



HHS Public Access

Author manuscript

Arterioscler Thromb Vasc Biol. Author manuscript; available in PMC 2015 June 15.

Published in final edited form as:

Arterioscler Thromb Vasc Biol. 2012 December ; 32(12): 3012–3023. doi:10.1161/ATVBAHA.112.254219.

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

Chetan P. Hans^{1,2,#}, Sara N. Koenig¹, Nianyuan Huang¹, Jeeyun Cheng¹, Susana Beceiro⁴, Anuradha Guggilam^{1,2}, Helena Kuivaniemi⁵, Santiago Partida-Sánchez^{2,4}, and Vidu Garg^{1,2,3,#}

¹The Center for Cardiovascular and Pulmonary Research and The Heart Center, Nationwide Children's Hospital, Columbus, Ohio 43205

²Department of Pediatrics, The Ohio State University, Columbus, Ohio 43205

³Department of Molecular Genetics, The Ohio State University, Columbus, Ohio 43205

⁴Center for Microbial Pathogenesis, Nationwide Children's Hospital, Columbus, Ohio 43205

⁵The Sigfried and Janet Weis Center for Research, Geisinger Clinic, Danville, PA

Abstract

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 (AngII)-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 AngII. Reconstitution of bone marrow-derived cells from *Notch1*^{+/-}; *Apoe*^{-/-} mice (donor) in lethally irradiated *Apoe*^{-/-} mice (recipient) decreased 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. Additionally, there was an overall reduction in the inflammatory burden in the aorta of the *Notch1*^{+/-}; *Apoe*^{-/-} mice as compared to the *Apoe*^{-/-} mice. Lastly, pharmacologic inhibition of Notch1 signaling also prevented AAA formation and progression in *Apoe*^{-/-} mice.

#Corresponding Authors: Chetan P. Hans, Heart Center and Center of Cardiovascular and Pulmonary Research, Nationwide Children's Hospital, 700 Children's Drive, Room WB4235, Columbus, Ohio 43205. Phone: 614-355-5744, Fax 614-355-5725, chetan.hans@nationwidechildrens.org; Vidu Garg, Heart Center and Center of Cardiovascular and Pulmonary Research, Nationwide Children's Hospital, 700 Children's Drive, Room WB4221, Columbus, Ohio 43205. Phone: 614-355-5740, Fax 614-355-5725, vidu.garg@nationwidechildrens.org.

Disclosures

Chetan P. Hans and Vidu Garg have applied for a patent related to this work.

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.

Keywords

aneurysm; Notch1 signaling; inflammation; macrophages

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% males over 65 years of age.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 non-surgical 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 histologic 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 IL-6 and MCP-1 in the aortic wall.^{8, 11, 12} Inflammatory macrophages cause activation of matrix metalloproteinases (MMPs) 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 four 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* suffer 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 AngII-induced mouse model of AAA. Although AngII-infusion studies have reported striking differences as compared to AAA pathophysiology in human patients, it resembles the human AAA in male propensity, localized dilation of aorta and histological features of the aortic injury.²² Here, we show that the Notch1 signaling pathway is activated in the aneurysmal aorta of the AngII-treated *Apoe*^{-/-} mice, and that haploinsufficiency or pharmacologic 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 Supplement.

Generation of *Notch1*^{+/-};*Apoe*^{-/-} Mice

Apoe^{-/-} and *Notch1*^{+/-};*Apoe*^{-/-} littermates were generated as described in the Supplemental Methods. 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). Non-aneurysmal 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, Michigan, USA.

Angiotensin-II Infusion and DAPT Treatment

Mini-osmotic pumps containing AngII (1000ng/min/kg) or saline were implanted subcutaneously in anesthetized male mice (8–10 weeks old) following standard protocol. A group of *Apoe*^{-/-} mice (n=10) were injected with a Notch inhibitor (DAPT).^{23, 24}

Transabdominal Ultrasound Imaging

For *in vivo* imaging of the abdominal aorta, two 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 (HE), elastin and immunohistochemistry (IHC) with antibodies to NICD, mouse monocyte-macrophage marker (Moma2), and monocyte chemotactic protein-1 (mcp1), as described.^{25, 26} Specificity of NICD, Moma2 and Mcp1 staining was determined using non-specific IgG against the source of host species.

Bone Marrow Transplantation Studies and Quantification of Leukocytes

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 and were injected into the tail vein of 7–8 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 (FACS) after staining with macrophage (Cd11b and Cd14) and lymphocyte markers (Cd3, Cd4, Cd8).

Macrophage Migration and Proliferation Studies

Mice were injected intraperitoneally with sterile thioglycollate 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 μ M polycarbonate filter transwell membrane plates (upper chamber) and lower chamber media was supplemented with 100 nM n-formyl-met-leu-phe, (FMLP)^{27, 28}. 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 LPS and Ifn- γ (20 ng/ml) for M1 polarization or Il-4 (20 ng/ml) for M2 polarization.

RNA Isolation and qRT-PCR

The suprarenal aorta of approximately 5 mm in length was frozen in RNAlater, and mRNA 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 RT-PCR (qRT-PCR).

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 and Log rank test was used depending on data distribution.

