Inhibition of Notch1 Signaling Reduces Abdominal Aortic Aneurysm in Mice by Attenuating Macrophage-Mediated Inflammation

Chetan P. Hans, Sara N. Koenig, Nianyuan Huang, Jeeyun Cheng, Susana Beceiro, Anuradha Guggilam, Helena Kuivaniemi, Santiago Partida-Sánchez, Vidu Garg

Objective—Activation of inflammatory pathways plays a critical role in the development of abdominal aortic aneurysms (AAA). Notch1 signaling is a significant regulator of the inflammatory response; however, its role in AAA is unknown.

Methods and Results—In an angiotensin II–induced mouse model of AAA, activation of Notch1 signaling was observed in the aortic aneurysmal tissue of Apoe−/− mice, and a similar activation of Notch1 was observed in aneurysms of humans undergoing AAA repair. Notch1 haploinsufficiency significantly reduced the incidence of AAA in Apoe−/− mice in response to angiotensin II. Reconstitution of bone marrow–derived cells from Notch1+/−;Apoe−/− mice (donor) in lethally irradiated Apoe−/− mice (recipient) decreased the occurrence of aneurysm. Flow cytometry and immunohistochemistry demonstrated that Notch1 haploinsufficiency prevented the influx of inflammatory macrophages at the aneurysmal site by causing defects in macrophage migration and proliferation. In addition, there was an overall reduction in the inflammatory burden in the aorta of the Notch1+/−;Apoe−/− mice compared with the Apoe−/− mice. Last, pharmacological inhibition of Notch1 signaling also prevented AAA formation and progression in Apoe−/− mice.

Conclusion—Our data suggest that decreased levels of Notch1 protect against the formation of AAA by preventing macrophage recruitment and attenuating the inflammatory response in the aorta. (Arterioscler Thromb Vasc Biol. 2012;32:3012-3023.)

Key Words: aneurysm ■ inflammation ■ macrophages ■ Notch1 signaling

Abdominal aortic aneurysm (AAA) is characterized by extensive remodeling of the aortic wall, which results in a weakened and dilated aorta that is prone to rupture.1–4 The incidence of AAA is associated with the clinical risk factors of smoking, hypercholesterolemia, and hypertension, and it affects ≈8% of men aged >65 years.1 Rupture of an AAA carries a risk of death up to 90% and is among the leading causes of death in the United States.5 Currently, there are no available nonsurgical therapies to treat AAA.6,7

A hallmark of AAA in both diseased humans and animal models is the presence of extensive inflammatory macrophages and lymphocytes by histological examination.8 These infiltrating cells exacerbate tissue injury by releasing cytokines, chemokines, and adhesion molecules.9,10 Human aneurysmal aortas, along with mouse models of AAA, demonstrate upregulation of interleukin-6 and monocyte chemotactic protein 1 (MCP1) in the aortic wall.8,11,12 Inflammatory macrophages cause activation of matrix metalloproteinases, resulting in both the degradation of collagen and its associated collagenous matrix, along with elastin fragmentation and smooth muscle cell apoptosis.13 These pathways act in synergy to progressively weaken the aortic wall, predisposing it to rupture.2,3,14

The Notch signaling pathway is important in a wide spectrum of developmental processes, but recently it has been implicated in the molecular pathogenesis of cancer and cardiovascular disease.15,16 The Notch signaling pathway consists of a family of 4 Notch receptors (1–4) that interact with the Jagged and Delta family of ligands. In the canonical signaling pathway, Notch1 is activated after receptor–ligand binding at the cell surface, resulting in the release and translocation of the Notch1 intracellular domain (NICD) to the nucleus where it functions as a transcriptional activator.17 Mice lacking Notch1 display experience embryonic lethality with profound cardiovascular defects, whereas heterozygous deletion of Notch1 is associated with mild aortic valve calcification.18,19 Notch1 signaling has also been shown to be critical for the development and activation of lymphocytes and macrophages in various cell culture studies.15,20,21 The role of Notch1 signaling in inflammatory aneurysmal disease, however, has not been addressed.
To obtain insight into the role of Notch1 signaling in aortic aneurysm development, we generated Notch1+/−; Apoe−/− mice and conducted our studies in the angiotensin II (AngII)–induced mouse model of AAA. Although AngII infusion studies have reported differences compared with AAA pathophysiology in human patients, it resembles the human AAA in male propensity, localized dilation of aorta, and histological features of the aortic injury and is a commonly used model. Here, we show that the Notch1 signaling pathway is activated in the aneurysmal aorta of the AngII-treated Apoe−/− mice and that haploinsufficiency or pharmacological inhibition of Notch1 significantly reduces the incidence of AAA by a macrophage-mediated mechanism.

**Materials and Methods**

A detailed description of the Methods is available in the online-only Data Supplement.

**Generation of Notch1+/−; Apoe−/− Mice**

Apoe−/− and Notch1+/−; Apoe−/− littermates were generated as described in the Methods in the online-only Data Supplement. Animal experiments were approved by Institutional Animal Care and Use Committee at the Research Institute at Nationwide Children’s Hospital.

**Human Aortic Tissue Samples**

Tissue specimens were collected from the infrarenal abdominal aorta of patients undergoing AAA repair (n = 3). Nanoeurysmal infrarenal aortic tissue samples (n = 3) were collected at the time of autopsy of individuals with no evidence of AAA. The collection of the human tissues was approved by the Institutional Review Board of Wayne State University, Detroit, MI.

**AngII Infusion and DAPT Treatment**

Mini-osmotic pumps containing AngII (1000 ng/min per kg) or saline were implanted subcutaneously in anesthetized male mice (8–10 weeks old) following the standard protocol. A group of Apoe−/− mice (n = 10) were injected with a Notch inhibitor, N-[N-(3,5-difluorophenacetyl]-L-alanyl)

---

**Transabdominal Ultrasound Imaging**

For in vivo imaging of the abdominal aorta, 2-dimensional (B-mode) transabdominal ultrasound images were obtained at weekly intervals after the implantation of osmotic pumps using a VisualSonics Vevo2100 imaging system (Ontario, Canada) with a mechanical transducer (MS400).

**Histology and Immunostaining**

Experimental animals were euthanized and the aortas were dissected, fixed in 10% formalin, and maximum aortic diameters measured. Serial sections were stained with hematoxylin and eosin, elastin, and immunohistochemistry (IHC) with antibodies to NICD, mouse monocyte-macrophage marker (MOMA2), and MCP1, as described. Specificity of NICD, MOMA2, and MCP1 staining was determined using nonspecific IgG against the source of host species.

**Bone Marrow Transplantation Studies and Quantification of Leukocytes**

Recipient mice were irradiated with single dose of 1000 rad from a cesium source. Bone marrow–derived cells were obtained from the tibia and femur of donor mice and were injected into the tail vein of 7- to 8-week-old irradiated recipient mice (1 × 10^7 cells/mL).

**Cell Isolation and Flow Cytometry**

After 7 days of AngII infusion, macrophages were isolated from the abdominal aorta, and lymphocytes were isolated from the abdominal aorta, spleen, and peripheral blood. Cells were sorted with fluorescence-activated cell sorting after staining with macrophage (Cd11b and Cd14) or lymphocyte markers (Cd3, Cd4, Cd8).

**Macrophage Migration and Proliferation Studies**

Macrophages were injected intraperitoneally with sterile thiglycollate broth to elicit in vivo macrophage proliferation. For the scratch assay, cells were grown as a monolayer and then scraped to create an injury. To assess proliferation, immunostaining was performed on these fixed cells with Ki67. For chemotactic-induced migration, cells were grown in 8 µmol/L polycarbonate filter transwell membrane plates (upper chamber), and lower chamber media was supplemented with 100 nmol/L n-formyl-met-leu-phe. Bone marrow–derived macrophages (BMDM) were isolated from femurs and tibias of Apoe−/− and Notch1+/−; Apoe−/− mice. BMDM were stimulated for 24 hours with 100 ng/mL lipopolysaccharide (LPS) and interferon-γ (20 ng/mL) for M1 polarization or IL-4 (20 ng/mL) for M2 polarization.

