Macrophage Notch ligand Delta-like 4 promotes vein graft lesion development: implications for the treatment of vein graft failure

Jun-ichiro Koga[#][1], Toshiaki Nakano[#][1], James E. Dahlman[2,3,4], Jose-Luiz Figueiredo[1,5], Hengmin Zhang[5], Julius Decano[5], Omar F Khan[2,3,4], Tomiharu Niida[1], Hiroshi Iwata[5], Jon C. Aster[6], Hideo Yagita[7], Daniel G. Anderson[2,3,4,8], C. Keith Ozaki[9], and Masanori Aikawa[1,5,10]

[1]The Center for Excellence in Vascular Biology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA

[2]David H. Koch Institute for Integrative Cancer Research, Cambridge, MA

[3]Harvard-MIT Division of Health Sciences and Technology, Cambridge, MA

[4]Institutes for Medical Engineering and Science, Massachusetts Institute of Technology, Cambridge, MA

[5]the Center for Interdisciplinary Cardiovascular Sciences, Brigham and Women's Hospital, Harvard Medical School, Boston, MA

[6]Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA

[7]Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan

[8]Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA

[9]Division of Vascular and Endovascular Surgery, Department of Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA

[10]Channing Division of Network Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA.

[#] These authors contributed equally to this work.

Abstract

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 two biotherapeutics for Delta-like ligand 4 (Dll4), a Notch ligand: 1) blocking antibody; and 2) macrophage- or endothelial cell (EC)-targeted small-interfering RNA (siRNA). Dll4 antibody administration for 28 days inhibited vein graft lesion

Address for correspondence: Masanori Aikawa, M.D., Ph.D.; Center for Excellence in Vascular Biology, Brigham and Women's Hospital, Harvard Medical School; 77 Avenue Louis Pasteur, Boston, MA 02115; Phone: +1-617-730-7777; Fax: +1-617-525-4400, maikawa@rics.bwh.harvard.edu.

development in LDL-receptor deficient (Ldlr−/−) mice, and suppressed macrophage accumulation and macrophage expression of pro-inflammatory 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, while EC-targeted Dll4 siRNA produced no effects. Gain-of-function and loss-of-function studies suggested in vitro that Dll4 induces pro-inflammatory molecules in macrophages. Macrophage Dll4 also stimulated smooth muscle cell (SMC) proliferation and migration and suppressed their differentiation.

Conclusion—These results suggest that macrophage Dll4 promotes lesion development in vein grafts via macrophage activation and crosstalk between macrophages and SMC, supporting the Dll4-Notch axis as a novel therapeutic target.

Keywords

bypass grafting; inflammation; antibody; RNAi; biotherapy

Introduction

Vein graft failure is a global health burden with no effective medical solutions.[1] Due to the pandemic of atherosclerotic peripheral artery disease (PAD) 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 (SVG) are widely used for PAD because they remain patent longer than artificial conduits.[3] Approximately 50% of lower extremity SVG, however, become occluded or narrowed within a year.[4] When PAD grafts fail, the only available therapeutic options are devastating limb amputation or invasive and expensive angioplasty or surgical revascularization. Coronary artery SVG also fail at high rates.[5] Although current therapies such as statins can reduce the onset of complications of arterial diseases (e.g., myocardial infarction),[6] no effective medical solutions are available for vein graft failure.

The Notch pathway, involving ligands (Delta-like ligand 1 [Dll1], Dll3, Dll4, Jagged1, 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 grafts also tend to contain macrophages,[2, 14] 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, the present study used two clinically-relevant biotherapeutics: 1) Dll4 blocking antibody; and 2) Dll4 siRNA encapsulated in macrophage- or endothelial cell (EC)-targeted lipid nanoparticles (LNP).

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 SVG contained many cells expressing Dll4 (**Figures 1A; Supplemental Figure I**). In the failed grafts, some CD68-positive intimal macrophages were immunoreactive to Dll4 antibody (**Supplemental Figure IB**). In high-cholesterol/high-fat-fed Ldlr−/− mice, IVC implanted into the carotid artery developed more advanced lesions than in wild-type mice.[15] The neointima of vein grafts in Ldlr−/− mice showed features similar to those of advanced arterial plaques prone to rupture, including foam cell accumulation, microvessels, and intraplaque hemorrhage (**Supplemental Figure II**), supporting previous reports on a similar model in hypercholesterolemic ApoE3*Leiden mice by the Paul Quax group.[16, 17] Vein grafts of Ldlr−/− mice expressed higher levels of Dll4 mRNA compared to native IVC of Ldlr−/− or wild-type mice (qPCR, **Figure 1B**). In mouse vein grafts, Dll4 localized primarily to intimal macrophages, while smooth muscle cell (SMC) expression of Dll4 was minimal (Day 28, double immunofluorescence, **Figure 1C**). Ligand binding promotes the cleavage of Notch receptors and release of the intracellular domain.[9] 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, while few if any smooth SMC and EC were stained positively (**Supplemental Figure III**). Dll4 and NICD were almost undetectable in the native IVC (**Supplemental Figure IVA**). But the amounts of immunoreactive Dll4 and NICD in the intima of mouse vein grafts increased in parallel over time (**Supplemental Figures IVA and IVB**), indicating acceleration of Notch signaling activation during the lesion development. Furthermore, the amounts of Dll4 and NICD correlated positively with the wall area (**Supplemental Figure IVC**). 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[11, 18, 19] was administered to Ldlr−/− mice twice a week for 28 days. Reduced amounts of NICD following antibody administration indicate that Dll4 mediates Notch activation in vein graft lesions (**Figure 1D**). Dll4 blockade produced no effects on serum levels of total cholesterol (801.0 ± 51.5 vs. 867.0 ± 15.7 mg/dL), triglycerides (216.2 ± 19.3 vs. 249.3 ± 36.1 mg/dL), and body weight (29.6 ± 0.7 vs. 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]

Histologic assessment demonstrates that 28 days of Dll4 antibody treatment decreased the area and thickeness 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 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 (**Figures 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 cells (PCNA) in the intima (**Figure 2D**). Proliferation of macrophages and SMC may contribute to the pathogenesis of vascular diseases.[20-23] Blockade of Dll4 reduced proliferating macrophages and SMC, as demonstrated by double immunofluorescence with PCNA (**Supplemental Figure V**). Inflammation in the adventitia may contribute to the pathogenesis of vascular diseases.[24] Dll4 antibody therapy, however, did not reduce adventitial macrophages significantly (**Supplemental Figure VI**).

To examine whether Dll4 plays a major pathological role in the early processes of venous responses to the arterial environment and ask a more clinical question whether a shorter antibody administration after surgery is also effective, we administered Dll4 antibody only for 7 days after graft implantation. This one-week Dll4 blockade did not reduce lesion size at Day 28 (**Supplemental Figure VIIA**). It should be noted, however, that this short treatment produced a statistically significant reduction of intimal macrophage accumulation, which was sustained until 21 days later (Day 28) without continued antibody administration (**Supplemental Figure VIIB**). We previously reported that Dll4 blockade for 12 weeks improves glucose tolerance and insulin sensitivity.[11] As demonstrated in the present study, Dll4 blockade for 7 or 28 days significantly reduced macrophage accumulation in vein grafts. To address whether improved glucose metabolism may have contributed to the beneficial effects of Dll4 suppression on vein grafts, we examined glucose tolerance and insulin sensitivity 7 days after the initiation of antibody administration. Seven-day Dll4 antibody treatment caused no effects on these metabolic parameters (**Supplemental Figure VIII**).

