Delta-Like 4 Induces Notch Signaling in Macrophages
Implications for Inflammation

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Background—Activated macrophages contribute to the pathogenesis of inflammatory diseases such as atherosclerosis. Although Notch signaling participates in various aspects of immunity, its role in macrophage activation remains undetermined.

Methods and Results—To explore the role of Notch signaling in inflammation, we examined the expression and activity of Notch pathway components in human primary macrophages in vitro and in atherosclerotic plaques. Macrophages in culture express various Notch pathway components including all 4 receptors (Notch1 to Notch4). Notch3 selectively increased during macrophage differentiation; however, silencing by RNA interference demonstrated that all receptors are functional. The ligand Delta-like 4 (Dll4) increased in macrophages exposed to proinflammatory stimuli such as lipopolysaccharide, interleukin-1β, or minimally-modified low-density lipoprotein in a Toll-like receptor 4– and nuclear factor-κB–dependent fashion. Soluble Dll4 bound to human macrophages. Coincubation of macrophages with cells that expressed Dll4 triggered Notch proteolysis and activation; increased the transcription of proinflammatory genes such as inducible nitric oxide synthase, pentraxin 3 and Id1; resulted in activation of mitogen-activated protein kinase, Akt, and nuclear factor-κB pathways; and increased the expression of Dll4 in macrophages. Notch3 knockdown during macrophage differentiation decreased the transcription of genes that promote inflammation, such as inducible nitric oxide synthase, pentraxin 3, Id1, and scavenger receptor-A. These in vitro findings correlate with results of quantitative immunohistochemistry, which demonstrated the presence of Dll4 and other Notch components within macrophages in atherosclerotic plaques.

Conclusion—Dll4-triggered Notch signaling may mediate inflammatory responses in macrophages and promote inflammation. (Circulation. 2007;115:2948-2956.)

Key Words: atherosclerosis ■ DLL4 protein, human ■ inflammation ■ macrophages ■ receptors, Notch

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Proinflammatory macrophages contribute importantly to a wide variety of pathological states including cancer, neurologic disorders such as Alzheimer’s disease, and cardiovascular diseases ranging from atherosclerosis, in-stent stenosis, and arterial and valvular calcification to heart failure.1-5 Macrophages adapt to the local microenvironment and acquire various functions associated with physiological and pathological processes. In the context of atherosclerosis, activated macrophages participate critically in every stage of lesion progression, from fatty streak formation to the onset of acute thrombotic complications. Matrix-degrading enzymes and prothrombotic molecules elaborated from activated macrophages may promote plaque disruption and subsequent thrombosis.1,6-9 Moreover, macrophage proliferation may contribute to development of the inflamed plaque.10 Additionally, macrophages secrete various proinflammatory cytokines such as interleukin-1β (IL-1β) that induce atherothrombosis-associated molecules and the activation of endothelial cells and smooth muscle cells. Thus, macrophages participate in an amplification cascade that sustains...
inflammatory responses in the atherosclerotic plaque and promote its structural instability and thrombogenicity.

Clinical studies have established that lipid-lowering therapy reduces the onset of acute coronary events,\textsuperscript{11,12} possibly in part through attenuation of inflammation and macrophage activation.\textsuperscript{1,7,10} However, despite effective lipid lowering, cardiovascular events remain a significant clinical problem. Further understanding of mechanisms that trigger macrophage activation could lead to more effective therapeutic strategies for atherosclerosis and its acute complications.

The Notch pathway mediates juxtacrine signaling that requires cell-to-cell contact and critically determines the growth, differentiation, and survival of various cell types in diverse tissues.\textsuperscript{13,14} The Notch family members (Notch1 to 4) are large type I transmembrane receptors that undergo proteolytic processing by a furin-like convertase during transit to the cell surface.\textsuperscript{15} Binding of a ligand (eg, Delta-like 1 (Dll1), Delta-like 3 (Dll3), Delta-like 4 (Dll4), Jagged1, or Jagged2) triggers sequential receptor cleavage by a disintegrin and metalloproteinase domain (ADAM)-type metalloproteinases and \gamma-secretase, which results in the liberation and nuclear translocation of Notch intracellular domain (Notch\textsuperscript{ICD}).\textsuperscript{16} Notch\textsuperscript{ICD} association with the sequence-specific DNA-binding factor RBP-J\textsuperscript{κ}/CBF-1 leads to the formation of a transcriptional activator complex that induces the transcription of Notch target genes.

