Promotes SMC Migration, Proliferation, and dedifferentiation**

SMC migration and proliferation may contribute to the development of vein graft lesions. Notch signaling requires the direct cell–cell contact via the ligand–receptor binding. SMCs in the
intima of atherosclerotic plaques and vein grafts are, however, surrounded by extracellular matrix and generally lack membrane contacts with neighboring SMCs, whereas such direct contacts are common in plaque macrophages, suggesting that direct physical interactions between SMC and macrophages via Dll4–Notch binding may hardly occur. Nevertheless, we examined the effects of Dll4 binding to SMC in vitro using primary human saphenous vein SMC. Immobilized recombinant Dll4 attachment did not affect the number of human saphenous vein SMCs both in 0.5% and 10% fetal bovine serum (Figure IXA in the online-only Data Supplement). Blockade of Dll4 binding also produced no effects on the growth of human saphenous vein SMC (Figure IXB in the online-only Data Supplement). These results indicate that direct Dll4 binding may not play a major role in SMC biology. Therefore, we performed indirect coculture experiments to examine whether macrophage expression of Dll4 induces SMC migration, proliferation, and dedifferentiation by soluble factors in a paracrine fashion. Conditioned media from RAW264.7 cells transfected with Dll4 plasmid accelerated SMC migration (Figure 7A), increased SMC number (Figure 7B), and suppressed their expression of myosin heavy chain, the strictest SMC differentiation marker (Figure 7C). PDGF-BB induces SMC migration, proliferation, and dedifferentiation. Notch activation by enforced expression of Dll4 or immobilized recombinant Dll4 induced PDGF-B expression in RAW264.7 cells (Figure 7D and 7E), indicating that PDGF-BB may mediate the effects of Dll4 via macrophage–SMC crosstalk. Other proinflammatory factors, which Dll4 induces in macrophages, may also contribute to this interaction (Figure 6).

**Role of EC-Derived Dll4 in the Development of Vein Graft Lesions**

Among human primary macrophages, human saphenous vein SMC, and human saphenous vein EC, Dll4 mRNA levels were highest in human saphenous vein EC under the quiescent state (Figure XI in the online-only Data Supplement). LPS markedly induced Dll4 only in primary macrophages (Figure XI in the online-only Data Supplement). Endothelium also appeared positive for Dll4 in the failed human vein grafts (Figure I in the online-only Data Supplement). In human saphenous vein EC, blockade of Dll4 suppressed MCP-1, IL-1β, IL-6, and VCAM-1 expression (Figure XB in the online-only Data Supplement). In contrast, immobilized Dll4 induced EC expression of IL-1β and VCAM-1 (Figure XC in the online-only Data Supplement). Dll4 antibody treatment reduced the number of adventitial microvessels (Figure XI in the online-only Data Supplement). We therefore explored a new mechanism, by which EC-derived Dll4 participates in the pathogenesis of vein graft disease using Dll4 siRNA formulated in EC-targeted LNP (Figure 8A). Despite the high silencing efficacy, EC-targeted Dll4 suppression produced no effects on several parameters for the development of vein graft lesions (intima area, intima thickness, lumen diameter, media/intima thickness, and vessel diameter; Figure 8B and 8C).
These results further support a major role for macrophage Dll4 in vein graft disease.

**Discussion**

Although many mechanisms have been proposed and validated for arterial diseases, the pathogenesis of vein graft disease remains obscure. Using 2 different biotherapies—blocking antibody and macrophage-targeted siRNA, this study demonstrates novel mechanisms by which macrophage Dll4 promotes the development of vein graft lesions. Accumulating evidence has established that macrophages contribute to various mechanisms for arterial diseases, including plaque rupture. The role of macrophages in vein graft disease, however, remains elusive. Failing vein grafts in patients exhibit macrophage...
Clinical evidence has linked biomarkers of inflammation with vein graft failure. Preclinical studies proposed the role of macrophages in neo-intima formation in vein grafts. However, no medical therapies are currently available to target vein graft inflammation, which has driven our current efforts.

