DAPT, a potent Notch inhibitor regresses actively growing abdominal aortic aneurysm via divergent pathways

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Abstract

Abdominal aortic aneurysm (AAA) is a localized pathological dilation of the aorta exceeding the normal diameter (~20 mm) by more than 50% of its original size (≥30 mm), accounting for approximately 150,000–200,000 deaths worldwide per year. We previously reported that Notch inhibition does not decrease the size of pre-established AAA at late stage of the disease. Here, we examined whether a potent pharmacologic inhibitor of Notch signaling (DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester)), regresses an actively growing AAA. In a mouse model of an aneurysm (Apoe<sup>−/−</sup> mice; n=44); DAPT (n=17) or vehicle (n=17) was randomly administered at day 14 of angiotensin II (AngII; 1 μg/min/kg), three times a week and mice were killed on day 42. Progressive increase in aortic stiffness and maximal intraluminal diameter (MILD) was observed in the AngII + vehicle group, which was significantly prevented by DAPT (P<0.01). The regression of aneurysm with DAPT was associated with reduced F4/80<sup>+</sup>Cd68<sup>+</sup> (cluster of differentiation 68) inflammatory macrophages. DAPT improved structural integrity of aorta by reducing collagen fibrils abnormality and restoring their diameter. Mechanistically, C–C chemokine receptor type 7 (Ccr7)<sup>+</sup>F4/80<sup>+</sup> dendritic cells (DCs), implicated in the regression of aneurysm, were increased in the aorta of DAPT-treated mice. In the macrophages stimulated with AngII or lipopolysaccharide (LPS), DAPT reversed the expression of pro-inflammatory genes Il6 and Il12 back to baseline within 6 h compared with vehicle (P<0.05). DAPT also significantly increased the expression of anti-inflammatory genes, including c-Myc, Egr2, and Arg1 at 12–24 h in the LPS-stimulated macrophages (P<0.05). Overall, these regressive effects of Notch signaling inhibitor emphasize its therapeutic implications to prevent the progression of active AAAs.

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Author Contribution

C.P.H. designed the study. N.S. and R.D. performed experiments and analyzed data. N.S. and C.P.H. wrote the manuscript. J.M.B., J.T. and G.A. contributed in the TEM-related experiment. All authors read, edited and approved the manuscript.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.
Introduction

Abdominal aortic aneurysm (AAA) is a chronic degenerative disease in aged population. AAA is defined as a local dilation of the abdominal aorta exceeding the normal diameter by 1.5-times [1]. Due to failures of the effective medical therapies and limited surgical treatments, the mortality rate due to aortic rupture is increasing in small and actively growing AAA [2,3]. These limitations emphasize the need to understand the key regulators at an active stage of the disease to halt the progression of AAA.

The progression of an active AAA is a complex remodeling process of the aortic wall involving both medial and adventitial layers [4–6]. Past two decades of research has identified that infiltration of immune cells and vascular apoptosis contributes to progression of AAA at early stages of the disease [7,8]. Macrophages are known to undergo phenotypic changes in response to various environmental cues, classified as either pro-inflammatory (M1) or anti-inflammatory (M2) based on gene signatures and secretion of inflammatory mediators [9–11]. Using RNA-Sequencing and subsequent validation using the Gene Ontology database and Kyoto Encyclopedia of Genes and Genomes enrichment analysis, we have shown that Notch1 haploinsufficiency favors the transition of naïve (Mφ) macrophages to the anti-inflammatory (M2) phenotype by novel pathways belonging to macrophage polarization, inflammatory response, and extracellular matrix (ECM) pathway [12]. We have previously shown that Notch inhibition prevents the development of early AAA in angiotensin II (AngII)-mouse model by macrophage-dependent mechanism(s) [13–15]. However, anti-inflammatory drugs failed to regress the actively growing aneurysm [16–18]. Thus, it is important to examine the novel pathways that play critical role in progression of the disease and may involve more than inflammation. In addition to potential anti-inflammatory properties, here we have examined the therapeutic efficacy of Notch inhibitor (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; DAPT, a γ-secretase inhibitor) on novel pathways including ECM degradation, apoptosis, and recruitment of non-macrophage cell types in AAA.

We utilized an experimental model of AngII-induced AAA and inhibited Notch signaling at day 14 of AngII infusion by administering DAPT for next 28 days. The animals with DAPT (or vehicle) treatment were sacrificed at day 42 and analyzed for various cellular and molecular biomarkers of AAA. Based upon our results, we concluded that inhibition of Notch signaling by DAPT attenuates the progression of active AAA via multifactorial pathways including the transition of M1 to M2 phenotype, decreased Il6 expression and recruitment of C–C chemokine receptor type 7 (CCR7)^F4/80^- cells, ultimately leading to decrease in intraluminal diameter and reduced ECM remodeling in the AAA tissues.

Materials and methods

Mice, aneurysm model, experimental groups, and DAPT treatment

The in vivo studies were performed at the Center for Cardiovascular Research, Nationwide Children’s Hospital (Columbus, OH) and at the Dalton Cardiovascular Research Center, University of Missouri (Columbia, MO). All the animal-related experiments were approved by the Animal Care and Use Committee (ACUC; #8799) at the University of Missouri and
the Institutional Animal Care and Use Committee (IACUC; #AR11–00031) of the Research Institute at Nationwide Children’s Hospital. All the animal experiments conformed to the NIH guidelines (Guide for the Care and Use of Laboratory Animals). Male mice were studied for the *in vivo* studies because of the low incidence of AngII-induced AAA in female mice as described [19].

