

## Monocyte Chemotactic Protein-1–Interleukin-6–Osteopontin Pathway of Intra-Aneurysmal Tissue Healing

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**Background and Purpose**—We have previously demonstrated that the local delivery of monocyte chemotactic protein-1 (MCP-1) via an MCP-1–releasing poly(lactic-co-glycolic acid)–coated coil promotes intra-aneurysmal tissue healing. In this study, we demonstrate that interleukin-6 (IL-6) and osteopontin are downstream mediators in the MCP-1–mediated aneurysm-healing pathway.

**Methods**—Murine carotid aneurysms were created in C57BL/6 mice. Drug-releasing coils (MCP-1, IL-6, and osteopontin) and control poly(lactic-co-glycolic acid) coils were created and then implanted into the aneurysms to evaluate their intra-aneurysmal–healing capacity. To investigate the downstream mediators for aneurysm healing, blocking antibodies for IL-6 receptor and osteopontin were given to the mice implanted with the MCP-1–releasing coils. A histological analysis of both murine and human aneurysms was utilized to cross-validate the data.

**Results**—We observed increased expression of IL-6 in MCP-1-coil–treated aneurysms and not in control-poly(lactic-co-glycolic acid)-only–treated aneurysms. MCP-1–mediated intra-aneurysmal healing is inhibited in mice given blocking antibody to IL-6 receptor. MCP-1–mediated intra-aneurysmal healing is also inhibited by blocking antibody to osteopontin. The role of IL-6 in intra-aneurysmal healing is in recruiting of endothelial cells and fibroblasts. Local delivery of osteopontin to murine carotid aneurysms via osteopontin-releasing coil significantly promotes intra-aneurysmal healing, but IL-6–releasing coil does not, suggesting that IL-6 cannot promote aneurysm healing independent of MCP-1. In the MCP-1–mediated aneurysm healing, osteopontin expression is dependent on IL-6; inhibition of IL-6 receptor significantly inhibits osteopontin expression in MCP-1–mediated aneurysm healing.

**Conclusions**—Our findings suggest that IL-6 and osteopontin are key downstream mediators of MCP-1–mediated intra-aneurysmal healing. (*Stroke*. 2017;48:00-00. DOI: 10.1161/STROKEAHA.116.015590.)

**Key Words:** antibodies, blocking ■ interleukins ■ intracranial aneurysm ■ monocyte chemoattractant protein-1 ■ osteopontin

Cerebral aneurysms (CAs) occur in up to 5% of the population in the United States and up to 7% of all strokes are caused by CA rupture,<sup>1–3</sup> which is associated with up to 50% death or dependency. Histological analysis of completely cured CAs obtained at autopsy compared with incompletely cured CAs obtained at surgery demonstrate that intra-aneurysmal tissue repair with collagen, macrophages, neutrophils, smooth muscle cells (SMCs), and endothelial cells is needed to achieve a cure for CAs.<sup>4</sup> We have studied the mechanism of intra-aneurysmal tissue repair in a murine carotid aneurysm model<sup>5</sup> and demonstrated previously that local delivery of monocyte chemotactic protein-1 (MCP-1) via a poly(lactic-co-glycolic acid) (PLGA)–coated platinum coil that releases MCP-1 promotes intra-aneurysmal tissue healing.<sup>6</sup> Although MCP-1 and its role in tissue repair and remodeling have been studied in other disease models such as wound healing<sup>7,8</sup> and myocardial infarction,<sup>9</sup> CAs differ significantly in that the tissue healing must occur and be induced within an

intraluminal intra-aneurysmal space with no native matrix on which inflammatory cells can infiltrate and connective tissue can proliferate. Therefore, an intraluminal scaffold must be surgically introduced to initiate an inflammatory response and promote the tissue repair and remodeling cascade, which in this model is the MCP-1–releasing coil.

We have demonstrated that intravascular MCP-1–mediated inflammatory tissue healing is macrophage inflammatory protein (MIP)-1 $\alpha$  and MIP-2 dependent. When MIP-1 $\alpha$  or MIP-2 was blocked, MCP-1–mediated aneurysmal healing in our murine carotid aneurysm model was significantly reduced. Although we hypothesized the postulated pathway by MCP-1 in the inflammatory tissue-healing cascade, the downstream mediators of MCP-1 in aneurysmal healing have not been clarified. Some studies showed that MCP-1 activates inflammatory cells followed by the recruitment of monocytes/macrophages that release a variety of cytokines.<sup>10–13</sup> Additional studies were needed to further define the pathway

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of MCP-1–mediated inflammatory aneurysmal tissue healing. The detailed mechanisms and the pathway of downstream mediators in this specific MCP-1–mediated aneurysm healing, however, remain unclear. In this study, we demonstrate that interleukin-6 (IL-6) and osteopontin are downstream mediators in the MCP-1–mediated aneurysm-healing pathway.

## Methods

### Animals

All animal procedures were performed under the approval of the University of Florida Animal Care and Use Committee and guidelines. In all experiments, female C57BL/6 mice (6–10 weeks old) were used (Charles River, Wilmington, MA).

### Human Aneurysm Specimens

The studies of human aneurysm specimens and control superficial temporal arteries were performed under the approval of University of Florida Institutional Review Board. Patients signed informed institutional review board research consent before undergoing aneurysm surgery, and aneurysms and control superficial temporal arteries were collected at the time of surgery.

### Drug-Releasing Coil

Drug (cytokine)-releasing coils were created as described previously.<sup>6</sup> Briefly, bare platinum coils were dipped into 10 mg/mL of each protein (MCP-1, IL-6, and osteopontin; R&D Systems, Minneapolis, MN) in 50:50 PLGA and dichloromethane anhydrous with Mg(OH)<sub>2</sub>. Control PLGA-only coils were created by dipping bare platinum coils into an aqueous suspension of PBS in 50:50 PLGA and dichloromethane anhydrous without protein.

