Brief Review

Delta-Like Ligand 4-Notch Signaling in Macrophage Activation

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Abstract—The Notch signaling pathway regulates the development of various cell types and organs, and also contributes to disease mechanisms in adults. Accumulating evidence suggests its role in cardiovascular and metabolic diseases. Notch signaling components also control the phenotype of immune cells. Delta-like ligand 4 (Dll4) of the Notch pathway promotes proinflammatory activation of macrophages in vitro and in vivo. Dll4 blockade attenuates chronic atherosclerosis, vein graft disease, vascular calcification, insulin resistance, and fatty liver in mice. The Dll4–Notch axis may, thus, participate in the shared mechanisms for cardiometabolic disorders, serving as a potential therapeutic target for ameliorating these global health problems. (Arterioscler Thromb Vasc Biol. 2016;36:2038-2047. DOI: 10.1161/ATVBAHA.116.306926.)

Key Words: atherosclerosis ■ fatty liver ■ inflammation ■ macrophage ■ metabolic disease ■ veins

Chronic inflammation contributes to the pathogenesis of vascular diseases, such as atherosclerosis, stenosis after percutaneous coronary intervention, and vein graft failure.1–3 The initial steps of atherogenesis include endothelial cell (EC) activation in response to various stimuli such as dyslipidemia, leading to increased expression of adhesion molecules and chemokines. Activated EC mediates the recruitment of circulating monocytes into the vascular wall, where monocytes differentiate into macrophages. By taking up lipids, these phagocytes become foam cells, a key feature of atherosclerotic plaques. Various stimuli such as oxidized lipids and cytokines may promote proinflammatory activation of macrophages. Factors derived from activated macrophages may further promote the activation of neighboring cell types in the vessel wall, including macrophage themselves, EC, and smooth muscle cells (SMC), amplifying excessive proinflammatory responses. The accumulating evidence from over decades of research suggests that macrophages play key roles in the initiation and the development of atherosclerosis and the onset of its acute thrombotic complications. Furthermore, the plasticity and heterogeneity of macrophages and the balance of their phenotypes (proinflammatory versus anti-inflammatory populations) may be determined by each microenvironment and then, in turn, help to determine the progression and the nature of atherosclerotic lesions. Fine-tuning key regulators of macrophage activation may provide a way to effectively attenuate atherogenesis. The development of such therapies requires better understanding of the mechanism for macrophage activation in each of the specific disease contexts and stages.

The Notch signaling pathway plays various roles in the development of the cardiovascular system and of the diseases of this system in adults.4 The Notch pathway is composed of ligands (Delta-like ligand [Dll1], Dll3, Dll4, Jagged1, and Jagged2) and cell surface receptors (Notch1–4). Direct cell-to-cell contact via the binding of a ligand to a receptor triggers downstream responses. We previously demonstrated that Dll4-mediated Notch signaling promotes proinflammatory activation of human macrophages in vitro and accelerates the development of atherosclerosis, vein graft lesions, vascular calcification, and metabolic diseases in mouse models.5–7 This review briefly summarizes the role of Notch signaling in vascular diseases and then focuses on Dll4 in macrophage activation and vascular inflammation.

Notch Signaling Pathway

The Notch pathway regulates the aspects of embryonic development and differentiation of various cell types and organs.4 Figure 1 illustrates the canonical Notch signaling pathway. In mammals, ligands of the Jagged (Jagged1 and Jagged2) and Delta-like (Dll1, Dll3, and Dll4) families interact with Notch family receptors (Notch1, Notch2, Notch3, and Notch4) expressed on a neighboring cell. Notch receptors exist on the cell surface as a proteolytically cleaved heterodimer consisting of a large ectodomain and a membrane-tethered intracellular domain. Notch signaling occurs when a ligand binds to the extracellular domain of a Notch receptor. This binding triggers a cascade of enzymatic cleavages of the receptor by ADAM (a disintegrin and metalloproteinase) family members (eg, ADAM10, ADAM17) and the γ-secretase complex.
resulting in the release of the Notch intracellular domain (ICD). Notch ICD translocates to the nucleus, where it forms a complex with the DNA-binding protein RBP-Jκ (recombination signal binding protein for immunoglobulin kappa J region). With the absence of Notch signaling, RBP-Jκ acts as a transcriptional repressor and interacts with corepressor proteins. Activation of the signaling mechanism and nuclear translocation of Notch ICD seem to replace corepressors with coactivators, such as Mastermind-like protein 1. The Notch ICD/RBP-Jκ/Mastermind-like protein 1 complex then leads to the transcriptional activation of the genes targeted by Notch.