Results

Notch1 is Activated in Response to AngII in *Apoe*^{-/-} Mice

To determine if Notch1 signaling is altered in the formation and progression of AAA, we examined 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 to *Apoe*^{-/-} mice treated with saline at day 7 (Figure 1A–F) and day 28 (Figure 1G–L). 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 (IF) using the monocyte and macrophage marker (Moma2) (Supplemental Figure IA–F in the online-only data). At day 28, expression of NICD protein was extended to both the adventitial and medial layers of the aneurysmal abdominal aorta (Figure 1J–L and Supplemental Figure IG–L). Consistent with immunostaining, qRT-PCR 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 as compared to saline treated controls at day 7 and day 28

(Supplemental Figure II A–D). Consistent with our observations in mice, increased NICD signaling was also observed in the infrarenal abdominal aorta from patients undergoing AAA repair (Figure 1P–R) as compared to age-matched controls (Figure 1M–O) 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 if *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} Gross examination and transabdominal ultrasound imaging at 28 days of AngII demonstrated luminal expansion in suprarenal region of the abdominal aorta of *ApoE*^{-/-} mice (Figure 2E, K), while no such luminal expansion in this region was observed in the *Notch1*^{+/-};*ApoE*^{-/-} mice (Figure 2F, L). As expected, *Notch1*^{+/-} and wild type mice treated with AngII and all mice treated with saline did not develop luminal expansion (Figure 2 and Supplemental Figure III). A similar rate of mortality was observed between *ApoE*^{-/-} mice and *Notch1*^{+/-};*ApoE*^{-/-} mice in response to AngII infusion (Supplemental Figure IV). No significant blood pressure (BP) differences were observed between *ApoE*^{-/-} and *Notch1*^{+/-};*ApoE*^{-/-} mice with AngII infusion suggesting that the protective effects of *Notch1* haploinsufficiency were not related to BP (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 2 E, M). In comparison, the maximal aortic width of suprarenal aorta was significantly reduced in the *Notch1*^{+/-};*ApoE*^{-/-} mice treated with AngII as compared to *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 or saline-treated controls (Figure 2M). In total, 80% (8/10) of *ApoE*^{-/-} mice treated with AngII developed AAA, defined as a 50% increase in maximal aortic width, compared to 20% (2/10) of *Notch1*^{+/-};*ApoE*^{-/-} mice treated with AngII ($P < 0.05$; Figure 2N). Notably, except marginal adventitial thickening, no other characteristic features of AAA were observed in the *Notch1*^{+/-};*ApoE*^{-/-} mice (Figure 2Q, T, W).

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, S, V). 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, T, W). Histological examination of *Notch1*^{+/-} mice treated with AngII or saline-treated controls also did not show any evidence of AAA (Figure 2O, R, U and Supplemental Figure III). Active caspase-3 immunostaining was prominent in the aortic smooth muscle cell (aSMC) enriched medial layer of aorta in the *ApoE*^{-/-} mice infused with AngII as compared to *Notch1*^{+/-};*ApoE*^{-/-} mice (Supplemental Figure V, which was not surprising as apoptosis of aSMCs is a characteristic feature of AAA).²⁹ 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.^{2, 30, 31} We performed bone marrow transplantation (BMT) experiments to address the question if *Notch1* haploinsufficiency in bone marrow-derived cells alters aneurysmal development in *Apoe*^{-/-} mice (Figure 3; Supplemental Figure VI). After optimizing the irradiation and validating the repopulation procedure, the *Apoe*^{-/-} (Group I, n=12) and *Notch1*^{+/-};*Apoe*^{-/-} (Group II, n=12) 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 with *Notch1*^{+/-};*Apoe*^{-/-} bone marrow-derived cells (Group I) significantly decreased the progression of luminal expansion as compared to Group II (Figure 3; Supplemental Figure VI). *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 non-irradiated *Apoe*^{-/-} mice (Group IV; data not shown).³² *Notch1* haploinsufficiency did not affect total leukocyte count, 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 day 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 (Supplemental Figure VII). At both day 7 and day 28 of AngII infusion, the expression of Moma2 in the abdominal aorta of *Notch1*^{+/-};*Apoe*^{-/-} mice was decreased as compared to *Apoe*^{-/-} mice as determined by IHC (Figures 4A–H). 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–P, Supplemental Figure VIII). 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 MCP-1 positive inflammatory cells (arrowheads in Figure 4F, H, N, P).

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, 30, 33} To determine if *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 pro-inflammatory mediators by qRT-PCR 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 pro-inflammatory mediators (*Vegf*, *iNOS*, *Icam1*, *Vcam1*) was found in the abdominal aorta of *Notch1*^{+/-};*ApoE*^{-/-} mice as compared to *ApoE*^{-/-} mice (Figure 4Q).

To determine if 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 up-regulate expression of Notch1 and its downstream targets and mimic inflammatory response of AAA in a 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 about 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 FACS 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 as compared to *ApoE*^{-/-} mice after 7 days of AngII infusion (Figure 5A–C). *Notch1* haploinsufficiency did not alter the differentiation of Cd4⁺ or Cd8⁺ lymphocytes at the site of injury, although total number of Cd3⁺ T lymphocytes was significantly decreased in the suprarenal aorta of *Notch1*^{+/-};*ApoE*^{-/-} mice as compared to *ApoE*^{-/-} mice after 7 days of AngII infusion as determined by FACS analysis and immunostaining (Supplemental Figure IX). Interestingly, no significant difference was observed in the absolute number of Cd3⁺ T cells in the spleen (Supplemental Figure IX) or peripheral blood (data not shown) of these experimental mice.