### Murine Carotid Aneurysm and Coil Implant

Murine carotid aneurysms were created in C57BL/6 mice as described previously.<sup>5,6</sup> Briefly, the right common carotid artery is exposed and then 10 U/mL of porcine pancreatic elastase solution (Worthington Biochemical Corp, Lakewood, NJ) in PBS is applied for 20 minutes. After elastase, the right common carotid artery is occluded distally using a heat cauterization apparatus. The aneurysms are fully grown

in 3 weeks. Fully mature murine carotid aneurysms were treated with drug-releasing or control PLGA-only coils as described previously.<sup>6</sup> Briefly, the right common carotid artery is exposed and temporarily ligated by 3-0 suture proximal and distal. A 27 gauge needle or microsurgical knife is used to make a small incision. A coil is inserted into the aneurysm after which the incision is closed using a heat cauterization apparatus. Animals were euthanized with 4% paraformaldehyde in PBS by cardiac perfusion at 3 days, 1 week, 2 weeks, or 3 weeks after the coil implant. For cytokine array analysis, animals were euthanized without 4% paraformaldehyde. Animals were randomly assigned to the experimental group.

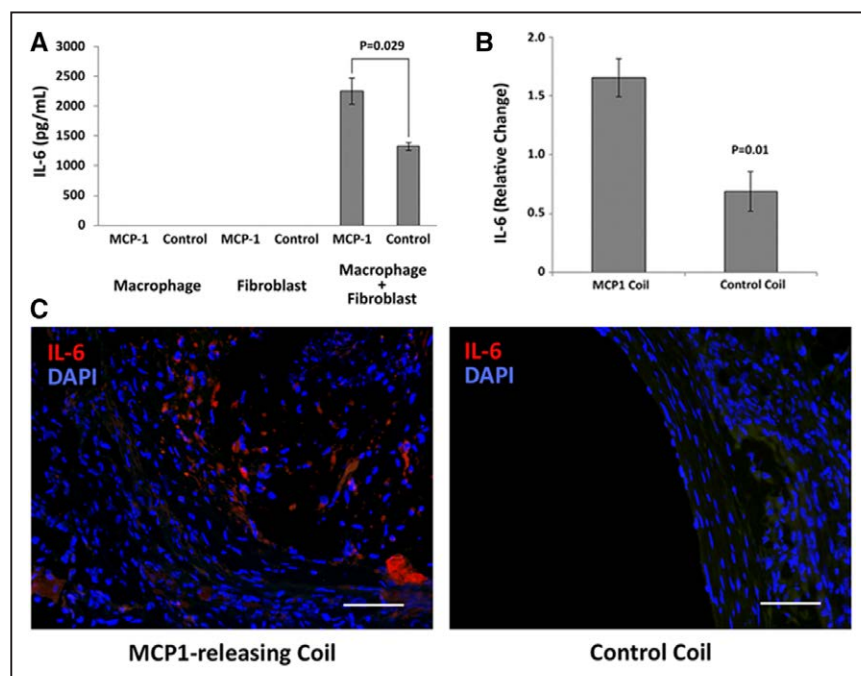
### Cytokine and Receptor Blocking In Vivo

IL-6 receptor–blocking antibody (200 µg/mL per animal, IP; Genentech), osteopontin–blocking antibody (100 µg/mL per animal, IP; R&D Systems), or their control isotype-matched antibodies were given to mice 2 days before the MCP-1–releasing coil implant and every 48 hours for up to 3 weeks. Mice were randomly selected from the cage and received blocking antibodies or isotype-matched IgG control, which were blindly labeled with numbers. The mouse surgeon and data collectors were blinded.

### In Vitro Cell Assays

In vitro intercellular cell signaling assay was performed using macrophages (J774, ATCC) and fibroblasts (3T3, ATCC). Briefly, macrophages alone, fibroblasts alone, or cocultured macrophages and fibroblasts were cultured in 24-well plates and grown to 80% confluence followed by supplementing 10 ng/mL of MCP-1 protein (R&D Systems) or control PBS. Forty-eight hours later, culture medium was collected and IL-6 levels were measured using an IL-6 ELISA kit (R&D Systems).

The recruitment effect of IL-6 on ECs (HUVEC, C-003-5C; Invitrogen), macrophages (J774, ATCC), SMCs (MOVAS, ATCC), and fibroblasts (3T3, ATCC) was studied in vitro by cell migration assay. Oris Pro Cell Migration Assay kit was used for cell migration assay. All cells were cultured with media supplemented with fetal bovine serum and grown to 100% confluence followed by serum starvation for 12 hours in 96-well cell culture plates. The assay was started with 50 ng/mL of IL-6 protein or PBS supplemented in culture medium. All procedures followed the manufacturer's instructions. Twenty-four hours after the assay started, cell migratory area was measured using Image Pro software.