We refer readers to review other articles for more detailed descriptions for the mechanisms of canonical and noncanonical Notch pathways.4,8–10

**Notch Signaling in Cardiovascular Development and Congenital Diseases**

**Artery Versus Vein Differentiation and Angiogenesis**

The Notch pathway regulates arteriovenous differentiation.11 In zebra fish, Notch signaling-deficient embryos exhibited a poorly formed dorsal aorta and posterior cardinal vein with accompanying arteriovenous malformations.12 These embryos exhibited the loss of expression of arterial markers, such as ephrinB2. Studies of mammalian cells in culture showed that the Notch pathway is downstream of the vascular endothelial growth factor (VEGF) pathway.13 Dll4-mediated Notch signaling induced ephrinB2 expression in cultured EC14 and leads to the differentiation of arterial EC. In Dll1, loss of function mutant embryos, generation of the Notch1 intracellular domain, and expression of arterial markers such as neuropilin1, VEGF receptor 2, and ephrinB2 were lost. Dll1 is also required as a critical Notch ligand for maintaining arterial identity of EC.15

Notch signaling plays a primary role in regulating formation and function of endothelial tip cells during angiogenesis.16 Dll4 signaling on the tip cells regulates VEGF receptor expression on the adjacent Notch receptor-expressing cells and differentiates them into stalk cells. Dll4–Notch signaling suppressed tip cell numbers, filopodia extension, and branching of angiogenic sprouts during angiogenesis.17 In contrast to Dll4, Jagged1 overexpression enhanced angiogenesis and resulted in increased number of tip cells.18 The inhibition study of Jagged-type or Dll-type Notch ligands in EC using Notch decoys showed that blockade of Jagged1/Jagged2–Notch1 signaling suppressed angiogenic sprouting, whereas blockade of Dll1/Dll4–Notch1 signaling caused endothelial hypersprouting.19 In another report, overexpression of Jagged1 in EC led to increased vessel density and maturation during wound healing, although Dll4 blockade increased vascular density but decreased mural cells.20 Therefore, Jagged-type and Dll-type Notch ligand may have the opposite roles in angiogenesis.

Taken together, Notch signaling mediated by Dll1 and Dll4 is essential for arterial differentiation via VEGF-A during vascular morphogenesis. Notch signaling crossregulated by Jagged-type versus Dll-type ligands determines the balance between angiogenic growth and arterial maturation.
Cardiac Development
Notch signaling contributes to cardiomyocyte differentiation, valve development, ventricular trabeculation, and outflow tract development. Notch activation showed to decrease myocardial gene expression within the early in *Xenopus* heart field.21 Notch effector RBP-Jκ deficiency in ES cells increased cardiomyogenic differentiation in the embryoid body.22 As described above, Notch inhibits cardiogenesis in early development; however, it is required for cardiomyocyte differentiation during subsequent cardiac development. Notch signaling is active in the formation of ventricular trabeculation starting in early development.23 Notch signaling also regulates cardiac valve development.24 Endocardial cells undergo epithelial–mesenchymal transformation and form the valve primordia.25 The Notch-Hey-Bmp2/4 pathway promotes epithelial–mesenchymal transformation and subsequent completion of valve tissue development.26 During the development of the outflow tract including the pulmonary artery and aorta, neural crest-derived tissues express the Jagged1 and Jagged2 ligands while the endothelium expresses Dll1 and Dll4.27 The endothelium expresses Notch1 and Notch4 receptors and neural crest-derived cells surrounding the aortic arch express Notch2 and Notch3. A complex interplay among Notch signaling components seems, therefore, to regulate cardiac development.