**RNA Isolation and Quantitative Reverse Transcriptase Polymerase Chain Reaction**

The suprarenal aorta of ≤5 mm in length was frozen in RNA-later reagent, and mRNA was extracted using TRIzol reagent after homogenizing tissue with TissueLyser II. Macrophages were washed and RNA extracted using RNeasy kit (Qiagen). cDNA was synthesized and subjected to real-time quantitative reverse transcriptase polymerase chain reaction.

**Statistical Analysis**

Statistical comparisons were performed using either Student t test or 1-way ANOVA followed by the Bonferroni multiple comparison test. GraphPad PRISM V5.0 (San Diego, CA) was used for these comparisons, and P < 0.05 was considered significant. For the statistical analysis of actual incidence, the Fisher exact test was performed using the SAS software (Cary, NC). To analyze the bone marrow transplantation (BMT) data for the 2-group comparisons at each time point, 2-sample t or Wilcoxon sum rank test and log rank test were used depending on the data distribution.

**Results**

**Notch1 Is Activated in Response to AngII in Apoe−/− Mice**

To determine whether Notch1 signaling is altered in the formation and progression of AAA, we analyzed the expression of NICD, the active form of Notch1, in the abdominal aorta of AngII-induced mouse model of AAA. NICD immunoreactivity was increased in the aneurysmal aorta of AngII-treated Apoe−/− mice compared with Apoe−/− mice treated with saline at day 7 (Figure 1A–1F) and day 28 (Figure 1G–1L). At day 7, increased NICD signaling was restricted to the vicinity of vascular injury and was localized with inflammatory macrophages as determined by double immunofluorescence using the monocyte and macrophage marker (MOMA2; Figure 1A–1F in the online-only Data Supplement). At day 28, expression of NICD protein was extended to both the adventitial and medial layers of the aneurysmal abdominal aorta (Figure 1J–1L; Figure 1G–1L in the online-only Data Supplement). Consistent with immunostaining, quantitative reverse transcriptase polymerase chain reaction data demonstrated a significant increase in mRNA expression of Notch1 and its downstream target, Hey1, in the abdominal aorta of...
Apoe\(^{-/-}\) mice treated with AngII compared with saline-treated controls at day 7 and day 28 (Figure IIA–IID in the online-only Data Supplement). Consistent with our observations in mice, increased NICD signaling was also observed in the infrarenal abdominal aorta from patients undergoing AAA repair (Figure 1P–1R) compared with age-matched controls (Figure 1M–1O) and appeared to be localized to inflammatory cells. Overall, activation of the Notch1 signaling pathway is observed in both mouse and human models of AAA.

**Notch1 Haploinsufficiency Reduces the Occurrence of AAA in AngII-Induced Mouse Model**

Next, we examined whether Notch1 haploinsufficiency affects the incidence of AAA in the AngII-induced mouse model of AAA. Notch1\(^{+/+}\), Apoe\(^{-/-}\), and Notch1\(^{+/+}\):Apoe\(^{-/-}\) mice were treated with AngII or saline for 28 days using published protocols.\(^{2,14}\) Transabdominal ultrasound imaging and gross examination at 28 days of AngII demonstrated luminal expansion in the suprarenal region of the abdominal aorta of Apoe\(^{-/-}\) mice (Figure 2E and 2K), whereas no such luminal expansion in this region was observed in the Notch1\(^{+/+}\):Apoe\(^{-/-}\) mice (Figure 2F and 2L). As expected, Notch1\(^{-/-}\) and wild-type mice treated with AngII and all mice treated with saline did not develop luminal expansion (Figure 2; Figure III in the online-only Data Supplement). A similar rate of mortality was observed between Apoe\(^{-/-}\) mice and Notch1\(^{+/+}\):Apoe\(^{-/-}\) mice in response to AngII infusion (Figure IV in the online-only Data Supplement). No significant blood pressure differences were observed between Apoe\(^{-/-}\) and Notch1\(^{+/+}\):Apoe\(^{-/-}\) mice with AngII infusion, suggesting that the protective effects of Notch1 haploinsufficiency were not related to blood pressure (data not shown).

Macroscopic measurement of the external diameter of suprarenal aorta demonstrated a significant increase in maximal aortic width of Apoe\(^{-/-}\) mice treated with AngII versus saline-treated Apoe\(^{-/-}\) mice (Figure 2M). In comparison, the maximal aortic width of suprarenal aorta was significantly
Reduced in the Notch1+/−;Apoe−/− mice treated with AngII compared with Apoe−/− mice on similar treatment (Figure 2M). In fact, the aortic width of Notch1+/−;Apoe−/− mice in response to AngII was not significantly different from Notch1+/− mice treated with AngII (P<0.05; Figure 2N).

Histologically, Apoe−/− mice treated with AngII demonstrated cellular and architectural changes of typical AAA, including thrombus formation, adventitial remodeling, inflammatory cell infiltration, and elastin degradation (Figure 2P, 2S, and 2V). The aortae of Notch1+/−;Apoe−/− mice infused with AngII displayed a well-defined lumen, with no elastin degradation and minimal infiltration of inflammatory cells (Figure 2Q, 2T, and 2W). Histological examination of Notch1+/− mice treated with AngII or saline-treated controls also did not show any evidence of AAA (Figure 2O, 2R, and 2U; Figure III in the online-only Data Supplement). Notably, except for marginal adventitial thickening, no other characteristic features of AAA were observed in the Notch1+/−;Apoe−/− mice (Figure 2Q, 2T, and 2W). Active caspase-3 immunostaining was prominent in the aortic smooth muscle cell (aSMC)–enriched medial layer.

Figure 2. Notch1 haploinsufficiency reduces the incidence of abdominal aortic aneurysms (AAA) in Apoe−/− mice after 28 days of angiotensin II (AngII) infusion. Representative aortas from mutant mice treated with saline (A–C) and AngII (D–F) are shown. E, Aneurysm (red arrow) is observed in the suprarenal aorta of Apoe−/− mice treated with AngII but is absent in Notch1+/− mice (D) and reduced in Notch1+/−;Apoe−/− mice treated with AngII (F). No aneurysms were seen in saline-treated groups (A–C). Transabdominal ultrasound images (G–L) show that Notch1+/−;Apoe−/− mice have decreased abdominal aortic luminal diameter compared with Apoe−/− mice (K) when infused with AngII. Dashed yellow lines outline the expansion of lumen in K. M, Quantitative measurement of maximal aortic width (mm) in all experimental mice. Mean and SD are shown. Each individual animal is represented by a symbol. ***P<0.001 when comparing Apoe−/− with AngII vs Apoe−/− treated with AngII. N, Table showing incidence of AAA in Notch1+/−, Apoe−/−, and Notch1+/−;Apoe−/− mice treated with saline or AngII. *P<0.05 when comparing Apoe−/− with AngII vs Notch1+/−;Apoe−/− treated with AngII. O–T, Transverse sections of abdominal aorta were stained with hematoxylin and eosin (n=6 for each genotype). Adventitial remodeling, along with inflammation, elastin degradation and thrombus, was found in the aorta of Apoe−/− mice infused with AngII (P and S). No changes except mild adventitial thickening was observed in Notch1+/− (O and R) and Notch1+/−;Apoe−/− mice (Q and T) infused with AngII. U–W Elastin staining demonstrated elastin fragmentation in Apoe−/− mice (V) that was not seen in Notch1+/− (U) and Notch1+/−;Apoe−/− mice (W). (R,S,T) represent high magnification images of boxed region in (O,P,Q), respectively. Scale bar, 1 mm (A–F) and 50 µm (O–W). LRA indicates left renal artery.
of aorta in the Apoe−/− mice infused with AngII compared with Notch1+/−;Apoe−/− mice (Figure V in the online-only Data Supplement), which was not surprising as apoptosis of aSMCs is a characteristic feature of AAA. These data demonstrate that Notch1 haploinsufficiency significantly decreased the occurrence of pathological sequela associated with AAA in an established mouse model.