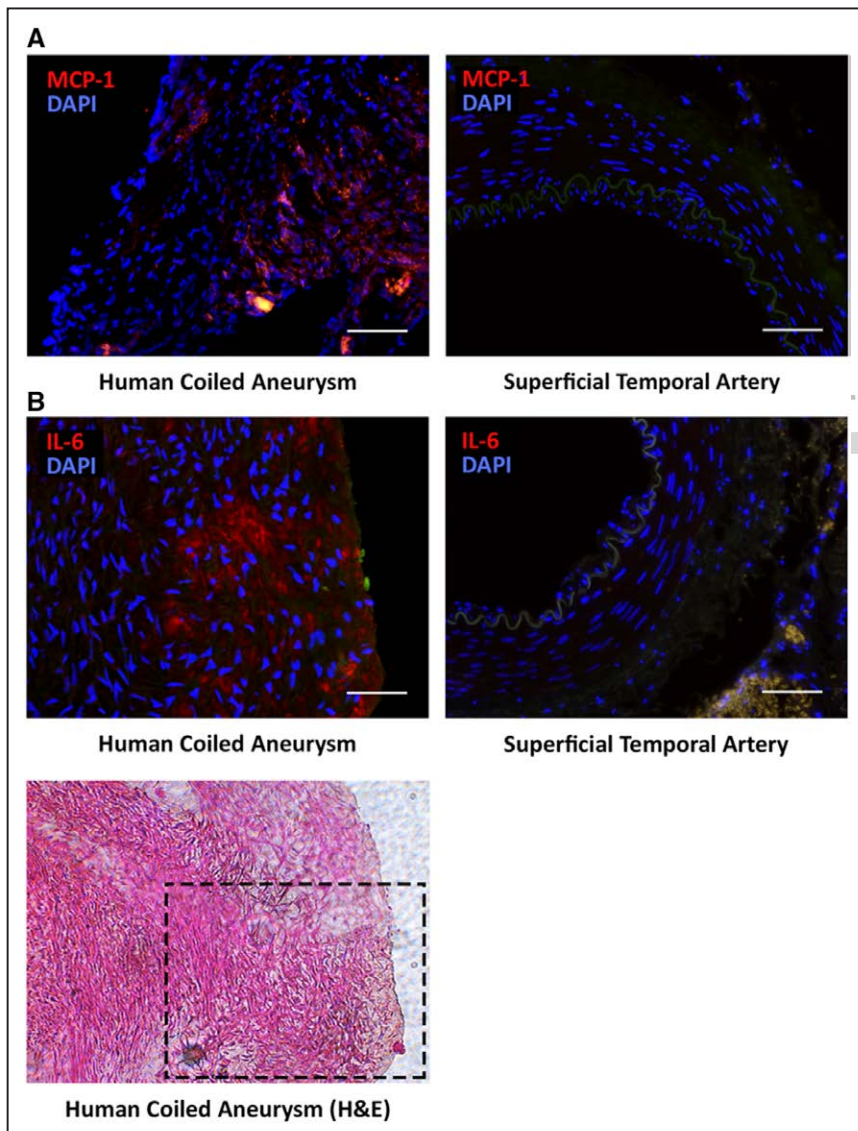
**Figure 1.** Monocyte chemoattractant protein-1 (MCP-1) induces interleukin (IL)-6 expression both in vitro and in vivo. **A**, In vitro intercellular cell signaling assays were performed with macrophages alone, fibroblasts alone, or macrophages–fibroblasts coculture exposed to MCP-1 or control PBS. Significantly increased secretion of IL-6 was observed in macrophage–fibroblast cocultured medium, which are stimulated by MCP-1 (2250.0±219.9 mg/mL) compared with control PBS-exposed cocultured medium (1325.5±63.0 mg/mL). IL-6 secretion was not detected in single-cultured cells medium. (n=8 each group). **B**, Three weeks after coil implant, the coiled aneurysms were harvested. Cytokine array analysis of MCP-1–releasing coil–treated murine carotid aneurysms (n=5) reveal significantly higher expression of IL-6 (1.7±0.2) than that of PLGA-control-coil–treated aneurysms (n=5; 0.7±0.2; P=0.01). **C**, Immunofluorescent staining of representative sections of aneurysms implanted with MCP-1–releasing coil and PLGA control coil. DAPI=nuclei. Scale bar, 50 µm.

### Immunohistochemical Analysis

All human samples were fixed using 4% paraformaldehyde solution after collection and transferred to 70% ethanol solution for paraffin block embedding. All mouse samples were fixed in 4% paraformaldehyde solution after collection and transferred to 30% sucrose solution for optimal cutting temperature frozen embedding. The blocks were sectioned using a microtome or cryostat into 5- $\mu$ m sections. Immunohistochemistry was performed on mouse and human aneurysms and control specimens. To evaluate aneurysm formation, the following antibodies were used for immunohistochemistry: mouse MCP-1, mouse IL-6, mouse osteopontin, human MCP-1, human IL-6, and human osteopontin.

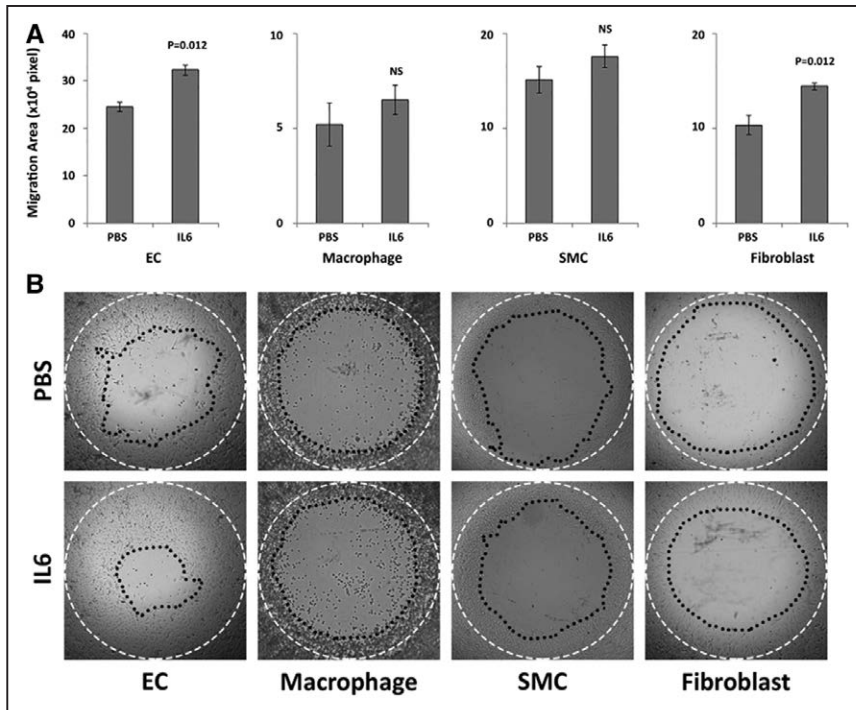
For the human coiled aneurysms and the superficial temporal artery samples, the respective fluorescent intensities of the expressions of MCP-1, IL-6, and osteopontin were analyzed. Samples were collected using systematic random sampling to ensure biasfree analysis. The tissues were sectioned into 5- $\mu$ m sections through the use of a microtome. A total of 5 sections, with 1 collected every 100  $\mu$ m (20 sections), were then used for analysis. The fluorescent intensity of their expression was analyzed using ImageJ software. Immunohistochemistry for temporal cascade analysis was performed. Coiled aneurysms were harvested at 3 days, 1 week, 2 weeks, and 3 weeks after coil treatment implant (n=5 mice for each time point). All samples were fixed using a

4% paraformaldehyde solution after collection and transferred to 30% sucrose solution for optimal cutting temperature frozen block embedding. Various stereological techniques were used to ensure biasfree analysis. Samples were collected using systematic random sampling. The tissues were cross sectioned into 5- $\mu$ m sections through the use of a cryostat from the distal part of the aneurysm. Once the blade made contact with the coil, the tissues were sectioned every 100  $\mu$ m. A total of 5 sections, with 1 collected every 100  $\mu$ m (20 sections), were then used for analysis. An immunohistochemistry analysis was performed on the mouse aneurysms. To evaluate the temporal cascade of inflammatory cells in aneurysm, the following antibodies were used for the immunohistochemistry analysis: anti-mouse IL-6, anti-mouse CD45, anti-mouse NIMP-R14 (neutrophil), anti-mouse F4/80, anti-mouse iNOS, anti- $\alpha$  smooth muscle actin, and anti-fibroblast-specific protein 1. Stereological counting rules were used for the cell counts. After the staining, 5 microscopic high-resolution images per slides were obtained using a  $\times 20$  objective lens by blinded observer. All 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). For IL-6 and  $\alpha$ -smooth muscle actin, the fluorescent intensity of their expression was analyzed using ImageJ software.