The key novel findings demonstrated in this study include (1) the expression of Dll4 by macrophages in the intima of human and mouse vein grafts; (2) increased Dll4 expression and NICD accumulation during the development of experimental vein graft lesions; (3) positive correlations between the graft wall area and Dll4 expression or NICD accumulation; (4) reduced vein graft lesions after Dll4-targeted biotherapeutics (blocking antibody and macrophage-selective Dll4 siRNA); (5) the role of Dll4–Notch signaling in macrophage and SMC growth in vein grafts; (6) the effects of Dll4 blockade on macrophages being independent of metabolic effects; (7) the potential role of Dll4 in EC activation; (8) no substantial differences in vivo role for EC-derived Dll4 in vein graft disease as demonstrated by EC-targeted Dll4 siRNA; and (9) the role of Dll4-expressing macrophages in SMC dedifferentiation, migration, and proliferation via a paracrine mechanism.

The previous studies including our own suggested that Notch-signaling components, including Dll4, contribute to various biologies of hematopoietic cells. However, the mechanistic evidence for the role of Dll4 in inflammation of cardiovascular organs remains scant.

Macrophage polarization, as often classified by at least 2 subpopulations: a proinflammatory (M1) and an anti/noninflammatory (M2), is associated with various cardiovascular diseases. In vein grafts, Dll4 blockade reduced the expression of multifunctional proinflammatory IL-1β and TNF-α, typical M1 molecules, suggesting the broad antiatherogenic effects of Dll4 antibody via suppression of a positive feedback loop of sustained macrophage activation and providing insight into the clinical impact of this therapy. Examining the relative contribution of macrophage-derived Dll4 used the macrophage-targeted LNP C12-200. Dll4 silencing in macrophages via C12-200 decreased intimal thickening and macrophage burden.

Our in vitro evidence in this study suggests a role for Dll4 in EC activation. Therefore, we used EC-targeted LNP 7C1 to explore an additional potential mechanism for vein graft disease mediated by EC-derived Dll4. Despite an excellent silencing efficacy in endothelium, Dll4 siRNA formulated in 7C1 produced no effects on the development of vein graft lesions, as quantitatively determined by several parameters. These results provide another line of evidence for the pivotal role of Dll4 expressed by macrophages in the development of inflamed vein grafts.

**Figure 8.** The relative contribution of endothelial cell (EC)-derived Delta-like ligand 4 (Dll4) in vein graft lesion development as examined via small-interfering RNA (siRNA) delivery to endothelium. EC-targeted lipid nanoparticles (7C1) containing control siRNA (Ctrl) or Dll4 siRNA (siRNA) were injected via tail vein. A, Dll4 mRNA levels were quantified by real-time polymerase chain reaction in the endothelium isolated by laser capture microdissection. B, Masson–Trichrome staining of vein grafts at day 28. Scale bars, 500 µm. C, Bar graphs demonstrate the results of quantitative morphometric analysis (each n=6).
primary human SMC than those in macrophages or EC. Dll4 binding to primary SMC did not induce their proliferation. In addition, previous studies demonstrated a lack of membrane contact between SMC surrounded by extracellular matrix, whereas direct contact between macrophages is common. Notch-signaling activation requires direct cell-to-cell contact that allows ligand–receptor binding. Thus, Dll4–Notch interaction between neighboring SMC or between SMC and macrophages may not occur so frequently in vascular lesions. Instead, a series of experiments reported in this study suggest a novel paracrine mechanism by which macrophage Dll4 activates neighboring SMC (Figure XIIA in the online-only Data Supplement).