Eight-week-old *Apoe*−/− mice (B6.129P2) were purchased from The Jackson Laboratory and inbred to generate sufficient numbers of mice (*n=*44) to perform the study in two cohorts (Supplementary Figure S1). Aneurysmal studies were performed on these mice by infusing AngII following published protocols [14,20]. Briefly, mice were anesthetized in a closed chamber with 1–2% isoflurane in oxygen for 2–5 min until immobile. Each mouse was then removed and taped on a heated (37 ± 2°C) procedure board with 1.0–1.5% isoflurane administered via nosecone during minor surgery. Mini osmotic pumps containing AngII (1 μg/min/kg; Sigma–Aldrich) were implanted subcutaneously as described [14]. At day 14, mice were randomly divided into two groups. A group of mice was administered DAPT (10 mg/kg dissolved in 10% ethanol, 90% corn oil; Sigma–Aldrich) three times a week for the next 28 days as described [13]. The other group received vehicle (10% ethanol + 90% corn oil; Sigma–Aldrich) alone. The *in-vivo* dose effects of Notch inhibitor in various mouse disease models including traumatic brain injury and aging has been well studied [21–23]. We chose 10 mg/kg dose as described in our previous studies and literature [14,21–23]. This concentration was effective in reducing the aneurysm formation in AngII-induced mouse model of AAA and the findings were consistent with the data obtained using *Notch1* haploinsufficient mice [14]. The timing and duration of DAPT was carefully chosen to inhibit Notch signaling during the active stage of AAA as described in various studies [8,14,20,24,25]. The mice were monitored daily to assess abnormal behavior or sudden death. All mice were killed at day 42 such that they received AngII for first 28 days and vehicle or DAPT for last 28 days. Mice were killed with an overdose of anesthetics ketamine (150–200 mg/kg) and xylazine (20 mg/kg) intraperitoneally.

**Transabdominal ultrasound imaging, pulse wave velocity, distensibility, and radial strain measurements**

For abdominal aorta measurements, 40 MHz high-frequency array transducer (Vevo MS550D) was used to collect B-mode, M-Mode and ECG-based kilohertz Visualization (EKV) mode images by the imaging system (Vevo 2100, VisualSonics) as described [14,26]. *In vivo* aortic stiffness was measured locally in the abdominal aorta by pulse wave velocity (PWV) technique by analyzing EKV data collected at various days of AngII infusion using Vevo Vasc software as described previously [26,27]. The measurements for maximal intraluminal diameter (MILD), PWV, distensibility, and radial strains were conducted following the two-man principles who were blinded to the study groups.

**AAA classification**

The aortae were dissected, cleaned off all the exogenous adipose tissue, and imaged as described [26]. AAA complexity was determined by Daugherty’s classification by measurement of the aortic diameter and histological features [8,20,28]. The incidence of
aneurysm was defined as dilation of the suprarenal aorta more than 50% of the MILD compared with original size [20].

Histology, immunohistochemistry, RNA isolation, and qRT-PCR

A group of the abdominal aortae from experimental mice were fixed overnight in 10% formalin, rinsed with PBS, and processed as described previously [15]. For the histological (Hematoxylin/Eosin, Trichrome, and Verhoeff-Van Gieson stain) and immunohistochemistry (IHC) analyses, the images were obtained in the areas near elastin fragmentation in the aorta to characterize the features of stability in these regions [14]. For IHC, the abdominal aortae were stained with antibodies for F4/80 (1:200; ab100790, Abcam), Il6 (1:200, AF-406-NA, R&D Systems), cleaved caspase 3 (1:200; 9661S, Cell Signaling), Notch1 intracellular domain (NICD, 1:400; ab8925, Abcam), and monocyte chemotactic protein 1 (Mcp1, 1:400; ab7202, Abcam) as described [15]. The intensity of the immunostaining was evaluated by obtaining four to six images from random areas of interest at 40× from each tissue (n=6–8) and quantified using the Fiji version of ImageJ following the software directions [29]. The specificity of all the antibodies was confirmed using appropriate IgG controls in place of primary antibodies or using only secondary antibody as described [26]. For RNA isolation, the suprarenal aorta of approximately 5 mm in length was frozen in RNAlater, homogenized with sonic dismembrator (Model CL-334, Thermo Fisher), and total RNA was extracted using fibrous RNAeasy kit (Qiagen) as described [26]. The primer sequences for genes are available on request. Throughout the study, we use gene symbols available from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/).

Transmission electron microscopy for mouse aortic tissue samples

Samples were prepared at the Electron Microscopy Core Facility, the Ohio State University and University of Missouri following a modified version of National Center for Microscopy and Imaging Research (NCMIR) methods for 3D EM [30]. Briefly, segments of suprarenal abdominal aorta from the experimental mice (n=4) were dissected, fixed in 4% glutaraldehyde for at least 24 h, and processed for routine transmission electron microscopy (TEM) as previously described[1]. Aortic pieces were osmium tetroxide post-fixed, dehydrated in a graded series of ethanol, and embedded in Spurr’s epoxy resin (Electron Microscopy Sciences). Thin sections were cut and imaged using a JOEL JEM-1400 TEM equipped with an Olympus Veleta digital camera. To determine the percent abnormal collagen, ImageJ (NIH) was used to trace total collagen area and total abnormal collagen area from TEM images of remodeled adventitia. This fraction determined for each image (n=6 per sample) was used to determine percent abnormal collagen in each sample. Fibril diameter measurements were determined using ImageJ (NIH) using longitudinal sections of collagen fibrils at magnifications ranging from 25 to 50 kx. At least n=100 fibrils were analyzed for each group by using multiple images and TEM sections as described previously [31].

Isolation of bone marrow-derived macrophages and DAPT treatment

Bone marrow-derived macrophages (BMDMs) were isolated from 6- to 8-week-old male Apoe−/− mice using a previously established protocol [13,14]. Total RNA was extracted after treatments using the RNeasy kit (Qiagen) following the manufacturer’s instructions. For
gene expression studies, BMDMs were serum-starved for 2 h followed by stimulation with low lipopolysaccharide (LPS; 10 ng/ml and IFN-γ; 10 ng/ml) or high LPS (100 ng/ml and IFN-γ; 20 ng/ml) for 3 h. A group of BMDMs were treated with AngII (2 μM) for 3 h. After completion of incubation time, a portion of cells was kept for gene expression studies of various pro- and anti-inflammatory macrophage markers. Remaining cells were washed twice with PBS and kept with RPMI 1640 (1% FBS + 1% penicillin and streptomycin) containing either DMSO or DAPT (10 μM) for various time intervals (6 or 12 h) as described in Supplementary Figure S10A.

