Estrogen Deficiency Promotes Cerebral Aneurysm Rupture by Upregulation of Th17 Cells and Interleukin-17A Which Downregulates E-Cadherin

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Background—Estrogen deficiency is associated with the development of cerebral aneurysms; however, the mechanism remains unknown. We explored the pathway of cerebral aneurysm development by investigating the potential link between estrogen deficiency and inflammatory factors.

Methods and Results—First, we established the role of interleukin-17 (IL-17)A. We performed a cytokine screen demonstrating that IL-17A is significantly expressed in mouse and human aneurysms ($P=0.03$). Likewise, IL-17A inhibition was shown to prevent aneurysm formation by 42% ($P=0.02$) and rupture by 34% ($P<0.05$). Second, we found that estrogen deficiency upregulates T helper 17 cells and IL-17A and promotes aneurysm rupture. Estrogen-deficient mice had more ruptures than control mice (47% versus 7%; $P=0.04$). Estradiol supplementation or IL-17A inhibition decreased the number of ruptures in estrogen-deficient mice (estradiol 6% versus 37%; $P=0.04$; IL-17A inhibition 18% versus 47%; $P=0.018$). Third, we found that IL-17A-blockade protects against aneurysm formation and rupture by increased E-cadherin expression. IL-17-inhibited mice had increased E-cadherin expression ($P=0.003$). E-cadherin inhibition reversed the protective effect of IL-17A inhibition and increased the rate of aneurysm formation (65% versus 28%; $P=0.04$) and rupture (12% versus 0%; $P=0.22$). However, E-cadherin inhibition alone does not significantly increase aneurysm formation in normal mice or in estrogen-deficient mice. In cell migration assays, E-cadherin inhibition promoted macrophage infiltration across endothelial cells ($P<0.05$), which may be the mechanism for the estrogen deficiency/IL-17/E-cadherin aneurysm pathway.

Conclusions—Our data suggest that estrogen deficiency promotes cerebral aneurysm rupture by upregulating IL-17A, which downregulates E-cadherin, encouraging macrophage infiltration in the aneurysm vessel wall. (***J Am Heart Assoc***. 2018;7: e008863. DOI: 10.1161/JAHA.118.008863.)

Key Words: aneurysm • e-cadherin • estrogen • IL-17 • macrophage

C erebral aneurysm (CA) rupture is devastating, resulting in 50% mortality and 30% morbidity.[1] Estrogen deficiency has been linked to aneurysm formation and rupture. Approximately 70% to 75% of aneurysms occur in women,[2] with postmenopausal women having the highest risk for aneurysm rupture.[3,4] Several studies have investigated estrogen deficiency and CA[5–8]; however, the mechanism remains unknown. Understanding the mechanisms of aneurysms, particularly estrogen signaling, is needed to identify novel therapeutic targets.

Although the ultimate cause of CAs is not known, infiltration of inflammatory cells, such as monocytes, macrophages, neutrophils, and lymphocytes into the vessel wall, is known to be one of the key phases of CA formation and development. Infiltration of inflammatory cells is the main source of inflammatory cytokines, which play key roles in immune cell regulation, activation, and migration. These inflammatory cells also secrete collagenases and proteinases, which promote aneurysm formation and development. These inflammatory responses cause vascular phenotypic changes and contribute to aneurysm formation and development. Inflammation and macrophage infiltration are hallmarks of human CAs.[9–13] In this study, we identify a potential link

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Clinical Perspective

What Is New?

- We have established support for the link between estrogen deficiency and T helper 17 cells and interleukin-17 (IL-17) in cerebral aneurysms.
- Phenotypic changes in endothelial cells when exposed to hemodynamic conditions at bifurcation aneurysms were observed, and the expression of IL-17A receptor was noted.
- There is a link between IL-17 and E-cadherin, namely that IL-17 suppresses E-cadherin in cerebral aneurysm formation and rupture.

What Are the Clinical Implications?

- Although further study is needed to clarify the IL-17A and E-cadherin pathway in estrogen deficiency, our findings may provide potential novel therapy to prevent or halt aneurysm development, growth, or rupture in human patients.

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between estrogen deficiency and inflammatory factors involved in aneurysm formation.

Materials and Methods

The data, analytical methods, and study materials will not be made available to other researchers for purposes of reproducing the results or replicating the procedure.

Human Aneurysms

All human studies were performed according to a protocol approved by the University of Florida's Institutional Review Board. Patients participating in the study gave informed consent before undergoing craniotomy and aneurysm clipping surgery. Aneurysms and control superficial temporal arteries were harvested at surgery (n=5 each; Table S1). The samples were immediately fixed with 4% PFA.

Murine CA Model

All animal experimentation was performed in accordance with the University of Florida's Institutional Animal Care and Use Committee, and ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. The timeline for each model is shown in Figure S1.

CAs were induced in 8- to 12-week-old female C57BL/6 mice (Charles River Laboratories, Wilmington, MA) as previously described.[14] Briefly, the left common carotid artery and right renal artery were ligated in mice fed a hypertensive diet (8% NaCl diet with 0.12% beta-aminopropionitril; Harlan Laboratories, Indianapolis, IN). One week after arterial ligation, 10 μL of 1.0 U of porcine pancreatic elastase solution (Worthington Biochemical Corp, Lakewood, NJ) diluted in 1 mL of 0.1 mol/L of PBS (Invitrogen, Carlsbad, CA) was stereotactically injected into the right basal cistern at 1.2 mm rostral of bregma, 0.7 mm lateral of midline, and 5.3 mm deep to the surface of the brain. Angiotensin II (Bachem, Torrence, CA) was continually infused by a subcutaneous osmotic pump (Alzet, Cupertino, CA) at a dose of 1000 ng/kg/min in PBS to increase blood pressure (Figure S2). In this model, ≈70% to 80% of mice develop aneurysms with most occurring in the left circle of Willis (COW).[14] Mice that did not develop aneurysms were used for control. Three weeks postelastase, mice underwent cardiac perfusion with 4% PFA. COW with or without aneurysms were harvested and postfixed with 4% PFA for 24 hours before being transferred into 70% EtOH. Dehydrated samples were paraffin embedded and sectioned into 5-μm sections onto glass slides.

To study aneurysm rupture, a higher dose of elastase solution (10 μL, 5.0 U/mL) was administered (100% of mice develop aneurysms and ≈60% of the aneurysms rupture) and mice underwent PFA cardiac perfusion 5 weeks postelastase.[14]

Mice exhibiting neurological symptoms (inactivity, circling paresis, or ≥15% weight loss) before PFA cardiac perfusion (<3 weeks postelastase in aneurysm model, <5 weeks postelastase in rupture model) were immediately euthanized, and brains were inspected for evidence of hemorrhage or aneurysm rupture.

Murine Estrogen Deficiency Model

Estrogen deficiency was induced in C57BL6 female mice by ovariectomy (OVE) or by estrogen E2-receptor (E2R) blockade. Microsurgical OVE or control sham operation were performed concurrently with right renal artery ligation in our murine aneurysm model. For E2R blockade, pure estrogen receptor antagonist (ICI 182.780; Tocris Bioscience, Bristol, UK) or control 100 μL of DMSO/PBS (1:1) solution was given subcutaneously 48 hours pre-elastase (150 μg/animal) and every 48 hours (150 μg/animal) for 3 weeks postelastase.

Supplementation

Beta-estradiol (reconstituted in a PBS-ethanol solution [9:1]; Sigma-Aldrich, St. Louis, MO) or control vehicle (PBS-ethanol solution) was administered by subcutaneous Alzet Osmotic pump (0.25 μg/h per mouse) to OVE female mice starting day 0 of elastase. These experiments were not performed in E2R-blocked mice because we presumed that E2 supplementation would not overcome competitively inhibited E2Rs.

Cytokine Arrays

Three weeks after elastase, the COW of mice with and without aneurysms were harvested and total protein was extracted using a radioimmunoprecipitation assay buffer (Sigma-Aldrich). Cytokine arrays were performed using a kit suitable for mice (RayBiotech, Norcross, GA). Densitometry was performed on film developed using the cytokine array kit to identify differentially expressed cytokines in murine COW with aneurysms and control murine COW. Using the same kit and same densitometry methods, cytokine arrays were performed on the COW of mice treated with an anti-IL-17 (interleukin-17) antibody or isotype-matched immunoglobulin (Ig) control (rat IgG2A) to identify potential downstream mediators of IL-17.

Serum IL-17 and T Helper 17 Cells

Serum IL-17 concentrations were measured by enzyme-linked immunosorbent assay and circulating T helper 17 (Th17) cells were quantified by fluorescence-activated cell sorting from blood collected from submandibular vein at 7, 14, and 21 days after elastase in OVE, E2R-blocked, and control C57BL6 female mice. Serum was isolated from collected blood samples and kept in −20°C freezer until assayed by a quantikine mouse IL-17 enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN). Mononuclear cells were isolated using Ficoll-Paque (GE Healthcare, Little Chalfont, UK) and immunostained using mouse anti-CD4, and mouse anti-CD196 antibodies (eBiosciences, San Diego, CA).