We further examined the effects of Dll4 suppression on the inflammatory burden in vein grafts. Dll4 antibody-treated vein grafts contained lower levels of IL-1β, TNF-α, and PDGF-B mRNA compared to control grafts (**Figure 3A**). To examine whether the reduced expression of these factors merely resulted from diminished macrophage number, or whether Dll4 blockade also reduced macrophage activation, we performed qPCR on macrophages isolated from vein grafts. Dll4 antibody treatment decreased macrophage expression of IL-1β and TNF-α, molecules typical of a pro-inflammatory "M1" phenotype (**Figure 3B**).[13] In contrast, Dll4 antibody therapy increased arginase 1 that represents non/anti-inflammatory "M2" polarization (**Figure 3B**). A reduction of Hey2, a prototypical Notch target gene, indicates that Dll4 antibody indeed suppressed Notch signaling in macrophages. These results indicate that Dll4 suppression diminishes the pro-inflammatory 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 co-injected two 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 (**Supplemental Figure VII**). MMP produced by activated macrophages impair

collagen content and structures.[12, 22] Picrosirius red staining viewed under a circularly polarized microscope showed no significant difference in the content of total fibrillar collagen between two 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 days 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 non-targeting 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 pro-inflammatory 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 of Dll4 induced prototypical Notch target genes Hes1 and Hey1 (**Figure 6A**). Enforced expression of Dll4 induced pro-inflammatory molecules typical of "M1" macrophages (e.g., IL-1β, TNF-α; **Figure 6B**). In contrast, Dll4 blocking antibody exerted opposing effects (**Figure 6C**). Furthermore, Dll4 blockade inhibited the expression of pro-inflammatory 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 de-differentiation

SMC migration and proliferation may contribute to the development of vein graft lesions.[21] 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, while such direct contacts are common in plaque macrophages,[14, 27, 28] 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 (HSVSMC). Immobilized recombinant Dll4 (rDll4) attachment

did not affect the number of HSVSMCs both in 0.5% and 10% FBS (**Supplemental Figure IXA**). Blockade of Dll4 binding also produced no effects on the growth of HSVSMC (**Supplemental Figure IXB**). These results indicate that direct Dll4 binding may not play a major role in SMC biology. Therefore, we performed indirect co-culture experiments to examine whether macrophage expression of Dll4 induces SMC migration, proliferation, and de-differentiation 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 (SM-MHC), the strictest SMC differentiation marker (**Figure 7C**).[29] PDGF-BB induces SMC migration, proliferation, and de-differentiation.[30, 31] Notch activation by enforced expression of Dll4 or immobilized rDll4 induced PDGF-B expression in RAW264.7 cells (**Figures 7D and 7E**), indicating that PDGF-BB may mediate the effects of Dll4 via macrophage–SMC crosstalk. Other pro-inflammatory factors, which Dll4 induces in macrophages, may contribute this interaction (**Figure 6**).

The role of EC-derived Dll4 in the development of vein graft lesions

Among human primary macrophages, HSVSMC, and human saphenous vein EC (HSVEC), Dll4 mRNA levels were highest in HSVEC under the quiescent state (**Supplemental Figure XA**). LPS markedly induced Dll4 only in primary macrophages (**Supplemental Figure XA**). Endothelium also appeared positive for Dll4 in the failed human vein grafts (**Supplemental Figure I**). In HSVEC, blockade of Dll4 suppressed MCP-1, IL-1β, IL-6, and VCAM-1 expression (**Supplemental Figure XB**). In contrast, immobilized Dll4 induced EC expression of IL-1β and VCAM-1 (**Supplemental Figure XC**). Dll4 antibody treatment reduced the number of adventitial microvessels (**Supplemental Figure XI**). 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 7C1 (7C1-Dll4 siRNA).[32] Dll4 by 7C1-Dll4 siRNA reduced Dll4 mRNA in endothelium by > 70% (**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; **Figures 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 two different biotherapies — blocking antibody and macrophage-targeted siRNA, the present 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.[6, 12, 13] The role of macrophages in vein graft disease, however, remains elusive. Failing vein grafts in patients exhibit macrophage accumulation[14] and signs of rupture.[33, 34] Clinical evidence has linked biomarkers of inflammation with vein graft failure.[35] Preclinical studies proposed the role of macrophages

in neointima formation in vein grafts.[16, 36, 37] However, no medical therapies are currently available to target vein graft inflammation, which has driven our current efforts.

The key novel findings demonstrated in the present 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 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 de-differentiation, 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.[10, 11, 38-43] However, the mechanistic evidence for the role of Dll4 in inflammation of cardiovascular organs remains scant.[10, 11] Macrophage polarization, as often classified by at least two subpopulations: a pro-inflammatory ("M1") and an anti/non-inflammatory ("M2"), is associated with various cardiovascular diseases.[13, 44] In vein grafts, Dll4 blockade reduced the expression of multifunctional pro-inflammatory IL-1β and TNF-α, typical "M1" molecules, suggesting the broad anti-atherogenic 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 the present 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.

Phenotypic modulation of SMC contributes to the pathogenesis of arterial diseases.[29] SMC activation may also participate in the pathogenesis of vein graft disease.[21] The present study therefore explored the novel mechanism that Dll4 in SMC promotes activation of this vascular cell type. Dll4 expression levels were, however, much lower in quiescent or activated 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, while direct contact between macrophages is common.[27, 28] 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 the present study suggest a novel paracrine mechanism by which macrophage Dll4 activates neighboring SMC (**Supplemental Figure XIIA**).

To maximize clinical significance of our study, we used two 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 (e.g., reductions in C-reactive protein levels) with no substantial adverse effects,[50] leading to a longer, larger 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 provides the novel evidence that the Dll4-Notch axis contributes to the pathogenesis of vein graft lesion development (**Supplemental Figure XIIA**). 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 (**Supplemental Figure XIIB**), a major clinical problem with no medical solutions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Owens CD, Ho KJ, Conte MS. Lower extremity vein graft failure: A translational approach. Vasc Med. 2008; 13:63–74. [PubMed: 18372442]

2. Fowkes FG, Rudan D, Rudan I, Aboyans V, Denenberg JO, McDermott MM, Norman PE, Sampson UK, Williams LJ, Mensah GA, Criqui MH. Comparison of global estimates of prevalence and risk factors for peripheral artery disease in 2000 and 2010: A systematic review and analysis. Lancet. 2013; 382:1329–1340. [PubMed: 23915883]

3. Twine CP, McLain AD. Graft type for femoro-popliteal bypass surgery. Cochrane Database Syst Rev. 2010:CD001487. [PubMed: 20464717]

4. Conte MS, Bandyk DF, Clowes AW, et al. Results of PREVENT III: A multicenter, randomized trial of edifoligide for the prevention of vein graft failure in lower extremity bypass surgery. J Vasc Surg. 2006; 43:742–751. discussion 751. [PubMed: 16616230]

5. Fitzgibbon GM, Kafka HP, Leach AJ, Keon WJ, Hooper GD, Burton JR. Coronary bypass graft fate and patient outcome: Angiographic follow-up of 5,065 grafts related to survival and reoperation in 1,388 patients during 25 years. J Am Coll Cardiol. 1996; 28:616–626. [PubMed: 8772748]

6. Libby P, Aikawa M. Stabilization of atherosclerotic plaques: New mechanisms and clinical targets. Nat Med. 2002; 8:1257–1262. [PubMed: 12411953]

7. Bray SJ. Notch signalling: A simple pathway becomes complex. Nat Rev Mol Cell Biol. 2006; 7:678–689. [PubMed: 16921404]

8. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: Cell fate control and signal integration in development. Science. 1999; 284:770–776. [PubMed: 10221902]

9. Thurston G, Noguera-Troise I, Yancopoulos GD. The delta paradox: Dll4 blockade leads to more tumour vessels but less tumour growth. Nat Rev Cancer. 2007; 7:327–331. [PubMed: 17457300]

10. Fung E, Tang SM, Canner JP, Morishige K, Arboleda-Velasquez JF, Cardoso AA, Carlesso N, Aster JC, Aikawa M. Delta-like 4 induces notch signaling in macrophages: Implications for inflammation. Circulation. 2007; 115:2948–2956. [PubMed: 17533181]