Congenital Diseases
Evidence has linked Notch signaling with congenital heart diseases. Notch1 mutations cause bicuspid aortic valve and aortic valve calcification.28,29 Jagged1 mutations are also related with Alagille syndrome and tetralogy of Fallot.30 Notch3 seems to play an important role in the function and survival of vascular SMC. Cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is an inherited condition that causes stroke and vascular dementia.31 Degeneration of SMC with accumulation of granular osmiophilic material is a typical characteristic of CADASIL. Notch3 ectodomain accumulates in the cerebral microvasculature in CADASIL patients. Notch 3 CADASIL mutations promote SMC abnormalities.32 Combined Notch1 and Notch3 mutations cause pericyte dysfunction, leading to features typical of CADASIL.33

Mechanisms for Macrophage Activation
Macrophage activation plays a central role in atherosclerotic vascular diseases.34 Activated macrophages express a variety of proinflammatory mediators that contribute to the initiation and development of acute complications of atherosclerosis. Macrophage expression of potent chemokines such as CCL2/MCP-1 (chemokine (C-C motif) ligand 2/monocyte chemotractant protein 1) contributes to enhancing infiltration of monocytes/macrophages into the vessel wall. Accumulation of macrophages expressing proteases (eg, matrix metalloproteinase [MMP]–family collagenases) may degrade extracellular matrix components in atherosclerotic plaques (such as fibrillar collagen) and reduce their mechanical strength, leading to physical disruption (plaque rupture) and acute thrombosis.34,35 

Evidence suggests that macrophages are a heterogeneous population.36 A well-established concept of macrophage polarization has helped to understand that the balance of a proinflammatory phenotype (M1) and a non/anti-inflammatory phenotype (M2) regulates normal homeostasis of various organs.36 The M1/M2 imbalance (eg, M1 dominance in atherosclerotic lesions) in a microenvironment may promote inflammatory disorders. Interferon-γ (IFNγ) or lipopolysaccharides (LPS) typically induces M1 activation in vitro as gauged by increased expression of proinflammatory factors, such as CCL2/MCP-1, interleukin-1β (IL-1β), IL-6, IL-12, tumor necrosis factor-α (TNF-α), and inducible NOS (iNOS). IL-4 or IL-10 promotes M2 macrophage activation with the increased expression levels of arginase 1, MRC1 (mannose receptor, C type 1), and IL-10. IL-10 also represents proresolving proteins that promote the resolution of inflammation.37 M2-like macrophages may also contribute to the regression of atherosclerosis.38 The evidence suggests that 2 macrophages may further be classified into at least 4 subgroups depending on a stimulus: M2a, M2b, M2c, and M2d.39–37 M2a macrophages, induced by IL-4 and IL-13, express high levels of MRC1 and may contribute to the tissue repair. M2b macrophages, induced by stimulation with immune complexes and Toll-like receptor agonist or IL-1 receptor ligands, produce both anti-inflammatory (IL-10) and proinflammatory (IL-1β), IL-6, and TNF-α cytokines. IL-10 and glucocorticoids induce M2c macrophages, which exhibit anti-inflammatory activities and release pentraxin-3, IL-10 and transforming growth factor (TGF)-β. M2c macrophages also express Mer receptor kinase that is essential in supporting the efferocytotic function.40 M2d macrophages are induced by Toll-like receptor agonists through the adenosine A2A receptor, produce IL-10 and VEGF, and provide proangiogenic property. Accumulating evidence suggests that there are additional macrophage phenotypes (eg, M4, Mox, and Mhem).3,37,38 The existence of several subsets may indicate the multidimensional nature of macrophage activation rather what has been suggested under the M1/M2 dichotomy.39,41 A recently proposed new nomenclature, thus, reflects specific stimuli, for example, M(IFNγ) and M(LPS) rather than M1 for proinflammatory macrophages; M(IL-4) and M(IL-10) for anti-inflammatory or proresolving phenotypes instead of M2.41 In vivo situations (particularly diseased organs in humans) should involve >1 instigator. Multiple downstream signaling pathways may be activated in parallel and intertwined through complex cross talk. Combination and relative contributions of such pathways or coexisting phenotypes may vary among different disease contexts and depend on the stage or site of each disease. Overall, the balance of cross-regulated signaling mechanisms or multiple macrophage subsets may, thus, determine the state of inflammation. Although the significance of macrophage heterogeneity in human vascular disease remains incompletely understood, this framework of the macrophage phenotypes helps to clarify specific molecular mechanisms by which each stimulator affects mediators and downstream genes. Using M(IFNγ) and M(IL-4) in global proteomics of human and mouse macrophages, we recently identified 2 members of the poly (ADP-ribose) polymerase (PARP)
family—PARP9 and PARP14—as potential regulators of the balance between pro- and anti-inflammatory subpopulations.42