Previous studies of the role of the Notch pathway in immune cell types have focused mainly on lymphocytes. Notch signaling participates in lymphocyte development, maturation, activation, and transformation.\textsuperscript{14,17,18} However, Notch signaling can also influence myeloid cell differentiation,\textsuperscript{19,20} and its expression and role remain undetermined in macrophages, a key cell type in inflammation and many other diseases.\textsuperscript{1–6} Previous immunohistochemical and ultrastructural studies clearly demonstrated direct membrane contact between adjacent macrophages,\textsuperscript{2,22} which supports a role for homotypic juxtacrine communication between macrophages in inflamed tissues. Here we provide the evidence that Dll4 expression increases in activated human macrophages and that Dll4 binding induces proinflammatory responses. Our findings suggest that the Dll4-Notch pathway participates in inflammatory states characterized by macrophage activation.

**Methods**

**Cell Cultures**

Human peripheral blood mononuclear cells were isolated by density gradient centrifugation and cultured in RPMI-1640 that contained 5% human serum.\textsuperscript{10} In stimulation assays, confluent macrophages were treated with Ultra-pure lipopolysaccharide (LPS; InvivoGen, San Diego, Calif), cytokines, or minimally modified LDL (mmLDL). Coculture experiments used a murine stromal cell line overlaid on human primary macrophages for the indicated duration (see Figure, online Data Supplement).

**Reverse Transcription and Quantitative Polymerase Chain Reaction**

TaqMan quantitative polymerase chain reaction (PCR) was performed on GeneAmp 5700 (Applied Biosystems, Foster City, Calif).

Quantitative PCR detection of human Dll4, toll-like receptor 4 (TLR4), inducible nitric oxide synthase (iNOS), pentraxin 3 (PTX3), and Id1 was performed on Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, Calif) (see Table in the online Data Supplement for oligonucleotide sequences). Quantitative PCR values were normalized to GAPDH. Relative fold changes were calculated by the comparative threshold cycles (C\textsubscript{T}) method, 2\textsuperscript{ΔΔC\textsubscript{T}}.

**Transfection and RBP-J\textsuperscript{κ}/CBF-1 Luciferase Reporter Assay**

200 nM of small interfering RNA (siRNA) was applied to human macrophages with cationic lipid-mediated transfection. Plasmids that contained a RBP-J\textsuperscript{κ}/CBF-1 firefly-luciferase reporter gene and a TK- Renilla luciferase internal control reporter gene were cotransferred into RAW264.7 cells by nucleofection (amaxa, Gaithersburg, Md). After coculture of RAW264.7 cells with MS5-Dll4 or MS5-GFP cells for 48 hours, luciferase activities were determined in whole cell lysates by use of the Dual-Luciferase Reporter Assay System (Promega, Madison, Wis).

**Dll4.Fc Binding Assay**

Dll4.Fc protein was generated from human full-length Dll4 cDNA subcloned into human IgG1 fusion protein vector, pEd.Fc (M.J. Tavares, PhD, et al, unpublished observations, 2006). Dll4.Fc binding assay was assessed on human macrophages. After blockade of nonspecific binding, macrophages were incubated for 30 minutes at 4°C with 1 μg Dll4.Fc or control Fc fragment prebound to 0.5 μg biotinylated anti-human goat IgG at 15° to 20°C, followed by Streptavidin-phycocerythrin (2.5 μg/mL) for 45 minutes at 4°C.

**Immunohistochemistry and Western Blotting**

Immunohistochemistry was performed on fresh frozen sections of discarded human carotid endarterectomy specimens, collected in accordance with a protocol approved by the Institutional Review Board of the Brigham and Women’s Hospital. For Western blotting, 80 μg of sample protein was loaded into each lane. After incubation with primary antibodies, blots were incubated with horseradish peroxidase–tagged secondary antibodies and stained with an ECL detection kit (Perkin Elmer, Waltham, Mass).