Since 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 if 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, towards the injury site after 24h (Figure 5D–H). After 24h, decreased

proliferation was observed in *Notch1*^{+/-}; *ApoE*^{-/-} macrophages as compared to *ApoE*^{-/-} macrophages as determined by staining with proliferation marker (Ki67; Figure 5I–M). Increased Ki67 staining localized with macrophages was also observed in the abdominal aorta of *ApoE*^{-/-} mice, whereas no such co-expression was observed in the aorta of *Notch1*^{+/-}; *ApoE*^{-/-} mice (Supplemental Figure XA–F). In a transwell culture system, FMLP (a potent and specific chemotactic agent)-induced migration of primary macrophages was significantly reduced with *Notch1* haploinsufficiency (Supplemental Figure XG). To determine, if *Notch1* haploinsufficiency affects proliferation of primary macrophages in vivo, thioglycollate injection was used to elicit 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 (Supplemental Figure XH). Recent studies have suggested integral role for *Notch1* to regulate M1 polarization of naïve macrophages mediated by synthesis of interferon regulatory factor 8 (Irf8) protein.³⁷ As expected, expression of Irf8 was upregulated in the abdominal aorta of *ApoE*^{-/-} mice in response to AngII at day 7 whereas *Notch1* haploinsufficiency prevented upregulation of Irf8 (Supplemental Figure X I, J). To determine whether *Notch1* regulates polarization of macrophages, we evaluated markers for inflammatory M1-associated genes in response to LPS/Ifn- γ stimulation and M2-associated genes in response to Il4 stimulation in the bone-marrow derived macrophages (BMDM). Increased expression of *Il6*, *Il12*, *Tnf- α* and *iNOS* genes was observed in the BMDM of *ApoE*^{-/-} mice in response to LPS/Ifn- γ whereas no change was seen with *Notch1* haploinsufficiency (Figure 5N). More importantly, in response to Il4, the expression of arginase1 (*Arg1*) and macrophage galactose N-acetyl-galactosamine specific lectin 2 (*Mgl2*) was higher in BMDM from *Notch1*^{+/-}; *ApoE*^{-/-} mice as compared to *ApoE*^{-/-} mice suggesting that *Notch1* deficient macrophages are polarized towards the M2 fate (Figure 5O). The M1 polarization of macrophages in response to LPS/Ifn- γ was further confirmed by FACS analysis (Supplemental Figure X; M, N). M1 polarization of macrophages in *ApoE*^{-/-} mice was associated with increased Irf8 staining, which was reduced by *Notch1* haploinsufficiency (Supplemental Figure X; K, L). 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.

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

While 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 if protective effects of *Notch1* haploinsufficiency on AAA formation are not secondary to the embryonic functions of *Notch1* and to investigate therapeutic potential of *Notch1* inhibition, we examined whether pharmacologic inhibition of *Notch1* protects against the formation of AAA in adult *ApoE*^{-/-} mice infused with AngII. Mice were treated with DAPT (10 mg/kg) three times a week, starting one week prior to 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 if 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 (Supplemental Figure XI). Treatment with DAPT resulted in a significant reduction in the aortic diameter and incidence of AAA at both time intervals as compared to untreated *Apoe*^{-/-} mice and was similar to *Notch1*^{+/-}; *Apoe*^{-/-} mice (Figure 6A–D). Histologic analysis of the aorta of the *Apoe*^{-/-} mice treated with DAPT 7 days prior to AngII infusion (Figure 6F, I) demonstrated normal aortic wall architecture without infiltration of inflammatory cells as compared to untreated mice (Figure 6E, H). 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, J). Further, pharmacologic inhibition of Notch (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 down regulated the inflammatory response of these macrophages in response to LPS as compared to 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 was observed with DAPT treatment as compared to vehicle-treated *Apoe*^{-/-} mice (Supplemental Figure XII A–B). These studies demonstrate that the reduced incidence of AAA seen with genetic deficiency of *Notch1* are 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, pharmacologic 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 (Supplemental Figure XIII). 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.

While 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*

progression or stimulate the regression of established AAA, there is a critical need to develop pharmacologic interventions that can selectively target one or more features of AAA. A number of 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 a potential therapeutic agents for cancer and leukemia.^{49, 50} Based upon recent findings that Notch inhibition decreases macrophage infiltration⁴⁵ and directly regulates macrophage polarization³⁷, it is plausible that targeting Notch1 signaling may hold a promising target-based therapy for developing AAA. Beneficial effects of pharmacologic inhibition of γ -secretase have been reported to blunt the inflammatory response in a mouse model of atherosclerosis and reduce plaque formation⁵¹ and also to prevent vascular inflammation in an experimental model of giant cell arthritis.⁴⁸ Further investigations in other animal models of AAA (elastase and CaCl₂ infusion) are needed to determine the clinical utility of Notch1 inhibition as a pharmacologic therapy.^{11, 14, 52}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to 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 C.P.H. and funding from the Research Institute at Nationwide Children's Hospital and NIH/NHLBI to V.G.