IL-17 Blockade, E-Cadherin Blockade, and Combination

Separate studies were performed to test the effect of the following on aneurysm formation and/or aneurysm rupture: (1) IL-17 blockade; (2) combined blockade of Il-17 and E-cadherin; and (3) E-cadherin blockade. To study the effect on aneurysm formation, antagonist was given 48 hours pre-elastase and every 48 hours for 3 weeks postelastase. To study the effect on aneurysm rupture, antagonist was given 1 week postelastase every 48 hours for up to 5 weeks.

Mice designated to receive IL-17 blockade were given subcutaneous rat anti-mouse IL17 antibody (R&D Systems; MAB421) or isotype-matched Ig control (rat IgG2A [R&D Systems; MAB006]; 15 μg/animal) every 48 hours (15 μg/animal).

Mice designated to receive IL-17 and E-cadherin combined blockade were given rat anti-mouse IL-17 antibody and E-cadherin–blocking antibody (Sigma-Aldrich; DECMA-1) or rat anti-mouse IL-17 antibody and isotype-matched Ig control (rat IgG1 [R&D Systems; MAB005]) (15 μg/animal each) every 48 hours (15 μg/animal each).

Mice designated to receive E-cadherin blockade were given E-cadherin–blocking antibody (Sigma-Aldrich; DECMA-1) or isotype-matched Ig control (rat IgG1 [R&D Systems; MAB005]; 15 μg/animal each) every 48 hours (15 μg/animal each).

Sample Size in Murine Model

The following number of animals were used in each group for analysis: IL-17 neutralization experiments using the aneurysm model with IL-17 antibody (n=14) and IgG control (n=14); IL-17 neutralization experiments using the rupture model with IL-17 antibody (n=19) and IgG control (n=18); estrogen deficiency blood collection experiments with OVE (n=15), sham (n=15), E2R blocker (n=14), and vehicle control (n=16); estrogen deficiency experiments with OVE (n=15), sham (n=18), E2R blocker (n=18), and vehicle control (n=18); estrogen deficiency experiments with E2 supplementation (n=18) and vehicle (n=19); estrogen deficiency with IL17 neutralization experiments with OVE+IL17 antibody (n=18), OVE+IgG control (n=17), E2R blocker+IL17 antibody (n=18) and E2-receptor blocker+IgG control (n=19); and E-cadherin and IL17 inhibition experiments with E-cadherin anti-body+IL17 antibody (n=19) and E-cadherin antibody+IgG control (n=16). For all cytokine array experiments, 3 animals in each group were used.

Randomization and Blinding

All cages (cage card number, etc,) were recorded in Excel sheets by the individual who is not a surgeon, and mice were randomly selected by nonsurgeon from cages to receive treatment or control. Syringes were labeled with numbers to blind the administrator of the treatment. The treatment assignment for each numbered syringe was kept in a password-protected locked master data sheet.

Blood Pressure Monitoring

Mouse blood pressures were measured using the noninvasive CODA mouse and rat tail−cuff blood pressure monitoring system (Kent Scientific, Torrington, CT), according to manufacturer's instructions. Mice were anesthetized with isoflurane and blood pressure was recorded 2 days before renal and carotid artery ligation (baseline) and 1 week after beginning treatment or control (post-treatment). Each animal's blood pressure was measured 5 times at each time point to obtain an accurate mean value for data analysis.

In Vitro Parallel Plate Flow Chamber Model

We have previously described an in vitro parallel plate flow chamber model that mimics the flow dynamics of human

bifurcation saccular CAs or human straight arteries. This model was validated by computational flow dynamic studies and takes into account fluid velocity and wall shear stress gradients present in aneurysms or normal arteries.[15] Normal healthy human carotid artery endothelial cells (Cell Applications, Inc, San Diego, CA) were pooled from 3 donors and seeded on coverslips cut from tissue culture plastic dishes (Techno Plastic Products, Trasadingen, Switzerland) at a density of 20 000 cells/cm^2 from passages 2 to 5 and grown to confluence. Endothelial cells were exposed to pulsatile shear stress for >75 hours and ultimately exposed to shear stress at 10 dyne/cm^2 for 72 hours in bifurcation aneurysm or straight artery parallel-plate flow chambers at 5% CO_2 and 37°C.

Cell Migration Assay

A cell migration assay was performed utilizing human umbilical vein endothelial cells (C-003-5C; Invitrogen) and mouse J774 macrophages (ATCC, Manassas, VA). Human umbilical vein endothelial cells were cultured in a cell culture dish until 100% confluent and seeded onto the upper chamber of Transwell permeable supports (Corning Life Sciences, Corning, NY; 3422) at a concentration of 50 000 cells per well on a 24-well cell culture cluster dish (Corning Life Sciences; 3526). Human umbilical vein endothelial cells were cultured on the upper chamber of Transwell permeable supports for 24 hours, followed by serum starvation for another 24 hours. Macrophages were starved separately in cell culture dishes for 24 hours and then seeded on Transwell Permeable supports containing previously-starved human umbilical vein endothelial cells at a concentration of 20 000 cells per well. The bottom chambers were filled with 0.6 mL of serum-free medium with monocyte chemoattractant protein-1 (R&D Systems; 10 ng/mL). Media containing anti-E-cadherin–blocking antibody (n=5; EMD Millipore, Billerica, MA; 15 μg/mL) or control media (n=5) were added to the upper chamber of the Transwell permeable supports. Macrophages were left to migrate for 48 hours at 37°C 5% CO_2.

Permeable supports were stained using immunohistochemistry following the 48-hour incubation. Supports were rinsed in PBS and fixed using 4% PFA. Supports were placed in 0.6 mL of Proteinase K solution (IHC World, Ellicott City, MD; IW-1101) for 2 minutes. They were then rinsed in TBST and placed in 0.6 mL of 4% horse serum for 1 hour. Supports were then placed in 0.6 mL of 1:1000 anti-CD11b monoclonal antibody (BD Pharmingen, San Jose, CA) for 1 hour. They were then rinsed in TBST and placed in 0.6 mL of 1:500 donkey anti-rat IgG Alexa Fluor 594 secondary antibody (Invitrogen) for 1 hour. Supports were then rinsed in TBST, and the upper chambers of the membranes were swabbed using cotton-tip applicators to remove excess cells that failed to migrate.

Supports were then mounted in 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) on 30-mm glass-bottom dishes (Wilco Wells BV, Amsterdam, the Netherlands). Stereological counting rules were used for the cell counts. After the staining, 5 microscopic high-resolution images per support were obtained using a ×40 objective lens. Images were not overlapped with other fields, and all image files were blindly named. Cells were counted by 2 blinded observers using Image Pro software (Media Cybernetics, Rockville, MD).

Immunohistochemistry

Immunohistochemistry for murine samples was performed with the following antibodies with heat-induced antigen retrieval: anti-neutrophil (Abcam, Cambridge, MA; ab2557), anti-IL-17 (Abcam; ab79056), and anti-F4/80 (AbD Serotec, Oxford, UK; MCA497). Immunohistochemistry for human samples was performed with anti–IL-17 antibody (Abcam; ab79056). Primary antibodies were detected using species appropriate Alexa Fluor secondary antibodies. Sections were mounted in VectaShield with 4′,6-diamidino-2-phenylindole mounting medium (Vector Laboratories) before imaging. Positive controls and concentration-matched Ig controls were included with each immunoassay (data not shown). Human and murine samples were imaged with an Olympus IX71 inverted fluorescent scope (Olympus Inc, Center Valley, PA) fitted with a Retiga 2000R CCD Camera (QImaging, Surrey, BC, Canada). Image analysis was carried out by observers blinded to the clinical details of the human aneurysm samples or mouse treatments.

Immunohistochemistry was performed on human carotid artery endothelial cells from the flow chamber models. An antibody against IL17A receptor (Abcam) was used after acetone/methanol (50:50) fixation followed by Alexa Fluor 594–conjugated donkey anti-rabbit secondary antibody (Life Technologies, Carlsbad, CA) staining. Stained cells from the bifurcation aneurysm and straight artery chambers were imaged using an Olympus IX71 fluorescent microscope (Olympus). Relative fluorescence units of each image were analyzed by observers blinded to the flow chamber using Image-Pro software (Media Cybernetics).

Sample Size and Statistical Analysis

All sample-size calculations were performed on the non-normalized (raw) scale using the SDs of the response variables observed in pilot studies. The goal of each calculation was to select a sample size that would yield an 80% chance (power) of detecting a biologically relevant difference between the control and treatment groups at a 2-sided significance level of 0.05. After the calculation, we increased the sample size to 15 to 20 animals per group to ensure adequate power in the event that some mice died.

Experiments were performed using sample size (n), and, unless otherwise indicated, experiments were not replicated.

Data are given as the mean with 95% confidence intervals. Fisher's exact test was performed to test for an association between aneurysm formation and rupture in each treatment group. Because the sample size for the 2-group comparisons was small and possibly a non-normal distribution, a nonparametric Mann–Whitney U test or the exact version of the Wilcoxon rank-sum test was used to determine whether group differences existed. GraphPad Prism software (version 7; GraphPad Software Inc, La Jolla, CA) and SAS software (version 9.4; SAS Institute, Cary, NC) were used for the analysis.