Dendritic cells, aortic rings isolation, and co-culture assay
Bone marrow-derived dendritic cells (DCs) were isolated from the Apoe−/− mice as described [32]. Briefly, bone marrow-derived cells were cultured with recombinant mouse GM-CSF (rmGM-CSF; 20 ng/ml) for 6 days. The culture media with fresh GM-CSF were replenished at day 3. Purity of DCs were confirmed by surface expression of CD11c by flow cytometry. Some cells were treated with DMSO or DAPT for 24 h to analyze the expression of CCR7 by flow cytometry. For co-culture assay, aortic rings were harvested from abdominal aorta of Apoe−/− mice as described previously [26, 33] and were co-cultured with DCs. Briefly, aortic rings were stimulated with AngII for 48 h and co-cultured with DCs with or without DAPT for 48 h further. Finally, aortic rings were washed with PBS and used for histology by fixing in formalin or for gelatin zymography with lysis in RIPA buffer and protein extraction.

Gelatin zymography
The protein samples obtained from aortic rings were separated in 7.5% polyacrylamide gels containing SDS and 1 mg/ml gelatin as described [26, 33]. After electrophoresis, gels were washed (2 × 30 min) with zymogram renaturation buffer (Bio-Rad) while on gentle rotation. Gels were further kept in zymogram development buffer (Bio-Rad) for 30 min at room temperature with gentle shaking and further at 37°C for overnight. Finally, gels were stained with Coomassie Blue stain, destained until bands started to appear, and images were captured using gel documentation system (ChemiDoc XRS+, Bio-Rad).

Phagocytosis assay and ELISA
BMDMs were serum-starved and then treated with either low LPS or vehicle for 3 h. Thereafter, cells were washed and treated with either DAPT, IL6 antibody (1:1000; ab6672) or recombinant IL6 protein (25 ng/ml, R&D systems) for 24 h. Cells were washed and incubated with fluorescein-conjugated Zymosan A bioparticles (20 particles/cell, Invitrogen) for 1 h. Unengulfed bioparticles were removed by washing with cold PBS. After fixation, images were taken on a microscope (Olympus). The extent of phagocytosis was determined using Gen 5 software [34]. Mean area of engulfed fluorescent particles was measured for at least 100 cells in different fields and phagocytic index; mean area of engulfed particles/cell was calculated. For ELISA, macrophages were plated on 24-well plates, pre-treated with DAPT (10 μM) for 1 h, and then treated with low LPS for 12 h. The supernatant was collected to measure soluble Il6 protein using mouse IL6 ELISA kit (R&D systems) as an indicator of soluble Il6 receptor according to manufacturer’s instructions.
Human AAA tissue samples and double immunofluorescence

Full-thickness aortic wall tissue specimens were collected from the infrarenal abdominal aorta from patients undergoing AAA repair operations (n=6; white men aged 60–75 years) at the Harper University Hospital in Detroit, Michigan as described previously [13,35]. Non-aneurysmal infrarenal aortic samples (n=6; white men aged 58–78 years) were collected at autopsies and used as non-AAA controls. The research has been carried out in accordance with the World Medical Association Declaration of Helsinki. The collection of the human tissues was obtained after informed consent and approved by the Institutional Review Board of Wayne State University in Detroit, Michigan. For immunofluorescence, NICD (1:400, ab8925) or IL6 (AF-406NA) antibodies were used as described previously [14]. For mice double immunofluorescence (DIF), aortic tissue was analyzed for Ccr7 (1:400, ab1657) and F4/80 using DIF. Images were captured using LionHeart fx microscope [34]. Fluorescence intensity was quantified using Gen 5 software (BioTek). Negative control was performed by substituting primary antibody with nonspecific IgG from the same animal host as described [26].

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software, Inc., CA) and SPSS. All the data were assessed for normality and equal variance using Shapiro–Wilk test and Levene test, respectively. Unpaired two-tailed Student’s t test was used to determine statistical difference between two groups for normally distributed continuous variables. Data without normal distribution were analyzed by non-parametric Mann–Whitney test or Kruskal–Wallis test. For comparison of multiple groups, ANOVA followed by Tukey’s multiple comparison analysis or two-way ANOVA followed by Bonferroni post hoc tests were used. Log-rank test or Fisher’s exact test was used for AAA incidences and classification. Data are presented as median ± interquartile range for the PWV, MILD, and maximal external aortic diameter (MEAD). For rest of the quantitation, mean ± SEM was calculated. Pearson’s correlation coefficient was used to calculate correlation analyses. P<0.05 was considered statistically significant for all tests.

Results

Notch inhibition regresses active AAA

To examine the effects of Notch inhibition on the progression of actively growing AAA, male Apoe−/− mice were administered AngII (1 μg/min/kg) for 28 days by subcutaneous implantation of osmotic pumps. At day 14 of AngII, mice were randomly divided into two groups. One group was given pharmacologic inhibitor of Notch (DAPT) subcutaneously, three times a week for next 28 days, while the other group received vehicle alone (Supplementary Figure S1). All mice were sacrificed on day 42 such that these mice received AngII for first 28 days and vehicle or DAPT for last 28 days. MILD and aortic stiffness (as determined by PWV) were two primary indicators to predict the growth and stability of AAA. The incidence of aneurysm was defined as dilation of the suprarenal aorta more than 50% of the MILD compared with original size [20]. AngII significantly increased MILD (1.53 ± 0.28 vs. 0.94 ± 0.4 mm; P<0.001) and PWV (1.59 ± 0.27 vs. 1.18 ± 0.11 m/s; P<0.001) in the abdominal aorta at day 14 compared with baseline (Supplementary Figure
S2A,B). The active AAAs at day 14 of AngII infusion were characterized by dilated aortae with perpetual influx of inflammatory cells, visible fragments of elastin fibrils and apoptosis of vascular smooth muscle cells (vSMCs; Supplementary Figure S3). Evidently, after this stage, the AAAs undergo remodeling process with increased degradation of ECM [8].