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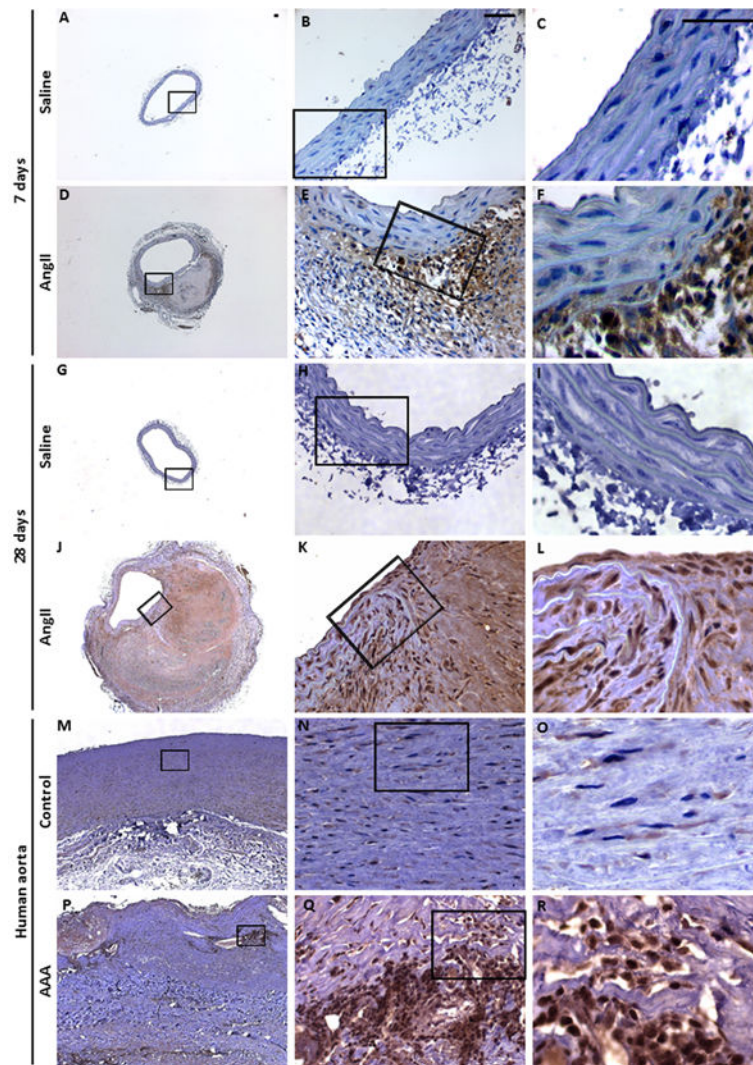


Figure 1. Increased expression of Notch1 intracellular domain in aneurysmal aorta of *Apoe*^{-/-} mice and human AAA

At day 7 of AngII infusion, transverse sections of the aneurysmal abdominal aorta from *Apoe*^{-/-} mice (n=5) demonstrate increased expression of Notch1 intracellular domain (NICD) by immunohistochemistry treated with AngII (D–F) as compared to saline treated controls (A–C). At day 28, NICD immunoreactivity extended to the medial layer (J–L) as compared to controls (G–I). Increased NICD signaling was also observed in the infrarenal abdominal aorta from patients with AAA (P–R) as compared to age-matched controls (M–O). Scale bar, 50 μ m.