Results

Role of IL-17A in CA Growth and Rupture

There is growing evidence that CAs develop and rupture because of an inflammatory process,[9,10] and macrophage infiltration in the artery wall is a hallmark of aneurysms.[11–13] We sought to identify important inflammatory mediators in aneurysm development. We performed a cytokine screen array on protein from the COW of mice that developed aneurysms and mice that did not develop aneurysms in our previously described murine aneurysm model.[14] In this model, ≈70% to 80% of mice develop aneurysms.[14] Mice that do not develop aneurysms were used as control. These control mice were not sham operated. They were subjected to the same conditions, arterial ligation and elastase, as the mice that developed aneurysms, and are therefore a perfectly matched control.

IL-17 was the cytokine with the greatest difference in expression between mice that developed aneurysms compared with mice that did not. IL-17 was significantly upregulated in mice that developed aneurysms ($P=0.05$; Figure 1A).

We cross-validated IL-17 expression in murine aneurysms with immunohistochemistry. There was significantly greater expression of IL-17A in the COW with aneurysms than in the COW without aneurysms (Figure 1B). We further performed immunohistochemistry on human aneurysms. There was significant IL-17A expression in human aneurysms compared with control human superficial temporal arteries (Figure S3). This was equally true for both ruptured and unruptured aneurysms.

We studied the effect of IL-17A neutralization on development of murine CAs and rupture. Mice inhibited with IL-17A antibody had significantly fewer aneurysms than control isotype-matched Ig-treated mice (5 of 14 mice versus 12 of 14 control Ig-treated mice; $P=0.02$; Figure 2A). There was no difference in blood pressure between groups of mice: IL-17–blocked mice that developed aneurysms (baseline, 94.53±5.77; post-treatment, 136.29±9.14); IL-17–blocked mice that did not develop aneurysms (baseline, 95.01±2.65; post-treatment, 123.84±10.25; ($P=$NS [not significant]); Ig control mice that developed aneurysms (baseline, 106 ±1.61; post-treatment, 133.41±2.92); and Ig control mice that did not develop aneurysms (baseline, 90.12±4.19; post-treatment, 123.413±2.92; $P=$NS).

We have previously shown that altering the dose of elastase and waiting 1 week to allow aneurysms to develop before a treatment intervention enables us to study the effect of a treatment on preventing rupture of a developed aneurysm.[14] Treatment after aneurysm formation is a more clinically relevant model than pretreatment models. We studied the therapeutic effect of post-aneurysm IL-17 neutralization. IL-17A neutralization 1 week after aneurysms have formed prevents rupture (5 of 19 mice versus 11 of 18 control Ig-treated mice; $P<0.05$; Figure 2B). There was no difference in the blood pressure between groups of mice: IL-17-blocked mice with rupture (baseline, 96.22±3.83; post-treatment, 118.32±6.82); IL-17-blocked mice without rupture (baseline, 94.98±3.36; post-treatment, 117.69±5.62; $P=$NS); Ig control mice with rupture (baseline, 89.6±2.62; post-treatment, 116.2±17.42); and Ig control mice without rupture (baseline, 98.37±3.47; post-treatment, 120.5±3.06; $P=$NS).

Aneurysms form at arterial bifurcation sites. We demonstrated that IL-17A acts specifically on endothelial cells at bifurcation sites. We performed immunohistochemistry on human carotid artery endothelial cells in our in vitro parallel-plate flow chamber model, which mimics hemodynamic stress at bifurcations or at straight arteries.[15] We have previously validated this model by computational flow dynamic studies, which take into account fluid velocity and wall shear stress gradients present in aneurysms or normal arteries.[15] Endothelial cells at bifurcations significantly express IL-17A receptor compared with endothelial cells in straight segments by relative fluorescence units (bifurcation, 55 992.5±5427.9; straight, 26 444.9±3156.0; $P<0.03$; Figure 3A).

Estrogen Deficiency Upregulates IL-17A and Promotes CA Rupture

Next, we investigated the link between estrogen deficiency and the IL-17A aneurysm inflammatory pathway. Estrogen-deficient female mice, either ovariectomized or E2R-blocked, show higher levels of serum IL-17 ($P=0.04$) and circulating Th17 (CD4 and CD196 positive) cells compared with sham-operated or vehicle-treated control mice, respectively ($P=0.04$; Figure 4A). Th17 cells were only observed within 1 week of elastase, likely because they are an early player in immune cell infiltration during aneurysm formation.

Estrogen-deficient female mice, either ovariectomized or E2R blocked, did not have more aneurysms than control sham-operated or vehicle-treated mice: OVE 87% (13 of 15) versus sham 72% (10 of 15), $P=$NS, and E2R-blocked 100% (18 of 18) versus vehicle 83% (15 of 18), $P=$NS.
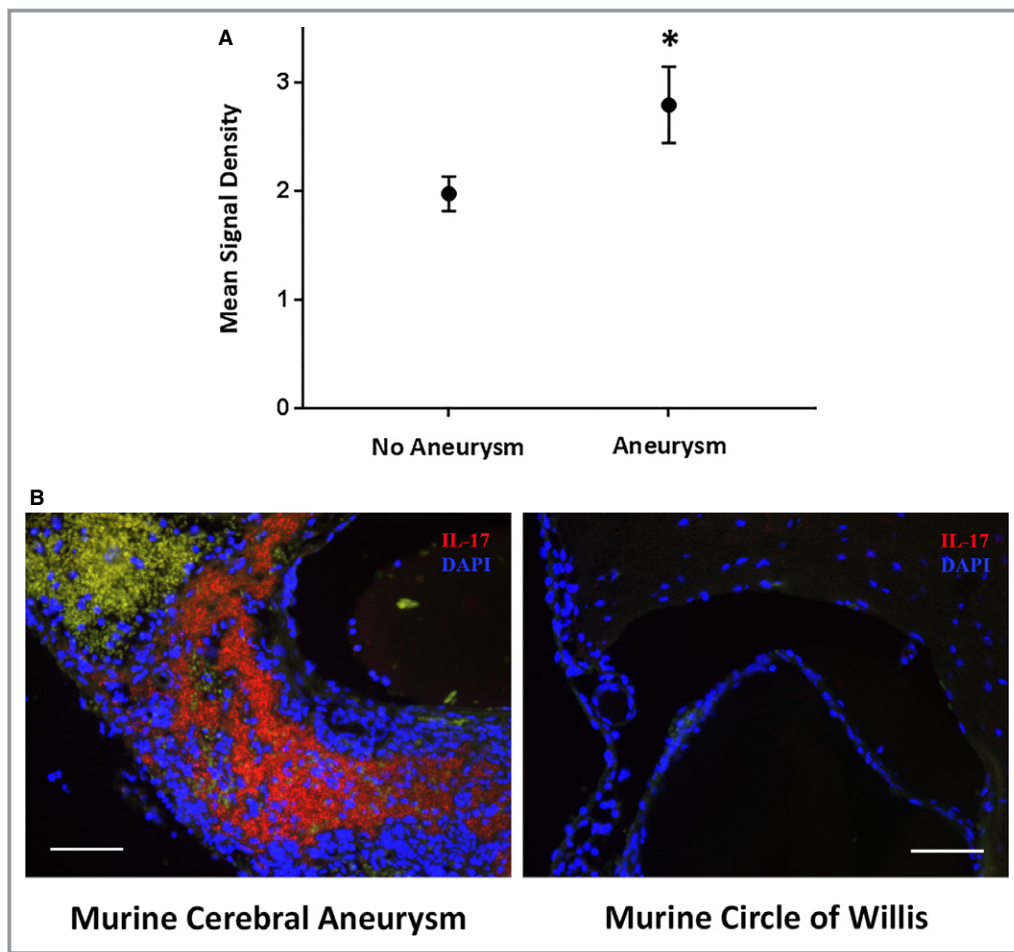
Figure 1. IL-17A expression in murine cerebral aneurysms. A, IL-17A expression by antibody-pair–based assay (cytokine array) is significantly higher in murine cerebral aneurysms (CAs; n=3) compared with murine circle of Willis (COW; n=3; *P=0.05) in a murine cerebral aneurysm model. In this model, ≈70% to 80% of mice develop CAs, so the CAs from mice that developed CAs were compared with the COW of mice that did not develop CAs subjected to the same model conditions. B, Murine CA (representative section displayed left panel) and murine COW (representative section displayed right panel) were immunostained for anti-IL17 (red) and DAPI (blue) and demonstrate robust expression of IL-17A in CAs, but not in COW, from mice subjected to the same model conditions. Green represents autofluorescence. Scale bar=50 μm. DAPI indicates 4′,6-diamidino-2-phenylindole; IL-17, interleukin-17.