**Notch1 Haploinsufficiency in Bone Marrow–Derived Cells Prevents AngII-Induced AAA Formation**

The influx of inflammatory cells, consisting of macrophages and lymphocytes, to the site of aneurysm formation is critical for the development of AAA. We performed bone marrow transplantation (BMT) experiments to address the question of whether Notch1 haploinsufficiency in bone marrow–derived cells alters aeurysmal development in Apoe−/− mice (Figure 3; Figure VI in the online-only Data Supplement). After optimizing the irradiation and validating the repopulation procedure, the Apoe−/− (group I, n=12) and Notch1+/−;Apoe−/− (group II, n=12) mice were irradiated and reconstituted with bone marrow–derived cells harvested from Notch1+/−;Apoe−/− and Apoe−/− mice, respectively. Two additional groups of mice were also studied to serve as control groups: irradiated Apoe−/− mice were repopulated with bone marrow–derived cells harvested from Apoe−/− mice (group III, n=8) and Apoe−/− mice without BMT (group IV, n=6). Transabdominal ultrasound measurements and gross examination demonstrate that infusion of AngII into Apoe−/− mice...

**Figure 3.** Notch1 haploinsufficiency in bone marrow–derived cells decreased abdominal aortic aneurysm (AAA) incidence and mortality rate in Apoe−/− mice. Bone marrow–derived cells from Apoe−/− mice increased AAA disease in Notch1+/−;Apoe−/− (group II; Apoe−/− → Notch1+/−;Apoe−/−, n=12) mice compared with Apoe−/− mice that received bone marrow–derived cells from Notch1+/−;Apoe−/− mice (group I; Notch1+/−;Apoe−/− → Apoe−/−, n=12). The representative transabdominal ultrasound images at day 0 (A and B), day 7 (C and D), day 14 (E and F), day 21 (G and H), and day 28 (I and J), demonstrating an increase in the suprarenal luminal diameter in group II compared with group I. K, Significant increase in the rate of luminal expansion in group II (red) compared with group I (green) after second week of angiotensin II (AngII) infusion (L). Significantly increased number of disease events, defined by either 50% increase in aortic diameter or sudden death. Representative images of the whole aorta from group I and group II mice are shown in M and N. Scale bar, 1 mm. LRA indicates left renal artery. ***P<0.001; **P<0.01; and *P<0.05.
with Notch1+/:Apoe− bone marrow–derived cells (group I) significantly decreased the progression of luminal expansion compared with group II (Figure 3; Figure VI in the online-only Data Supplement). Notch1+/:Apoe− mice repopulated with bone marrow–derived cells from Apoe− mice (group II) not only augmented progression of luminal expansion but also increased aortic rupture–associated mortality during 28 days of AngII infusion (P<0.001; Figure 3L). Consistent with other studies, BMT in irradiated Apoe− mice (group III, n=8) led to lower incidence of AngII-induced AAA compared with nonirradiated Apoe− mice (group IV; data not shown).32 Notch1 haploinsufficiency did not affect total leukocyte, monocyte, or lymphocyte count in the peripheral blood (data not shown). Overall, the BMT studies suggest that protective effects of Notch1 haploinsufficiency are preserved after transplantation of bone marrow–derived cells from Notch1+/:Apoe− mice into Apoe− mice.

**Notch1 Haploinsufficiency Reduces Macrophage Infiltration at Site of AAA**

Monocyte recruitment and macrophage infiltration occur during early and later stages of AAA development; therefore, we examined the suprarenal abdominal aorta at days 7 and 28 of AngII infusion for the expression of Moma2, a marker of activated macrophages. Similar to AngII exposure for 28 days (Figure 2), the aorta of Apoe− mice infused with AngII for 7 days showed signs of luminal expansion and elastin degradation, which was absent in Notch1+/:Apoe− mice (Figure VII in the online-only Data Supplement). At both day 7 and day 28 of AngII infusion, the expression of Moma2 in the abdominal aorta of Notch1+/:Apoe− mice was decreased compared with Apoe− mice as determined by IHC (Figure 4A–4H). Mcp1 plays a critical role in the recruitment and infiltration of macrophages in response to external stimuli. IHC of the aneurysmal region revealed a significantly increased expression of Mcp1 in the inflammatory cells of Apoe− mice, which was abrogated in the aorta of Notch1+/:Apoe− mice at both 7 and 28 days of AngII treatment (Figures 4I–4P; Figure VIII in the online-only Data Supplement). Of note, although marginal Mcp1 expression was observed in the SMC-enriched medial layer of Notch1+/:Apoe− mice, the adventitial thickening observed in the abdominal aortic region of Notch1+; Apoe− mice was devoid of Mcp1-positive inflammatory cells (arrowheads in Figure 4F, 4H, 4N, and 4P).

AngII is postulated to play a central role in initiating inflammation in the aorta by increasing the expression of chemokines, adhesion molecules, and cytokines in macrophages.2,3,33 To determine whether Notch1 haploinsufficiency reduces the inflammatory response in the AngII model of AAA, we examined the mRNA expression levels of a panel of cytokines, chemokines, and proinflammatory mediators by quantitative reverse transcriptase polymerase chain reaction in the aortas of Apoe− and Notch1+/:Apoe− mice treated with AngII for 28 days. Significant reduction of the expression of chemokines and cytokines (Mcp1, Il6, Cxcl10) and the proinflammatory mediators (Vegf, iNOS, Icam1, Vcam1) was found in the abdominal aorta of Notch1+/:Apoe− mice compared with Apoe− mice (Figure 4Q).

To determine whether a similar decreased inflammatory response occurs ex vivo, we isolated primary macrophages from the peritoneal cavity of Apoe− and Notch1+/:Apoe− mice and subjected them to LPS stimulation (100 ng/mL) for 3 hours. LPS, a potent activator of macrophages, is known to upregulate expression of Notch1 and its downstream targets and mimic inflammatory response of AAA in as similar fashion.15,34,35 The expression of Mcp1, Il6, Tnf-α, and iNOS in macrophages from Notch1+/:Apoe− mice was ≈60% less than in macrophages from Apoe− mice in response to LPS treatment (Figure 4R). As expected, the expression of Notch1 was also reduced by ≈60% in the macrophages from Notch1+/:Apoe− mice (data not shown). In summary, Notch1 haploinsufficiency decreases macrophage infiltration and resultant inflammatory response, as measured by cytokine and chemokine expression, in response to external stress.

**Notch1 Haploinsufficiency Causes Defects in Macrophage Migration and Proliferation by Differentially Regulating M1/M2 Polarization**

Given the limitations of IHC in determining the specificity and difficulty in quantification, we assessed direct infiltration of active macrophages and lymphocytes at the site lesion by fluorescence-activated cell sorting analysis. Consistent with our IHC observations, the suprarenal abdominal aorta of Notch1+/:Apoe− mice contained a decreased number of inflammatory macrophages with Cd11b and Cd14 staining compared with Apoe− mice after 7 days of AngII infusion (Figure 5A–5C). Notch1 haploinsufficiency did not alter the differentiation of Cd4+ or Cd8+ lymphocytes at the site of injury, although the total number of Cd3+ T lymphocytes was significantly decreased in the suprarenal aorta of Notch1+; Apoe− mice compared with Apoe− mice after 7 days of AngII infusion as determined by fluorescence-activated cell sorting analysis and immunostaining (Figure IX in the online-only Data Supplement). Interestingly, no significant difference was observed in the absolute number of Cd3+ T cells in the spleen (Figure IX in the online-only Data Supplement) or peripheral blood (data not shown) of these experimental mice.