Notch Signaling in Macrophage Activation
In the past decade, we have tested the hypothesis that Notch signaling participates in macrophage activation. Fung et al5 reported that, in response to a proinflammatory stimulus—LPS, IL-1β or minimally modified low-density lipoprotein, primary human macrophages, derived from peripheral blood mononuclear cells, acquire the ability to express Dll4, which had previously been considered as a EC-specific Notch ligand (Figure 2A). This may indicate that a proinflammatory subpopulation(s) of macrophages, for example, M1, M(LPS), may contain greater amounts of Dll4 on the cell surface. The same study further demonstrated that Dll4 binding triggers Notch signaling, leading to various responses such as the induction of iNOS expression and the nuclear factor κB (NF-κB) pathway, features typical of proinflammatory macrophage activation5 (Figure 2B). Many studies have demonstrated the cross talk between Notch and NF-κB pathways at multiple levels.43–45 We also reported that Dll4 binding to the macrophage-like cell line RAW264.7 decreased the levels of the endogenous NF-κB inhibitor IκBα, suggesting NF-κB activation6 and that pharmacological NF-κB suppression abrogated Dll4-triggered CCL2/MCP-1 induction. These results indicate that Notch signaling induces proinflammatory macrophage activation in part via the NF-κB pathway. Other reports also showed that in macrophages, LPS and other Toll-like receptor ligands induce Notch1 and Notch2 expression, and that Notch signaling increases the expression of TNF-α.

Figure 2. The role of Delta-like ligand 4 (Dll4) in proinflammatory macrophage activation and atherogenesis. A, Lipopolysaccharide (LPS) or IL-1β treatment increased the expression of Dll4 protein in human primary macrophages. B, Coculture experiments of human primary macrophages with the mouse stroma cell line expressing human Dll4 (MS5-Dll4) demonstrated that Dll4 binding promoted the activation of nuclear factor κB (NF-κB) pathway and the expression of proinflammatory genes, such as iNOS. MS5-GFP cells served as control. C and D, The effects of Dll4 blockade on atherogenesis. C, Hematoxylin and eosin staining of the aortic arch. D, Immunostaining for macrophages (Mac3) in plaques. n=7 to 8 (early phase) and 14 to 15 (late phase). Panels A and B reproduced from Fung et al5 with permission of the publisher. Copyright ©2007, Wolters Kluwer Health, Inc. Panels C and D reproduced from Fukuda et al6 with permission of the publisher. Copyright ©2012, National Academy of Sciences. GFP indicates green fluorescent protein.
and iNOS. Another study reported that Notch-RBP-J signaling controls the transcription factor IRF8 (interferon regulatory factor 8) and induces M1 macrophage genes. Fung et al. reported that macrophages from Notch1−/− mice showed decreased induction of IL-6, IL-12, and TNF-α in response to LPS/IFN-γ. Interestingly, we demonstrated that Dll4 binding to human primary macrophages promotes the expression of Dll4 itself, indicating that Dll4-mediated Notch signaling may accelerate a positive feedback loop of proinflammatory activation of macrophages. Collectively, these studies indicate that Notch signaling may promote an excessive proinflammatory microenvironment in cardiovascular and other tissues and contribute to disease mechanisms.

We, thus, investigated whether the suppression of Dll4–Notch signaling reduces proinflammatory macrophage activation. Fung et al. found that siRNA silencing of Notch1, Notch2, Notch3, or Notch4 reduces Dll4-triggered iNOS induction in human primary macrophages. Fukuda et al. demonstrated that blockade of Dll4 by anti-Dll4 antibody decreased the proinflammatory macrophage phenotype in the epididymal fat of metabolically challenged mice. Koga et al. showed that in lesion macrophages isolated from mouse vein grafts, Dll4 antibody decreased the proinflammatory genes IL-1β and TNF-α and increased anti-inflammatory arginase 1. This in vitro and in vivo evidence indicates that Dll4-mediated Notch signaling may skew the vascular microenvironment toward the dominance of proinflammatory macrophages over anti-inflammatory or proresolving macrophages.