**Figure 2.** Monocyte chemoattractant protein-1 (MCP-1) and interleukin (IL)-6 expressions in healed human coiled cerebral aneurysms. Immunofluorescent staining of human cerebral aneurysms that have been previously coiled demonstrate (A) MCP-1 and (B) IL-6 expression in the tissue ingrowth of the coiled part of the aneurysm. DAPI=nuclei. Scale bar, 50  $\mu$ m. Hematoxylin-eosin staining of an adjacent section of a coiled aneurysm demonstrates cell orientation in the healed part of tissue (**bottom**). Black dashed line indicates the area where immunofluorescence was performed for IL-6.





**Figure 3.** Interleukin (IL)-6 induces in vitro migration of endothelial cells (ECs) and fibroblasts. IL-6 chemotaxis for ECs, macrophages, smooth muscle cells (SMCs), and fibroblasts was tested by Oris Pro Cell Migration and Invasion assay kit with 50 ng/mL of IL-6 protein or without protein (PBS; n=8 each group). **A**, After the assay was performed, the migratory cells were measured using Image Pro software. The assay revealed that IL-6 induces ECs and fibroblast migration but not macrophages and SMCs. **B**, Representative images of migration assays in each group. White dashed line indicates premigration area (diameter=2 mm); and black dotted line, postmigration area.

### Cytokine Array Analysis

Protein was extracted from aneurysm samples using radio-immunoprecipitation assay lysis and extraction buffer with proteinase inhibitor. Cytokine array was performed with a RayBio Mouse Cytokine Antibody Array Kit (RayBiotech, Norcross, GA).

### Statistical Analysis

Continuous responses are summarized by means, SDs, and 95% confidence intervals, as well as by medians and ranges. Because of small sample sizes and possible non-normal distribution of responses for continuous variables, the nonparametric Mann-Whitney *U* test was used to detect shifts in the distribution of responses in experimental groups relative to control groups for single comparison. A 2-way ANOVA model was used to compare mean transformed responses between IgG control and anti-IL-6-treated animal groups observed at 3 or 4 different time points. Separate groups of animals were observed at each time point. Animal group, observation day, and the interaction between animal group and observation day were modeled as fixed effects.

## Results

### MCP-1 Induces IL-6 Expression in Macrophages and Fibroblasts Coculture

Intercellular cell signaling assays were performed to determine downstream mediators of MCP-1 and the role of cell-cell interactions. We cocultured lipopolysaccharide-activated macrophages with fibroblasts and exposed them to their culture media containing MCP-1 or control PBS. Expression of IL-6 by ELISA was not detected in the culture medium of macrophages alone or fibroblasts alone; however, secretion of IL-6 by macrophages-fibroblasts coculture was significantly upregulated. MCP-1 compared with control PBS demonstrated significantly increased secretion of IL-6 in culture medium ( $P=0.029$ ; Figure 1A).

### IL-6 Is Expressed in MCP-1-Treated Murine Aneurysms and Healed Human Coiled Aneurysms

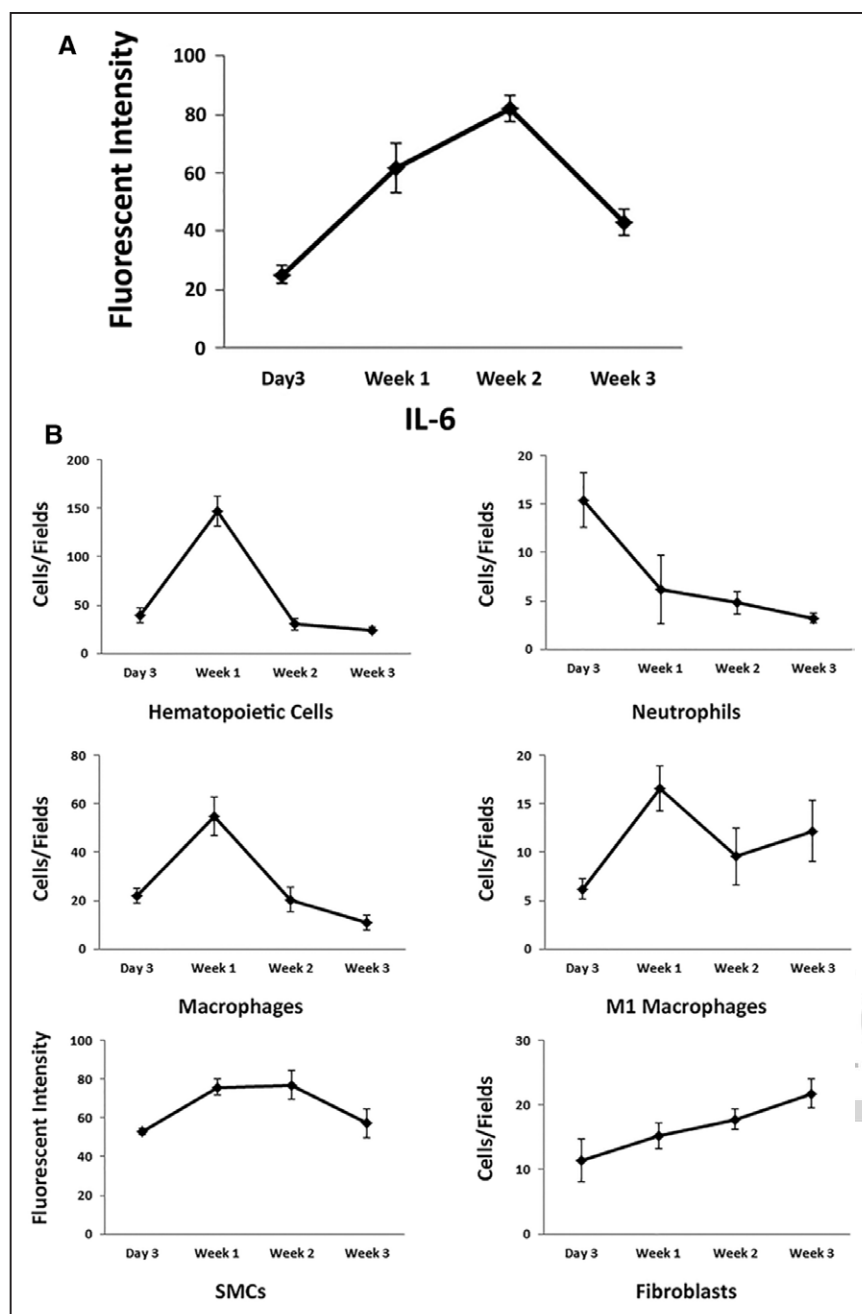
Cytokine array analysis was performed on MCP-1-coil-treated murine carotid aneurysms compared with control PLGA-only-coil-treated murine carotid aneurysms 3 weeks after coil treatment and demonstrated significantly increased IL-6 expression in the MCP-1-treated aneurysms compared with PLGA-only-treated aneurysms ( $P=0.01$ ; Figure 1B). Immunofluorescent staining demonstrates IL-6 expression in the intra-aneurysmal tissue ingrowth (n=5; Figure 1C). Human CAs that have been previously coiled (n=2) demonstrate MCP-1 and IL-6 expression in the tissue ingrowth of the coiled part of the aneurysm, but not in human superficial temporal artery (n=5; Figure 2, Figure I and Table I in the online-only Data Supplement).