Because it has been shown that total lymphocyte deficiency had no detectable effect on the development of AAA in the AngII mouse model,36 we focused on the effects of Notch1 haploinsufficiency on macrophage functions. We first examined whether Notch1 signaling has direct effects on macrophage migration or proliferation. In a classical scratch assay, Notch1 haploinsufficiency significantly decreased the migration of peritoneal-derived primary macrophages toward the injury site after 24 hours (Figure 5D–5H). After 24 hours, decreased proliferation was observed in Notch1+; Apoe− macrophages compared with Apoe− macrophages as determined by staining with proliferation marker (Ki67; Figure 5I–5M). Increased Ki67 staining localized with macrophages was also observed in the abdominal aorta of Apoe− mice, whereas no such coexpression was observed in the aorta of Notch1+; Apoe− mice (Figure 5A–5F in the online-only Data Supplement). In a transwell culture system, n-formyl-met-leu-phe (a potent and specific chemotactic agent)–induced migration of primary macrophages was significantly reduced with Notch1 haploinsufficiency (Figure XG in
Figure 4. Minimal macrophage recruitment and monocyte chemotactic protein-1 (MCP1) expression in the aorta of Notch1+/−;Apoe−/− mice after 7 and 28 days of angiotensin II (AngII) infusion. Immunohistochemistry showing decreased expression of MOMA2, a macrophage marker, in the aneurysmal tissue at day 7 and day 28 of AngII-treated Notch1+/−;Apoe−/− mice (B, F and D, H) compared with ApoE−/− mice (A, E and C, G). Decreased MCP1 expression in the abdominal aorta of Notch1+/−;ApoE−/− mice (J, N and L, P) in response to AngII compared with ApoE−/− mice (I, M and K, O). MOMA2 and MCP1 expression was specifically decreased in adventitial layer (F, H, N, and P; arrowheads). Q, Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) demonstrates reduced mRNA levels of Mcp1, Il6, Cxcl10, Vegf, iNos, Icam1, and Vcam1 in the abdominal aorta of Notch1+/−;Apoe−/− mice compared with ApoE−/− mice treated with AngII for 28 days (*n=3, in triplicate). R, qRT-PCR demonstrates reduced expression of a similar panel of inflammatory mediators in lipopolysaccharide (LPS)-stimulated primary macrophages isolated from the peritoneal cavity of Notch1+/−;Apoe−/− mice compared with ApoE−/− mice. Macrophages from 5 mice were pooled for each experiment; data shown represent 3 experiments performed in triplicate. (E-H) are high magnification images of (A-D) and (M-P) are high magnification images of (I-L) ***P<0.001; **P<0.01; *P<0.05. Mean and SD are shown. Scale bar, 50 µm.
To determine whether Notch1 haploinsufficiency affects proliferation of primary macrophages in vivo, thioglycollate injection was used to elicit the recruitment of macrophages to the peritoneal cavity. Notch1 haploinsufficiency resulted in the diminished influx of macrophages by almost 50% at day 4 of the thioglycollate infusion (Figure XH in the online-only Data Supplement). Recent studies have suggested an integral role for Notch1 to regulate M1 polarization of naive macrophages mediated by synthesis of interferon regulatory factor 8 protein. As expected, expression of interferon regulatory factor 8 was upregulated in the abdominal aorta of Apoe−/− mice in response to AngII at day 7, whereas Notch1 haploinsufficiency prevented upregulation of interferon regulatory factor 8 (Figure XI and XJ in the online-only Data Supplement). To determine whether Notch1 regulates polarization of macrophages, we evaluated markers for inflammatory M1-associated genes in response to LPS/interferon-γ stimulation in the BMDM. Increased expression of Il6, Il12, Tnf-α, and iNOS genes was observed in the BMDM of Apoe−/− mice compared with Apoe−/− mice. Notch1 haploinsufficiency increased M2 polarization–associated genes in BMDM in response to IL4 (20 ng/mL) compared with Apoe−/− mice (n=3). Scale bar, 50 µm. ***P<0.001; **P<0.01; and *P<0.05.
with increased interferon regulatory factor 8 staining, which was reduced by Notch1 haploinsufficiency (Figure XK and XL in the online-only Data Supplement). Taken together, the results demonstrate that Notch1 haploinsufficiency causes defects in the migration and proliferation functions of macrophages by differentially regulating M1/M2 polarization of macrophages, thus preventing them from infiltrating the site of aneurysm formation in the AngII mouse model.

Pharmacological Inhibition of Notch Signaling Attenuates Aneurysm Development in an AngII-Induced Mouse Model of AAA

Although the BMT studies and flow cytometry data demonstrate a specific role for Notch1 signaling in macrophages, Notch1 is known to be necessary for the proper development of the vasculature.18 To determine whether the protective effects of Notch1 haploinsufficiency on AAA formation are not secondary to the embryonic functions of Notch1 and to investigate the therapeutic potential of Notch1 inhibition, we examined whether pharmacological inhibition of Notch1 protects against the formation of AAA in adult Apoe−/− mice infused with AngII. Mice were treated with DAPT (10 mg/kg) 3x a week, starting 1 week before initiating the AngII infusion or 3 days after the AngII infusion and continuing for 28 days.23,24 These time periods were chosen to determine whether DAPT mimics the effects of Notch1 haploinsufficiency on AAA formation and to determine its therapeutic potential to prevent the progression of AAA after a small aortic dilation is established (Figure XI in the online-only Data Supplement). Treatment with DAPT resulted in a significant reduction in the aortic diameter and incidence of AAA at both time intervals compared with untreated Apoe−/− mice and was similar to Notch1−/−;Apoe−/− mice (Figure 6A–6D). Histological analysis of the aorta of the Apoe−/− mice treated with DAPT 7 days before AngII infusion (Figure 6F and 6I) demonstrated normal aortic wall architecture without infiltration of inflammatory cells compared with untreated mice (Figure 6E and 6H). Although marginal adventitial thickening was observed in Apoe−/− mice treated with DAPT treatment 3 days after AngII infusion, no visible inflammation was observed (Figure 6G and 6J). Furthermore, pharmacological inhibition of Notch1 (DAPT) had similar defects in the migration and proliferation of macrophages in a murine macrophage cell line (RAW 264.7 cells; data not shown). DAPT also downregulated the inflammatory response of these macrophages in response to LPS compared with non-DAPT–treated cells (data not shown). Significantly decreased gene expression of Notch1 and its downstream Hey1 expression in the abdominal aorta of these mice were observed with DAPT treatment compared with vehicle-treated Apoe−/− mice (Figure XIIA–XIIIB in the online-only Data Supplement). These studies demonstrate that the reduced incidence of AAA seen with genetic deficiency of Notch1 is not the result of developmental differences in the aortic wall and also demonstrate a potential therapeutic strategy for treatment of AAA.

Discussion

The Notch1 signaling pathway has been implicated in numerous developmental processes and disease states.15,16,18 Here, we provide the first evidence that Notch1 signaling is activated in the abdominal aorta from the AngII-induced mouse model of AAA and in patients with AAA. We also demonstrate that Notch1 haploinsufficiency significantly reduces the incidence of AAA in Apoe−/− mice infused with AngII. The protective effects of Notch1 haploinsufficiency on AAA are mediated by decreased recruitment of inflammatory macrophages at the site of aneurysm. Bone marrow studies demonstrate that Notch1 haploinsufficiency in inflammatory cells is sufficient to reduce the development of AAA. Gene expression studies demonstrate that Notch1 haploinsufficiency results in decreased expression of Il6, Mcp1, and adhesion molecules, which play critical roles in macrophage recruitment at the site of injury in response to stress. Flow cytometry confirmed the selective recruitment of inflammatory macrophages in the suprarenal aorta in response to AngII. Notch1 inhibition reduces M1 polarization of macrophages and promotes their M2 fate, thus causing defects in macrophage migration and proliferation. Consistent with this, pharmacological inhibition of Notch signaling by DAPT attenuated dilation of the abdominal aorta in the AngII-induced model of AAA. These findings suggest that Notch1 actively participates in the process of inflammation by directly regulating cytokines and chemokines critical for macrophage recruitment in the pathogenesis of aneurysm (Figure XIII in the online-only Data Supplement). Deficiency of Notch1, by preventing this inflammatory cascade, preserves the anti-inflammatory environment and normal aortic wall architecture, thereby attenuating dilation of the suprarenal abdominal aorta.