To maximize clinical significance of our study, we used 2 scientifically validated and clinically relevant techniques to suppress Dll4—RNAi mediated by LNP and antibody administration. The gene silencing by siRNA oligos formulated in LNP, a robust and well-established research tool for investigating the role of macrophage gene expression in vivo, enabled us to selectively target Dll4 in macrophages or EC, as documented in the previous studies.25,26,32,45,46 It should be noted that the delivery of siRNA in such LNP has already been used in humans and proven safe and effective, contributing to the generation of promising clinical data.47–49 In addition, antibody therapies for chronic diseases have become widely available in the clinic. For instance, a phase IIb clinical trial on 4 months of anti–IL-1β monoclonal antibody treatment presented anti-inflammatory effects (eg, reductions in C-reactive protein levels) with no substantial adverse effects,24 leading to a larger, longer cardiovascular outcome study. In the present study, Dll4 antibody therapy for only 7 days exerted beneficial effects on lesional macrophages in vein grafts. The use of such clinically relevant therapeutics suggests how our preclinical findings could be translated into the clinical development of Dll4-targeted therapies for vein grafts. In addition, noninvasive ultrasonography of vein grafts, a routine procedure in the clinic, in live mice supports the microscopic findings. These lines of evidence indicate the clinical translatability of our mouse study.

In conclusion, we provide the novel evidence that the Dll4–Notch axis contributes to the pathogenesis of vein graft lesion development (Figure XIIA in the online-only Data Supplement). Complementary in vivo experiments using macrophage- or EC-targeted siRNA demonstrate the relative contribution of macrophages to the development of vein graft lesions mediated by Dll4. The study has identified Dll4 as a new promising therapeutic target for vein graft failure (Figure XIIIB in the online-only Data Supplement), a major clinical problem with no medical solutions.

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Disclosures
None.

References


Significance

Although vein graft failure is a major clinical problem, no medical therapies are available. Using clinically relevant biotherapies—blocking antibody and small-interfering RNA–loaded nanoparticles, we demonstrate Delta-like ligand 4 promotes inflammation and lesion development in mouse vein grafts, providing novel mechanisms and new therapeutic solutions for this disease.
In the article by Koga et al, which appeared in the November 2015 issue of the journal (Arterioscler Thromb Vasc Biol. 2015;35:2343–2353. DOI: 10.1161/ATVBAHA.115.305516), a correction was needed.

For the top right panel of Supplemental Figure IV-A, a section of a part of the carotid artery, adjacent to the vein graft, was accidentally used. To correct this error, the authors have replaced this image by another one at the level of the vein graft (see below). They further repeated color image analysis and statistics for Figures IV-B (left panel; \( P < 0.05 \)) and IV-C (left panel; \( r = 0.87, P < 0.05 \)). The revised quantitative data are similar to the original results.

The authors apologize for the error.