However, estrogen-deficient female mice have significantly more aneurysm ruptures. Ovariectomized and E2R-blocked female mice had significantly more aneurysm ruptures than control sham-operated or vehicle-treated control mice: OVE 47% (7 of 15) versus sham 7% (1 of 15), P=0.04, and E2R-blocked 44% (8 of 18) versus vehicle 0% (0 of 18), P=0.003 (Figure 4B).

The higher risk of rupture in the estrogen-deficient ovariectomized mice is reversed by E2 supplementation: E2 supplementation 6% (1 of 18) versus control vehicle 37% (7 of 19), P=0.04 (Figure 4C). E2 supplementation was not performed in E2R-blocked mice because we presumed E2Rs would still be blocked despite supplemental estrogen.

We studied the therapeutic effect of post-aneurysm IL-17 neutralization to prevent rupture in ovariectomized and E2R-blocked estrogen-deficient female mice. IL-17 neutralization 1 week after aneurysms have formed prevents rupture in ovariectomized mice at a percentage of 11% (2 of 18 mice) versus 35% (6 of 17 control-treated mice), P=0.018, and in E2R-blocked mice at a percentage of 0% (0 of 18 mice) versus 47% (9 of 19 control-treated mice), P=0.002 (Figure 4D).

IL-17A Downregulates E-Cadherin and E-Cadherin Suppression Produces Aneurysms and Promotes Rupture

Next, we investigated inflammatory mediators downstream from IL-17. We performed cytokine screen array experiments with our murine aneurysm model on protein from the COW of IL-17A–blocked mice compared with control (isotype-matched
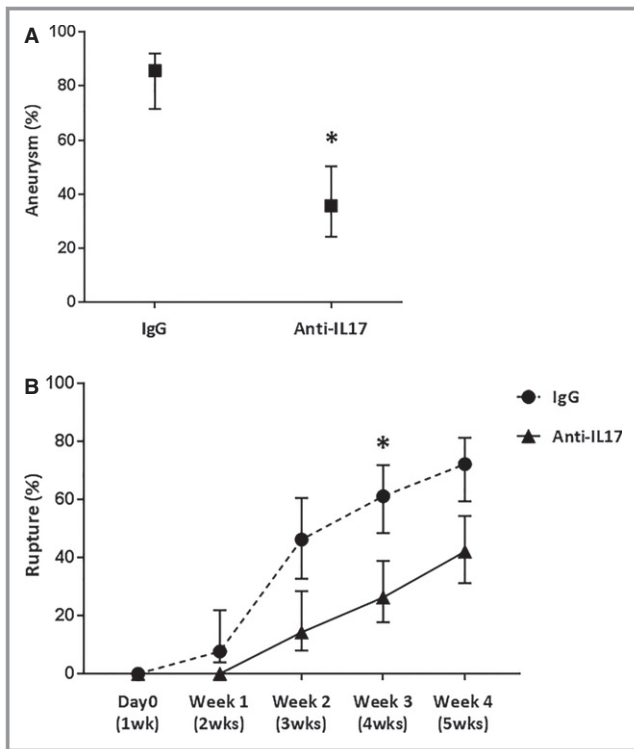
Figure 2. IL-17 neutralization prevents cerebral aneurysm (CA) growth and rupture. A, IL-17 neutralization with rat anti-mouse IL17 antibody (n=14) prevents CA growth compared with isotype-matched Ig control (n=14; 36% [5 of 14] vs 86% [12 of 14]; *P=0.02) in a murine CA model. B, IL-17 neutralization with rat anti-mouse IL17 antibody (n=19) prevents CA rupture compared with control isotype-matched Ig (n=18; 26% [5 of 19] vs 61% [11 of 18]; *P<0.05) in a murine CA rupture model. Timeline of rupture rate is depicted by number of weeks after IL-17 blocker or Ig control administered (number of weeks after CA formation by elastase in parentheses). IgG indicates immunoglobulin G; IL-17, interleukin-17.

IgG)-treated mice. E-cadherin was the protein with the greatest difference in expression between IL-17A–blocked and control-treated mice. E-cadherin was significantly upregulated in IL-17A–blocked mice (P=0.05; Figure 5A).

E-cadherin is a calcium-dependent cell-cell adhesion glycoprotein responsible for cellular adhesion between epithelial cells. Downregulation or inhibition of E-cadherin allows for greater infiltration of inflammatory cells into the vascular wall, which is believed to be the mechanism by which aneurysms develop. We demonstrated that this function of E-cadherin is suppressed by IL-17 in aneurysm formation. IL-17 neutralization significantly suppresses monocyte and neutrophil infiltration at the murine COW compared with Ig control (Figure 5B).

We studied the effect of selective E-cadherin inhibition on development of murine CAs and rupture. Selective E-cadherin inhibition reverses the protective effect of IL-17A inhibition.

Selective E-cadherin inhibition of IL-17A–blocked mice reversed the protective effect of IL-17A blockade on aneurysm development: 65% (11 of 17 IL-17–blocked E-cadherin–blocked mice) versus 28% (5 of 18 IL-17–blocked control Ig mice), P=0.04. E-cadherin inhibition tends toward reversing the protective effect of IL-17A blockade on rupture: 12% (2 of 17 IL-17–blocked E-cadherin–blocked mice) versus 0% (0 of 18 IL-17–blocked control Ig mice), P=0.22 (Figure 6).

We confirmed that there is a marginal decrease (−20%) in E-cadherin expression in tissue from ovariectomized female mice compared with tissue from sham mice (P=0.08; Figure S4). Selective E-cadherin inhibition alone in estrogen-normal female mice did not significantly increase aneurysm formation: 75% (6 of 8 E-cadherin–blocked mice) versus 85.7% (12 of 14 control Ig mice) P=0.60; and did not significantly increase aneurysm rupture: 37.5% (3 of 8 E-cadherin–blocked mice) versus 42.9% (6 of 14 control Ig mice), P=1.0.

Furthermore, selective E-cadherin inhibition alone in ovariectomized female mice did not significantly increase aneurysm formation or rupture: 77.8% (7 of 9 E-cadherin–blocked mice) versus 82.4% formation (14 of 17 control Ig mice), P=1.0; and 22.2% (2 of 9 E-cadherin–blocked mice) versus 35.3% rupture (6 of 17 control Ig mice), P=0.67.

There was also no significant difference noted in E2R-blocked female mice. Selective E-cadherin inhibition alone in E2R-blocked female mice did not significantly increase aneurysm formation or rupture: 71.4% (5 of 7 E-cadherin–blocked mice) versus 100% formation (19 of 19 control Ig mice), P=0.06; and 14.3% (1 of 7 E-cadherin–blocked mice) versus 47.4% rupture (9 of 19 control Ig mice), P=0.19.

E-cadherin forms tight junctions between endothelial cells, and inhibition of E-cadherin promotes macrophage infiltration. In cell migration assays, E-cadherin inhibition promotes macrophage infiltration across endothelial cells (P<0.05), which may be the mechanism for the IL-17/E-cadherin aneurysm pathway (Figure 6C).

Discussion

Estrogen deficiency is associated with aneurysms. Previous studies have shown that estrogen-deficient rodents produce more aneurysms and that estrogen supplementation reverses this effect.[5–8] These previous studies do not corroborate what we observe clinically. In humans, aneurysms occur more often in women (70–75%) pre- *and* postmenopause.[2] However, aneurysm ruptures occur most often in postmenopausal women.[3,4]