Ultrasound imaging analysis at weekly interval showed a progressive increase in MILD (1.97 ± 0.22 mm; P<0.01) and PWV (1.90 ± 0.26 m/s; P<0.001) until day 42 in the AngII + vehicle-treated mice (Figure 1A–C). With DAPT treatment, significant reduction in the progression of MILD (1.51 ± 0.35 mm, P<0.05,) and PWV (1.52 ± 0.35 m/s; P<0.01) was observed at day 42 than AngII + vehicle (Figure 1A–C). Distensibility and radial strain were decreased at day 42 in the AngII + vehicle and AngII + DAPT groups compared with baseline; however, no significant difference was observed between these two groups (Figure 1D,E). No significant changes in PWV, distensibility, and radial strain were observed in the control mice treated with vehicle alone (data not shown). At day 42, MEAD of the suprarenal aorta was significantly higher in AngII + vehicle mice than controls (2.04 ± 0.53 vs. 0.90 ± 0.05 mm; P<0.001, Figure 1F,G). With Notch inhibition, ~25% reduction in the MEAD was observed (1.61 ± 0.44 mm, P<0.05; Figure 1F,G). In the AngII + vehicle group, 37.5% (6/16) mice developed type II AAA and 56.3% (9/16) mice developed type III AAA (Figure 1H). On the contrary, 68.8% (11/16) mice in the DAPT-treated mice depicted type II AAA (P=0.07) and 18.8% as type III AAA (3/16; P<0.01). One mouse in each subgroup died from acute aortic rupture (type IV) after 14 days as ascertained thorough post-mortem examination (Figure 1H). Consistent with our previous studies, significant increase in the mRNA expressions of Notch1, its upstream ligand, Jagged1, and its downstream target Hey1, Hey2, and HeyL was observed in the aortae of Apoe−/− mice in response to AngII than vehicle alone (Supplementary Figure S4A–H) [13,14]. The mRNA expression of Notch1 ligand Dll4 or Rbpj, the transcriptional effector of Notch signaling were not significantly altered by AngII infusion in the aortae. With DAPT administration, decreased expression of Notch1 and its downstream targets were observed (Supplementary Figure 4A–H). In addition, increased nuclear staining of NICD was observed in response to AngII as compared with vehicle only in the regions of elastin fragmentation, which was almost negligible in the DAPT-treated mice (Supplementary Figure S4I). DAPT did not affect the weight, survival, and lipid levels in these mice (data not shown). Taken together, pharmacologic inhibition of Notch signaling regressed the actively growing AAAs, decreased aortic stiffness and overall severity of disease.

As reported earlier [13], gastrointestinal toxicity, including goblet cell metaplasia and dilation of intestinal crypts/glands (yellow arrowheads) was observed in the treated mice by PAS staining (Supplementary Figure S5A). Interestingly, marginal proliferation of goblet cells (red arrowheads) and mucosal epithelial necrosis was also observed in the mice treated with AngII (Supplementary Figure S5A). No apparent toxic effects of DAPT were observed in the hepatic tissues of these experimental mice (Supplementary Figure S5B).

**Notch inhibition minimizes elastin and ECM degradation in AAA**

Progression of AAA is characterized by IL6-mediated degradation of ECM proteins including elastin and collagen [36]. Extensive dilation of the aortic lumen along with

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adventitial remodeling and fragmentation of the elastic lamellar architecture was observed in the medial aortic layer of AngII + vehicle group (Figure 2A–C). DAPT significantly decreased elastin fragmentation as determined by semi-quantitative analysis ($P<0.05$, Figure 2B,C). Among the panel of elastin cross-linking genes, DAPT significantly increased the expression of tropoelastin (Eln1, $P<0.01$), fibulin1 (Fbln, $P=0.052$), Fbln4 (P<0.01) and Fbln5 (P<0.001) as compared with AngII alone (Supplementary Figure S6A–L). Expression of lysyl oxidase (Lox) and its isoforms (2 and 3) were also significantly higher with DAPT treatment. Significantly higher expression of latent transforming growth factor β-binding proteins (LTBPs) 1 and 2 and Emilin 1 and 2 were observed with DAPT treatment. It is important to note that the basal expression of these set of genes was undetectable probably because of low expression, hence the comparison was determined between the AngII-treated groups only (Supplementary Figure S6A–L).

TEM was performed to examine collagen ultrastructure. At day 42, TEM showed a frequent occurrence of abnormal collagen fibrils with disrupted or unresolvable D-periods and small fibril diameter in AngII + vehicle treated mice as compared with controls (Figure 2D–F). However, DAPT mice had significantly fewer instances of these fibril abnormalities and largely had collagen fibrils with normal D-periodic banded structure and diameter (Figure 2D–F, $P<0.05$). Because of negligible collagen abnormality in the vehicle-only group, the comparison was analyzed between the AngII-treated groups only (Figure 2E), whereas collagen diameter was analyzed in the normal and abnormal regions of AngII-treated groups (Figure 2F). Abnormal collagen fibrils in the aortae of AngII + vehicle mice were found to have a significantly smaller diameter than either normal or abnormal fibrils in any other experimental group ($P<0.05$; Figure 2F). Together, these data suggest that the treatment with DAPT results in partial restoration of normal elastin and collagen fibrils, thereby resulting in aneurysm stability.