The Data Supplement file has been corrected and is available at http://atvb.ahajournals.org/content/35/11/2324.
Supplemental Figure I: Dll4 expression increases in human vein grafts. (A) Human SVG harvested for bypass surgery (control native vein, left) and failed SVG (middle and right). Sections were stained with anti-Dll4 antibody. Right images show high magnification images in rectangles. Scale bars indicate 100 µm (left and middle) and 50 µm (right). (B) Serial section of failed human SVG stained with anti-Dll4 and anti-CD68 antibodies. Scale bar indicates 100 µm.
Supplemental Figure II: The neointima of vein grafts in Ldlr-/- mice shows features associated with advanced atherosclerotic lesions. IVCs from donor Ldlr-/- mice were implanted into the carotid artery of recipient Ldlr-/- mice after 2 weeks of high fat diet feeding. Vein grafts were harvested 28 days after implantation and then stained by Masson-Trichrome staining. Scale bar indicates 200 µm.
Supplemental Figure III: Accumulation of Notch 1 intracellular domain (NICTD) in vein grafts. Immunostaining for the neoepitope of cleaved Notch1 detected NICTD in macrophages (A), SMC (B) and EC (C) at 28 days after vein grafting of mice. Upper panels show double staining of Mac-3 and NICTD. Middle panels show double staining of α-SMA and NICTD. Lower panels show double staining of CD31 and NICTD. Arrows indicate double-positive cells. The data represent 5 mice that showed similar results. Scale bars indicate 20 µm.
Supplemental Figure IV: The expression of Dll4 and Notch 1 intracellular domain (NICD) increase in mouse vein grafts. (A) Immunostaining of Dll4 and NICD domain in inferior vena cava (IVC), and vein grafts at days 7 and 28. Scale bars indicate 100 μm. (B) Bar graphs show quantification of Dll4 and NICD as percentage positive area in the intima in IVCs and vein grafts at days 7 and 28 (n=5 to 6). (C) Figures show the association of Dll4- or NICD-positive area with vessel wall area of the ultrasound data in vein grafts at day 28 (n=5). L, the lumen; A, the adventitia.
Supplemental Figure V: Macrophage and SMC proliferation in vein grafts of mice treated with control IgG or Dll4 blocking antibody for 28 days. (A) Upper panels show double staining of Mac-3 and PCNA. Arrows indicate double-positive cells. Lower panels show double staining of α-SMA and PCNA. (B) The percentage of PCNA-positive cells in Mac-3 or α-SMA-positive cells. N=5 or 6.
Supplemental Figure VI: No significant effect of Dll4 blockade on adventitial macrophages. Adventitial cells immunoreactive for Mac-3 in vein grafts were quantified at 28 days after implantation. N=9-10.
Supplemental Figure VII: One-week administration of Dll4 antibody at the early phase reduced macrophage burden. (A) Dll4 blocking antibody or control IgG was administered until 7 days after vein graft implantation in high-fat-fed Ldlr-/− mice. Vein graft was harvested 28 days after implantation and stained by Masson-Trichrome staining. Scale bars indicate 400 μm. Bar graphs demonstrate the results of morphometric analyses. n = 7 and 6. (B) Mac-3 staining of vein graft at Day 28. Macrophage accumulation was quantified as percentage of Mac-3–positive area in intimal layer. Scale bars indicate 200 μm. Bar graph shows the result of quantitative analysis. n = 4 and 6.
Supplemental Figure VIII: Glucose metabolism and insulin sensitivity of Dll4 Ab treatment mice after day 7. (A) Ldlr/- mice were treated with anti-Dll4 antibody (n=6) or control IgG (n=6) for 7 days and underwent (A) glucose tolerance test (GTT) and (B) Insulin tolerance test (ITT).
Supplemental Figure IX: The effects of Dll4 binding on SMC number (A) HSVSMCs were cultured over immobilized recombinant Dll4 (rDll4) (2 μg/mL). rDll4 did not increase the number of HSVSMCs both in 0.5% and 10% FBS. N=7. (B) Blockade of Dll4 binding did not inhibit 10% FBS-mediated HSVSMC growth. N=7. HSVSMC number was evaluated by MTS assay and the left axis of the graph shows optical density (O.D.).
Supplemental Figure X: The role of Dll4 on HSVECs. (A) Quantitative analyses of Dll4 mRNA levels with or without 3 hours of 10 ng/mL LPS stimulation. n = 4-6. (B) HSVECs were incubated with 10 µg/mL of IgG or Dll4 blocking antibody. mRNA was extracted 24 hours later and quantified by real-time PCR. Data are shown as relative expression normalized by HSVECs treated with IgG. n = 5. (C) HSVECs were plated on immobilized Dll4, and RNA was extracted 24 hours later. Bar graph shows the results of real-time PCR. Data are shown as relative expression normalized by HSVECs treated with PBS. n=4 to 7.
Supplemental Figure XI: The effects of Dll4 blockade on adventitial microvessels. Microvessels in the adventitia of vein grafts were quantified at 28 days after implantation. α-SMA positive luminal structures were considered as adventitial microvessels. Scale bar indicates 200 mm. N=6.
Supplemental Figure XII: Schematic views summarizing key novel findings and clinical perspectives. (A) Macrophage Dll4-Notch signaling as a key player of vein graft lesion development. Dll4 activates lesional macrophages, which can form a positive feedback loop of inflammation. Crosstalk between Dll4-Notch-activated macrophages and SMCs may modulate SMC phenotype and promote lesion development. Dll4 blocking antibody or macrophage-targeted Dll4 siRNA inhibits Dll4-mediated vascular inflammation and remodeling. SMC, smooth muscle cells; Mϕ, macrophages. (B) Clinical perspectives. Dll4 biotherapy — blocking antibody and macrophage-targeted siRNA — may serve as possible therapeutic options for the prevention of vein graft failure.
Supplemental Figure XIII: Properties of lipid nanoparticles. Particle diameter, weighted by volume, as measured by dynamic light scattering (DLS) after nanoparticle formulation. Control siRNA (Ctrl) and Dll4 siRNA (siDll4) particle sizes are consistent (N=3 formulations each).
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<td>Myh11</td>
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Materials and Methods