11. Fukuda D, Aikawa E, Swirski FK, Novobrantseva TI, Kotelianski V, Gorgun CZ, Chudnovskiy A, Yamazaki H, Croce K, Weissleder R, Aster JC, Hotamisligil GS, Yagita H, Aikawa M. Notch ligand Delta-like 4 blockade attenuates atherosclerosis and metabolic disorders. Proc Natl Acad Sci U S A. 2012; 109:E1868–1877. [PubMed: 22699504]

12. Aikawa M, Libby P. The vulnerable atherosclerotic plaque: Pathogenesis and therapeutic approach. Cardiovasc Pathol. 2004; 13:125–138. [PubMed: 15081469]

13. Moore KJ, Sheedy FJ, Fisher EA. Macrophages in atherosclerosis: A dynamic balance. Nat Rev Immunol. 2013; 13:709–721. [PubMed: 23995626]

14. Cox JL, Chiasson DA, Gotlieb AI. Stranger in a strange land: The pathogenesis of saphenous vein graft stenosis with emphasis on structural and functional differences between veins and arteries. Prog Cardiovasc Dis. 1991; 34:45–68. [PubMed: 2063013]

15. Yu P, Nguyen BT, Tao M, Campagna C, Ozaki CK. Rationale and practical techniques for mouse models of early vein graft adaptations. J Vasc Surg. 2010; 52:444–452. [PubMed: 20573477]

16. Eefting D, Bot I, de Vries MR, Schepers A, van Bockel JH, Van Berkel TJ, Biessen EA, Quax PH. Local lentiviral short hairpin rna silencing of CCR2 inhibits vein graft thickening in hypercholesterolemic apolipoprotein e3-leiden mice. J Vasc Surg. 2009; 50:152–160. [PubMed: 19563963]

17. de Vries MR, Niessen HW, Lowik CW, Hamming JF, Jukema JW, Quax PH. Plaque rupture complications in murine atherosclerotic vein grafts can be prevented by TIMP-1 overexpression. PLoS One. 2012; 7:e47134. [PubMed: 23071737]

18. Fukushima A, Sumi T, Ishida W, Ojima A, Kajisako M, Koyanagi A, Koyama N, Yagita H. Notch ligand Delta-like 4 inhibits the development of murine experimental allergic conjunctivitis. Immunol Lett. 2008; 121:140–147. [PubMed: 19027794]

19. Yamanda S, Ebihara S, Asada M, Okazaki T, Niu K, Ebihara T, Koyanagi A, Yamaguchi N, Yagita H, Arai H. Role of ephrinb2 in nonproductive angiogenesis induced by Delta-like 4 blockade. Blood. 2009; 113:3631–3639. [PubMed: 19218547]

20. Rosenfeld ME, Ross R. Macrophage and smooth muscle cell proliferation in atherosclerotic lesions of whhl and comparably hypercholesterolemic fat-fed rabbits. Arteriosclerosis. 1990; 10:680–687. [PubMed: 2403295]

21. Luscher TF. Vascular biology of coronary bypass grafts. Curr Opin Cardiol. 1991; 6:868–876. [PubMed: 10149593]

22. Aikawa M, Rabkin E, Sugiyama S, Voglic SJ, Fukumoto Y, Furukawa Y, Shiomi M, Schoen FJ, Libby P. An HMG-CoA reductase inhibitor, cerivastatin, suppresses growth of macrophages expressing matrix metalloproteinases and tissue factor in vivo and in vitro. Circulation. 2001; 103:276–283. [PubMed: 11208689]

23. Robbins CS, Hilgendorf I, Weber GF, et al. Local proliferation dominates lesional macrophage accumulation in atherosclerosis. Nat Med. 2013; 19:1166–1172. [PubMed: 23933982]

24. Majesky MW, Dong XR, Hoglund V, Mahoney WM Jr. Daum G. The adventitia: A dynamic interface containing resident progenitor cells. Arterioscler Thromb Vasc Biol. 2011; 31:1530–1539. [PubMed: 21677296]

25. Novobrantseva TI, Borodovsky A, Wong J, et al. Systemic rnai-mediated gene silencing in nonhuman primate and rodent myeloid cells. Mol Ther Nucleic Acids. 2012; 1:e4. [PubMed: 23344621]

26. Leuschner F, Dutta P, Gorbatov R, et al. Therapeutic siRNA silencing in inflammatory monocytes in mice. Nat Biotechnol. 2011; 29:1005–1010. [PubMed: 21983520]

27. Shimokama T, Haraoka S, Watanabe T. Immunohistochemical and ultrastructural demonstration of the lymphocyte-macrophage interaction in human aortic intima. Mod Pathol. 1991; 4:101–107. [PubMed: 2020653]

28. Vanderwal AC, Dingemans KP, Weerman MV, Das PK, Becker AE. Specialized membrane contacts between immunocompetent cells in human atherosclerotic plaques. Cardiovascular Pathology. 1994; 3:81–85. [PubMed: 25990852]

29. Aikawa M, Sivam PN, Kuro-o M, Kimura K, Nakahara K, Takewaki S, Ueda M, Yamaguchi H, Yazaki Y, Periasamy M, et al. Human smooth muscle myosin heavy chain isoforms as molecular markers for vascular development and atherosclerosis. Circ Res. 1993; 73:1000–1012. [PubMed: 7916668]

30. Holycross BJ, Blank RS, Thompson MM, Peach MJ, Owens GK. Platelet-derived growth factor-bb-induced suppression of smooth muscle cell differentiation. Circ Res. 1992; 71:1525–1532. [PubMed: 1423945]

31. Kenagy RD, Fukai N, Min SK, Jalikis F, Kohler TR, Clowes AW. Proliferative capacity of vein graft smooth muscle cells and fibroblasts in vitro correlates with graft stenosis. J Vasc Surg. 2009; 49:1282–1288. [PubMed: 19307078]

32. Dahlman JE, Barnes C, Khan OF, et al. In vivo endothelial siRNA delivery using polymeric nanoparticles with low molecular weight. Nat Nanotechnol. 2014; 9:648–655. [PubMed: 24813696]

33. Qiao JH, Walts AE, Fishbein MC. The severity of atherosclerosis at sites of plaque rupture with occlusive thrombosis in saphenous vein coronary artery bypass grafts. Am Heart J. 1991; 122:955–958. [PubMed: 1927881]

34. Motwani JG, Topol EJ. Aortocoronary saphenous vein graft disease: Pathogenesis, predisposition, and prevention. Circulation. 1998; 97:916–931. [PubMed: 9521341]

35. Owens CD, Ridker PM, Belkin M, Hamdan AD, Pomposelli F, Logerfo F, Creager MA, Conte MS. Elevated c-reactive protein levels are associated with postoperative events in patients undergoing lower extremity vein bypass surgery. J Vasc Surg. 2007; 45:2–9. discussion 9. [PubMed: 17123769]

36. Tatewaki H, Egashira K, Kimura S, Nishida T, Morita S, Tominaga R. Blockade of monocyte chemoattractant protein-1 by adenoviral gene transfer inhibits experimental vein graft neointimal formation. J Vasc Surg. 2007; 45:1236–1243. [PubMed: 17543688]

37. Jiang Z, Yu P, Tao M, Ifantides C, Ozaki CK, Berceli SA. Interplay of CCR2 signaling and local shear force determines vein graft neointimal hyperplasia in vivo. FEBS Lett. 2009; 583:3536–3540. [PubMed: 19822149]

38. Allman D, Aster JC, Pear WS. Notch signaling in hematopoiesis and early lymphocyte development. Immunol Rev. 2002; 187:75–86. [PubMed: 12366684]

39. Bigas A, Guiu J, Gama-Norton L. Notch and Wnt signaling in the emergence of hematopoietic stem cells. Blood Cells Mol Dis. 2013; 51:264–270. [PubMed: 23927968]

40. Mohtashami M, Shah DK, Kianizad K, Awong G, Zuniga-Pflucker JC. Induction of t-cell development by Delta-like 4-expressing fibroblasts. Int Immunol. 2013; 25:601–611. [PubMed: 23988616]