**Notch inhibition reduces inflammatory response in AAA**

To identify factors that influence the regression of actively growing AAA, cDNA from abdominal aortae of mice were subjected to qPCR for a panel of pro-inflammatory and anti-inflammatory cytokines. mRNA expression of Il6, iNos, Il12, and Cd38 decreased significantly in the AngII + DAPT mice at day 42 compared with AngII + vehicle (Figure 3A–D). Among the anti-inflammatory panel, expression of Arg1, Mgl2, and Egr2 increased significantly in the AngII + DAPT mice at day 42 compared with AngII + vehicle (Figure 3E–H). Notch inhibition led to significant reduction in immunostaining of F4/80 and Il6 positive cells in the adventitial region of aorta (Figure 3I–L). Inhibition of IL6 signaling has been shown to prevent AAA development by regulating matrix metalloproteinases (MMPs) [37]. Indeed, gene expression of Mmp9 significantly decreased in the abdominal aorta of AngII + DAPT mice at day 42 compared with AngII + vehicle (Supplementary Figure S7). In contrast, expression of Mmp3 and tissue inhibitor of metalloproteinases (Timps 1–3) significantly increased in the abdominal aorta of DAPT-treated mice compared with vehicle treatment. Consistent with reduced aortic accumulation of monocytes/macrophages, immunostaining for Mmp2 and Mmp9 were attenuated in the aortae from DAPT-treated mice as compared with vehicle treated mice (data not shown). Thus, reduction in pro-
inflammatory factors, MMPs expression, and increase in the anti-inflammatory factors may contribute to DAPT-mediated AAA suppression.

**Notch inhibition regresses AAA by decreasing the recruitment of pro-inflammatory macrophages**

The optimal conditions for the regression of AAA can be achieved by blocking the continuous influx of pro-inflammatory macrophages and preventing apoptotic cell death of vSMCs. Indeed, we observed a significant decrease in the expression of Mcp1 with DAPT treatment particularly in the regions of elastin fragmentation (red arrows in Figure 4A,D). Cluster of differentiation 68 (Cd68), a protein highly expressed by pro-inflammatory macrophages with phagocytic activities was increased with AngII in the areas of elastin fragmentation (red arrows in Figure 4B,E). With DAPT treatment, the expression of Cd68 was minimized in these regions. Active caspase-3, a marker of apoptotic cell death was increased in the vSMC-rich medial layer and in the adventitial layer of AngII + vehicle group. With DAPT treatment, marginal caspase-3 activity was observed in the adventitial layer, and the medial layer was completely devoid of active caspase-3 immunostaining (red arrows in Figure 4C,F). Overall, these data suggest that DAPT has protective effect on the progression of AAA via diverse pathways.

**Notch inhibition recruits CCR7⁺F4/80⁻ DCs to repair the vascular injury**

Next, we examined if decreased macrophage infiltration with DAPT is replaced by non-macrophage cell population to repair the aortic tissue from pre-existing damage. In the related vascular diseases such as atherosclerosis, the freshly recruited CCR7⁺ DCs have been shown to replace pro-inflammatory macrophages to initiate the regression; however, their roles in AAA are completely unknown [38,39]. DIF for CCR7 and F4/80 immunostaining revealed that in the AngII + vehicle group, the regions close to elastin fragmentation were populated with F4/80⁺ macrophages (green arrows in Figure 5B, lower panel). CCR7⁺ DCs were negligible in this region and did not correlate with F4/80⁺ macrophages in the AngII + vehicle group. In the DAPT treated mice, F4/80⁺ macrophages were minimal in the regions of elastin fragmentation (Figure 5C,D). In contrast, these areas were populated with CCR7⁺ DCs alongside the lumen. More importantly, these cells seem to be co-localized with the newly synthesized elastin (yellow arrows in Figure 5A–C) with no significant presence of F4/80⁺ macrophages. In the control aortae from saline-treated mice, the expression of F4/80⁺ macrophages or CCR7⁺ DCs was negligible (Figure 5A). To confirm the direct effects of Notch1 inhibition on CCR7⁺ DCs, bone marrow-derived cells were treated with recombinant mouse GM-CSF plus vehicle or DAPT for 6 days to differentiate into DCs. Analysis of these cells by flow cytometry (Figure 5E) indicated that DAPT increased the percentage of larger (FSC-higher) CCR7⁺ cells (29.4%) as compared with the vehicle (22.5%). More importantly, DAPT-treated DCs prevented elastin fragmentation (red arrows in Figure 5F) and decreased MMP2 activity in the medial layer of aortic rings in a co-cultured system (Figure 5G). Vehicle-treated DCs increased collagen contents in the adventitial region of aortic rings, which was reduced with DAPT treatment (Figure 5F). Overall, these results not only extend our previous studies, but also propose novel mechanism(s) by which CCR7⁺ DCs may induce the regression of actively growing AAA.
Notch inhibition reverses pro-inflammatory macrophages phenotype in IL6-dependent manner

Mechanistically, we conducted dose- and time-dependent effects of Notch inhibition on LPS (5, 25, 100 ng/ml) and IFN-γ (20 ng/ml) stimulated RAW cells, a mouse macrophage cell line in vitro. LPS increased the mRNA expression of Notch1 20–40 fold, peaked at 6–12 h in a dose-dependent manner, and reverted to baseline by 48 h (Supplementary Figure S8). Pretreatment of macrophages with DAPT reduced the expression of Notch1 in a dose-dependent manner at all the concentrations, but was more evident with high dose of LPS (100 ng/ml; Supplementary Figure S8). It is important to note that LPS mimics the pro-inflammatory functions of AngII signaling mediated through TLR4 signaling, which contributes to AAA formation and development [40, 41]. These data suggested that LPS activates Notch1 signaling at early time point and provided basis to target Notch inhibition for the in vitro reversal experiments.