Vein graft implantation

The Harvard Medical Area Standing Committee on Animals reviewed and approved all animal experimental protocols. Male LDL receptor–deficient (Ldlr/-) mice with C57Bl/6 background were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained with water ad libitum. High-fat diet containing 1.25% cholesterol (D12108; Research Diets, NJ) was started at 12 weeks of age, both in donor and recipient Ldlr/- mice. After 2 weeks high-fat diet feeding, vein graft implantation was performed as previously reported.1,2 Inferior vena cava (IVC) was harvested from age-matched donor Ldlr/- mice and implanted into the recipient right carotid artery with small cuffs made from polyetheretherketone (Zeus, Orangeburg, SC). After vein graft implantation, blood flow and pulsation of the graft was visually assessed to confirm successful grafting. For the selective blockade of Dll4, we intraperitoneally administered hamster anti-mouse Dll4 antibody (250 µg per injection) (HMD4-2, Bio X Cell, West Lebanon, NH)3-5 twice a week. Non-immune hamster IgG antibody (Bio X Cell) was administered to control mice with the same regimen.

Histopathology and immunohistochemistry

Vein grafts were harvested 28 days after vein graft implantation. After perfused fixation with 10% neutral buffered formalin, grafts were incubated with 15% and 30% sucrose for 24 hours each. Then, grafts were embedded and frozen in OCT compound. Sections for morphometric analysis were cut with 7 µm thickness. Lesion size was analyzed with 30 µm intervals after 300 µm trimming from the distal edge of proximal cuff. After Masson-Trichrome staining, 8 sections per each graft were analyzed and average values were used for statistical analysis. The border between intima and media/adventitia was determined by the internal elastic lamina as previously reported.1 The area of lumen, intima and media/adventitia were determined by Image J software. Several variables including intimal thickness, luminal diameter and vessel diameter were calculated based on these measured values as previously reported.1

Immunostaining was also performed with frozen sections cut with 7 µm thickness in mice. Macrophages were stained with rat anti-Mac-3 monoclonal antibody (BD Pharmingen, San Diego, CA). Vascular SMCs were stained with rabbit polyclonal anti-α-SMA actin antibody (Thermo Fisher Scientific Inc., Waltham, MA) or mouse monoclonal anti-human SMA (1A4) antibody (DAKO, Denmark). Dll4 was stained with rabbit polyclonal anti-Dll4 antibody (Abcam, Cambridge, MA) and cleaved Notch1 was stained with rabbit polyclonal anti-Notch1 (cleaved N-terminus) antibody (EMD Millipore, Billerica, MA). Proliferating cells were evaluated with mouse polyclonal anti-proliferation cell nuclear antigen (PCNA) antibody (DAKO) with “Mouse on Mouse” staining kit (Vector Laboratories) or rabbit anti-PCNA antibody (Abcam). In fluorescent immunostaining,
antibodies conjugated with Alexa Fluor 488 or 568 (Life technologies, Carlsbad, CA) were used as secondary antibodies. Isolated human saphenous vein grafts were used for histochemical analysis under the approval of institutional review board of Brigham and Women’s Hospital. Vein grafts were fixed with 10% neutral buffered formalin, embedded in paraffin and cut with 5 μm thickness. Dll4 was stained with rabbit polyclonal anti-Dll4 antibody (Abcam). Macrophage and SMC were stained with mouse monoclonal anti-human CD68 antibody and mouse monoclonal anti-human SMA (1A4) antibody (DAKO).