Although the role of the Notch signaling pathway as a critical regulator of cell fate during development is well established,16,38 there is emerging evidence that Notch signaling is also critical in the pathogenesis of a variety of inflammatory diseases. Although NOTCH1 mutations have been associated with bicuspid valve, aortic valve calcification, and thoracic aortic aneurysm, no study has exclusively examined the role of Notch1 in the pathogenesis of AAA.40–42 A recent study showed strong correlation of bicuspid valve with thoracic aortic aneurysm, but interestingly none of the patients in their study had AAA, suggesting different pathophysiology for thoracic aortic aneurysm and AAA.43 There is growing evidence that activation of the Notch1 signaling pathway regulates the expression of an inflammatory cascade that includes Il6 and Mcp1, along with a variety of key inflammatory genes such as iNos, Cxcl10, Icam1, and Vcam.15 A recent study suggests that Notch1 signaling alone is sufficient to switch on Il6 mRNA transcription in macrophages.12 Although deficiency of Il6 and MCP1 has also been shown to prevent AAA formation, the role of Cxcl110 receptor in AAA is conflicting. Although increased expression of Cxcl110 is reported in AngII-exposed Apoe−/− mice, deficiency of Cxcl110 has also been shown to augment AAA formation.44 Overall, our work provides the first evidence suggesting a role for Notch1 signaling in AAA, and further studies are required to decipher the specific role of these inflammatory factors in AAA.

Although remarkable progress has been made in the recent years in understanding the role of Notch1 signaling in the immune system, the complexity of Notch signaling because of its multiple receptors and ligands is not completely understood. Data obtained from our chemotactic studies and...
immunostaining combined with BMT studies support the hypothesis that Notch1 haploinsufficiency primarily interferes with the migration, proliferation, and activation of macrophages. Consistent with our findings, a recent study reported that myeloid-specific reduction in Notch1 decreases macrophage recruitment and localization during angiogenesis. However, we do not exclude the possibility that Notch1 regulates expression of proinflammatory cytokines in other cells, including aortic SMCs and endothelial cells, in a similar manner as in macrophages. It is worthwhile to mention here that modest levels of Mcp1 expression observed in the intimal endothelial layer and medial smooth muscle layer of the aorta in Notch1+/−;ApoE−/− mice seem to be insufficient for the recruitment of macrophages (Figure 4; Figure VIII in the online-only Data Supplement). Because monocyte infiltration seems to precede aSMC apoptosis in developing AAA, factors released by monocyte and macrophages may play a primary role in the overall perpetuation of inflammation by regulating the function of aSMCs and endothelial cells in the setting of AAA. Further studies are needed to decipher the crosstalk between macrophages and aSMCs in the setting of AAA.

In our study, Notch1 haploinsufficiency reduced infiltration of Cd3+ T lymphocytes at the site of aneurysm formation.
Consistent with other studies, differential effects of Notch1 haploinsufficiency on cytotoxic CD4+ or CD8+ T-lymphocyte differentiation in the aneurysmal aorta or spleen were not detectable in response to AngII, suggesting that Notch1 pathway activation is not a feature of all T cells involved in vascular inflammation and is disease-specific. Furthermore, total lymphocyte deficiency, achieved by developing Apoe−/− mice that lacked the recombination activating gene-1 gene, fails to affect the development of AAA in AngII mouse model, suggesting that lymphocytes are dispensable for AAA.

The clinical management of AAA is based on the control of primary risk factors, such as tobacco use, dyslipidemia, hypertension, atherosclerosis, and infection. To prevent the progression or to stimulate the regression of established AAA, there is a critical need to develop pharmacological interventions that can selectively target the features of AAA. Several strategies have been proposed to achieve the objective of impeding AAA progression, but available options fall short of this goal. Our data demonstrate that Notch1 is an important player in the inflammatory process in the setting of AAA and suggest that treatment with Notch1-specific inhibitors may be a potentially promising strategy for slowing aneurysm development. Notch1 inhibitors are potential therapeutic agents for cancer. Based on recent findings that Notch inhibition decreases macrophage infiltration and directly regulates macrophage polarization, it is plausible that targeting Notch1 signaling may hold a promising target-based therapy for developing AAA. Beneficial effects of pharmacological inhibition of γ-secretase have been reported to blunt the inflammatory response in a mouse model of atherosclerosis and reduce plaque formation and also to prevent vascular inflammation in an experimental model of giant cell arthritis. Investigations in other animal models of AAA (elastase and CaCl2 infusion) will further support the clinical use of Notch1 inhibition as a pharmacological therapy.

Acknowledgments

We thank Dave Dunaway in the Flow Cytometry Core, members of the Morphology Core at the Research Institute at Nationwide Children’s Hospital for technical support, Yongjie Miao for the statistical analysis of the data, and Drs P.A. Lucchesi and B. Lilly for helpful comments on the manuscript.

Sources of Funding

This work was supported by an American Heart Association-National Center Scientific Development Grant to Dr Hans and funding from the Research Institute at Nationwide Children’s Hospital and National Institutes of Health/National Heart, Lung, and Blood Institute to Dr Garg.

Disclosures

C.P. Hans and V. Garg have applied for a patent related to this work. The other authors have no conflicts to report.

References


Inhibition of Notch1 Signaling Reduces Abdominal Aortic Aneurysm in Mice by Attenuating Macrophage-Mediated Inflammation
Chetan P. Hans, Sara N. Koenig, Nianyuan Huang, Jeeyun Cheng, Susana Beceiro, Anuradha Guggilam, Helena Kuivaniemi, Santiago Partida-Sánchez and Vidu Garg

Arterioscler Thromb Vasc Biol. 2012;32:3012-3023; originally published online October 18, 2012;
doi: 10.1161/ATVBAHA.112.254219

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/32/12/3012

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2012/10/18/ATVBAHA.112.254219.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Supplemental Table and Figures

Title: Inhibition of Notch1 Signaling Reduces Abdominal Aortic Aneurysm in Mice by Attenuating Macrophage-Mediated Inflammation

Authors: Chetan P. Hans, Sara N. Koenig, Nianyuan Huang, Jeeyun Cheng, Susana Beceiro, Anuradha Guggilam, Helena Kuivaniemi, Santiago Partida-Sánchez and Vidu Garg
### Supplemental Table I. List of primer sequences used in qRT-PCR studies