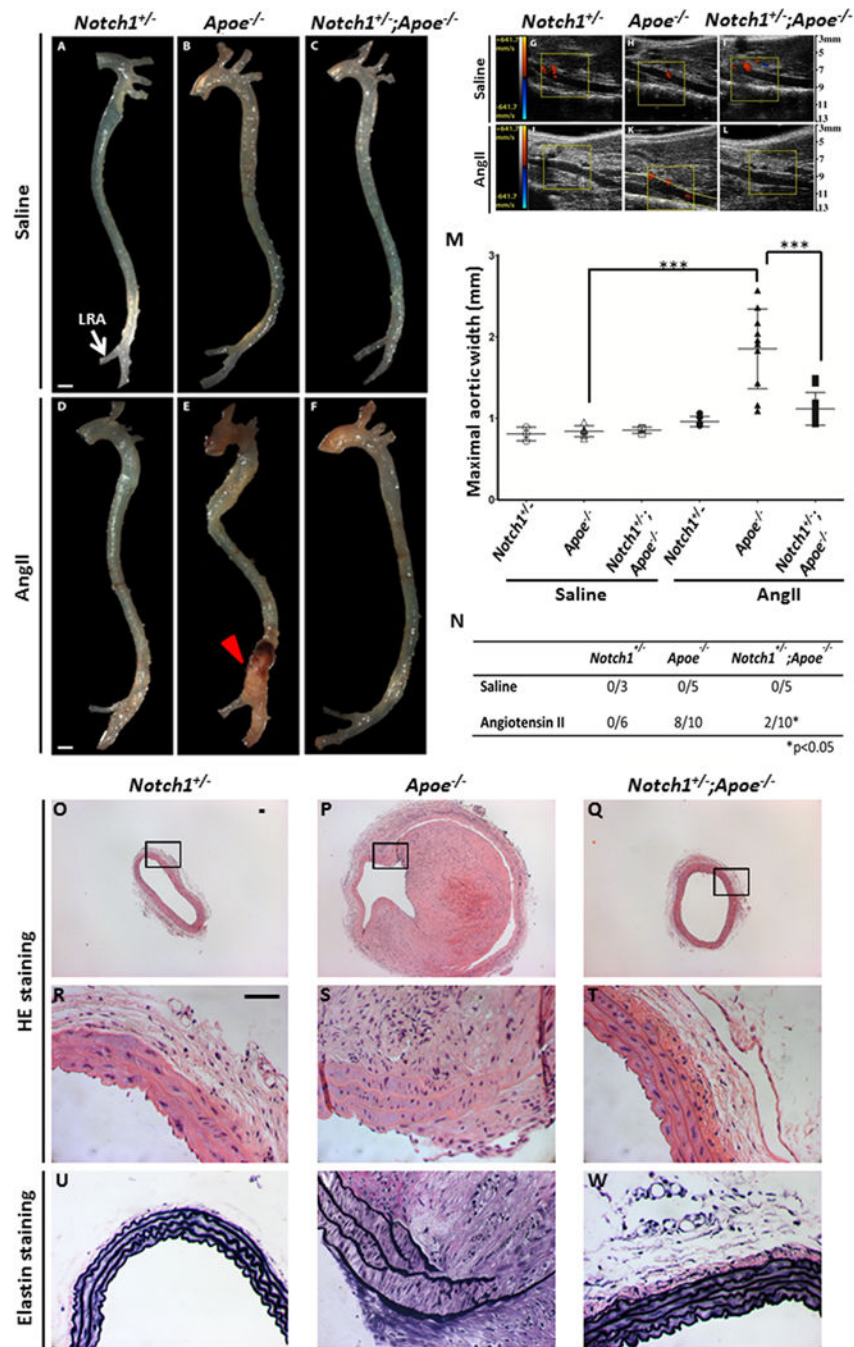


Figure 2. *Notch1* haploinsufficiency reduces the incidence of AAA in *ApoE*^{-/-} mice after 28 days of AngII infusion

Representative aortas from mutant mice treated with saline (A–C) and AngII (D–F) are shown. LRA, left renal artery. (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 (L) have decreased abdominal aortic luminal diameter as compared to *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 standard deviations are shown. Each individual animal is represented by a symbol. *** $P < 0.001$ when comparing *Apoe*^{-/-} with AngII versus *Apoe*^{-/-} with saline and *Apoe*^{-/-} with AngII versus *Notch1*^{+/-}; *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 versus *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 were found in the aorta of *Apoe*^{-/-} mice infused with AngII (P, S). No changes except mild adventitial thickening was observed in *Notch1*^{+/-} (O, R) and *Notch1*^{+/-}; *Apoe*^{-/-} mice (Q, 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). Scale bar, 1 mm (A–F) and 50 μm (O–W).

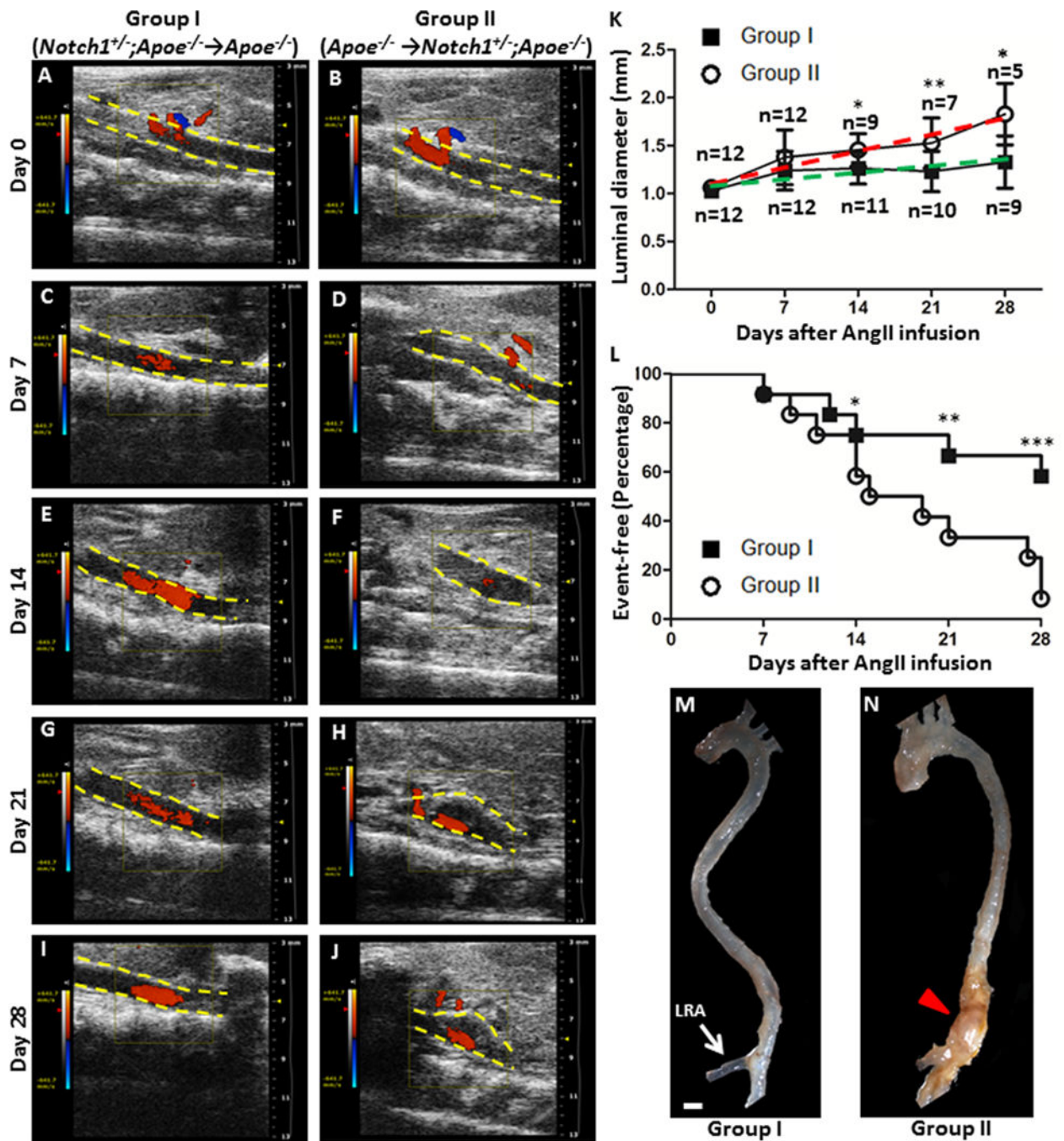


Figure 3. *Notch1* haploinsufficiency in bone marrow-derived cells decreased AAA incidence and mortality rate in *Apoe*^{-/-} mice