### IL-6 Induces Migration of Endothelial Cells and Fibroblasts and Not SMCs or Macrophages

Healed human CAs are characterized by inflammatory cells: fibroblasts, SMCs, endothelial cells, and macrophages.<sup>4</sup> IL-6 chemotaxis of fibroblasts, SMCs, endothelial cells, and macrophages was studied in in vitro cell migration assays with IL-6 and control serum-free medium. Cell migration assays demonstrate that IL-6 has a recruitment effect on the migration of endothelial cells (245086.1±9266.3 pixel versus 322835.4 versus 10720.9 pixel;  $P=0.01$ ) and fibroblasts (103259.5±10307.8 pixel versus 144382.4±3741.7 pixel;  $P=0.01$ ; Figure 3A). However, there were no effects on migration of SMCs and macrophages.

### Temporal Cascade of MCP-1-IL-6-Mediated Intra-Aneurysmal Healing

To define the temporal cascade of MCP-1-IL-6-mediated intra-aneurysmal healing, MCP-1-coil-treated murine carotid



**Figure 4.** Temporal cascade of inflammatory cells during monocyte chemoattractant protein-1 (MCP-1)—interleukin (IL)-6–mediated intra-aneurysmal healing in mice. Immunofluorescent staining and analysis on 3-d, 1-wk, 2-wk, and 3-wk murine MCP-1–releasing coil–treated aneurysm tissues were performed. **A**, IL-6 expression peaks 2 wk after coil treatment. **B**, Hematopoietic cells including macrophages appear as early as 3 d and peak 1 wk after coil treatment. Neutrophils peak at day 3 and decrease after. Smooth muscle cells (SMCs) peak 1 wk after coil treatment, but number of fibroblasts increases and peaks at 3 wk.

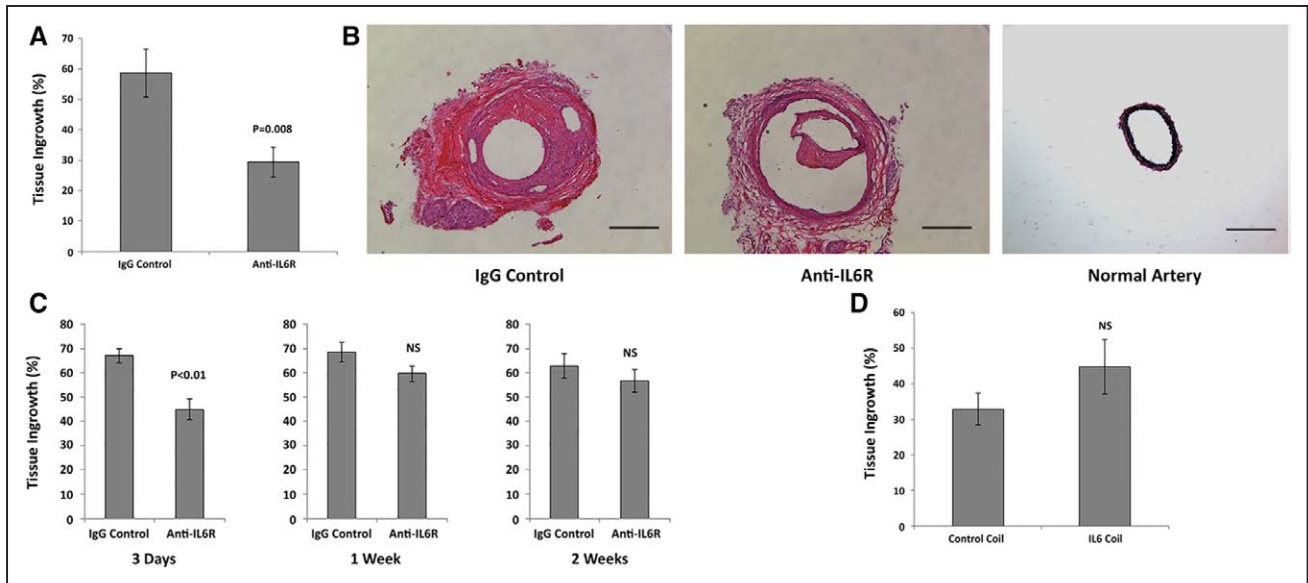
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aneurysms were harvested at 3 days, 1 week, 2 weeks, and 3 weeks after coil treatment, and immunofluorescent analysis was performed. IL-6 expression appeared at 3 days, reached highest levels at 2 weeks, and decreased after. CD45-positive hematopoietic cells, including neutrophils and macrophages, peaked at 1 week and then decreased after. Fibroblasts peaked at 3 weeks, and SMCs peaked at 2 weeks (Figure 4).