Treatment of BMDMs with LPS/IFN-γ for 3 h significantly increased the expression of IL6, II12, and iNos (pro-inflammatory cytokines) (Supplementary Figure S9A,B). Gene expression of c-Myc, Egr2, and Arg1 (anti-inflammatory cytokines) were not affected by LPS at this time interval. Next, we examined if DAPT can reverse the up-regulated expression of these pro-inflammatory cytokines in the pre-activated macrophages. BMDMs were stimulated with either AngII or LPS/IFN-γ for 3 h to trigger inflammatory response and then replaced with either DMSO or DAPT (10 μM) for various time intervals (6, 12, or 24 h; Supplementary Figure S10A). DAPT significantly decreased the expression of II6, II12, and iNos at 6 and 12 h compared with vehicle at both low- and high-LPS stimulation (Figure 6A–C, Supplementary Figure S10E–G). However, DAPT decreased only II6 mRNA expression, and not the II12 and iNos in the AngII-stimulated BMDMs (Supplementary Figure S10B–D). DAPT treatment significantly increased the expression of M2-genes including c-Myc and Egr2 in macrophages albeit differentially at late time points (12 and 24 h, respectively; Figure 6D–F and data not shown). Expectedly, LPS increased the secretion of II6 at 12 h in the media and DAPT significantly decreased it (Figure 6G), suggesting an increase in the activation of soluble II6 receptor. Notch inhibition also significantly decreased the downstream targets of II6 signaling including Adam17, gp130, and Stat3 (Figure 6H–J). DAPT significantly decreased the LPS-mediated phagocytic activity of the fluorescently labeled zymosan beads in an IL6-dependent manner (54%; Figure 6K,L). A similar trend was observed with the BMDMs from Notch1+/− mice although the phagocytic index was lower (Supplementary Figure S11). Increased NICD and IL6 immunostaining was observed in human AAA aortic tissues localized in the inflammatory areas compared with abdominal aortic tissues from age-matched non-AAA controls (Figure 6M,N). Merged images demonstrated a significant increase in the double-positive cells for NICD and IL6 (yellow arrowheads, Figure 6M,N). Taken together, these data suggest that Notch inhibition has the potential to reverse the pro-inflammatory phenotype of macrophages by regulating IL6 signaling.
Discussion

To examine the translational applications of Notch inhibition on the progression of actively growing AAA, we used *in vivo* and *in vitro* reversal of inflammatory response by DAPT, a potent pharmacologic Notch inhibitor. We provide evidence that DAPT halts the progression of actively growing aneurysms by limiting the inflammatory response and ECM degradation in the aortic wall. These protective effects of Notch inhibition were translated in the overall reduction in the intraluminal diameter and aortic stiffness of the damaged vascular wall. In the *in vitro* cell culture system, Notch inhibition reduces the expression of *Il6* and *Il12* and up-regulates the expression of M2-related genes including *c-Myc* and *Egr2* in the aorta and in the pro-inflammatory macrophages. Mechanistically, Notch inhibition, replaces pro-inflammatory macrophages with CCR7$^+$ DCs to mitigate the inflammatory responses and reduce apoptotic cell death of vSMCs. Together, these divergent pathways potentially synergize to reverse the progression of actively growing AAAs in an experimental mouse model.

As reported in our previous studies, aortic stiffening is an early change generating aortic wall stress that triggers aneurysmal growth and remodeling [42]. It is important to point out that the administration of DAPT at late stage stabilizes pre-established AAA by decreasing aortic stiffness without decreasing the aortic diameter [33]. In contrast, administration of DAPT in the actively growing AAA seems to be clinically more relevant and suggests the novel pathways to attenuate disease progression. Our novel findings that Notch inhibitor regresses the growth of actively growing AAA and provides stability to the AAAs has potential therapeutic implications.

Studies have shown strong correlation of aortic stiffness with inflammatory response and ECM degradation in mouse models of AAA [42]. We recently demonstrated functional deformities in the elastin and collagen fibril architecture in human AAA and mouse models of AAA [43,31]. Abnormal collagen fibrils with compromised D-periodic banding were observed in the excised human tissue and in remodeled regions of AAA in AngII infused mice. These abnormal fibrils were characterized by statistically significant reduction in depths of D-periods and an increased curvature of collagen fibrils (manuscript accepted in *Acta Biomaterialia*, 2020). Since, elastin and collagen degradation and inflammatory response were minimal with DAPT, we speculate Notch inhibition creates considerably less pro-inflammatory environmental milieu for the aortic tissue to regain its contractile properties. In support of our studies, Yoshimura et al. reported that SP600125 could block AAA progression and stimulate aneurysm regression by systemic pharmacologic inhibition of c-Jun N-terminal protein kinase (JNK), an intracellular signaling switch that controls MMPs production [44].

The infiltrating pro-inflammatory macrophages and other myeloid cells exacerbate vascular injury through release of cytokines and chemotactic factors (IL6, IL12, and MCP1), leading to further recruitment of macrophages and apoptotic cell death of vSMCs. IL6 is a multifunctional pro-inflammatory cytokine up-regulated during the AAA disease progression [45–47]. Recent findings suggest that Notch pathway is involved in IL6 signaling [48,49]. Pluripotent roles of IL6 in macrophage functions including recruitment,
infiltration, and phagocytosis have been reported [50,51]. Trans-activation of IL6 and downstream mediators, such as STAT3 and ADAM17 are increased in human AAA [52,53]. Accordingly, we show that DAPT reduced the expression of Adam17. Since shedding of soluble IL6 receptor is mainly governed by ADAM17 [54,55], we speculate that Notch inhibition reduces overall inflammatory response by preventing IL6 trans-signaling. In fact, IL6/STAT3 signaling pathway is a critical mediator of macrophage recruitment and infiltration [50]. Hence, reduction in MCP1 and IL6 may be sufficient to change the environment milieu of the aortic tissue toward tissue repair and recruitment of anti-inflammatory DCs [38,39]. Decreased phagocytic activity of macrophages with Notch inhibitor or IL6 inhibition is also in accordance with the published reports and propose a cross-talk between these two pathways [56]. Pro-inflammatory phagocytes drive inflammatory pathways throughout disease progression, resulting in expanding AAA in later stages of disease by degradation of ECM. Further studies are required to address the specific interactions of IL6 signaling with Notch pathway using antibody neutralization or gene deletion strategies in the context of AAA pathogenesis.