**Notch Signaling in Atherosclerosis**

In atherosclerosis, proinflammatory activation of macrophages may play a critical role in the lesion progression from the early phase of fatty-streak formation to plaque rupture and thrombus formation. We tested the hypothesis that Notch signaling promotes the progression of atherosclerosis in vivo. The expression of Dll4 mRNA and protein increased in the aorta of low-density lipoprotein receptor–deficient (Ldlr−/−) mice that were fed high-cholesterol/high-fat diet for 24 weeks. The blockade of Dll4–Notch signaling by neutralizing antibody tended to reduce MMP-9 expression in the aorta was also lower in the antibody group. Dll4 suppression inhibited the expression of MMP-9 and MMP-13, potent proteolytic enzymes responsible for collagen degradation and possibly plaque rupture and acute thrombosis. Moreover, neutralizing antibody tended to reduce MMP-9 expression in macrophages and overexpression of Dll4 increased MMP-9 in vitro. These results indicate that the Dll4–Notch axis may promote not only the progression of atherosclerosis but also the onset of its acute thrombotic complications.

T lymphocytes contribute to atherogenesis. Notch signaling plays critical roles in the maintenance and differentiation of T lymphocytes. The evidence has also implicated Notch signaling in the regulation of T-helper cell differentiation. Antigen presenting cells expressing Dll1 and Dll4 promote differentiation of T-helper 1 (type 1 T helper) cells, whose cytokines (Th1 cytokines, e.g., IFNγ and TNF-α) accelerate vascular inflammation. This mirrors what we observed in the relationship between Dll4–Notch and proinflammatory macrophage activation. In contrast, Jagged ligands on antigen presenting cells induce the differentiation of Th2 cells, which produce anti-inflammatory molecules, such as IL-4, and suppress vascular inflammation. How Notch signaling on T cells affects vascular inflammation remains unknown. T cells migrate into the artery wall, and T-cell activation amplifies inflammatory response in arteries during the progression of atherosclerosis. The role of Notch signaling in T-cell biology (e.g., Th1/Th2 balance) may, thus, contribute to the pathogenesis of vascular disease.

The pathogenesis of atherosclerosis involves SMC. Accumulating evidence suggests that Notch signaling regulates SMC biology. The evidence also suggests that Notch signaling regulates SMC differentiation and proliferation, leading to vascular lesion formation. During the lesion development, Notch pathway regulates the differentiation of bone marrow–derived cells into SMC-like cells. A recent report showed that Notch2 inhibits and Notch3 promotes PDGF (platelet-derived growth factor)-B–dependent SMC proliferation in human aortic SMC. In the same study, overexpression of Notch2 ICD suppressed ERK phosphorylation; however, Notch3 ICD increased ERK phosphorylation and led to PDGF-B–mediated SMC proliferation. Thus, in the context of SMC biology, the functions of Notch2 and Notch3 might differ.

Notch signaling promotes proinflammatory responses (IL-6, IL-8, IL-1α) in EC and induces senescence of EC. Notch1 inhibition reduces shear stress–induced IL-1β, IL-6, and ICAM-1 (intercellular adhesion molecule 1) expression in EC. EC Dll4 induces M1 macrophage activation. A recent study identified cross talk between Dll4–Notch and bone morphogenetic protein 9 pathways in EC homeostasis. TNF-α decreased Notch4 expression, while increasing Notch2 expression in human EC, leading to caspase activation and apoptosis. The same study provided mechanistic evidence that silencing of Notch4 and overexpression of Notch2 ICD similarly induced caspase activity in human EC. TNF-α–mediated caspase-dependent apoptosis in EC may, thus, involve these 2 Notch receptors in an opposite manner. The Notch pathway regulates the integrity of endothelium by controlling EC proliferation and recruiting EC precursors from the bone marrow. Briot et al. recently reported that high-fat diet and inflammatory lipids reduced the expression of Notch1 in EC, and decreased Notch1 expression increased monocyte attachment to EC. Taken together, Notch signaling in EC may regulate inflammation, apoptosis, and EC proliferation and contribute to atherogenesis.