### IL-6 Receptor Blockade Inhibits MCP-1–Mediated Intra-Aneurysmal Healing

Mice harboring carotid aneurysms treated with MCP-1–releasing coils were administered an antibody antagonist of IL-6 receptor (IL-6R) or IgG control. Inhibition of IL-6R by antibody significantly reduced MCP-1–mediated aneurysm tissue healing compared with control ( $58.6 \pm 7.9\%$

versus  $29.3 \pm 4.9\%$ ;  $P=0.008$ ; Figure 5A and 5B). To investigate the temporal sequence of when IL-6 is necessary during MCP-1–mediated intra-aneurysmal healing, IL-6R was blocked at different time points (3 days, 1 week, and 2 weeks after MCP-1-coil treatment). There was significantly decreased tissue ingrowth when IL-6R was blocked 3 days after coil implant ( $67.1 \pm 2.8\%$  versus  $44.9 \pm 4.3\%$ ;  $P<0.01$ ; Figure 5C). When IL-6R was blocked at both 1 and 2 weeks after the coil implant, however, the effect was noticeably smaller. Although there was no statistically significant difference at these later time periods, some biological inhibitory effects were noted. These results suggest that IL-6 has a more significant role in the earlier stages of MCP-1–mediated aneurysm healing as opposed to healing occurring after 1 to 2 weeks.



**Figure 5.** Monocyte chemoattractant protein-1 (MCP-1)-mediated aneurysm healing is interleukin (IL)-6 dependent. C57BL/6 mice harboring aneurysms implanted with MCP-1-releasing coils were administered an antibody antagonist of IL-6 receptor (anti-IL-6R; 200  $\mu$ g/mL/injection,  $n=10$  each) or IgG control ( $n=10$  each). **A**, IL-6R-blocked aneurysms had significantly reduced tissue ingrowth compared with IgG control. **B**, Hematoxylin and eosin staining of representative sections of MCP-1-releasing coil implanted aneurysms with anti-IL-6R, IgG control, or normal artery. Scale bar, 200  $\mu$ m. **C**, Temporal sequence of IL-6R blockade revealed that IL-6 has significant role in MCP-1-mediated aneurysm healing between 3 d and 1 wk after coil implant ( $n=10$  each). **D**, C57BL/6 mice harboring aneurysms implanted with IL-6-releasing coils did not improve intra-aneurysmal tissue healing ( $n=15$  each).

### IL-6 Does Not Promote Aneurysm Healing Independent of MCP-1

Murine carotid aneurysms were treated with IL-6-releasing coils or PLGA-only coils, and no significant difference in intra-aneurysmal tissue healing was seen. These results suggest that MCP-1-mediated inflammatory aneurysm healing is IL-6 dependent, but IL-6 does not promote aneurysm healing independent of MCP-1 (Figure 5D).

### Osteopontin Is a Downstream Mediator in the MCP-1-IL-6 Pathway of Intra-Aneurysmal Tissue Healing

To identify downstream mediators of the MCP-1-IL-6 aneurysm-healing pathway, cytokine array analysis was performed on MCP-1-coil-treated murine carotid aneurysms from mice administered anti-IL-6R or control IgG. Osteopontin expression was significantly decreased at 1 week after IL-6R blockade ( $P=0.001$ ; Figure 6A). The role of osteopontin in the MCP-1-IL-6 pathway was demonstrated by osteopontin blockade in MCP-1-coil-treated mice. MCP-1-coil-treated murine carotid aneurysms from osteopontin-blocked mice have significantly decreased intra-aneurysmal tissue ingrowth compared with control IgG-treated mice ( $62.5\pm 10.7\%$  versus  $33.3\pm 10.1\%$ ;  $P=0.028$ ; Figure 6B).

### Osteopontin Promotes Aneurysm Healing Independent of MCP-1

Murine carotid aneurysms were treated with osteopontin-releasing coils or PLGA-only coils, and osteopontin-releasing coil induced significantly greater aneurysmal tissue healing compared with the PLGA control coil ( $19.7\pm 3.7\%$  versus  $53.7\pm 5.2\%$ ;  $P=0.001$ ; Figure 6C). This suggests that

osteopontin promotes intra-aneurysmal healing independent of MCP-1. Human CAs that have been previously coiled demonstrate osteopontin expression in the tissue ingrowth of the coiled part of the aneurysms ( $n=2$ ) (Figure 6D; Figure I and Table I in the online-only Data Supplement).