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Notch1</td>
<td>5’-CCG TTA CAT GCA GCA GTT TC-3’</td>
<td>5’-AGC CAG GAT CAG TGG AGT TG-3’</td>
</tr>
<tr>
<td>Hey1</td>
<td>5’-TCT CAG CCT TCC CCT TTT C-3’</td>
<td>5’-CTT TCC CCT CCC TTG TTC TAC-3’</td>
</tr>
<tr>
<td>Mcp1</td>
<td>5’-CTG GAT CGG AAC CAA ATG AG-3’</td>
<td>5’-AAG GCA TCA CAG TCC GAG TC-3’</td>
</tr>
<tr>
<td>Il6</td>
<td>5’-CTA CCC CAA TTT CCA ATG CT-3’</td>
<td>5’-ACC ACA GTG AGG AAT GTC CA-3’</td>
</tr>
<tr>
<td>Tnf-α</td>
<td>5’-CCC ACT CTG ACC CCT TTA CT-3’</td>
<td>5’-TTT GAG TCC TTG ATG GTC GT-3’</td>
</tr>
<tr>
<td>Cxcl10</td>
<td>5’-CCC ACG TGT TGA GAT CAT TG-3’</td>
<td>5’-CAC TGG GTA AAG GGG AGT GA-3’</td>
</tr>
<tr>
<td>Vegf</td>
<td>5’-GGC TGC TGT AAC GAT GAA GC-3’</td>
<td>5’-TTA ACT CAA GCT GCC TCG C-3’</td>
</tr>
<tr>
<td>iNos</td>
<td>5’-CTC GGA GGT TCA CCT CAC TGT-3’</td>
<td>5’-TCC TG TCC AAG TGC TGC AGA-3’</td>
</tr>
<tr>
<td>Icam1</td>
<td>5’-GTG ATC CCT GGG CCT GGT G-3’</td>
<td>5’-GGA AAC GAA TAC ACG GTG ATG-3’ G</td>
</tr>
<tr>
<td>Vcam1</td>
<td>5’-TAC CAG CTC CCA AAA TCC TG-3’</td>
<td>5’-TCT GCT AAT TCC AGC CTC GT-3’</td>
</tr>
<tr>
<td>Il12</td>
<td>5’-GGA AGC AGC GCA GCA GAA TA-3’</td>
<td>5’-AAC TTG AGG GAG AAG TAG GAA TGG-3’</td>
</tr>
<tr>
<td>Arg1</td>
<td>5’-CTC CAA GCC AAA GTC CTT AGA G-3’</td>
<td>5’-AGG AGC TGT CAT TAG GGA CAT C-3’</td>
</tr>
<tr>
<td>Il10</td>
<td>5’-GTC CAG CCA GCC TTA TCG GA-3’</td>
<td>5’-ACC TGC TCC ACT GCC TTG CT-3’</td>
</tr>
<tr>
<td>Mgl1</td>
<td>5’-TGA GAA AGG CTT TAA GAA CTG GG-3’</td>
<td>5’-GAC CAC CTG TAG TGA TGT GGG-3’</td>
</tr>
<tr>
<td>Mgl2</td>
<td>5’-TTA GCC AAT GTG CTT AGC TGG-3’</td>
<td>5’-GGC CTC CAA TTC TTG AAA CCT-3’</td>
</tr>
</tbody>
</table>
**Supplemental Figure I. Increased expression of Notch1 intracellular domain localized with the macrophages in aneurysmal aorta of Apoe−/− mice.** At 7 day of AngII infusion, double immunofluorescence demonstrate strong co-expression of Moma2 (red) and NICD (green) in the adventitial region of Apoe−/− mice treated with AngII (D-F) as compared to saline treated mice (A-C). At day 28, NICD staining was localized with the adventitial macrophages (I; red arrow heads) and medial SMC layer (L; white arrow heads). Of note, red staining in the aortic medial layer (A, D, G, J) corresponds to non-specific autofluorescence of elastin fibrils. Aortas from three mice were examined and representative sections are shown. Scale bar represents 50 µm. All nuclei were stained by DAPI (blue; inserts B, E, H, K). (M-O) Negative controls for NICD staining with non-specific IgG showing specificity of the NICD.
Supplemental Figure II. Increased mRNA expression of Notch1 and its downstream Hey1 in aneurysmal aorta of Apoe<sup>-/-</sup> mice. Quantitative real-time PCR (qRT-PCR) demonstrates increased mRNA expression of Notch1 and Hey1 at 7 days (A, B) and 28 days (C, D) in the abdominal aorta of AngII treated Apoe<sup>-/-</sup> mice as compared to Apoe<sup>-/-</sup> mice treated with saline (n=3). ***P<0.001; **P<0.01.
Supplemental Figure III. No evidence of AAA was found in wild-type mice treated with AngII (A-C) or Notch1+/−; Apoe−/−, and Notch1+/−; Apoe−/− treated with saline (D-I). (n=3 mice for each group and representative images are shown). C, G, H and I are high magnification images of B, D, E and F respectively. Scale bar represents 1 mm (A) and 50 µm (B-I).
Supplemental Figure IV. *Notch1* haploinsufficiency did not affect mortality in *Apoe*⁻/⁻ mice in response to AngII. Expectedly, about 20% mortality was observed in *Apoe*⁻/⁻ mice in response to AngII. Similar mortality ratio was observed in *Notch1*⁺/⁻;*Apoe*⁻/⁻ mice. No mortality was observed in *Notch1*⁺/⁻ mice on wildtype background in response to AngII.
Supplemental Figure V. Decreased apoptotic cell death in the aorta of Notch1+/−;ApoE−/− mice. Active caspase-3 staining (A-F) shows decreased apoptosis in all layers of the aortic wall of Notch1+/− (A, D) and Notch1+/−;ApoE−/− mice (C, F) as compared to ApoE−/− mice (B, E). Scale bar represents 50 µm.
Supplemental Figure VI. Notch1 haploinsufficiency in bone marrow-derived cells decreased aortic luminal expansion in Apoe⁻/⁻ mice. The bone marrow derived cells from Apoe⁻/⁻ mice increased severity of the disease in Notch1⁺/⁻;Apoe⁻/⁻ (Group II; Apoe⁻/⁻ → Notch1⁺/⁻;Apoe⁻/⁻, n=12) mice as compared Apoe⁻/⁻ mice which received bone marrow derived cells from Notch1⁺/⁻;Apoe⁻/⁻ mice (Group I; Notch1⁺/⁻;Apoe⁻/⁻ → Apoe⁻/⁻, n=12). Luminal expansion at day 0 (A), day 7 (B), day 14 (C), day 21 (D) and day 28 (E) demonstrating an increase in the suprarenal luminal expansion in Group II as compared to Group I. **P<0.01; *P<0.05, #non-significant.
Supplemental Figure VII. Notch1 haploinsufficiency prevents formation of AAA at day 7. Visible vascular injury was noticed in the abdominal aorta of ApoE−/− mice in response to AngII (A), whereas no such evidence were found in the aorta of Notch1+/−;ApoE−/− mice (B) as further confirmed by HE staining (C-D) and elastin staining (E-F). Scale bar represents 50 µm.
Supplemental Figure VIII. (A) Quantification of Mcp1 immunoreactivity by Image Pro-plus showing significant reduction of Mcp1 expression in Notch1\textsuperscript{+/−};ApoE\textsuperscript{−/−} mice as compared to ApoE\textsuperscript{−/−} mice. (B,C) Negative controls for Moma2 and Mcp1 IHC using non-specific IgG. Means and standard deviations are shown. Scale bar represents 50 µm. **$P<0.01$.**
Supplemental Figure IX. Notch1 haploinsufficiency selectively promote infiltration of Cd3+ cells at the site of aneurysm formation without affecting differentiation into Cd4+ or Cd8+ T cells. (A-B) FITC positive Cd3 cells at the aneurysmal site which were significantly decreased in the abdominal aorta of Notch1+/−;Apoe−/− mice as shown in graph (C). (D, E) Marginal increase in Cd3 staining in the abdominal aorta of Apoe−/− mice which was absent in Notch1+/−;Apoe−/− mice at day 7 of AngII infusion. Cd3+ cell population in spleen is not affected by Notch1 haploinsufficiency (F-H). Notch1 haploinsufficiency did not affect differentiation of Cd3+ lymphocytes into cytotoxic Cd4+ or Cd8+ T cells (I-K). **P<0.01.
Supplemental Figure X. Effects of Notch1 haploinsufficiency on macrophage functions. (A-F) Notch1 haploinsufficiency decreased Ki67 positive macrophages in the abdominal aorta at day 7. (G) Notch1 haploinsufficiency caused defects in migration of macrophages in response to chemotactic agent (FMLP; 100 nM). (H) Notch1 haploinsufficiency decreased macrophage proliferation in the peritoneal cavity in response to thioglycollate infusion in Apoe−/− mice (n=12). (I-L) Increased expression of Notch1 dependent marker for M1 polarization (Irf8) in the aorta of Apoe−/− mice (I, J) and BMDM (K, L) in response to LPS/Ifn-γ stimulation. (M-N) FACS analysis demonstrating that Notch1 haploinsufficiency decreased M1 polarization of macrophages in response to LPS/Ifn-γ stimulation. Scale bar represents 50µm. ***P<0.001; **P<0.01.
Supplemental Figure XI. *Vascular injury in Apoe<sup>-/-</sup> mice at 3 days of AngII infusion.* Dilatation was observed in the abdominal aorta of *ApoE<sup>-/-</sup>* mice (B) within 3 days of Ang II infusion as compared to *Notch1<sup>+/+</sup>;ApoE<sup>-/-</sup>* mice (C). Scale bar represents 1 mm.
Supplemental Figure XII. Decreased mRNA expression of Notch1 (A) and its downstream Hey1 (B) with DAPT treatment by qRT-PCR. ***P<0.001, **P<0.01, *P<0.05.
Supplemental Figure XIII. Proposed model for the involvement of Notch1 signaling in the development of AAA.