Our study replicates what happens clinically: Estrogen-deficient female mice did not have significantly more aneurysms than control female mice, but estrogen-deficient female mice did have more aneurysm ruptures than control
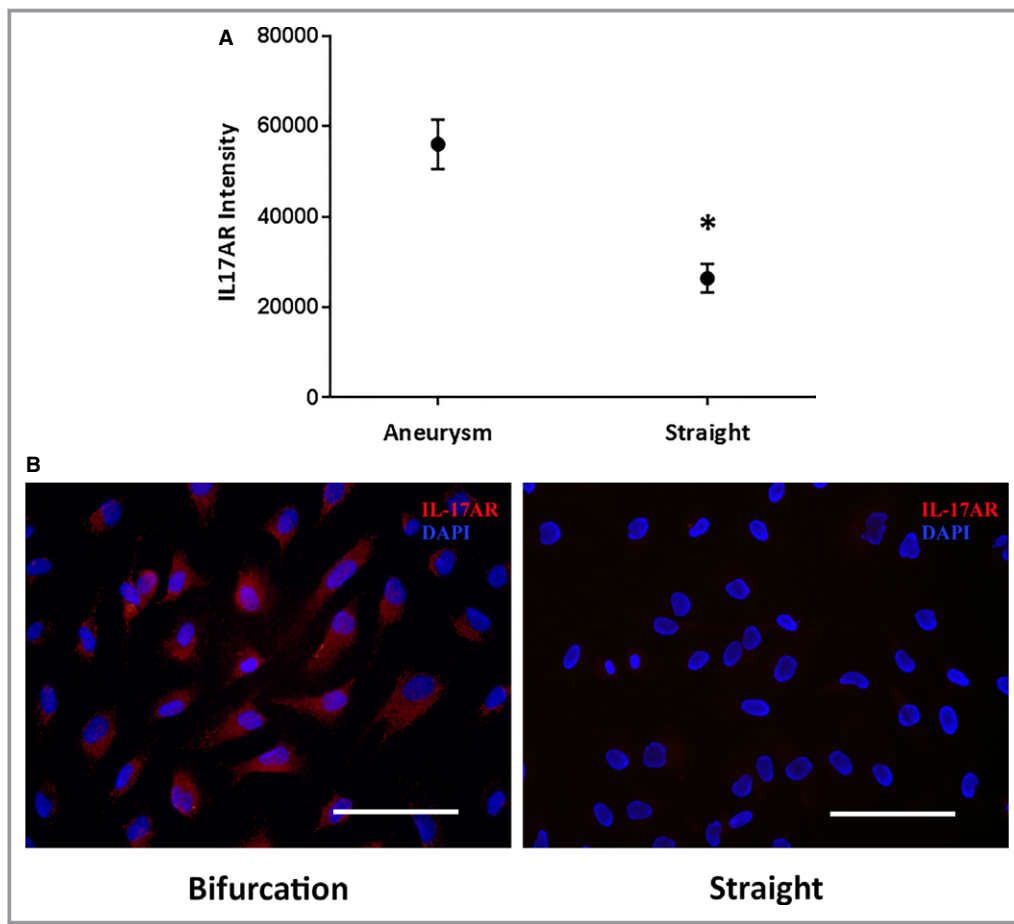
Figure 3. Endothelial cells at bifurcation aneurysms significantly express IL-17A receptor compared with those present in straight segments. Using a previously described in vitro parallel plate flow chamber model, endothelial cells were subjected to hemodynamic stresses at a bifurcation aneurysm vs a straight segment for 75 hours and then immunostained for anti-IL17A receptor. Relative fluorescence units (RFUs) were quantified and compared. A, Endothelial cells at a bifurcation aneurysm (n=4 flow chambers) significantly express IL-17A receptor compared with those present in straight segments (n=4 flow chambers) in RFUs (aneurysm, 55 992.5±5427.9; straight, 26 444.9±3156.0; *P=0.03). B, Endothelial cells from bifurcation aneurysm flow chambers (left panel) and from straight segment flow chambers (right panel) were immunostained for anti-IL17A receptor (red) and DAPI (blue). Endothelial cells from bifurcation aneurysm flow chambers demonstrate robust expression of IL17A receptor compared with endothelial cells from straight segment flow chambers. Scale bar=50 μm. DAPI indicates 4′,6-diamidino-2-phenylindole; IL-17, interleukin-17.

female mice. Estrogen supplementation does not reduce the number of aneurysms, but inhibits aneurysm rupture in estrogen-deficient female mice.

The mechanism for estrogen deficiency causing more aneurysm ruptures is not known. Experimental and human studies have demonstrated that aneurysms are the result of a vascular inflammatory process initiated by hemodynamic injury at bifurcations. Inflammatory cells, notably macrophages, infiltrate the vessel wall and contribute to matrix metalloproteinase–mediated degeneration of the extracellular matrix.[11–13] This process leads to an eventual weakening of vascular structural integrity, leading to aneurysm formation and rupture.[9,10]

Given the role of inflammation in aneurysms, we sought a link between estrogen deficiency and inflammation to better understand the mechanism for cerebral aneurysms.

We used a novel approach to find that link. Rather than using sham-operated controls, in our model, we were able to use mice subjected to the same model (hypertensive diet, renal and carotid artery ligation, intracranial elastase, and Angiotensin II) as experimental mice. By comparing mice that developed aneurysms with mice that did not, we were able to identify IL-17 as a link between estrogen deficiency and aneurysm inflammatory factors. We cross-validated this finding by demonstrating that IL-17A is significantly expressed in both murine and human aneurysms.
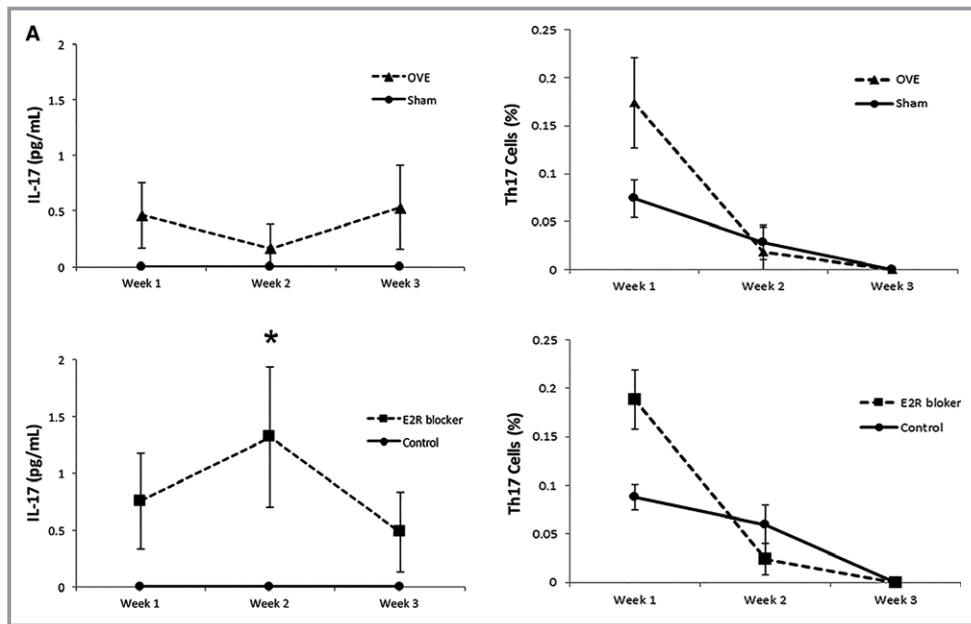
Figure 4. Estrogen deficiency-associated cerebral aneurysm (CA) rupture is by the IL-17–mediated pathway. A, Estrogen-deficient female mice, by ovariectomy (n=15; OVE, top row) or by estrogen E2-receptor blockade (n=14; bottom row), show higher levels of IL-17 and Th17 cells compared with sham-operated (n=15) or vehicle-treated (n=16) control mice, respectively. Levels of IL-17 were significantly higher at week 2 in E2 receptor-blocked mice compared with vehicle-treated control mice (*P=0.04). Levels of Th17 cells were significantly higher at week 1 in OVE mice compared with sham-operated mice (*P=0.04) and E2 receptor-blocked mice compared with vehicle-treated control mice (*P=0.04). B, Estrogen-deficient female mice, either OVE (n=15) or E2 receptor-blocked (n=18), in a murine CA model and murine CA rupture model did not have more CAs than control sham-operated (n=18) or vehicle-treated control mice (n=18; OVE 87% [13 of 15] vs sham 72% [10 of 15], NS, top left panel; E2R-blocked 100% [18 of 18] vs vehicle 83% [15 of 18], NS, bottom left panel), respectively, but had significantly higher risk of CA rupture (OVE 47% [7 of 15] vs sham 7% [1 of 15]; *P=0.04, top right panel; E2R-blocked 44% [8 of 18] vs vehicle 0% [0 of 18]; **P=0.003, bottom right panel). C, E2 supplementation in OVE mice (n=18) does not significantly change the number of CAs compared with vehicle-treated OVE mice (n=19; OVE E2 supplementation 67% [12 of 18] vs OVE vehicle 74% [14 of 19], NS, top panel). However, the higher risk of CA rupture in estrogen-deficient mice is reversed by E2 supplementation (n=18) compared with vehicle treatment (n=19; OVE E2 supplementation 6% [1 of 18] vs OVE vehicle 37% [7 of 19]; *P=0.04, bottom panel). D, IL-17 neutralizing antibody-treated animal reverses the higher risk of CA rupture observed in estrogen-deficient mice, OVE (n=18) or E2R-blocked (n=18), compared with isotype-matched Ig control-treated estrogen-deficient mice (OVE-IgG, n=17; E2RB-IgG, n=19; OVE IL17-blocked 11% [2 of 18] vs OVE Ig control 35% [6 of 17]; *P=0.018, top panel; E2R-blocked IL17-blocked 0% [0 of 18] vs E2R-blocked Ig control 47.4% [9 of 19]; **P=0.002, bottom panel). E2RB indicates E2-receptor blockade; IgG, immunoglobulin G; IL-17, interleukin-17; NS, not significant; Th17, T helper 17.

Other disease models have shown that estrogen deficiency leads to an increased population of IL-17–secreting Th17 cells by skewed T-cell differentiation and downregulation of forkhead box protein 3, which antagonizes Th17 cell differentiation.[16] This study demonstrates that estrogen deficiency is linked to CA rupture by increased number of Th17 cells and increased circulating levels of the inflammatory cytokine, IL-17A. IL-17A can induce pro-inflammatory cytokines such as IL-6 and TNF-α, which have been shown as mediators associated with hypertension and endothelial cell dysfunction.[17–19] In addition, it has been shown that endothelial cell dysfunction and hypertension are potentially associated with the binding of IL-17 to the endothelial cell receptor, leading to Rho-kinase-mediated endothelial nitric oxide synthase phosphorylation.[20]

Additionally, IL-17A seems to play an important role in aneurysms and aneurysm ruptures. IL-17 neutralization inhibits aneurysms and ruptures. Furthermore, IL-17A blockade inhibits ruptures in estrogen-deficient female mice.