**Cell isolation from vein graft**
Vein grafts were cut into small pieces and incubated with enzyme solution including 400 unit/ml collagenase type II and 0.75 unit/ml elastase (Worthington Biochemical, Lakewood, NJ) for 1 hour at 37 °C. After filtration through a 40-μm cell strainer (BD Pharmingen), cells were used for further experiments. In real-time PCR of isolated macrophages, F4/80 positive macrophages were isolated by magnetic sorting. PE-conjugated anti-F4/80 antibody (Biolegend, San Diego, CA) were used as the primary antibody to specifically select macrophages.

**Semi-quantitative real-time PCR**
RNA was extracted from tissues or cells by illustra RNAspin Mini Kit (GE Healthcare, Little Chalfont, UK), and cDNA was synthesized by High Capacity cDNA Reverse Transcription Kit (Life Technologies). Semi-quantitative real-time PCR was performed with a MyiQ single-color real-time PCR detection system (BioRad, Hercules, CA) and PerfeCTa SYBR Green SuperMix for iQ (Quanta Biosciences, Gaithersburg, MD). Primer designs are listed on supplemental table I. Data were calculated by ΔΔCT method and expressed in arbitrary units that were normalized by β-actin or GAPDH.

**Ultrasonography of vein grafts**
We performed the ultrasound biomicroscopic examination of vein grafts noninvasively in live mice at Day 28 before tissue harvesting. Mice were anesthetized using isoflurane and laid supine on a temperature regulated platform with four paws secured and in contact with the electrocardiographic electrodes for heart rate monitoring. The neck hair was removed, and pre-warmed ultrasound gel was used as an acoustic coupling medium. Using a VisualSonic Vevo 2100 Ultrasound Imaging System equipped with MS700 50-MHz linear array transducer (FUJIFILM VisualSonics, Inc, Toronto, ON, Canada), vein grafts were visualized and recorded in successive 2D B mode cross sectional views at 0.5 mm interval starting from proximal to distal portions. Ultrasound images were loaded into VevoVasc software (v.3.6.2.0, FUJIFILM VisualSonics), and vascular walls were traced using the
edge detection tool. Lumen area and vessel wall area were calculated by mean value of each vein grafts, and vessel wall volume was calculated by a cubic volume of a whole vessel.

Molecular imaging on macrophage activation
Intravital microscopy (IVM) was performed to evaluate macrophage accumulation and matrix metalloproteinase (MMP) activity in vivo, as previously reported.6 We used near-infrared fluorescent nanoparticles — Aminospark 750 (Ex/Em=753/773 nm) and MMP sense 680 (Ex/Em=680/700 nm) (PerkinElmer, Inc., Waltham, MA). These nanoparticles were administered via tail vein 24 hours before imaging. After surgical exposure of the vein graft, fluorescent signals from these nanoparticles were captured by confocal laser microscopy (Olympus FV1000).

Collagen hue analysis
In sections stained with picrosirius red (Polyscience, Inc., Warrington, PA), fibrillar collagen was observed under a circularly polarized microscope with green and red optic filters (HQ535/50m, D605/55m, Chroma), as previously described.6,7 As the thickness of collagen fiber increases, the color shifts from green to red. Collagen volume was quantified by image analysis software (NIS-Elements, Nikon).