Obviously, the sequence of events involved in the regression of AAA not only requires resolution of overt inflammation, but also requires restoration of the ECM proteins. LOX is essential to maintain the integrity and tensile strength of elastic and collagen fibers in vasculature [57]. Periadventitial treatment of aorta with JNK inhibitor and LOX gene modified smooth muscle progenitor cells has been shown to preserve elastic lamellar integrity and waviness suggesting its direct protective roles [58]. In our studies, increased vSMCs population and ECM biosynthesis with DAPT treatment was associated with increased expression of LOX and related ECM-crosslinking genes. In the light of literature showing Notch1 regulates the JNK signaling pathway [59,60], it will be interesting to examine if Notch1 signaling preserves elastin and collagen lamellar integrity in the aneurysmal tissue by inhibiting JNK pathways.

Among the novel mechanism of decreased macrophage contents in AAA undergoing regression, increased recruitment of CCR7\(^+\) DCs [38,61] has been proposed. The DCs are professional antigen-presenting cells that continuously scrutinize the microenvironment for antigens and local and systemic host responses against harmful agents [39,62,63]. In the context of atherosclerosis, mice with macrophages lacking lipoprotein receptor-related protein showed less pro-inflammatory macrophages in the plaque and increased CCR7-dependent egression of macrophages from the plaque [39]. The role of TGF\(\beta\) signaling in AAA pathogenesis has been shown to be pathogenic, protective or neutral, depending upon the stage of the disease, the environmental milieu of the vascular injury and cell-context dependency. TGF\(\beta\)2 also plays a critical role in promoting alternative activation of macrophages (M2-like polarization), which in turn show a pro-fibrotic phenotype by inducing collagen synthesis. In fact, we recently demonstrated that predominance of M2-like macrophages associated with IL12p40 deficiency increases Tgf\(\beta\)2-mediated proteolytic activities leading to AAA development [26]. We speculate that these differential roles of Tgf\(\beta\)2 may be cell-context dependent, which needs thorough investigations. It is also known that TGF\(\beta\) is required for the DCs to induce regulatory T cells, however, the complex interactions between DCs and the ECM in maintaining this balance to induce elastin synthesis during inflammation are completely unknown [64]. It will be interesting to
examine if the ECM biosynthesis and recruitment of CCR7+ DCs are directly regulated by Notch signaling or are associated with overall changes in the anti-inflammatory environmental milieu of the aortic tissue.

Notch1 signaling has also been implicated in age-related disorders including Alzheimer’s disease [65]. Notch signaling is involved in the nervous system, cardiovascular and endocrine functions, which can directly impact age-related diseases [66,67]. Thus, Notch inhibition might represent an innovative target for the age-related diseases. Novel therapeutic strategies are also proposed to inhibit Notch signaling in preventing the recurrence and cure of cancer [68]. Except for potential local side effects of DAPT on intestinal hyperplasia, no other side effects of DAPT on these mice were observed. The combined effects of Notch inhibitor with other anti-inflammatory drugs and steroids to enhance the efficiency has been suggested in the recent studies [69,70]. Further studies will highlight these potentially dose-related local side effects while maintaining therapeutic efficacy in experimental AAA [13,71]. In summary, our findings suggest that the regressive effects of DAPT on AAA progression could be mediated by multifactorial pathways including (i) restoration of aortic stiffness, (ii) collagen fibril maintenance, (iii) synthesis of elastin precursors in the ECM, (iv) inhibition of IL6 signaling, and (v) replacement of pro-inflammatory macrophages with DCs. Given the complexity of AAA progression, we propose that inhibition of Notch signaling may provide a novel therapeutic strategy to treat AAA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

| Abbreviation | Description                        |
|--------------|------------------------------------|
| AAA          | abdominal aortic aneurysm          |
| ACUC         | Animal care and use committee      |
| ADAM17       | ADAM metallopeptidase domain 17    |
| AngII        | angiotensin II                     |
| Apoe−/−      | apolipoprotein E-knockout          |
| BMDM         | bone marrow-derived macrophage     |
Ccr7/CCR7 C–C chemokine receptor type 7  
cDNA complementary DNA  
Cd68 cluster of differentiation 68  
DAPT N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyler ester  
DC dendritic cell  
ECM extracellular matrix  
EKV ECG-based kilohertz Visualization  
FSC Forward scatter  
IFN interferon  
IHC immunohistochemistry  
IL6 Interleukin-6  
JNK c-Jun N-terminal protein kinase  
Lox/LOX lysyl oxidase  
LPS lipopolysaccharide  
Mcp1/MCP1 monocyte chemotactic protein 1  
MEAD maximal external aortic diameter  
MILD maximal intraluminal diameter  
MMP matrix metalloproteinase  
NICD Notch1 intracellular domain  
PAS Periodic acid–Schiff  
PWV pulse wave velocity  
RIPA Radioimmunoprecipitation assay  
TEM transmission electron microscopy  
TLR4 Toll like receptor 4  
vSMC vascular smooth muscle cell

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Clinical perspectives

- Inhibition of Notch signaling reverses the progression of actively growing AAA by the improvement of aortic functions and partial restoration of normal elastin and collagen fibers.

- At the molecular level, Notch inhibition-induced protective effects on AAA progression are associated with the down-regulation of IL6 trans-signaling.