Bone marrow-derived cells from *Apoe*^{-/-} mice increased AAA disease in *Notch1*^{+/-}; *Apoe*^{-/-} (Group II; *Apoe*^{-/-} → *Notch1*^{+/-}; *Apoe*^{-/-}, n=12) mice as compared *Apoe*^{-/-} mice which 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, B), day 7 (C, D), day 14 (E, F), day 21 (G, H) and day 28 (I, J) demonstrating an increase in the suprarenal luminal diameter in Group II as compared to

Group I. (K) Significant increase in the rate of luminal expansion in Group II (red) as compared to Group I (green) after 2nd week of 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. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

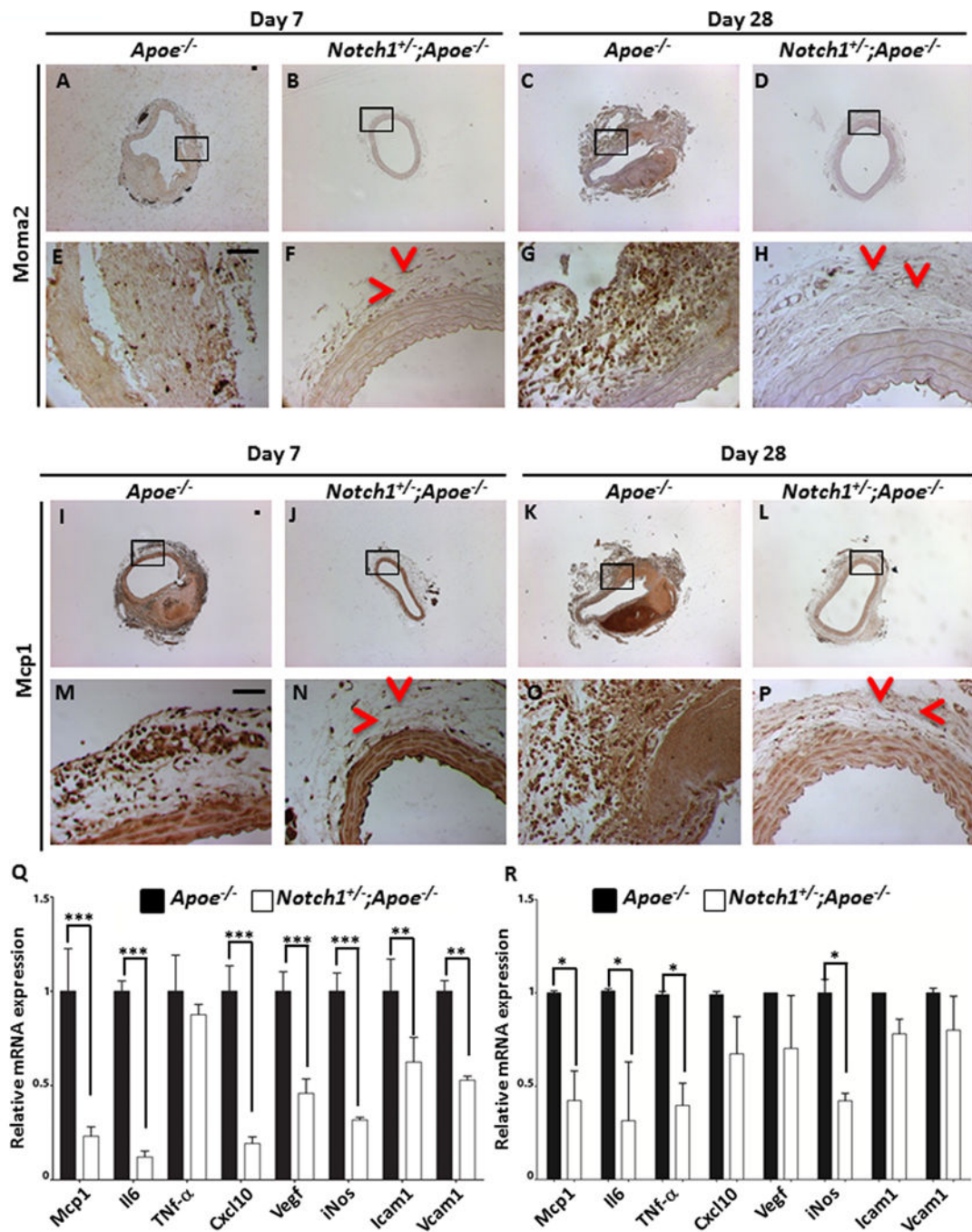


Figure 4. Minimal macrophage recruitment and Mcp1 expression in the aorta of *Notch1*^{+/-};*ApoE*^{-/-} mice after 7 and 28 days of AngII infusion