41. Mochizuki K, Xie F, He S, Tong Q, Liu Y, Mochizuki I, Guo Y, Kato K, Yagita H, Mineishi S, Zhang Y. Delta-like ligand 4 identifies a previously uncharacterized population of inflammatory dendritic cells that plays important roles in eliciting allogeneic t cell responses in mice. J Immunol. 2013; 190:3772–3782. [PubMed: 23440416]

42. Laranjeiro R, Alcobia I, Neves H, Gomes AC, Saavedra P, Carvalho CC, Duarte A, Cidadao A, Parreira L. The Notch ligand Delta-like 4 regulates multiple stages of early hemato-vascular development. PLoS One. 2012; 7:e34553. [PubMed: 22514637]

43. Wood S, Feng J, Chung J, Radojcic V, Sandy-Sloat AR, Friedman A, Shelton A, Yan M, Siebel CW, Bishop DK, Maillard I. Transient blockade of delta-like notch ligands prevents allograft rejection mediated by cellular and humoral mechanisms in a mouse model of heart transplantation. J Immunol. 2015; 194:2899–2908. [PubMed: 25687759]

44. Swirski FK, Libby P, Aikawa E, Alcaide P, Luscinskas FW, Weissleder R, Pittet MJ. Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. J Clin Invest. 2007; 117:195–205. [PubMed: 17200719]

45. Courties G, Heidt T, Sebas M, et al. In vivo silencing of the transcription factor IRF5 reprograms the macrophage phenotype and improves infarct healing. J Am Coll Cardiol. 2013

46. Leuschner F, Courties G, Dutta P, et al. Silencing of ccr2 in myocarditis. Eur Heart J. 2014

47. Fitzgerald K, Frank-Kamenetsky M, Shulga-Morskaya S, et al. Effect of an rna interference drug on the synthesis of proprotein convertase subtilisin/kexin type 9 (PCSK9) and the concentration of serum ldl cholesterol in healthy volunteers: A randomised, single-blind, placebo-controlled, phase 1 trial. Lancet. 2014; 383:60–68. [PubMed: 24094767]

48. Kanasty R, Dorkin JR, Vegas A, Anderson D. Delivery materials for sirna therapeutics. Nat Mater. 2013; 12:967–977. [PubMed: 24150415]

49. Coelho T, Adams D, Silva A, et al. Safety and efficacy of RNAi therapy for transthyretin amyloidosis. N Engl J Med. 2013; 369:819–829. [PubMed: 23984729]

50. Ridker PM, Howard CP, Walter V, Everett B, Libby P, Hensen J, Thuren T, CANTOS PilotInvestigative Group. Effects of interleukin-1beta inhibition with canakinumab on hemoglobin a1c, lipids, c-reactive protein, interleukin-6, and fibrinogen: A phase IIb randomized, placebo-controlled trial. Circulation. 2012; 126:2739–2748. [PubMed: 23129601]

Translational Perspective

Although vein graft failure is a major clinical problem, no medical therapies are available. Using clinically relevant biotherapies — blocking antibody and siRNA-loaded nanoparticles, we demonstrate Dll4 promotes inflammation and lesion development in mouse vein grafts, providing novel mechanisms and new therapeutic solutions for this disease.
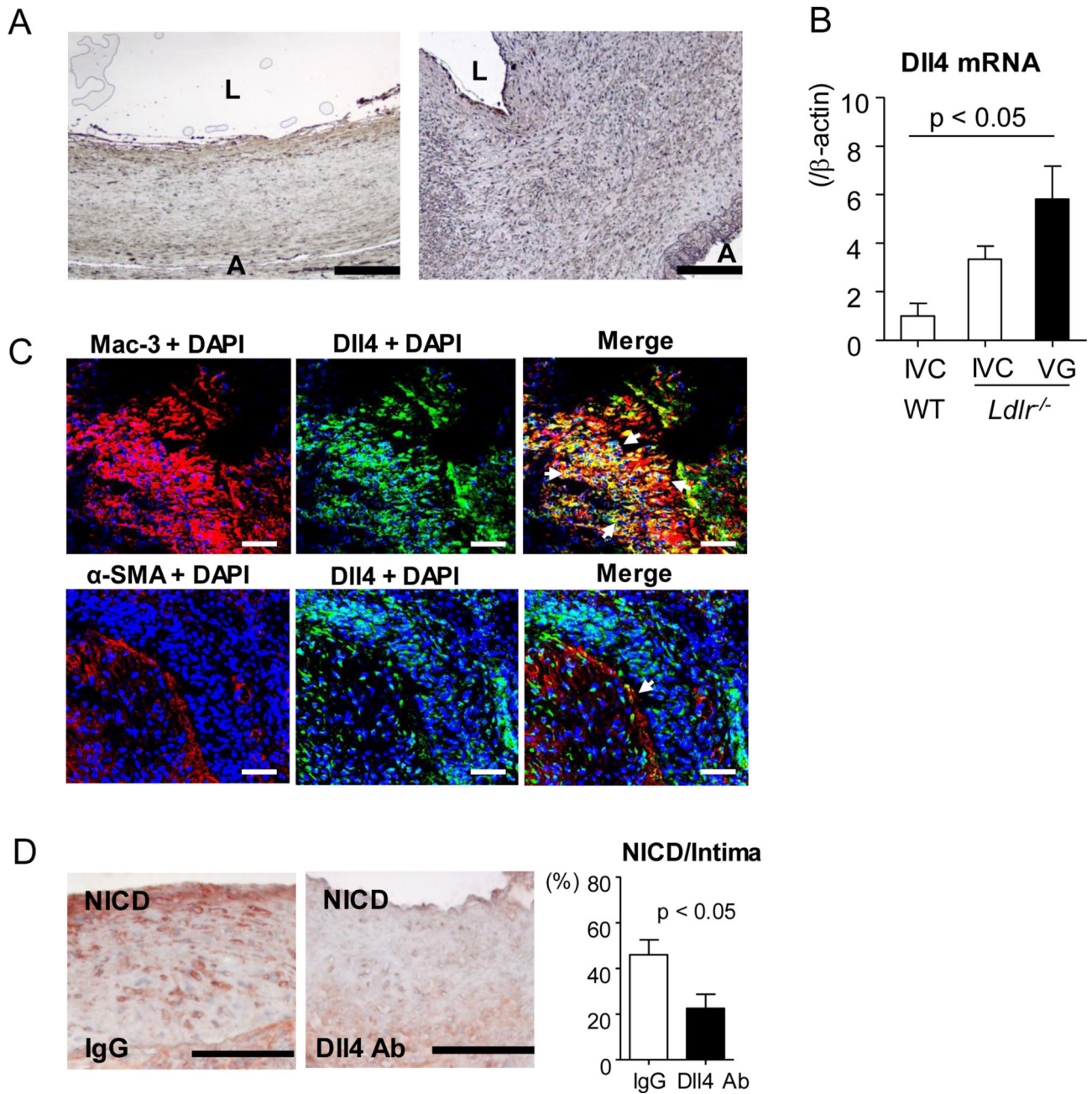
Figure 1. Dll4 expression increases in human and mouse vein grafts
(A) Human SVG harvested for bypass surgery (control native vein, left) and failed SVG
(right). Sections were stained with anti-Dll4 antibody. Scale bar indicates 300 μm. L, lumen;
A, adventitia. Similar data on additional samples (4 control veins; 4 failed SVG) are shown
in Supplemental Figure I. (B) Dll4 mRNA in mouse vein grafts (VG) analyzed 28 days after
grafting. Data are shown as relative expression normalized by native IVCs from wild type
(WT) mice. n = 5 to 8. (C) Serial section of mouse vein graft stained with anti-Dll4, anti-
Mac-3, and anti-α–SMA antibodies. Arrowheads indicate cells positive for both Mac3 and
Dll4. Scale bar indicates 200 μm. The data represent 5 mice that showed similar results. (D)
Immunostaining of cleaved Notch1 intracellular domain (NICD) at 28 days after vein