- Notch1 activation
- Inflammatory cytokines (IL-6, TNF-α), Chemokines (MCP-1) and adhesion molecules (ICAM-1, VCAM-1)
- Macrophage activation, migration & recruitment
- MMP activation, SMC apoptosis and ECM degradation
- Abdominal Aortic Aneurysm
SUPPLEMENTAL METHODS

Generation of Notch1\(^{+/−}\);ApoE\(^{+/−}\) mice. Six to eight week old ApoE\(^{+/−}\) female mice in a C57BL/6J background and Notch1\(^{+/−}\) male mice (C57BL/6J background; Jackson Laboratory, Bar Harbor, ME) were crossbred to generate Notch1\(^{+/−}\);ApoE\(^{+/−}\) mice which were interbred to obtain Notch1\(^{+/−}\);ApoE\(^{−/−}\) mice. ApoE\(^{−/−}\) mice were crossbred with Notch1\(^{+/−}\);ApoE\(^{−/−}\) mice to obtain ApoE\(^{−/−}\)(n=10) and Notch1\(^{+/−}\);ApoE\(^{−/−}\) (n=10) littermates. Mice were kept on a 12h/12h light/dark cycle with standard chow. Genotyping was performed according to the protocol from the Jackson Laboratories. Because of the variation of the extent of disease between male and female animals, only male mice were used throughout the studies. The mice (8-10 weeks old) were randomly assigned to receive saline or angiotensin II (AngII). Animal experiments were approved by Institutional Animal Care and Use Committee at the Research Institute at Nationwide Children’s Hospital.

Angiotensin-II infusion and DAPT treatment. Mini osmotic pumps (Model 2004; Alzet, Cupertino, CA) containing AngII (1000 ng/min/kg) or saline were implanted subcutaneously in the neck region of anesthetized mice following standard protocol.\(^1\) Briefly, mice were anesthetized in a closed chamber with 3% isoflurane in oxygen for 2 to 5 minutes until immobile. Each mouse was then removed, and taped on a heated (35-37°C) procedure board with 1.0-1.5% isoflurane administered via nosecone during minor surgery. Three independent experiments were performed to determine the effect of Notch1 haploinsufficiency on the formation of AAA. The effects of pharmacologic inhibition of Notch were tested on AAA formation in two independent experiments, one of which was performed simultaneously with studies on Notch1\(^{+/−}\);ApoE\(^{−/−}\) mice. Mice (n=10) were injected with a Notch inhibitor, DAPT (N-
N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (10 mg/kg dissolved in 10% ethanol, 90% corn oil), three times a week subcutaneously starting one week before the implantation of osmotic pump and continuing for an additional 28 days. DAPT experiment was repeated twice, first with a set of initial study with Apoe<sup>+/−</sup> and Notch<sup>+/−;ApoE<sup>−/−</sup></sup> mice (n=3 for each group) and second time with Apoe<sup>−/−</sup> mice alone (n=6 for Apoe<sup>−/−</sup> mice with DMSO and n=7 for Apoe<sup>−/−</sup> mice with DAPT). A portion of data from Apoe<sup>−/−</sup> mice (n=3) is shared in Figure 2 and Figure 7.

**Human infrarenal aortic tissue samples.** Full thickness aortic wall tissue specimens were collected from the infrarenal abdominal aorta from patients undergoing AAA repair (N = 3; Caucasian males of 67, 70 and 72 years of age) operations at the Harper University Hospital, Detroit, Michigan, USA. Non-aneurysmal infrarenal aortic samples (N = 3; Caucasian males of 53, 53 and 78 years of age) were collected at autopsies. Samples were incubated in phosphate-buffered formalin and embedded in paraffin for histological analyses. The collection of the human tissues was approved by the Institutional Review Board of Wayne State University, Detroit, Michigan, USA.

**Blood pressure measurements, in vivo imaging and quantification of aortic width.** The arterial blood pressure (BP) was measured in ApoE<sup>−/−</sup> and Notch<sup>+/−;ApoE<sup>−/−</sup></sup> mice by the standard noninvasive tail-cuff method (BP-2000 System, Visitech Systems, Apex, NC) by a single person. The mice were allowed to acclimate to the device for 5 days and then measurements were performed during the day after 48 hours (h) of AngII infusion. Twenty BP measurements were obtained and averaged for each mouse. For in vivo imaging of the abdominal aorta, two dimensional (B-mode) ultrasound images were obtained at weekly intervals after the
implantation of osmotic pumps using a VisualSonics Vevo2100 imaging system (Ontario, Canada) with a mechanical transducer (MS400). After 28 days, mice were deep anesthetized with ketamine/xylazine (60 and 3 mg/kg, respectively) the aortas were dissected, fixed in 10% formalin, and maximum aortic diameters measured.

**Histology and immunostaining.** The abdominal aortae from the mice were embedded in paraffin and serial sections (5µm) were obtained. Sections were stained with hematoxylin and eosin (HE), elastin (Sigma, St. Louis, Mo) and immunohistochemistry (IHC) with antibodies to NICD (1:200; Abcam, Cambridge, MA), mouse monocyte-macrophage marker (MOMA-2;1:100, Abcam, Cambridge, MA), IRF8 (1:400; Abcam, Cambridge, MA), CD3 (1:400; Abcam, Cambridge, MA), MCP-1 (1:200; Abcam, Cambridge, MA) and active caspase-3 (Cell Signaling, Danvers, MA, USA), as described. Specificity of these antibodies was determined using non-specific IgG against the source of host species. Briefly, serial sections were deparaffinized, and antigen retrieval and blocking was performed. For IHC, Vector ABC biotin kit and Vectastain DAB substrate (Vector Laboratories, Burlingame, CA) were employed for the development of reaction and tissues were counterstained with hematoxylin. MCP-1 positive areas were quantified with Image Pro plus software using average values of positive immunostaining per microscopic field (5 fields/slide, n=3 mice/genotype).

**Cell Isolation and flow cytometry.** Macrophages were isolated from the abdominal aorta and lymphocytes were isolated from the abdominal aorta, spleen and peripheral blood of ApoE^{-/-} and Notch1^{+/-};ApoE^{-/-} mice after 7 days of AngII infusion. Briefly, the aortas were removed from anesthetized mice (ketamine/xylazine; 60 and 3 mg/kg, respectively), minced into 3-to 4-mm pieces, and placed in 1-ml digestion solution containing 0.6 units/ml Liberase Blendzyme 3 (Roche) and 50µg/ml porcine pancreatic elastase (Sigma-Aldrich) in DMEM media. After
digestion, cells were washed in FACS buffer (0.5% BSA and 0.02% NaN₃ in DMEM) at 300 g for 5 min and subjected to FACS after staining with macrophage (CD11b and CD14). Spleens were aseptically removed and teased apart between two sterile slides. Cells were isolated and resuspended in 1 ml RBC lysis buffer (pH 7.2). After 1 min, cells were washed with RPMI 1640 medium twice and pelleted to remove cellular debris. Cells were washed and resuspended in FACS binding buffer and stained with lymphocyte (CD3, CD4, CD8) markers (eBiosciences, San Diego, CA). Blood was obtained from by cardiac puncture and erythrocytes lysed with RBC lysis solution added to the blood in a 3:1-ratio for 5 min at room temperature. Cells were centrifuged at 1,000 g for 3 min to remove the RBC lysis solution, and the leukocyte pellet was resuspended and washed in FACS binding buffer and stained with lymphocytes markers as explained. FACS studies were performed on BD (Becton Dickinson) LSRII flow cytometer with DiVa software, and data was analyzed with Flow Jo software (Ashland, OR).