**Statistical Analysis**

GAPDH-normalized C\textsubscript{T} values of control and various treatment groups were compared statistically with the Mann-Whitney U test. Individual relative fold changes were calculated with the Equation 2\textsuperscript{ΔΔC\textsubscript{T}} and illustrated in figures as mean relative fold changes ± SEM. Pearson’s correlation coefficient (R) with 2-tailed test of significance was used to determine bivariate correlations.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Notch3 Increases During Monocyte-Macrophage Differentiation**

To explore the possible role of the Notch pathway in macrophages, we used real-time reverse transcription–PCR to examine the expression of Notch pathway components during the differentiation of human monocytes to macrophages in culture. Differentiation was gauged by the expression levels of macrophage scavenger receptor A, a macrophage marker (Figure 1A).\textsuperscript{23} At day 10 in culture, macrophages (n=4) expressed mRNAs for multiple Notch receptors and ligands (mean PCR C\textsubscript{T}: Notch1, 30.77; Notch2, 26.89; Notch3, 28.45; Notch4, 33.91; Dll1, 34.79; Dll3, 36.45; Dll4, 40.00; Jagged1, 28.38; Jagged2, 35.75). Notably, expression levels of Dll4 were lower than those of other Notch ligands.
Macrophages also expressed ADAMs that participate in receptor cleavage, and Fringe proteins that modulate ligand-mediated signaling (mean PCR Ct: ADAM10, 25.31; ADAM17, 28.43; Lunatic Fringe, 33.87; Manic Fringe, 27.62; Radical Fringe, 26.59). Differentiation was accompanied by a marked rise in Notch3 mRNA, which increased 10.1±5.0 fold and 16.4±11.4 fold by days 7 and 10, respectively (P<0.05 for both). In contrast, Notch1 and Notch4 mRNA expression decreased at days 7 and 10 (P<0.05 for both), whereas Notch2 expression was unchanged. Jagged2, Manic Fringe, and Radical Fringe also increased during macrophage differentiation. Western blots showed increased expression of full-length Notch3 protein at day 10 (Figure 1B), which corroborated the mRNA findings. Relative to their intrinsic GAPDH expression, human primary macrophages expressed more Notch3 mRNA than human aortic smooth muscle cells and radial artery endothelial cells (both P<0.05 versus macrophages) (Figure 1C).

Proinflammatory Stimuli Induces Dll4 Expression in Macrophages

We used LPS (Ultra-pure LPS, InvivoGen) to broadly ascertain the effects of a proinflammatory stimulus on the Notch pathway in human primary macrophages. LPS stimulation (100 ng/mL) for 3 hours led to a dramatic induction of Dll4 mRNA in 24 different macrophage donors (3776.3±1717.1 fold increase, P=3.08±10^{-7}) (Figure 2A). Dll4 expression was triggered by LPS in a time- and dose-dependent manner (Figure 2B and 2C). The expression of Notch receptors did not change substantially with LPS treatment (Figure 2D). LPS increased mRNA levels of Jagged1 (6.1±1.2 fold, P<0.01) and ADAM17 (3.0±0.7 fold, P<0.05; n=5) (Figure 2D).

We also examined the effects of other proinflammatory stimuli implicated in atherogenesis. mmLDL and IL-1β increased Dll4 mRNA expression (68.7±36.3-fold and 130.9±61.7-fold, respectively, at 3 hours; P<0.01 for both) in macrophages, whereas tumor necrosis factor α, interferon γ, and granulocyte macrophage-colony stimulating factor had no significant effect (Figure 3).

Western blot analysis showed that LPS (Figure 4A) and IL-1β (Figure 4B) also increased expression of Dll4 protein. Furthermore, although the mRNA and protein levels of Notch3 were not increased, in Western blots stained with an antibody specific for the intracellular domain of Notch3, we observed that LPS induced a shift in the Notch3 polypeptides from 280 kDa (the size of newly synthesized, unprocessed Notch3) to 100 kDa (the size of furin-processed Notch3) (Figure 4C). These findings suggest that LPS increases the furin-processing of Notch3, an event that is predicted to enhance both the surface expression of Notch3 and therefore its availability to ligand.15

TLR4 Silencing and Nuclear Factor-κB Inhibition Limits Dll4 Induction by LPS

TLR4 serves as a receptor for LPS,24,25 TLR4 siRNA treatment silenced TLR4 mRNA expression in human macrophages (P<0.05 versus control siRNA) (Figure 5A), and decreased LPS-induced Dll4 mRNA expression (P<0.05 versus LPS + control siRNA) (Figure 5A). To examine the possible role of the nuclear factor-κB (NF-κB) pathway downstream of TLR4 in LPS-induced Dll4 expression, we used a cell-permeable peptide, SN50, that inhibits nuclear translocation of the active NF-κB complex that contains the p50 subunit.26 SN50 substantially reduced Dll4 expression at 100 μg/mL (95.8±3.3%; P<0.05 versus LPS only group) (Figure 5B), whereas SN50M, the control peptide, did not affect Dll4 expression.
Dll4 Binding to Macrophages Triggers Notch Signaling