grafting. Scale bars indicate 100 μm. Bar graph shows quantification of Notch signal activation evaluated as a percentage NICD-positive area in intima. n = 4.
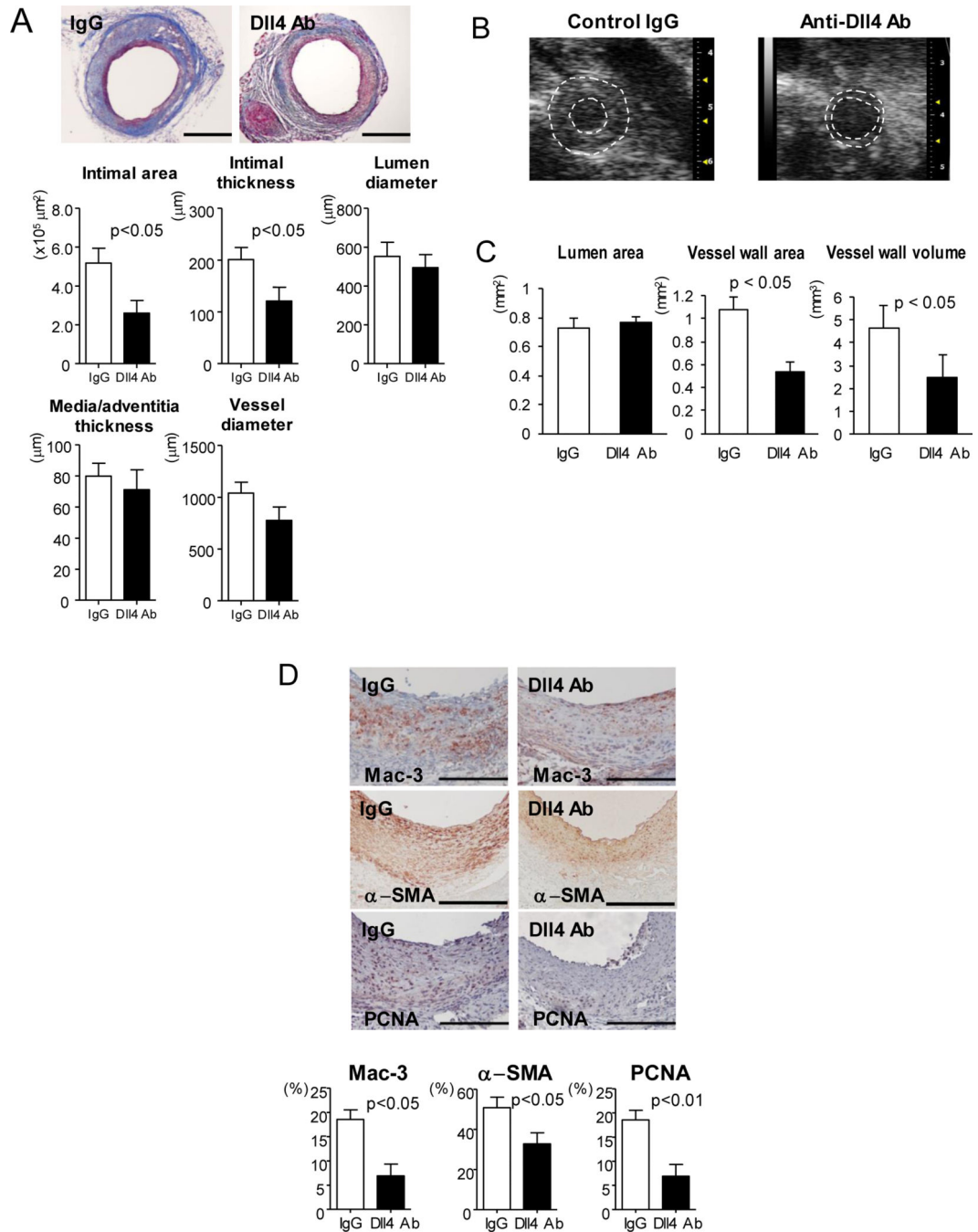
Figure 2. Antibody blockade of Dll4 for 28 days inhibits lesion formation in vein grafts
(A) Vein graft harvested 28 days after implantation from control IgG or Dll4 antibody-treated animals and results of morphometric analyses. Scale bar indicates 400 μm. n = 10 and 9. (B) Ultrasonographic images of vein grafts treated with control IgG (left) and anti-Dll4 antibody (right) 28 days after implantation. The white dotted line indicates vessel wall area. (C) Lumen and vessel wall area (mm^2), and vessel wall volume (mm^3) of ultrasonographic images in control IgG or Dll4 antibody treated vein grafts (n=5 and 3). (D) Immunostaining of Mac-3, α–SMA, and PCNA at day 28 and quantitative data shown as

percentages of staining positive area (Mac-3, α–SMA) and PCNA positive nucleus in the intima. Scale bar indicates 200 μm. n = 4 to 7.
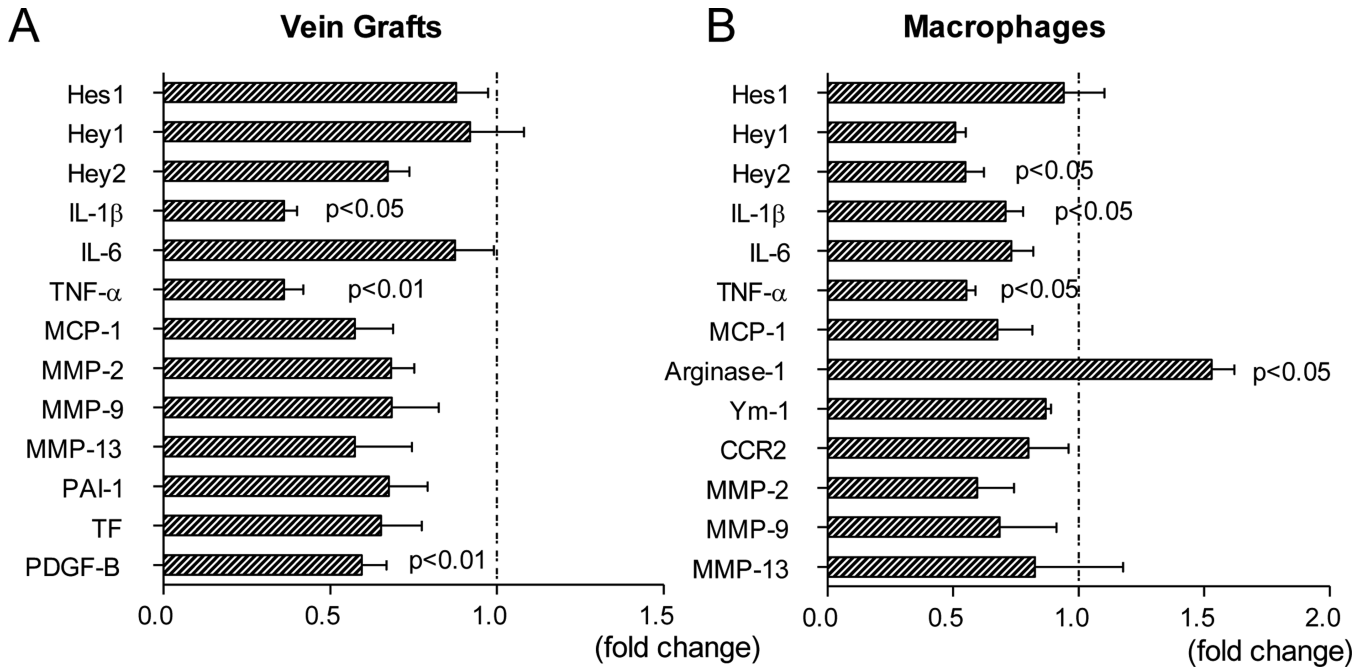
Figure 3. Antibody blockade of Dll4 for 28 days inhibits the expression of pro-inflammatory molecules in vein grafts and lesional macrophages