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) as compared to *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 as compared to *ApoE*^{-/-} mice (I, M and K, O). Moma2 and Mcp1 expression was specifically decreased in adventitial layer (F, H, N, P; arrowheads). (Q) qRT-PCR demonstrates reduced

mRNA levels of *Mcp1*, *Il6*, *Cxcl10*, *Vegf*, *iNOS*, *Icam1* and *Vcam1* in the abdominal aorta of *Notch1^{+/-};Apoe^{-/-}* mice as compared to *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 LPS-stimulated primary macrophages isolated from the peritoneal cavity of *Notch1^{+/-};Apoe^{-/-}* mice as compared to *Apoe^{-/-}* mice. Macrophages from 5 mice were pooled for each experiment; data shown represents 3 experiments performed in triplicate. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$. Means and standard deviations are shown. Scale bar, 50 μm .

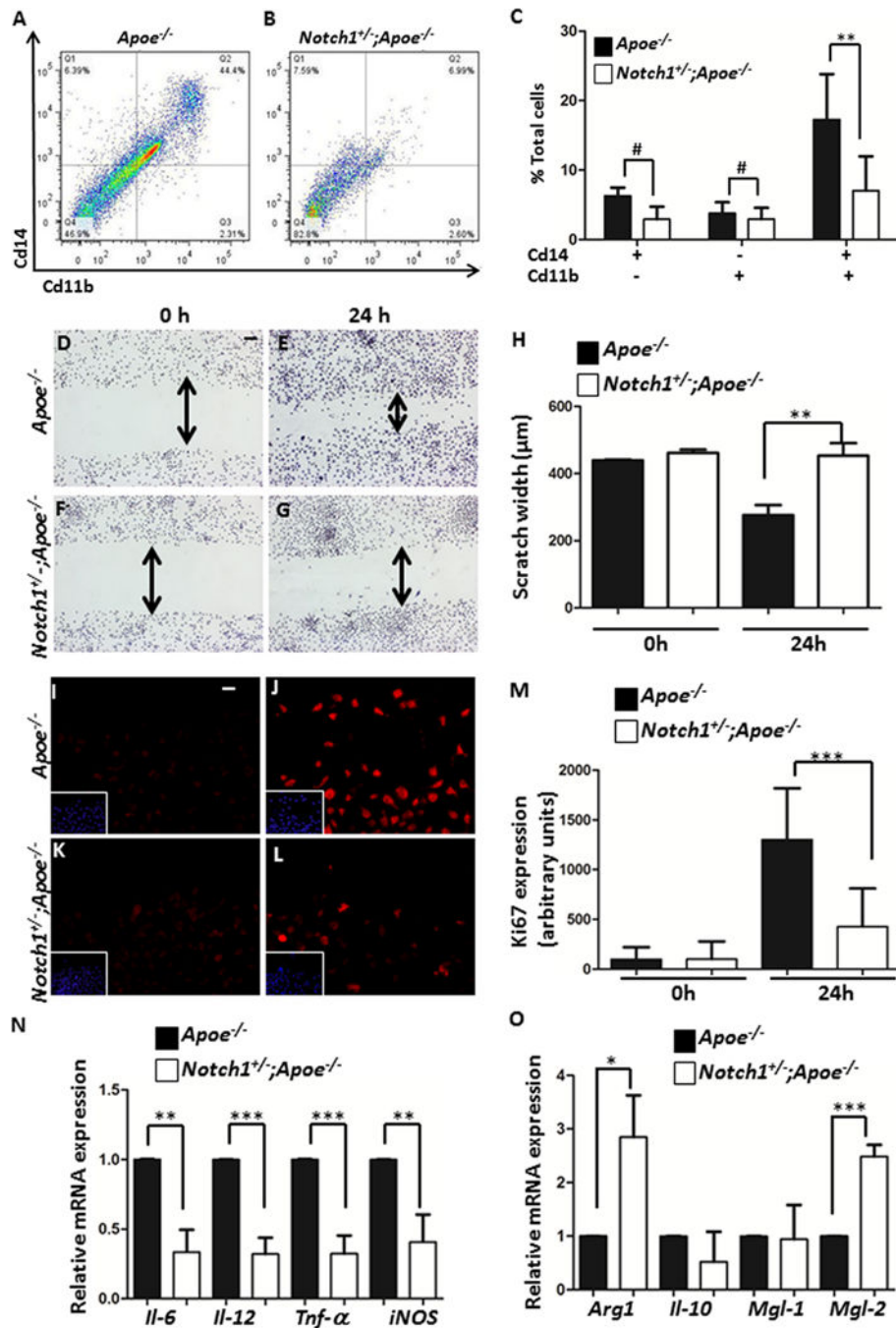


Figure 5. Notch1 haploinsufficiency decreases macrophage recruitment, migration and M1 polarization

(A–C) Flow cytometry demonstrates a decreased number of Cd11b⁺Cd14⁺ macrophages in the abdominal aorta of *Notch1*^{+/-};*Apoe*^{-/-} mice (B) as compared to *Apoe*^{-/-} mice (A). (C) Quantification of the FACS data (n=6 for each genotype). (D–G) Scratch assay showing decreased migration of *Notch1*^{+/-};*Apoe*^{-/-} macrophages to the site of injury after 24 h with quantification shown in (H). (I–L) Decreased proliferation of *Notch1*^{+/-};*Apoe*^{-/-} macrophages as detected by Ki-67 immunostaining. DAPI staining (blue) is shown in inserts

and quantification is in (M). (N) qRT-PCR demonstrates reduced expression of M1-polarization associated genes with LPS (100 ng/ml)/Ifn- γ (20 ng/ml) stimulation of BMDM of *Notch1*^{+/-};*ApoE*^{-/-} mice as compared to *ApoE*^{-/-} mice. (O) Notch1 haploinsufficiency increased M2-polarization associated genes in BMDM in response to Il4 (20 ng/ml) as compared to *ApoE*^{-/-} mice (n=3). Scale bar, 50 μ m. ***P<0.001; **P<0.01; *P<0.05.