**Notch Signaling in Calcification**

Vascular calcification is an independent predictor of cardiovascular disease. Calcification associates with plaque instability in atheroma and arterial stiffness. Accumulating evidence has implicated macrophages in cardiovascular calcification. Our study linked Dll4–Notch signaling with calcification in the aorta and aortic valves in Ldlr−/− mice (Figure 3A and 3B). The aortic expression of osteogenic regulators
Cbfa1/RUNX2, osteopontin, osteocalcin, and bone morphogenetic proteins decreased by neutralizing anti-Dll4 antibody treatment. In mouse primary macrophages, Dll4 blockade suppressed bone morphogenetic protein 2 (Msx2) gene expression, a key regulator of osteogenesis, and induced alkaline phosphatase activity and matrix mineralization in SMCs.71,72

Notch Signaling in Vein Graft Disease

Peripheral artery disease (PAD) is prevalent in ≈8 million people in the United States.73 Because of the diabetes mellitus pandemic, the incidence of PAD is projected to further increase. Autologous implantation of saphenous vein is a common surgical therapy for PAD. Approximately 50% of saphenous vein grafts for lower extremity PAD, however, become occluded or narrowed within a year.74 Despite its large impact as a global health problem, vein graft failure currently has no medical solutions. We examined whether blockade of Dll4–Notch signaling in macrophages suppresses the progression of vein graft diseases.7 Dll4 expression and Notch signaling increased in macrophages of human and mouse vein graft lesions. Neutralizing anti-Dll4 antibody therapy suppressed excessive body weight gain and reduced genes related to insulin sensitivity. Panels A, B, and D reproduced from Fukuda et al7 with permission of the publisher. Copyright ©2012, National Academy of Sciences. Panel C reproduced from Koga et al7 with permission of the publisher. Copyright ©2015, Wolters Kluwer Health, Inc.
These data suggest that macrophage-derived Dll4 promotes lesion development via macrophage activation and cross talk between macrophages and SMC in vein graft disease. The evidence suggests that the inhibition of Notch signaling using γ-secretase inhibitor or soluble Jagged-1 decreased SMC proliferation, increased apoptosis in SMC, redifferentiated the SMC phenotype, and led to attenuated intimal hyperplasia in vein grafts.75,76 Notch1 expression, but not Notch3 expression, increased in advanced vein grafts.79 These results suggest that Notch signaling in SMC also involves in the development of vein graft disease.

Notch Signaling in Metabolic Disease

Evidence has implicated inflammation in metabolic diseases.77,78 We, thus, examined whether Dll4-mediated Notch signaling affects various parameters of metabolic disorders.6 The expression of Dll4 increased in the adipose tissue of metabolically challenged Ldlr−/− mice on a high-cholesterol/high-fat diet. In this model, neutralizing anti-Dll4 antibody treatment reduced the accumulation of adipose tissue macrophages, increased insulin sensitivity, and decreased excessive body weight gain (Figure 3D). Dll4 blockade also increased the adipose expression of genes associated with insulin sensitivity—adiponectin, GLUT4 (glucose transporter type 4), C/EBPα (CCAAT-enhancer-binding protein alpha), and IRS-1 (insulin receptor substrate 1; Figure 3D). Blockade of Dll4 suppressed macrophage accumulation and the expression of CCL2/MCP-1 in adipose tissue. Interestingly, neutralizing anti-Dll4 antibody therapy also improved signs of fatty liver in the same model, for example, fat deposition, and size of adipocytes in fat with no modifications of plasma lipid profile. Among many cellular and molecular responses accompanied by increased adiposity, CCL2/MCP-1 and its receptor CC chemokine receptor 2 may be the most critical proinflammatory mediators.79 In our model, neutralizing anti-Dll4 antibody treatment suppressed CCL2/MCP-1 expression in adipose tissue. A recent study revealed the inhibition of Notch signaling in hepatocytes decreased insulin resistance.80 In another study, Notch signaling regulated the plasticity of white and beige adipocytes, increased metabolic rate, and improved glucose tolerance and insulin sensitivity in adipocyte-specific Notch1 inactivation mice.81 These lines of evidence suggest that Notch signaling merits evaluation as a novel therapeutic target for metabolic diseases.