mRNA expression of molecules associated with inflammation, macrophage phenotype, matrix degradation, and thrombogenicity were quantified in vein grafts (C) and F4/80 positive macrophages isolated from vein grafts (D) 7 days after graft implantation. Data are represented as fold change by Dll4 antibody relative to control IgG. PAI-1, plasminogen activator inhibitor-1; TF, tissue factor; CCR2, C-C chemokine receptor type 2. n = 3 to 7.
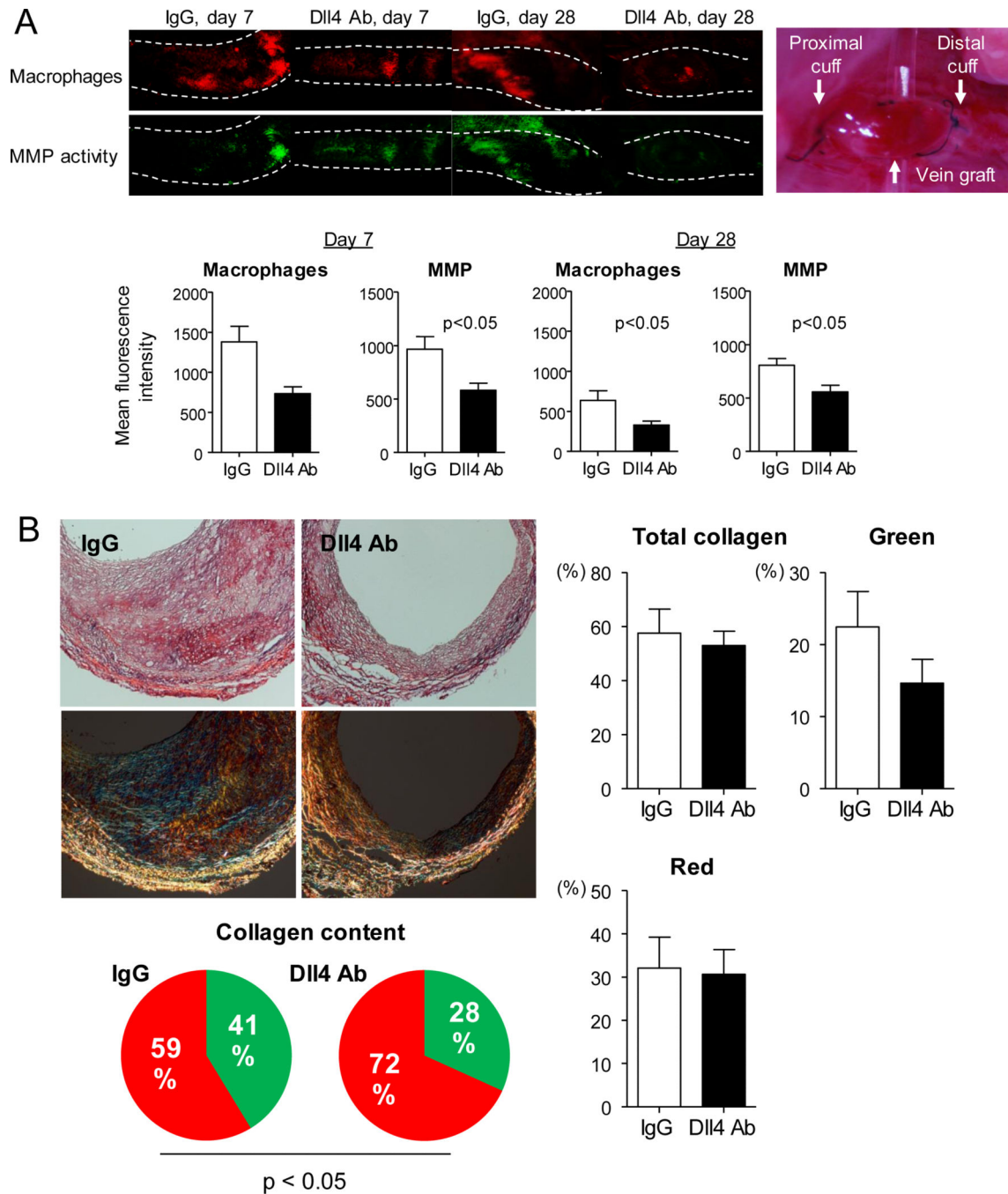
Figure 4. Blockade of Dll4 attenuates macrophage activation and collagen thinning

(A) Live molecular imaging of macrophage functions. Fluorescent probes (AminoSPARK 750 for phagocytic activity and MMPSense 680 for MMP activity) were co-injected 24 hours before intravital microscopy. Dll4 antibody therapy reduced macrophage MMP activity in vein grafts as early as 7 days after implantation. n = 4 to 11.. (B) Collagen content in the intima of vein grafts was quantified by picrosirius red staining under a polarized microscopy. Photomicrographs demonstrate representative samples without (top) or with

(bottom) polarized light. Circle graphs indicate a ratio of green (thin) vs. red (thick) collagen fibers. n = 6 and 5.
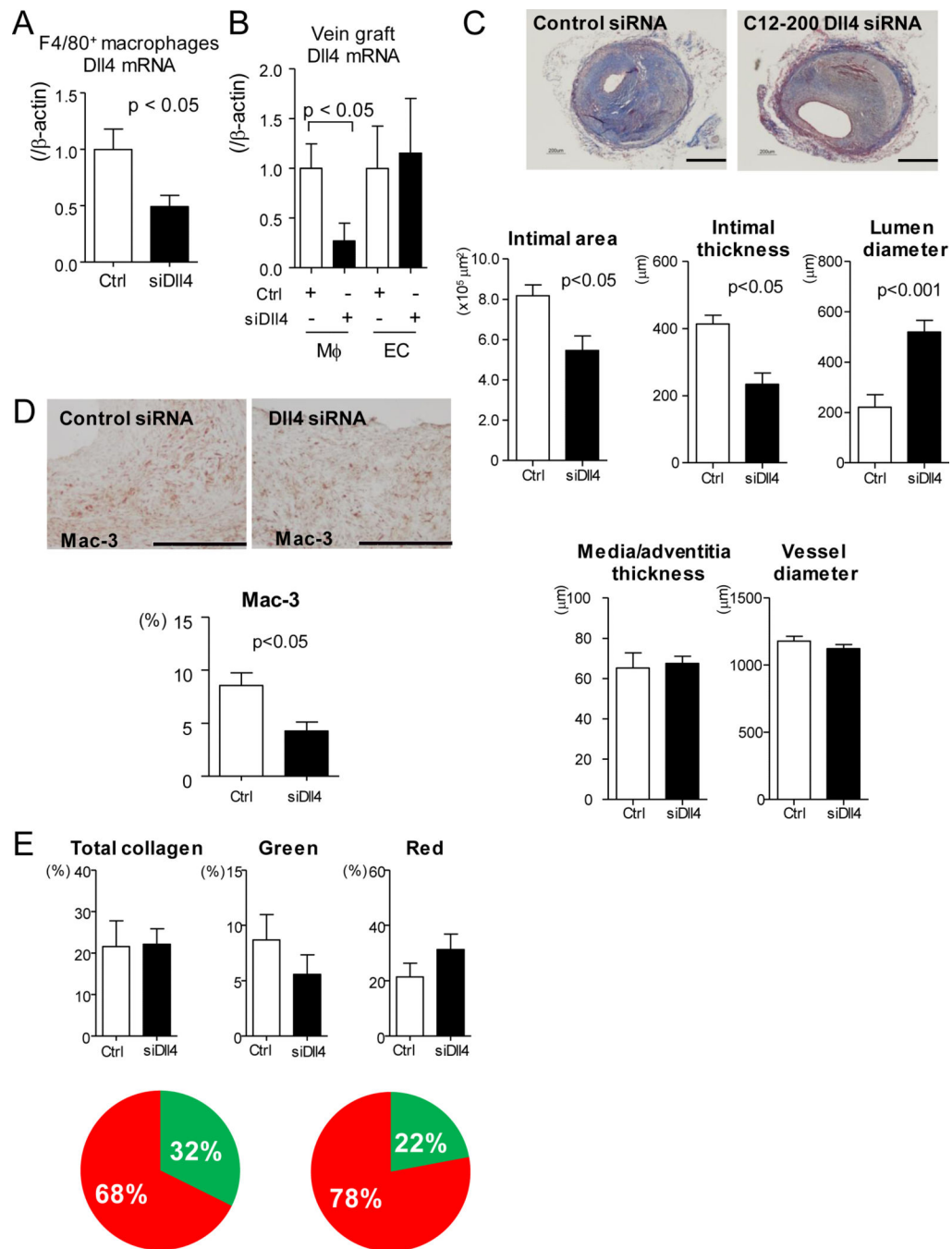
Figure 5. The relative contribution of macrophage Dll4 in vein graft lesion development as examined via siRNA delivery to macrophages