- Our study demonstrates for the first time that the regression of AAA with Notch inhibition is associated with a reduction in pro-inflammatory macrophages and recruitment of CCR7$^+$F4/80$^-$ DCs.

- These studies highlight a better understanding of the molecular mechanisms of AAA regression and propose Notch inhibition as a viable therapeutic strategy to limit the progression of AAA.
Figure 1. DAPT treatment regresses mid-stage AAA progression

(A) Representative transabdominal ultrasound images showing the MILD at weekly interval after AngII infusion. DAPT treatment was started at day 14. Dotted yellow lines outline the lumen. (B) Quantification of MILD as measured by ultrasound (n=16). (C–E) PWV (m/s), distensibility (1/Mpa) and radial strain (%) at various days of AngII and DAPT treatments as measured by Vevo Vasc analysis (n=8). (F) Representative images of suprarenal aorta in control, AngII + vehicle and AngII + DAPT showing MEAD. (G) Quantification of MEAD of suprarenal aorta (mm) (n=16). (H) Aneurysm severity (type I–IV) scored using a standard...
classification system. Type IV refers to a ruptured aneurysm. Student’s $t$ test followed by Bonferroni post hoc analysis was used for the individual time points in (B–E). Tukey’s multiple comparisons test was used for data analysis in (G) and Fisher’s exact test was used in (H). *$P<0.05$; ***$P<0.001$; ns, non-significant. Scale bar = 1 mm in (F).
Figure 2. DAPT restores the structural integrity of the active AAA
Representative histological images showing H&E (A) and elastin staining (B) in the experimental groups. (C) Quantification of elastin fragmentation (n=6). (D) Representative TEM images comparing collagen bundles/fibers and d-spacing of collagen molecules. Low magnification images show D-periodic banded collagen fibrils with straight contours, while abnormal collagen fibrils showed undulating or wavy contours. Average fibril diameters were compared for normal and abnormal fibrils for all samples as indicated. (E,F) Quantification of abnormal collagen fibrils and diameter. Scale bar = 1 mm in (A,B; upper panel), 50 μm in (B; lower panel). All scale bars are 200 nm in (D). Tukey’s multiple comparisons test was used for data analysis in (C,E,F). *P<0.05; **P<0.01; ***P<0.001.
Figure 3. Notch inhibition reduces pro-inflammatory cytokines and increases anti-inflammatory cytokines in the aorta

(A–D) mRNA expression of pro-inflammatory cytokines (Il6, Il12, iNos, and Cd38) in the aorta of experimental mice (n=3). (E–H) mRNA expression of anti-inflammatory cytokines (Arg1, Mgl2, cMyc, and Egr2) in the aorta of experimental mice (n=3). (I,J) Representative IHC images showing immunoreactivity of F4/80 and Il6 in the suprarenal aorta. (K,L) Quantification of F4/80 and Il6 immunostaining (n=6). Paired two-tailed Student’s t test was performed for (A–H) and ANOVA followed by Tukey’s multiple comparison analysis was
performed for (K,L). *$P<0.05$; **$P<0.01$; ***$P<0.001$; Scale bar = 50 μm. Abbreviation: lm, lumen.
Figure 4. Notch inhibition reduces macrophage infiltration and apoptotic cells death in the aorta (A–C) Representative IHC images showing the immunostaining of Mcp1 (A), CD68 (B), and active caspase-3 (C) in the aorta. (D–F) Quantification of immunostaining for Mcp1, CD68, and active caspase 3 respectively (n=4–6). (D–F) Tukey’s multiple comparisons test was used for data analysis in (D–F). *P<0.05; ***P<0.001; ns, non-significant. Scale bar = 50 μm in (A–C). Abbreviation: lm, lumen.
Figure 5. DAPT increases the expression of Ccr7⁺F4/80⁻ DCs in the aorta
(A–C) Representative DIF images showing F4/80 (red), Ccr7 (green), and DAPI (blue) immunostaining in the aorta of control, AngII + vehicle and AngII + DAPT respectively (4× and 40×). (D) Relative fluorescence intensity graphs of Ccr7 positive area in the aorta of experimental mice. (E) Representative flow cytometry images showing CCR7⁺ DCs with and without DAPT. (F) Representative trichrome staining in the aortic rings co-cultured with DCs in the presence or absence of DAPT. (G) Representative gelatin zymography image showing MMP activity in the aortic rings treated with AngII and co-cultured with DCs in the presence or absence of DAPT. ANOVA followed by Tukey’s multiple comparison analysis was performed for D. *P<0.05; **P<0.01; ***P<0.001; scale bar = 50 μm. Abbreviation: lm, lumen.
Figure 6. Notch inhibition decreases IL6 in macrophages

(A–F) Expression of pro-inflammatory genes (Il6, Il12, and iNos; A–C) and anti-inflammatory genes (Arg1, c-Myc, and Egr2; D–F) with vehicle or DAPT (10 μM) treatment at 6 h (A–C) and 12 h (D–F) of low LPS (10 ng/ml + IFN-γ 10 ng/ml) stimulation in Apoe −/− BMDMs. (G) Measurement of soluble IL6 in the supernatant of macrophages treated with low LPS for 12 h in presence of DAPT (10 μM) as measured by ELISA. (H–J) Gene expression of Adam17 (H), gp130 (I), and Stat3 (J) in macrophages treated with low LPS. Briefly, cells were treated with LPS for 3 h, washed and then further treated with DAPT for...
6 h for gene expression analysis. (K) Representative images showing phagocytosis of zymosan particles by BMDMs in various conditions. (L) Quantitative analysis of the phagocytosis. (M) NICD and IL6 expression and their co-localization in the aorta of healthy and AAA human patients as determined by immunofluorescence. (N) Quantification of relative fluorescence intensity of IL6 and NICD. *P<0.05, **P<0.01, ***P<0.001 in paired two-tailed Student’s t test.