Aneurysms are known to form at bifurcations, and it is believed that the hemodynamic stresses at bifurcations lead to arterial wall weakening, producing aneurysms and
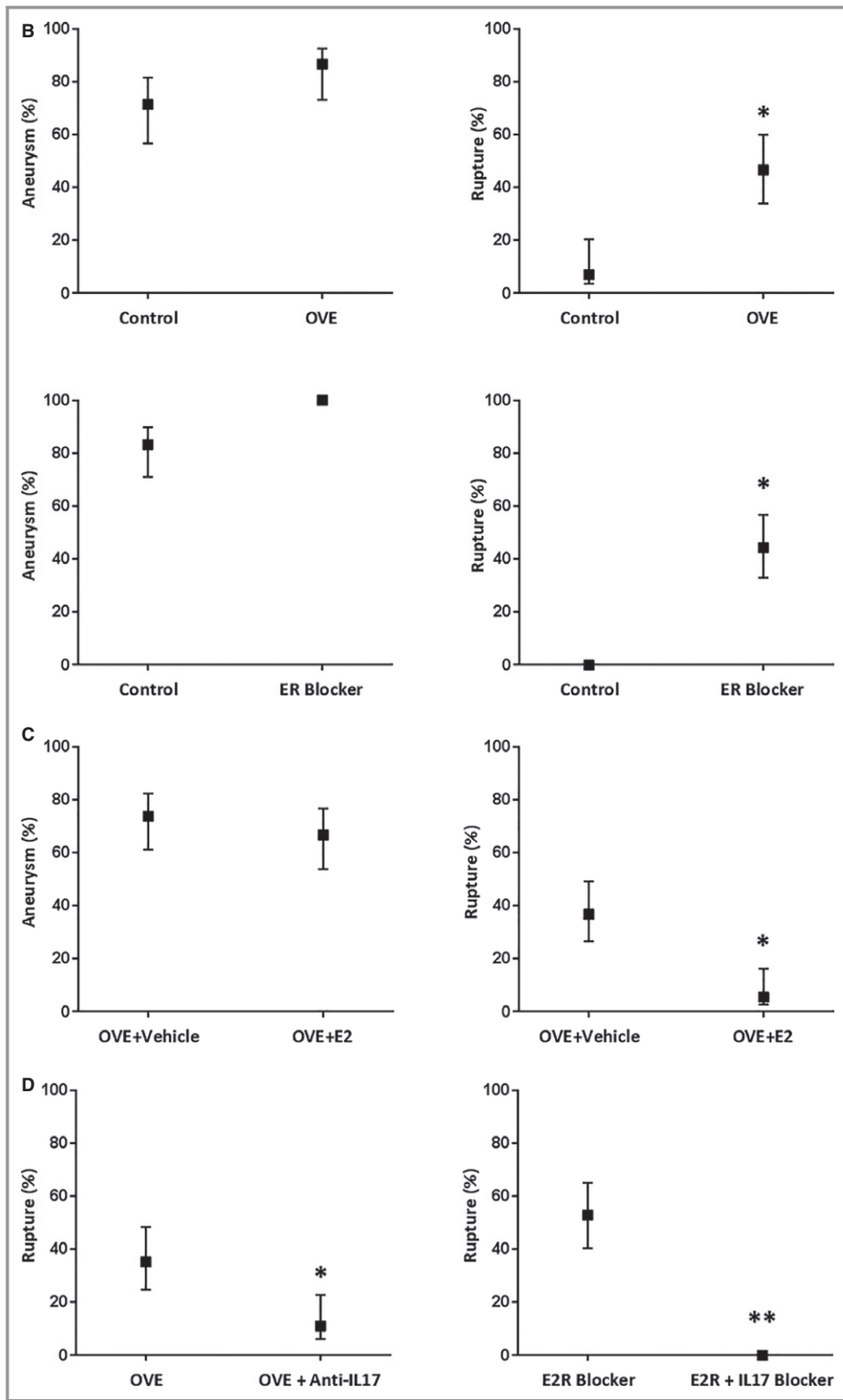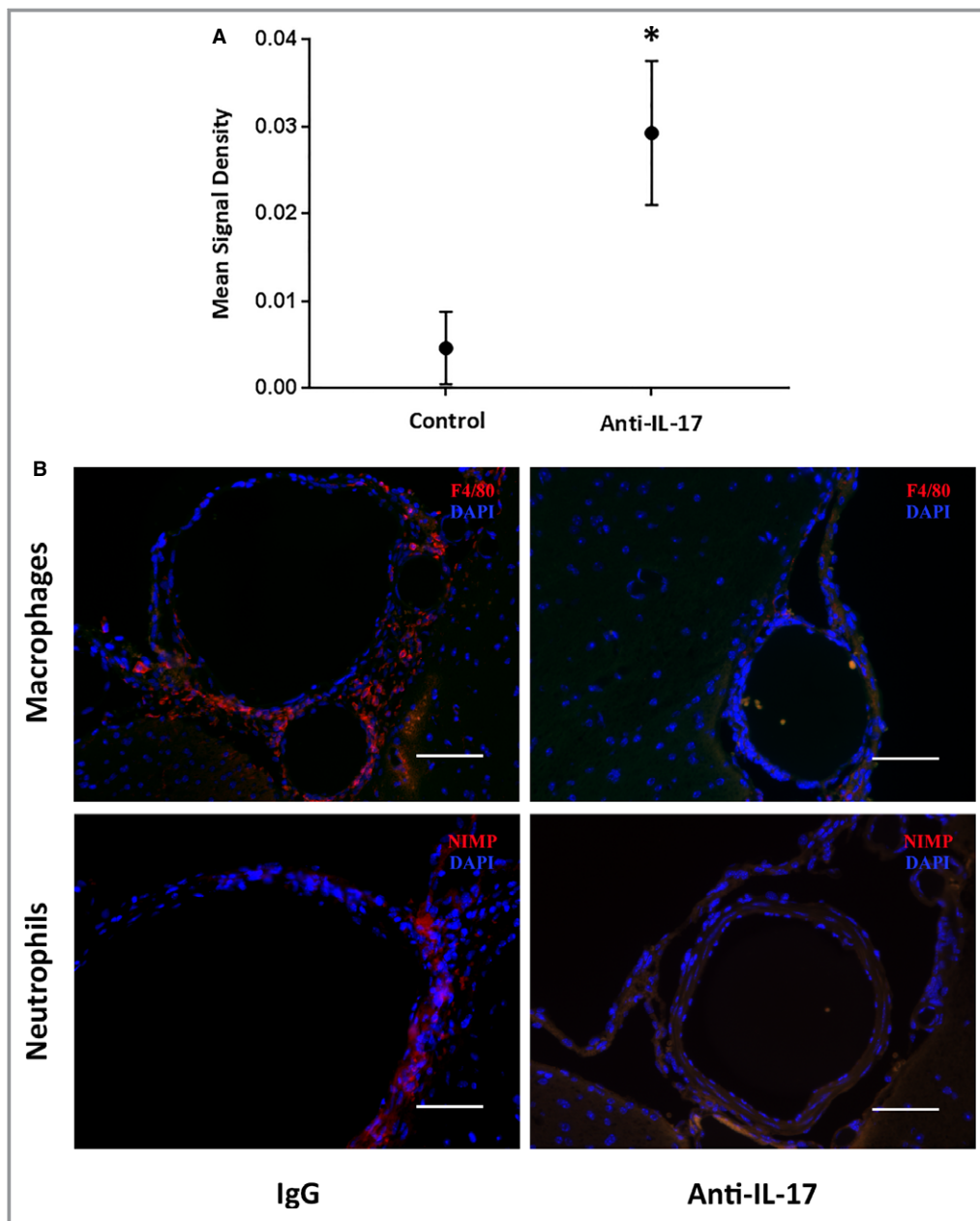
Figure 4. Continued.