To examine whether Dll4 binds to macrophages and triggers Notch signaling, we performed 4 assays. First, we detected significant binding of Dll4.Fc-biotinylated IgG complex to human macrophages as compared with the control Fc-biotinylated IgG complex or Streptavidin-phycoerythrin alone (Figure 6A). Second, other experiments were conducted with feeder cell lines stably transfected with a vector that expressed GFP alone (MS5-GFP) or Dll4-GFP (MS5-Dll4); these feeder cells are much less adherent to culture dishes than are macrophages, which makes it possible to remove these cells before harvesting macrophages for analysis (see Figure in the online Data Supplement). Human primary macrophages cocultured with MS5-Dll4 generated Notch1ICD (Figure 6B), the activated form of Notch1. Accumulation of Notch1ICD was sensitive to compound E, a potent γ-secretase inhibitor (Figure 6C), which suggests that Dll4 activates the canonical Notch signaling pathway. Third, the Dll4.Fc-IgG complex, but not Fc-IgG, also induced Notch1ICD production (Figure 6D). Fourth, when cocultured with MS5-Dll4 cells, the RAW264.7 macrophage cell line showed a >10-fold increase in the activity of a Notch-sensitive luciferase reporter gene that contains multiple binding sites for Notch1.
Dll4-Notch Binding Induces Inflammatory Pathways and Genes in Macrophages

Of further interest, Dll4 binding increased phosphorylated extracellular signal-regulated kinases 1 and 2 and Akt in human primary macrophages (Figure 7A), which indicates that Notch signaling induces mitogen-activated protein kinase (MAPK) and Akt pathways in this cell type. Coculture with MS5-Dll4 also increased phosphorylated extracellular signal-regulated kinases 1 and 2 in macrophages after 48 hours triggered production of Notch1ICD, which indicates cleavage of Notch1 receptor. Compound E, a γ-secretase inhibitor, abolished Notch1 cleavage by MS5-Dll4. D, Soluble Dll4-Fc-IgG complex, but not control Fc-IgG, also induced Notch1 cleavage in macrophages (n=2). E, RBP-Jκ/CBF-1 luciferase reporter activity increased >10-fold in RAW264.7 macrophages after coculture with MS5-Dll4 (P<0.05 versus MS5-GFP, n=3). These data represent cultures from 3 different macrophage donors or 3 independent experiments on RAW264.7 that provided similar results. PE indicates phycoerythrin; Ctrl, control.

Dll4 Colocalizes With Macrophages in Human Atherosclerotic Plaques

Staining for Dll4 and Notch3, as examples of ligand and receptor expression, co-localized with CD68 (a macrophage marker) in the tunica intima of human atherosclerotic plaques (Figure 8A). Neither nonimmune IgG nor PBS showed positive staining (data not shown). Computer-assisted color image quantification followed by statistical regression analysis demonstrated that immunoreactivity for Dll4 correlated positively with CD68 staining (Figure 8B). Although immunostaining did not demonstrate clearly whether subpopulations of macrophages express Dll4, Notch3, or both, quantitative analysis correlated strongly Dll4 and Notch3 staining (Figure 8B). Staining for Notch3 and other ligands (ie, Dll1, Jagged1, and Jagged2) also correlated positively with CD68 staining (Figure 8B). Taken together, these data indicate the presence of multiple Notch signaling pathway components in macrophages found in atherosclerotic plaques.
Discussion

The present study affirms our hypothesis that the Notch pathway plays an important role in macrophages, a key cell type in inflammation and atherosclerosis. Evidence that supports this idea includes the expression of multiple Notch receptors and ligands in human macrophages; markedly increased Dll4 expression in human macrophages stimulated with LPS, mmLDL, or IL-1β, an event that likely involves TLR4 and NF-κB; the
Notch3, in human atherosclerotic plaques rich in macrophages. The presence of Notch pathway components, such as Dll4 and transcription of iNOS, PTX3, Id1, and Dll4 itself; and the ways in macrophages stimulated with Dll4; the Dll4-induced signaling; the induction of the MAPK, Akt, and NF-

The pathological participation of macrophages in tumor angiogenesis,27–30 the proliferation of hematopoietic cells,31–33 and induction of Dll4 mRNA by LPS in dendritic cells.34,35 Dorsch et al reported that adoptive transfer of constitutively active Dll4 had negligible effects on monocytes, but did not determine its effects on differentiated macrophages.32 Recent evidence suggests that Dll4 plays a vital role in the cardiovascular system. Haploinsufficiency of the mouse Dll4 gene is embryonic lethal with resulting gross vascular developmental abnormalities.36 Two seminal reports elaborated on the pathological role that Dll4 plays in tumor angiogenesis, whereupon blockade of Dll4 signaling to Notch by therapeutic anti-Dll4 antibody led to uncoupling and deregulation of the vascular supply required by the tumor for proliferation and survival.27,30 The pathological participation of macrophages in tumorigenesis, tumor angiogenesis, and invasion,3 reminiscent of intraluminal cell phenotypic modulation, plaque angiogenesis, remodeling, and instability in atherosclerosis,1,6–11 is increasingly recognized, and it remains to be seen whether Dll4 is involved.