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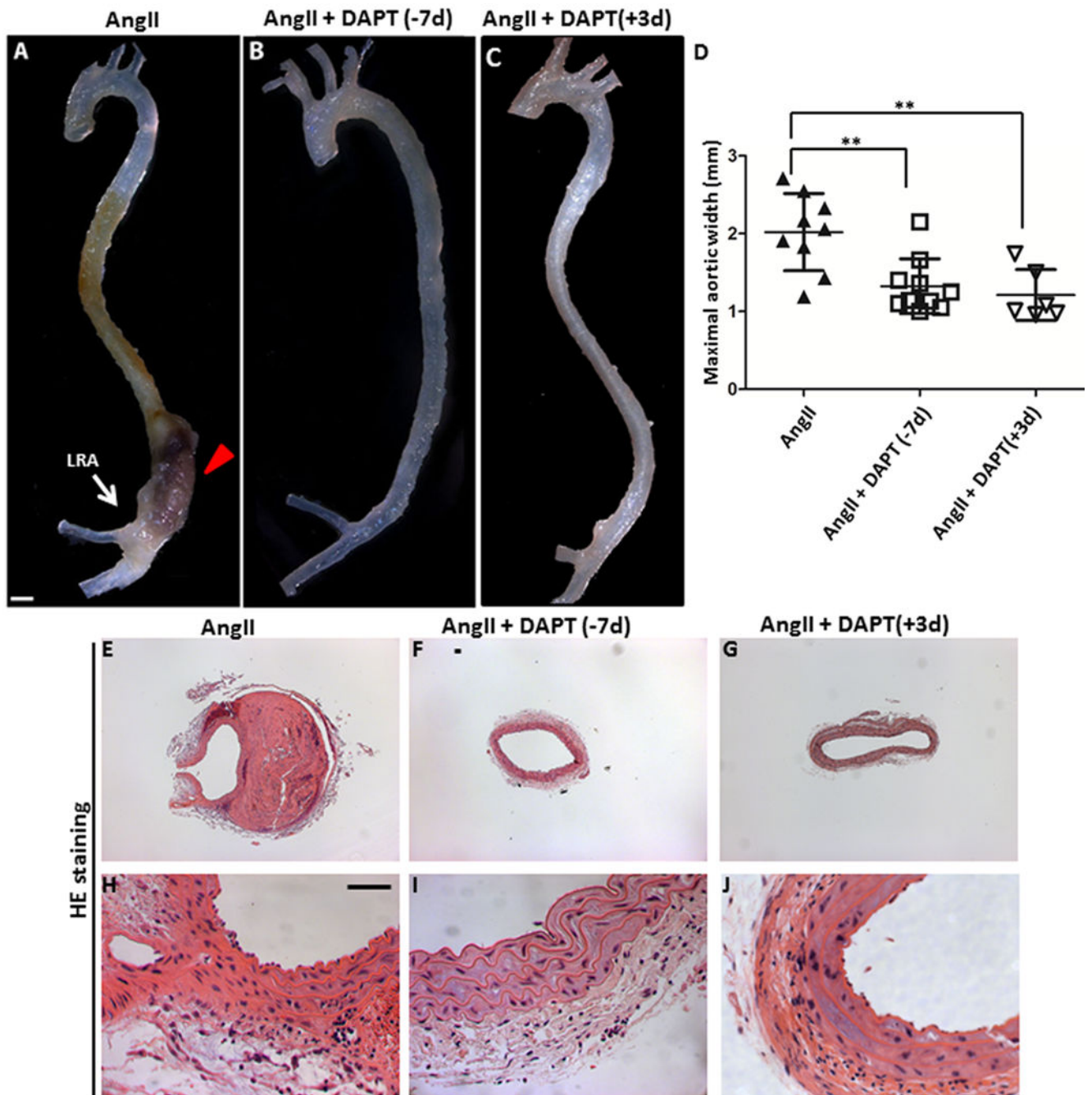


Figure 6. Pharmacologic inhibition of Notch signaling attenuates aneurysm development in an AngII-induced mouse model of AAA

Representative aortas from (A) *Apoe*^{-/-} (n=9), (B) *Apoe*^{-/-} mice treated with 10 mg/kg of DAPT 7 days prior to AngII infusion (n=10) and (C) *Apoe*^{-/-} mice treated with 10 mg/kg of DAPT 3 days after AngII infusion. All mice received AngII infusion for 28 days. Scale bar, 1mm. (D) Quantitative measurement of aortic width (mm) in these mice. Each symbol represents an individual animal. (E–J) Histologic sections show no evidence of aneurysm in

Apo^e^{-/-} mice that received DAPT (F, I, G, J). Scale bar, 50 μ m. Means and standard deviations are shown in D.

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