Preparation of human primary macrophages
Human primary macrophages were prepared as previously described.8 Briefly, blood mononuclear cells were isolated by density gradient centrifugation. Then, isolated cells were plated on culture dishes and adherent cells were cultured with RPMI1640 (Life Technologies) containing 5% human serum and 1% penicillin/streptomycin for 10 to 14 days before experiments.

Gain-of-function and loss-of-function experiments in mouse primary macrophages
Peritoneal macrophages were prepared for in vitro gain-of-function and loss-of-function studies as previously described.9,10 Brewer thioglycollate medium (BD Diagnostic Systems, Sparks, MD) was injected into the peritoneal cavity of C57Bl/6 mice 4 days before macrophage collection. Ice-cold phosphate-buffered saline (PBS) was injected into the peritoneal cavity and cells were thus harvested. Then, the cells were washed with PBS and plated on 48-well culture dishes for further experiments. Cells were cultured with RPMI1640 containing 10% fetal bovine serum (FBS) and overnight starvation was performed with 0.5% FBS before each experiment. In gain-of-function experiments, Dll4 plasmid (0.4 μg/well) or control plasmid was transfected with Lipofectamine 2000 (Life Technologies) according to the manufacture’s instruction and mRNA was quantified 24 hours later. Dll4 expression was verified by immunofluorescent imaging with rabbit anti-Dll4 antibody (Abcam) and Alexa Fluor 568 goat anti-rabbit IgG (Life Technologies). In loss-of-function experiments,
peritoneal macrophages were incubated with Dll4 blocking antibody (50 μg/mL) or isotype IgG for 4 hours, and then mRNA was extracted for quantitative analysis. In PDGF-B experiments, transient overexpression of Dll4 or Dll4 stimulation by immobilized Dll4 was performed to RAW264.7 cells. Dll4 immobilization was performed by overnight pre-incubation of cell culture plates with 2 μg/mL recombinant mouse Dll4 (R&D systems, Minneapolis, MN).

In culture experiments of human cells, saphenous vein SMCs (HSVSMCs) and saphenous vein endothelial cells (HSVECs) were isolated as previously described11,12. HSVSMCs were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies) containing 10% FBS, 1% non-essential amino acid solution, 1% pyruvate and 1% penicillin/streptomycin. HSVECs were cultured in M199 medium (Life Technologies) containing 20% FBS, 1% L-glutamine, 1% penicillin/streptomycin, 50 μg/ml endothelial cell growth supplement (Biomedical Technologies, Stoughton, MA), and 100 μg/ml heparin. In gain-of-function studies, these cells were plated on top of immobilized human Dll4 protein (2 μg/mL) (R&D systems). In loss-of-function studies, cells were incubated with 10 μg/mL mouse anti-human Dll4 antibody (MHD4-46).5 RNA was extracted at indicated time points.

**Indirect co-culture experiments**

Mouse aortic SMCs were prepared, as described previously,13 and cultured with DMEM containing 10% FBS, 1% non-essential amino acid solution, 1% pyruvate, and 1% penicillin/streptomycin. All experiments were performed with SMCs between 3 to 10 passages, and 3 hours starvation was performed before experiments with DMEM containing 0.1% FBS. To collect conditioned media, RAW264.7 cells were cultured with DMEM containing 0.1% FBS after transfection of Dll4 or control plasmid. Plasmid transfection was performed with lipofectamine 2000 according to the manufacturer’s instruction. Condition media were centrifuged at 1,500 g for 5 minutes and transferred to the culture dishes of SMCs 48 hours after transfection. To examine the SMC proliferation, 5 x 10^3 cells were plated on 96-well culture dishes. After 12 hours starvation, conditioned media were added into the culture dishes. 10% FBS was added as a positive control. Cell proliferative ability was evaluated 24 hours later by Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI).14 SMC migration was examined by the modified Boyden's chamber method with 8 μm pore chemotaxis chambers (Neuro Probe, Gaithersburg, MD). Conditioned media were placed in the lower chamber and 5 x 10^4 SMCs were placed on type I collagen pre-coated membrane. Cell migration was quantified as the number of SMCs migrated to the lower surface of the membrane after 4 hours incubation. Migrated cells were fixed with methanol and stained with 0.25 % crystal violet/50 % methanol. Then, number of cells was counted under microscope and for each sample, the average of 3 high power fields was used for analysis.14,15 The differential state of SMC was examined by quantification of SMC markers including α-SMA, smooth muscle 22-α
(SM22α), Calponin-1 and smooth muscle myosin heavy chain (SM-MHC). SMCs (8 x 10⁴ cells/well) were plated on a 24-well plate and incubated for 24 hours at 37 °C. After 3 hours starvation, SMCs were stimulated with conditioned media for 24 hours. Then, RNA was extracted for quantitative real-time PCR analysis.¹⁴