(A) Macrophage-targeted LNP containing control siRNA (Ctrl) or Dll4 siRNA (siDll4) were injected via tail vein, and F4/80 positive splenic macrophages were isolated. Dll4 mRNA was quantified by real-time PCR. n = 7. (B) Dll4 mRNA was quantified by real-time PCR in macrophages (Mϕ) or endothelial cells (EC) collected by laser capture microdissection. n = 5-6 sections. (C) Masson-Trichrome staining of vein grafts at day 28. Scale bars indicate 400 µm. n = 9 and 8. (D) Mac-3 immunostaining at day 28. n = 6. Scale bars indicate 200 µm. (E) Collagen analysis. Collagen content was analyzed 28 days after vein grafting by

picrosirius red staining under a polarized microscope. Collagen hue analysis measured thin (green) and thick (red) collagen fibers. n = 6 and 8.
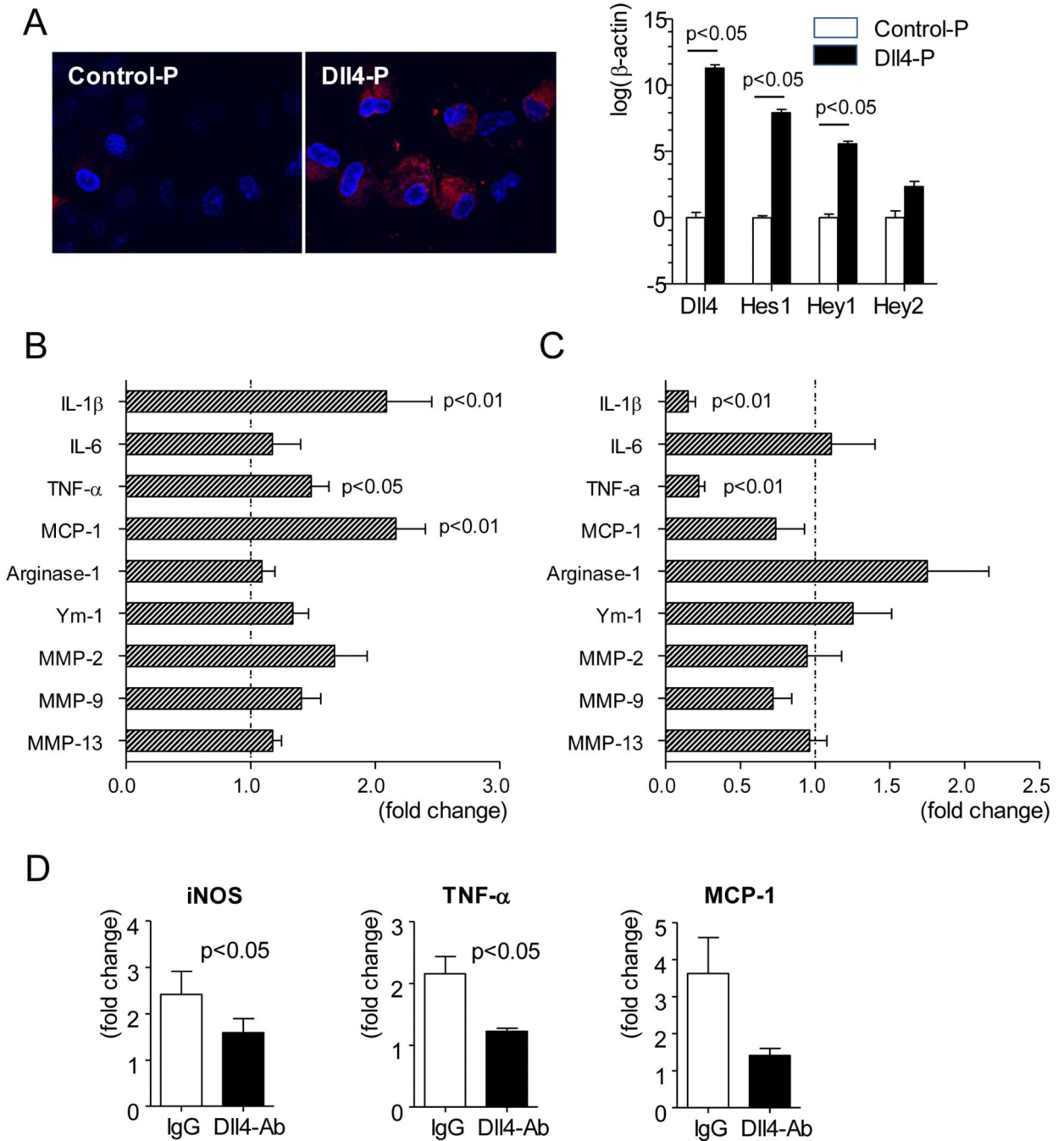
Figure 6. In vitro gain-of-function and loss-of-function studies in primary macrophages
(A and B) A gain-of-function study. Immunofluorescence image of peritoneal macrophages
24 hours after control plasmid (Control-P) or Dll4 plasmid (Dll4-P) transfection (A). Bar
graph shows quantitative analyses of Dll4 and prototypical Notch target genes (n = 3, B)
Data are represented as fold change by Dll4 plasmid transfection relative to control plasmid
transfection. n = 6. (C) A loss-of-function study. Data are represented as fold change by Dll4
blocking antibody relative to control IgG. n = 5 and 6. (D) mRNA expression after IFN-γ
stimulation. Peritoneal macrophages were incubated overnight with control IgG or Dll4