Figure 5. Mechanism of IL-17–mediated cerebral aneurysm (CA) growth and rupture is increased monocyte and neutrophil infiltration. A, E-cadherin expression by antibody-pair–based assay (cytokine array) is significantly higher in circle of Willis (COW) of mice treated with IL-17 neutralizing antibody with rat anti-mouse IL17 antibody (n=3) compared with mice treated with isotype-matched Ig control (n=3; *P=0.05). B, Murine CA from mouse treated with control isotype-matched Ig control (n=5) and murine COW from mouse neutralized for IL-17 with rat anti-mouse IL17 antibody (n=5) in the murine CA model were immunostained for monocytes using anti-F4/80 (red) or anti-neutrophil (red) and with DAPI (blue). These demonstrate significant monocyte and neutrophil infiltration in the vascular wall of murine CAs from mice treated with control Ig, but not in murine COW from mice neutralized for IL-17, suggesting that the mechanism for IL-17-mediated CA growth and rupture is by inflammatory cell infiltration in the vascular wall. Scale bar=50 μm. DAPI indicates 4′,6-diamidino-2-phenylindole; IgG, immunoglobulin G; IL-17, interleukin-17.

ruptures. We sought to establish a link between the estrogen deficiency/IL-17A pathway and hemodynamic stresses at bifurcations. We used a flow chamber model that replicates the hemodynamic stresses found at bifurcations and normal arteries. We demonstrate that endothelial cells at bifurcations selectively express significantly more IL-17A receptors than endothelial cells in normal straight arteries.
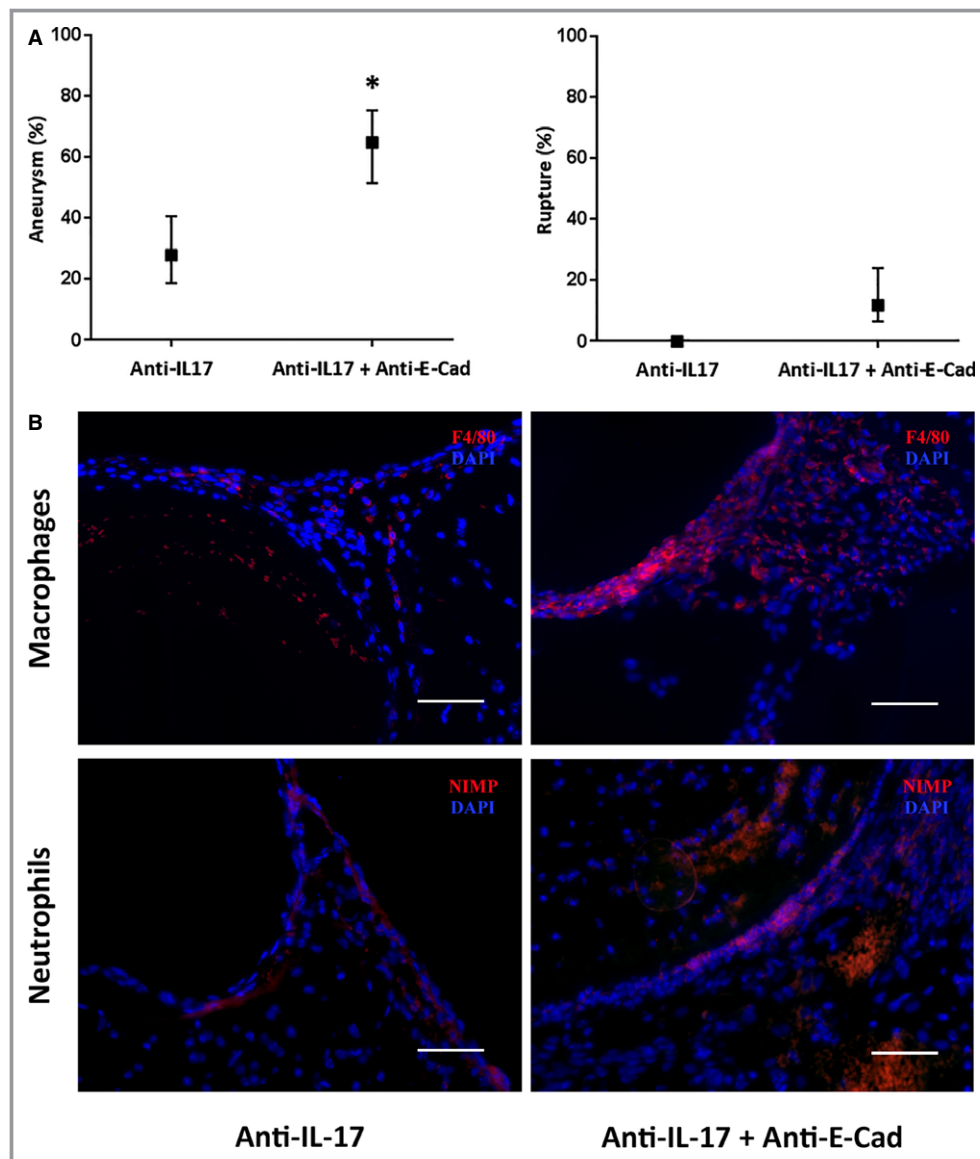
Figure 6. Selective E-cadherin inhibition reverses the protective effect of selective IL-17 inhibition on cerebral aneurysm (CA) growth and rupture. A, Mice neutralized for IL-17 with rat anti-mouse IL17 antibody were given E-cadherin blocking antibody (n=19) or isotype-matched Ig control (n=16) in the murine CA model and the murine CA rupture model. E-cadherin blocked in IL-17A neutralized mice reversed the protective effect of IL-17A neutralization on CA growth (IL17-blocked E-cad–blocked 65% [11 of 17] vs IL17-blocked Ig control 28% [5 of 18]; *P=0.04, left panel) and potential rupture (IL17-blocked E-cad–blocked 12% [2 of 17] vs IL17-blocked Ig control 0% (0 of 18); P=0.22, right panel) compared with isotype-matched Ig control-treated IL-17A–neutralized mice, suggesting that the IL-17 cerebral aneurysm pathway is mediated by E-cadherin. B, Murine CA from mouse treated with anti-IL-17 antibody (n=5) and treated with anti-IL-17 and anti-E-Cadherin (n=5) in the murine CA model were immunostained for monocytes using anti-F4/80 (red) or anti-neutrophil (red) and with DAPI (blue). Scale bar=50 μm. C, In cell migration assays, E-cadherin inhibition promotes macrophage infiltration across endothelial cells (P<0.05). Anti-E-Cad indicates anti-E-Cadherin; DAPI indicates 4′,6-diamidino-2-phenylindole; IgG, immunoglobulin G; IL-17, interleukin-17.

Based on these findings, the mechanistic theory we postulate thus far is that: (1) estrogen deficiency increases Th17 cells and circulating levels of IL-17A; and (2) circulating IL-17A acts selectively on endothelial cells at bifurcations because (3) these endothelial cells have been primed to selectively express IL-17A receptors, a phenotype caused by the hemodynamic stresses that occur at bifurcations as opposed to normal straight segments of arteries.
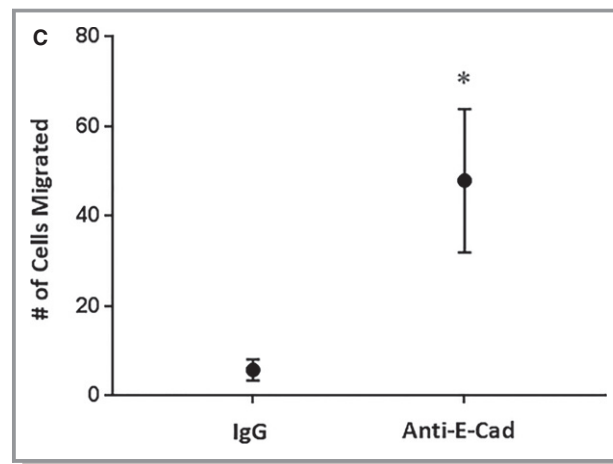
Figure 6. Continued

We next sought to identify what effect IL-17A has on endothelial cells that leads to aneurysm ruptures. We again used a cytokine screen array and compared mice that were IL-17 blocked with control-treated mice. We found significantly upregulated E-cadherin expression in IL-17–blocked mice.

E-cadherin is one of the cell adhesion molecules. There are several types of cadherin, which include N-cadherin and VE-cadherin. E- and N-cadherin are proteins, which mediate cell-cell adhesion in various types of cells. On the other hand, VE-cadherin is specific for vascular endothelial cell adhesion molecules.[21] E-cadherin suppression seems to play an important role in aneurysms and aneurysm ruptures. E-cadherin is a calcium-dependent cell-cell adhesion glycoprotein responsible for cellular adhesion between epithelial cells. Downregulation of E-cadherin disrupts the strength of adhesions between epithelial cells and allows for cell invasion and infiltration.[22]

E-cadherin's role in the pathobiology of aneurysms and ruptures is compatible with findings of inflammatory cell infiltration of the vascular wall of human aneurysms.[11–13] We demonstrate that E-cadherin blockade causes significant infiltration of macrophages in cell migration assays and in vivo at the murine COW. IL-17A blockade reverses the infiltration of monocytes and neutrophils.

Based on these findings, we summarize the mechanistic theory that we postulate as follows: (1) Estrogen deficiency increases Th17 cells and circulating levels of IL-17A; (2) circulating IL-17A acts selectively on endothelial cells at bifurcations because (3) bifurcation endothelial cells have been primed by hemodynamic stresses to phenotypically selectively express IL-17A receptors. The effect IL-17A has on bifurcation endothelial cells is that (4) IL-17A causes suppression of E-cadherin. E-cadherin is responsible for cellular adhesion between endothelial cells, so (5) suppression of E-cadherin allows for macrophage infiltration, which (6) causes degeneration of the extracellular matrix, producing artery wall weakening and eventually aneurysms and aneurysm ruptures.