Dll4–Notch Axis in Macrophages as a Shared Mechanism for Cardiometabolic Disease

A cluster of cardiometabolic disorders causes a global health burden. Metabolic diseases such as dyslipidemia and diabetes mellitus accelerate the development of coronary artery disease, PAD, and vein graft disease. The events caused by these vascular disorders (eg, acute myocardial infarction and vein graft failure) are major determinants of the clinical impact of metabolic diseases (eg, low quality of life and death). Such intertwined causal relationships among these cardiometabolic disorders may reflect the involvement of shared mechanisms, such as macrophage activation, and understanding these relationships may lead to the development of new therapies. Particularly, signaling pathways that promote a microenvironment where proinflammatory macrophage subsets dominate over anti-inflammatory or proresolving macrophages may be promising targets.

Clinical evidence has established that modifications of major risk factors of atherosclerotic vascular disease, such as the lowering of low-density lipoproteins by statins, prevent the onset of acute myocardial infarction. Preclinical and clinical evidence has established that anti-inflammatory effects of low-density lipoprotein lowering contributes to reduced risk.34,82 However, even potent statins do not prevent the events in all patients.83 The high residual risk has driven active research efforts to find other therapies. A few major clinical trials are currently testing the hypothesis that anti-inflammatory therapies improve cardiovascular outcomes.82 In addition, statins do not substantially reduce the incidence of some other cardiovascular disorders (eg, vein graft failure and aortic valve calcification). Overcoming these clinical challenges requires identifying novel disease mechanisms that lead to the development of new therapies.

We have, therefore, explored new triggering mechanisms of macrophage activation beyond lipids in the contexts of atherosclerotic vascular disorders.5,7,42,84 Our studies have...
proposed that Dll4-mediated inflammation represents a shared mechanism for arterial atherosclerosis and calcification, vein graft disease, insulin resistance, obesity, and fatty liver (Figure 4).\textsuperscript{5,7} According to our in vitro data, proinflammatory stimulants such as IL-1β induce macrophage expression of Dll4. In vitro and in vivo evidence identified downstream genes of Dll4–Notch signaling, including IL-1β, IL-6, CCL2/MCP-1, iNOS, NF-κB, and Dll4 itself. These lines of evidence suggest that excessive macrophage activation amplified by Dll4–Notch favors a sustained proinflammatory milieu in metabolically challenged organs.

What is the relative contribution of macrophage Dll4 in these diseases? Dll4 siRNA treatment via macrophage-targeted lipid nanoparticles retarded the development and inflammation of vein graft lesions,\textsuperscript{7} suggesting an important role of macrophage-derived Dll4 in vascular diseases. Dll4 blockade also reduced macrophage burden in the fat and liver, which may suggest a underlying shared mechanism for attenuating insulin resistance and fatty liver. It should be noted, however, that systemic Dll4 antibody treatment may also have suppressed activated Notch signaling in adipocytes and hepatocytes. Further investigations will be required, but the work by our group and others to examine the role of Notch signaling in macrophage activation and cardiometabolic disease was a critical first step toward the possible development of new therapeutics.

**Future Perspectives**

Ideal anti-inflammatory therapies may adjust the imbalance of various macrophage subpopulation and restore a relatively normal microenvironment by controlling excessive activation of proinflammatory programs without compromising anti-inflammatory or proresolving mechanisms. Discovering molecular switches that regulate seemingly complex signaling networks may provide promising therapeutic targets.\textsuperscript{42} We provided a proof of concept that Dll4–Notch is a therapeutic target for macrophage-mediated inflammatory diseases. Although it remains preliminary, our evidence indicated that Dll4 inhibition seems to attenuate the imbalance of macrophage heterogeneity by suppressing proinflammatory mediators and enhancing some of the anti-inflammatory molecules.\textsuperscript{5,7} To further establish the role of Dll4–Notch in regulating the balance of macrophage phenotypes or identify key molecular switches, it may be critical to establish better-defined models, use clinical samples for unbiased screening, and take more systemic approaches (eg, network analysis). Such strategies may help to better understand cross talk among proinflammatory, anti-inflammatory, and proresolving pathways and to predict potential effects of modulators of Notch signaling. Cardiometabolic disease is a devastating disorder. Despite all the challenges, research efforts to seek effective therapeutics must continue.

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**Disclosures**

None.

**References**


33. Kofler NM, Cuervo H, Uh MK, Murtomäki A, Kitajewski J. Combined deficiency of Notch1 and Notch3 causes pericyte dysfunction, modulating the transcrip-
Lipidol and mineralization of vascular smooth muscle cells: role of Msx2 gene


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