Our finding that LPS, mmLDL, or IL-1β, but not tumor necrosis factor α, interferon-γ, or granulocyte macrophage-colony stimulating factor, induces early expression of Dll4 concurs with current knowledge that members of the toll/IL-1 receptor superfamily share common adaptor proteins.25 TLR4 siRNA knockdown and pharmacological inhibition of NF-κB suggest that TLR4 and NF-κB mediate, at least in part, LPS-induced Dll4 expression (Figure 5). LPS and mmLDL are TLR4 ligands,24,25,37 and TLR4 may participate in various inflammatory diseases such as atherosclerosis and acute coronary events.38,39 Human primary macrophages from 24 donors displayed a wide variation in the magnitude of Dll4 induction by LPS (Figure 2A), which may be explained by genetic variants at TLR4.40 A more complete understanding of the role of the Toll/IL-1 receptor superfamily in Dll4 expression will require further investigation.

Accumulating evidence supports the existence of important but incompletely understood crosstalk between Notch and
other signaling pathways like MAPK, Akt, and NF-κB that regulate cell growth and inflammation.41–43 The present study demonstrates that Dll4 binds to and activates Notch in macrophages (Figure 6A through 6E) and promotes the activation of the MAPK, Akt, and NF-κB pathways (Figure 7A). Dll4 also triggered the transcription of genes such as iNOS, PTX3, and Id1 (Figure 7B and 7C) that may enhance plaque burden, progression, and thrombogenicity by contributing to a proinflammatory macrophage phenotype.44–46 Furthermore, whereas stimulation through the Toll/IL-1 receptor pathway (eg, by LPS or IL-1β) induces Dll4 expression in macrophages (Figures 2 and 3), Dll4-triggered Notch signaling increases expression of Dll4 itself (Figure 7E), which is reminiscent of a Dll4-induced positive feedback mechanism that links vascular endothelial growth factor signaling to Notch in endothelial cells.17

The initiation of Notch signaling requires receptor cleavage by γ-secretase.16 The present study shows that Dll4 binding to Notch on macrophages (Figure 6A) induces Notch (Notch1) cleavage that is suppressible by γ-secretase inhibition (Figure 6B and 6C). Dll4 can bind to Notch1, Notch3, and Notch4 in vitro,40 and Dll4 and Notch3 colocalized to macrophages within human atherosclerotic plaques (Figure 8). Notch3 also plays a critical role in vascular smooth muscle cell maturation and function.47,48 and, in other cell types, promotes the activation of MAPK and NF-κB pathways through uncertain mechanisms.41,43,46 Among Notch receptors, Notch3 expression preferentially increased during monocyte differentiation into macrophages (Figure 1), and Notch3 knockdown on day 5 of macrophage differentiation led to reduced expression of iNOS, PTX3, and Id1 in differentiated macrophages on day 10 (Figure 7D). However, siRNA silencing of each Notch receptor attenuated downstream expression of iNOS, PTX3, and Id1 (Figure 7C), and further work will be needed to delineate the contribution of each Notch receptor. The complexity of this question is heightened by the ability of each NotchICD to form higher order homodimers and possibly heterodimers with other NotchICDs on downstream target genes that contain paired RBP-Jκ/CFB1 binding sites,49 a response element that is found in a subset of Notch target genes, as well as genetic data that indicate that individual Notch receptors have both distinct and overlapping functions.13,50 Therefore, exploration of the functional evidence for the role of each receptor in macrophage activation and inflammation will require further investigations that use loss- and gain-of-function approaches in vitro and in vivo.

Taken together, our results support the idea that Notch signaling participates in juxtacrine homotypic communication between macrophages and also in the amplification of the proinflammatory milieu in inflamed tissues. Thus, further understanding of the Notch pathway in the context of macrophage biology likely will provide novel insights into the mechanisms of inflammation and new opportunities for rational therapeutic intervention.

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Disclosures
None.

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