A limitation of this study is that we used an animal aneurysm model, which is induced by elastase injection. It may not be the same as non-enzyme/chemical-induced aneurysms, and may not be relevant to naturally occurring human aneurysms. The process of naturally occurring CAs is still not known. We, however, have found that elastase and high blood pressure are necessary to produce the consistency of aneurysm formation in this model. Future study is needed to further clarify this pathway and provide more enhanced novel therapy to prevent or halt aneurysm development, growth, and rupture.

Sources of Funding

Disclosures

None.

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SUPPLEMENTAL MATERIAL

Human Aneurysm Samples

Sample ID	Gender	Age	Location	Size (mm)	IL-17
Aneurysm 1	M	68	ACOM	13	+
Aneurysm 2	F	68	MCA	6.6	+
Aneurysm 3	F	64	MCA	11.3	+
Aneurysm 4	F	48	ACOM	6	+
Aneurysm 5	M	61	MCA	8.6	+

ACOM = Anterior Communication Artery; MCA = Middle Cerebral Artery
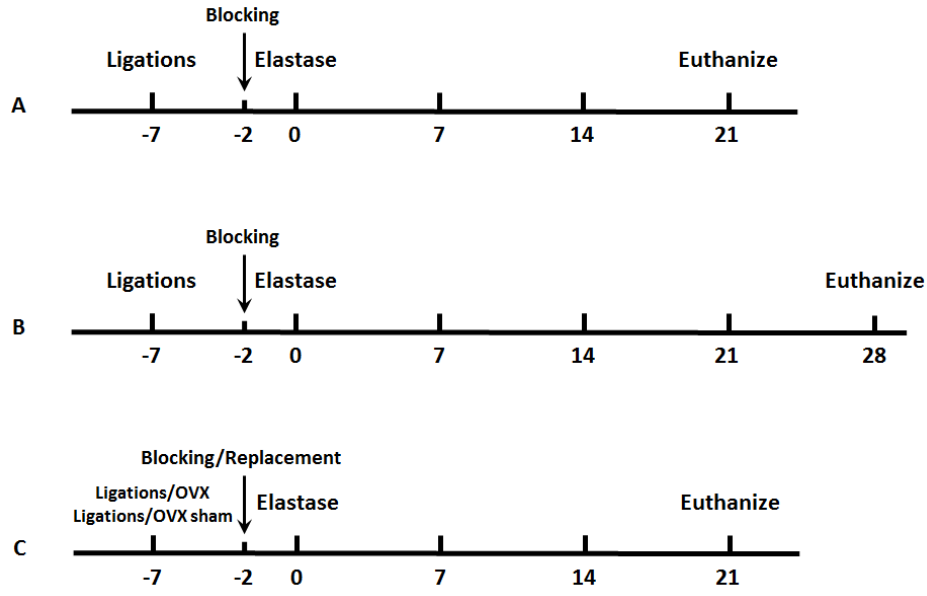
Table S1. Human Aneurysm Sample

Figure S1. Time line for murine aneurysm and rupture model

A) Aneurysm/Rupture model was used for Figure 1, 5 and 6. B) Rupture model was used for

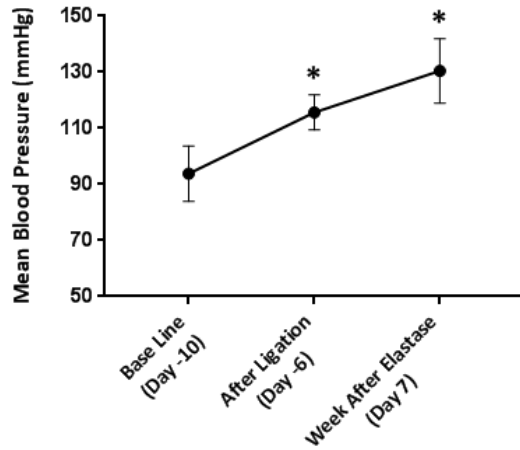Figure 2. C) OVE/OVE sham model was used for Figure 4.

Figure S2. Blood pressure measurement before and after surgery

Blood pressure was measured three days before (day -10) and one day after (day -6) ligation surgeries, and one week after elastase injection. Mean blood pressure were 93.8 +/- 9.9 mmHg, 115.7 +/- 6.2 mmHg, and 130 +/- 11.5 mmHg respectively.
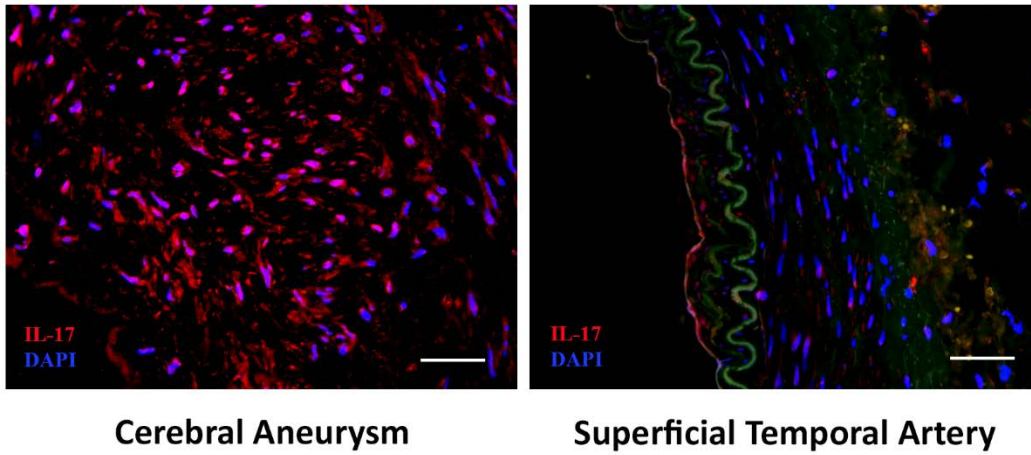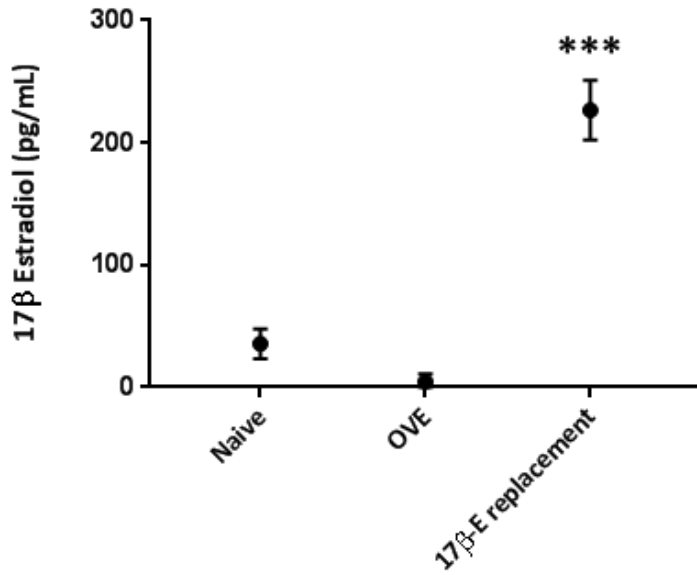
Cerebral Aneurysm   **Superficial Temporal Artery**

Figure S3. IL-17A expression in human cerebral aneurysms

Human CA and human superficial temporal artery were immnostained for anti-IL17 (red) and

DAPI (blue) and demonstrated robust expression of IL17A in CAs but not in superficial temporal
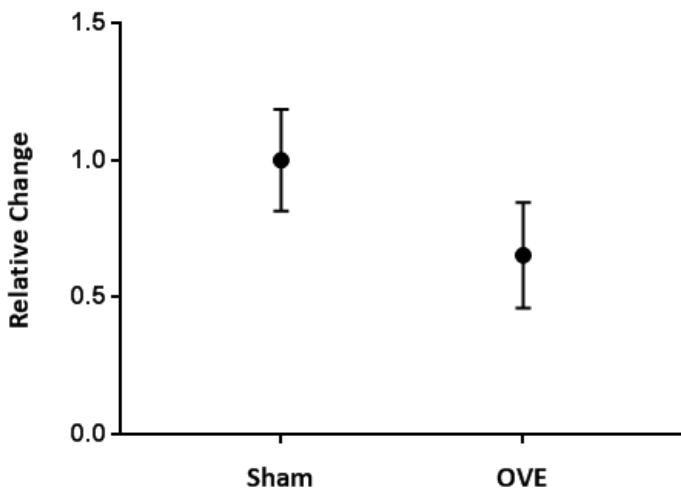
artery. Scale bar=50μL

a

b

Figure S4. 17β-estradiol level in blood serum is increased after E2 supplementation

a) Before and after OVE, 17β-estradiol level in blood serum were 35.6 +/- 24.4 pg/mL and 4.5

+/- 6.2 pg/mL respectively. The17β-estradiol level was increased to 226.6 +/- 24.5 pg/mL after

the E2 supplementation. b) Decreased E-cadherin expression was observed in overiectomized

mice compared with sham-operated mice by cytokine array (n=5 each. P=0.08)