### Discussion

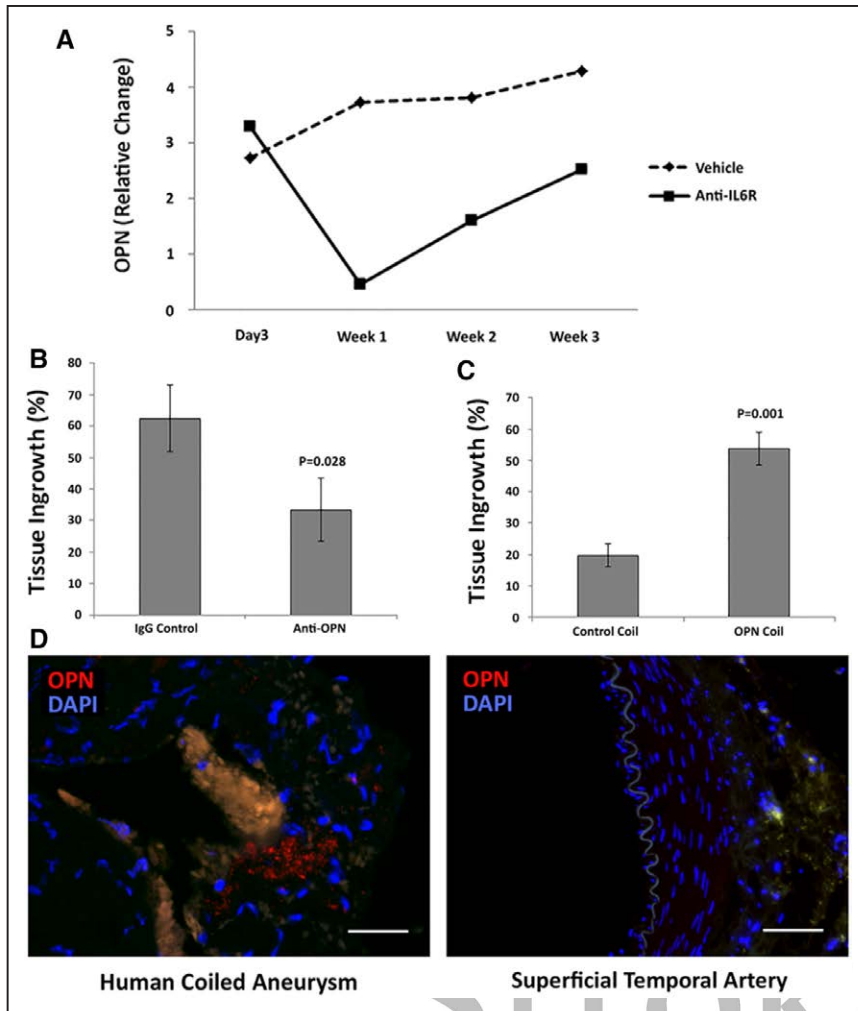
CAs occur in up to 5% of the general population, which is equal to up to 15 million people in the United States.<sup>1-3</sup> CA ruptures cause subarachnoid hemorrhages, which comprise up to 7% of all strokes<sup>14</sup> and result in 50% death or dependency. CAs can be treated endovascularly,<sup>15-18</sup> and after endovascular treatment, some CAs are healed.<sup>19-23</sup> However, not all CAs can be treated completely or recanalization of CAs can occur because of defective filling by coil or healed tissue.<sup>16</sup> The mechanisms of aneurysm healing after endovascular coiling are thought to be similar to other models of wound healing.

The earliest stage of the repair response is dominated by the inflammatory phase. During the inflammation and cell proliferation process of tissue healing, inflammatory cells, and other types of cells secrete chemokines such as MCP-1<sup>24</sup> to recruit adaptive immune cells to the site of healing. In addition, a number of cytokines, such as IL-6, have been primarily characterized as mediators of inflammatory and immunomodulatory reactions.<sup>25</sup>

### MCP-1, IL-6, and Osteopontin in Tissue Healing

MCP-1 is known as a mediator that recruits monocytes in several inflammation models and diseases.<sup>26,27</sup> It is also found in high levels in the early phase of wound repair.<sup>28,29</sup> MCP-1 is secreted by mononuclear cells and various vascular cells, such as endothelial cells and vascular SMCs.<sup>10,30</sup> The other





**Figure 6.** Monocyte chemotactic protein-1 (MCP-1)-interleukin (IL)-6-mediated aneurysm healing is osteopontin (OPN) dependent. **A**, Cytokine array analysis on 3-d, 1-wk, 2-wk, and 3-wk murine MCP-1-releasing coil-treated with IL-6R-blocked aneurysm tissues was performed. IL-6R blockade decreased expression of OPN in MCP-1-releasing coil-treated aneurysm (n=5 each). **B**, MCP-1-coil-treated murine aneurysms from mice administered an antibody antagonist of OPN (100  $\mu$ g/mL/injection; n=10) had significantly reduced intra-aneurysmal tissue ingrowth compared with the control group (n=10). **C**, OPN-releasing coil-treated aneurysms (n=15) have significantly increased aneurysm healing compared with PLGA-control-coil-implanted aneurysm (n=15;  $P=0.001$ ). **D**, Immunofluorescent staining of human healed aneurysms expresses OPN in the tissue ingrowth of the coiled part of the aneurysm. DAPI=nuclei. Scale bar, 50  $\mu$ m.



cytokine that has a crucial role in wound repair and healing is IL-6. IL-6 deficiency has been shown to delay and reduce wound healing,<sup>11,31</sup> which is caused by lack of matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinases (TIMP) modulation by IL-6.<sup>32</sup> IL-6 is expressed extracellularly according to our results (Figures 1C and 2B). IL-6 is known to be expressed by a variety of cell types, but the most important sources are macrophages and monocytes at inflammatory sites. Because we have confirmed monocyte/macrophage infiltration in our MCP-1-mediated aneurysm-healing model, it can be seen that they are the main source of IL-6 in the healed aneurysmal tissues. IL-6 has also been shown to have a role in the growth and differentiation of numerous cell types.<sup>33</sup> Recent studies suggest that IL-6 is involved in leukocyte trafficking and controlling the transition from innate to acquired immune responses.<sup>34</sup> MCP-1 and IL-6 have been thought to work independently, but recent studies have shown that they are codependent.<sup>35–37</sup> IL-6 has been shown to be a strong MCP-1 inducer in peripheral blood mononuclear cells.<sup>12,38,39</sup> Interestingly, on the other hand, MCP-1 is able to induce IL-6 release by human epithelial cells.<sup>35</sup> These observations suggest that MCP-1 and IL-6 can stimulate each other and are dependent on the environment and stage of tissue healing. We did not see significant tissue healing by IL-6-releasing coil independent of MCP-1. This may be

because IL-6 may not recruit a sufficient number of monocytes to the site of the aneurysm. IL-6 is an essential cytokine and has a key role in tissue healing; however, other chemotactic cytokines, specifically MCP-1, need to be present first at the site of injury. Osteopontin is expressed and secreted by cells in a variety of tissues, including vascular tissues, as well as in activated macrophages and lymphocytes. Osteopontin expression is observed in both pathological and pathophysiological conditions, and its synthesis can be induced in SMCs in various diseases.<sup>40</sup> Similar to our results in IL-6 expression, osteopontin in both our murine model and human samples was observed as an extracellular-expressing cytokine, and we were not able to identify which cell types were expressing it. Osteopontin is known as a secreted chemokine-like protein and an intracellular signaling complex that regulates cell adhesion, migration, and bone regeneration.<sup>41–43</sup> Although the detailed mechanism is not clear yet, it has been shown that osteopontin expression in fibroblasts leads to wound healing and fibrosis.<sup>44</sup> Osteopontin works as both a proinflammatory and anti-inflammatory cytokine during injury and tissue remodeling. Osteopontin has a key role in chemotaxis for monocytes/macrophages by supporting their adhesion as a proinflammatory cytokine.<sup>45</sup> Another key role of osteopontin is the recruitment and regulation of fibroblasts. In osteopontin

-null mice, the healed wound showed disorganization and a reduced number of collagen fibers.<sup>46,47</sup>

We observed that previously coiled human healed aneurysms express IL-6 and osteopontin. Bavinzski et al<sup>4</sup> showed that after the endovascular coiling treatment in human CAs, successful healing occurs in some cases. In our mouse model, however, we have not seen successful healing by PLGA-only coil implantation. This may be why we observed IL-6 and osteopontin expression in only MCP-1-releasing coil implanted murine aneurysms.