**Western blotting**

Total cellular protein was collected from RAW264.7 cells at 4°C in M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) containing 1% Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific). Protein concentration was determined by BCA protein assay and 20 µg of sample protein was then loaded onto each lane. Blots were stained with antibody specific for PDGF-B (LifeSpan BioSciences, Seattle, WA) and β-actin was used as an intrinsic control. Following incubation with HRP-tagged secondary antibody (Thermo Fisher Scientific), an ECL detection kit (Perkin Elmer) was used to reveal antibody binding.

**Formulation of lipid nanoparticles**

siRNA targeting mouse Dll4 sequence NM_019454, and non-targeting control siRNA (modified to avoid immune stimulation and off-target effects) were synthesized as previously described.¹⁶⁻¹⁸ Macrophage-targeted lipid nanoparticles were prepared and mixed with siRNA, as previously described,¹⁶¹⁹ to prepare siRNA encapsulated lipid nanoparticles. More specifically, the lipid-like material AF12 was mixed with cholesterol, polyethylene glycol (PEG), and disteroylphosphatidylcholine (DSPC) at a molar ratio of 50: 38.5: 1.5: 10. This mixture was combined with siRNA in a microfluidic device to produce nanoparticles.²⁰ After nanoparticle formulation, particle size was characterized with dynamic light scattering. Both control siRNA- and Dll4 siRNA-nanoparticles had an average diameter of 45 nm (Supplementary Figure XIII).

**In vivo kinetics study of lipid nanoparticles (LNP)**

Kinetics and cell specificity were analyzed with LNP incorporating Dll4 siRNA. In the kinetics study, 0.5 mg/kg particles were injected via tail vein and splenic macrophages were collected by magnet beads sorting (EasySep system, StemCell Technologies, Vancouver, Canada). In the specificity study, Ldlr⁻/⁻ mice were fed high-fat diet and vein graft implantation was performed as described above. AF12 nanoparticles incorporating control siRNA or Dll4 siRNA was administered 21 and 24 days after vein grafting. Vein grafts were harvested 28 days after grafting and snap-frozen in OCT compound. Endothelium and macrophages were isolated by the laser capture microdissection system (LMD6500, Leica, Germany) to examine the specificity and efficacy of siRNA treatment. Macrophage clusters were determined based on the Mac-3 immunostaining of the adjacent sections. The layer including luminal surface was considered as an endothelial layer. RNA was extracted from
dissected tissues and cDNA was synthesized as described above. cDNA was pre-amplified with PerfeCTa PreAmp SuperMix (Quanta Biosciences) and thenDll4 mRNA was quantified by real-time PCR. Validating the specificity and efficacy of 7C1 was performed using a similar protocol.

**Statistical analyses**

All data are reported as the mean ± SEM for continuous variables. We evaluated the normality of the data. In case they are expected not to follow Gaussian distribution, we performed non-parametric test (Mann-Whitney test) for a comparison between two groups. In case the data follows Gaussian distribution, statistical analysis of differences between two groups was performed by Student’s t test. Comparisons between three or more groups, we used one-way ANOVA followed by Bonferroni post-hoc test. Probability values less than 0.05 were considered to be statistically significant.

**References**