antibody, and then stimulated with 10 ng/mL IFN-γ for 4 hours. Bar graphs show results of real-time PCR. n = 4.
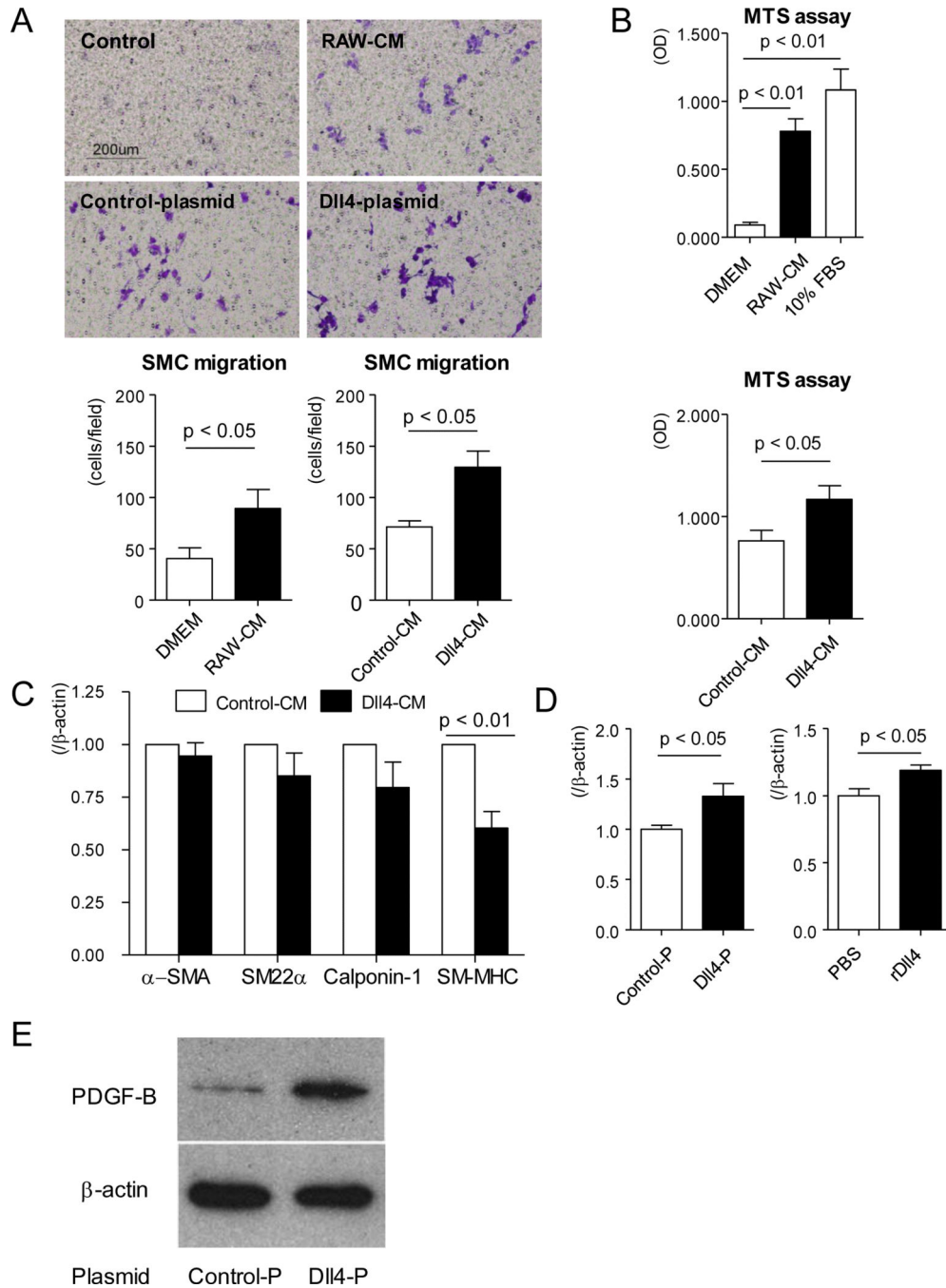
Figure 7. Macrophage–SMC crosstalk as a possible mechanism of vein graft lesion development
(A) SMC migration was examined by modified Boyden's chamber method. DMEM, control Dulbecco's modified Eagle's medium; RAW-CM, conditioned media from non-treated RAW264.7 cells; Control-CM, CM from control plasmid transfected RAW264.7 cells; Dll4-CM, CM from Dll4 plasmid transfected RAW264.7 cells. n = 4. (B) SMC proliferation induced by RAW-CM. RAW-CM increased SMC number compared with DMEM (above). Dll4-CM augmented CM-induced SMC growth (below). FBS; fetal bovine serum. n = 8. (C) mRNA level of SMC differentiation markers after 24 hours of incubation with CM. Data are

shown as relative expression normalized by SMC treated with Control-CM. n = 6. (D) mRNA expression levels of PDGF-B 24 hours after Dll4 plasmid transfection or immobilized Dll4 stimulation. Control-P, control plasmid; Dll4-P, Dll4 plasmid. n = 6 (left) and n = 7-8 (right). (E) Western blot of PDGF-B. Protein was extracted 24 hours after plasmid transfection.
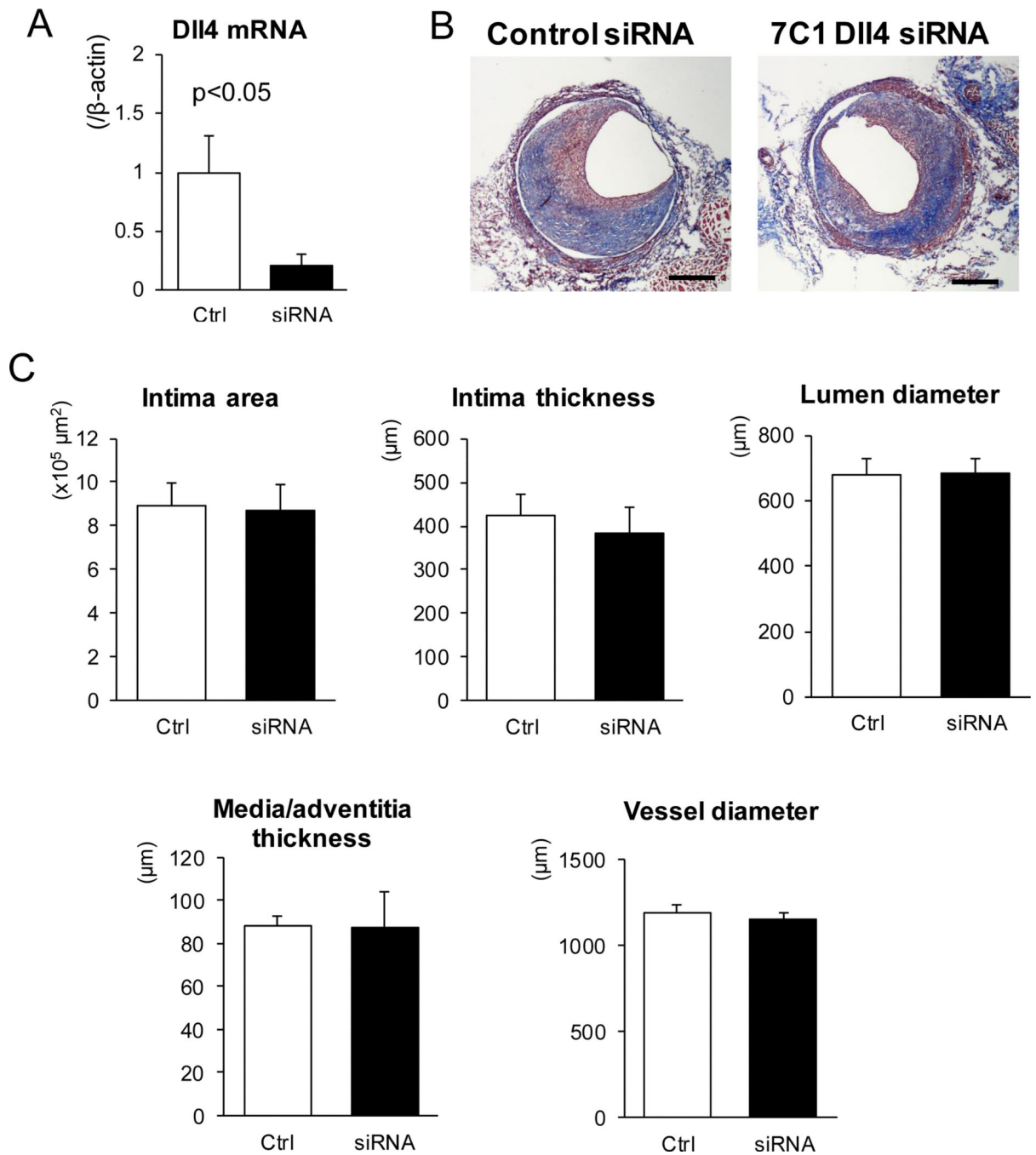
Figure 8. The relative contribution of endothelial cell (EC)-derived Dll4 in vein graft lesion development as examined via siRNA delivery to endothelium
EC-targeted lipid nanoparticles (7C1) containing control siRNA (Ctrl) or Dll4 siRNA (siRNA) were injected via tail vein. (A) Dll4 mRNA levels were quantified by real-time PCR in the endothelium isolated by laser capture microdissection. (B) Masson-Trichrome staining of vein grafts at day 28. Scale bars indicate 500 μm. (C) Bar graphs demonstrate the results of quantitative morphometric analysis (each n=6).