*Bone marrow transplantation studies and quantification of leukocytes.* Using the standard validated protocol, we performed bone marrow transplantation studies on mice in which both *Apoe<sup>−/−</sup>* and *Notch1<sup>+/−</sup>;* Apoe<sup>−/−</sup> mice were irradiated and repopulated with bone-marrow derived cells that were either *Notch1<sup>+/−</sup>;* Apoe<sup>−/−</sup> or Apoe<sup>−/−</sup> mice respectively. Mice were maintained on antibiotic drinking water for two weeks before the irradiation. After optimizing the irradiation and validating the repopulation procedure, the Apoe<sup>−/−</sup> (Group I, n=12) and *Notch1<sup>+/−</sup>;* Apoe<sup>−/−</sup> (Group II, n=12) were irradiated and reconstituted with bone marrow-derived cells harvested from *Notch1<sup>+/−</sup>;* Apoe<sup>−/−</sup> and Apoe<sup>−/−</sup> mice, respectively. Recipient mice were irradiated with single dose of 1000 Rads from a Cesium source. Bone marrow-derived cells were obtained from the tibia and femur of donor mice (*Apoe<sup>−/−</sup>* and *Notch1<sup>+/−</sup>;* Apoe<sup>−/−</sup>) and were injected into the tail vein of 7-8 weeks old irradiated recipient mice (1x10<sup>7</sup> cells/ml). Two additional groups of mice
were also studied to serve as control groups: irradiated Apoe<sup>−/−</sup> mice were repopulated with bone marrow-derived cells harvested from Apoe<sup>−/−</sup> mice (Group III, n=8) and Apoe<sup>−/−</sup> mice without BMT (Group IV, n=6). Four to five weeks after the irradiation, osmotic mini-pumps containing AngII were implanted in these mice.

**Macrophage migration and proliferation studies.** Apoe<sup>−/−</sup> or Notch1<sup>+/−</sup>;Apoe<sup>−/−</sup> mice (n=12) were injected intraperitoneally with 1.5 mL of 4% (wt/vol) sterile thioglycollate broth. After 4 days, peritoneal macrophages were isolated counted by Coulter counter after diluting in 5% acetic acid to lyse RBCs. RAW cells, an immortal murine macrophage cell line (264.7) were cultured following manufacturer’s instructions (ATCC, Manassas, VA) Cultured macrophages were treated with Notch inhibitor (DAPT) or DMSO (control) for 6 days prior to migration and proliferation studies<sup>9</sup>. For the scratch assay, cells were grown as a monolayer and then scraped to create an injury. To assess proliferation, immunostaining was performed on these fixed cells with Ki67 (1:200, Abcam, Cambridge, MA) and quantified with Image Pro plus software. For chemotactic-induced migration, cells were grown in 8 µm polycarbonate filter transwell membrane plates (upper chamber) and lower chamber media was supplemented with 100 nM n-formyl-met-leu-phe, (FMLP; Sigma, St. Louis, MO). After 24 h, the cells in the lower chamber were fixed, stained with Giemsa, and counted manually. Macrophages were harvested and pooled from three mice for each experiment. Experiments were performed in quadruplicate.

**Bone marrow–derived macrophages.** Bone marrow-derived macrophages (BMDMs) were isolated from femurs and tibias of Apoe<sup>−/−</sup> and Apoe<sup>−/−</sup>Notch<sup>+/−</sup> mice. Bone marrow was cultured in RPMI1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, with the addition of 20% L929-cell–conditioned medium. After 5 days of culture, cells were plated and used for experiments the next day. BMDMs were stained using biotin-
conjugated F4/80 (BM8, eBioscience) and a secondary streptavidin-Alexa Fluor 488 (Invitrogen, Carlsbad, CA) to confirm the purity of the macrophages in the population. For intracellular cytokine staining, BMDMs were stimulated with 100 ng/ml LPS E. coli O111:B4 (Sigma-Aldrich, Saint Louis, MO) and IFN-g (20 ng/ml, BioVision), or IL-4 (20 ng/ml, Biosource) for 24 hours. After treatment with the protein secretion inhibitor brefeldin A (10 μg/mL, Sigma-Aldrich) during the final 5 hours of stimulation, the cells were stained for surface markers, fixed and permeabilized using the fixation/permeabilization buffer (BD Biosciences), according to the manufacturer’s instructions. IL10 was detected using a PerCP Cy5.5-conjugated antibody (JES5-16E3, eBioscience) and IL-12 (p40/p70) was detected using a APC-conjugated antibody (C15.6, BD Biosciences). Data were collected on an LSRII (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

RNA Isolation and qRT-PCR. The suprarenal aorta of approximately 5 mm was cut from Apoe<sup>-/-</sup> and Notch1<sup>+/+</sup>;Apoe<sup>-/-</sup> mice at day 7 or 28 of AngII infusion, frozen in RNAlater, and RNA extracted using TRIzol reagent (Ambion, Austin, TX) after homogenizing tissue with TissueLyser II (Qiagen, Valencia, CA). Naïve macrophages were collected from the peritoneal cavity of ApoE<sup>-/-</sup> or Notch1<sup>+/+</sup>;ApoE<sup>-/-</sup> mice (n=3; pooled from 5 mice each) by standard protocol<sup>6,10</sup>. Isolated macrophages were cultured in RPMI containing 10% FCS for 3 days and then treated with 100 ng/ml LPS or diluent for 3 h in RPMI containing 1% FCS. RAW cells were pre-treated with DAPT (10 µM) or DMSO for 6 days prior to incubation with LPS. Following treatment, RAW cells and macrophages were washed and RNA extracted using RNAeasy kit (Qiagen, Valencia, CA). cDNA was synthesized using SuperScript VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, CA) and subjected to qRT-PCR by SYBR Green RT-PCR kit (Applied Biosystems, Foster City CA) using Applied Biosystems 7500 Fast Real-Time PCR.
System (Foster City CA). qRT-PCR was performed in triplicate and fold change determined by standardization to 18S rRNA. Expression levels of Notch1, Hey1 and 18S were determined by real-time PCR analysis using the TaqMan gene expression assays and TaqMan universal PCR master mix (Applied Biosystems; assay numbers, Mm_00435245, Mm_004688865 Mm_99999901 respectively) in 20 μl reaction volumes. The expression levels were standardized against 18S using the ΔΔCt method, and mean expression of the Apoe<sup>−/−</sup> mice with AngII infusion was set to 1. The primer sequences are detailed in Supplemental Table 1.

Statistical Analysis. Statistical comparisons were performed using either Student’s t-test or one-way ANOVA followed by the Bonferroni’s Multiple Comparison Test. GraphPad PRISM V5.0 (San Diego, CA) was used for these comparisons and a p<0.05 was considered significant. For the statistical analysis of actual incidence, Fisher’s exact test was employed using the SAS software (Cary, NC). To analyze the BMT data for the two group comparisons at each time point, two sample t or Wilcoxon Sum Rank test was used depending on data distribution. Log rank test was used to test the null hypothesis that there is no difference between two groups in the probability of disease. For the primary outcome of the effect of AngII on the luminal expansion over time, a linear mixed effects model was used for the analysis of repeated measurement. Mean effects of AngII averaged across time and trend of change of measurement over time were compared between Group I and Group II. Differences were considered statistically significant when P≤0.05 for single comparisons or after adjustment for multiple comparisons.
REFERENCES