### MCP-1-Mediated Aneurysm Healing Is Associated With IL-6 and Osteopontin Expression

Intra-aneurysmal tissue healing is the desired outcome of brain aneurysm therapies. We have demonstrated that MCP-1 has an inducing effect on the migration of inflammatory cells, which have key roles in the inflammatory tissue healing response.<sup>6</sup> In the present study, we demonstrate that IL-6 and osteopontin may have key roles in MCP-1-mediated aneurysm healing. Osteopontin has been reported to correlate with the expressions of IL-6.<sup>13,48</sup> Osteopontin can be induced in monocytes/macrophages after stimulation by IL-6 and other cytokines.<sup>49</sup> On the contrary, a study shows that osteopontin could upregulate the expression of IL-6.<sup>50</sup> These observations could explain the change of osteopontin expression by blocking IL-6 in aneurysm healing. Based on our studies, local delivery of MCP-1 via an MCP-1-releasing coil to aneurysms may cause the following intra-aneurysm healing/tissue ingrowth cascade: (1) MCP-1 initiates the induction of monocytes/macrophage and other inflammatory cells' recruitment and migration; (2) MCP-1 upregulates IL-6 expression in inflammatory cells and epithelial cells; (3) IL-6 induces osteopontin expression and secretion in macrophages and other inflammatory cells; and (4) osteopontin recruits monocytes/macrophages and fibroblasts, and acts as both pro- and anti-inflammatory cytokine. This suggests that the MCP-1-mediated healing pathway is regulated by IL-6 and osteopontin. Our result also suggests that osteopontin can work independently as an aneurysm-healing cytokine.

### Summary

Here, we demonstrate that intra-aneurysmal MCP-1-mediated inflammatory tissue healing is dependent on IL-6 and osteopontin. When IL-6 or osteopontin was blocked, the tissue ingrowth was significantly decreased. Correlation between MCP-1 and IL-6 in tissue healing has been shown, however, the pathway by which MCP-1 is dependent on osteopontin has not been shown and therefore requires further study.

### Limitations

In this study, we used a murine carotid aneurysm model. The carotid artery and cerebral artery have clear histological differences and therefore their respective responses to injury may differ. Coil-implanting experiments for research regarding CAs in animals have been replicated by other research groups using the carotid artery, as it is not possible to implant coils in cerebral arteries. Also, murine immunologic response to injury and healing differ from that of humans. Our results may

not be directly translated into treatment for human aneurysms, but the concept of the MCP-1-mediated aneurysm repair cascade is similar to that of CA healing in humans.

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### Disclosures

None.

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## Monocyte Chemotactic Protein-1–Interleukin-6–Osteopontin Pathway of Intra-Aneurysmal Tissue Healing

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Aneurysmal Tissue Healing**

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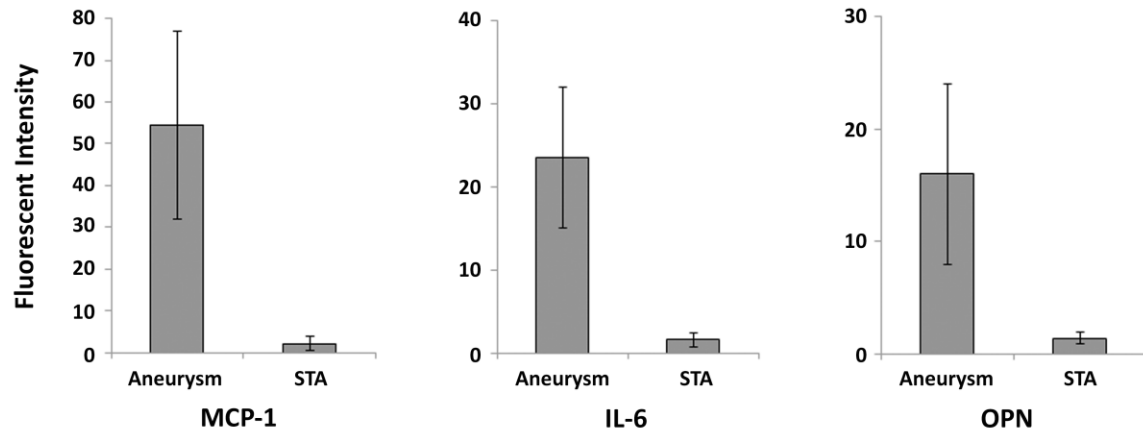
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Supplemental Table I. Immunostaining of human coiled aneurysms and control arteries

Sample	MCP-1	IL-6	OPN
Human Coiled Aneurysm #1	+	+	+
Human Coiled Aneurysm #2	+	+	+
STA #1	-	-	-
STA #2	-	-	-
STA #3	-	-	-
STA #4	-	-	-
STA #5	-	-	-

STA: Superficial Temporal Artery, +: positive, -: negative



**Supplemental Figure I. Quantification of MCP-1, IL-6 and OPN expression in human coiled aneurysms and control arteries.**

Immunofluorescent staining and analyses of human coiled aneurysms (n=2) and control STA (n=5) samples were performed. MCP-1, IL-6 and OPN expression were observed in human coiled tissues, but not in STA samples (MCP-1: 54.5 +/- 22.5 vs 2.2 +/- 1.6; IL-6: 23.5 +/- 8.5 vs 1.6 +/- 0.9; OPN: 16.0 +/- 8.0 vs 1.4 +/- 0.5). Representative images are shown in Figure 2 (MCP-1 and IL-6) and Figure 6 (OPN). STA: superficial temporal artery