DATA SUPPLEMENT

Neutrophil extracellular traps (NETs) promote the development of intracranial aneurysm rupture

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Expanded Materials & Methods

Histology

For the quantitative analysis of NETs and neutrophils, we collected the brain tissue samples from the mice treated with vehicle or Cl-amidine at 8 days after aneurysm induction (n = 5 from each group). The mice were transcardially perfused with phosphate-buffered saline (PBS), followed by 4% of paraformaldehyde. The brain tissues were post-fixed with 4% paraformaldehyde for 24 hours, then immersed in 15% sucrose for 24 hours, 30% sucrose for another 24 hours. The brain tissues were embedded in optimal cutting temperature compound (*OCT,* Tissue-Tek) at -80˚C. Sections were cut near the middle cerebral artery with a cryostat. Sections were immunohistochemically stained with antibodies for NETs (citrullinated histone H3 (H3-Cit), ab5103, Abcam, Cambridge, MA) and neutrophils (Ly-6G, BD, San Jose, CA). Sections were incubated in primary antibody overnight at 4°C, followed by incubation with corresponding biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA) and with a complex of avidin-biotin-horseradish peroxidase (Vector Laboratories). Immunoreactivity was visualized by incubating the sections with 0.05% 3,3'-diaminobenzidine (DAB, Vector Laboratories). Nuclei were visualized by counterstaining with aqueous hematoxylin. We chose the field with the highest number of cells around the middle cerebral artery for each sample and quantified the number of the infiltrated cells by cell counting (positive immunohistochemically stained cells) per high-power field (×40).

Real-time PCR detection of pro-inflammatory cytokines

We collected total RNA samples from cerebral arteries (Circle of Willis, including aneurysms) 8 days after aneurysm induction as previously described.[1, 2] We chose 8 days after elastase injection for these analyses to capture the molecular and histological changes that occur before aneurysms start rupturing. Our previous studies showed that aneurysms start rupturing around 7 - 8 days after aneurysm induction.[3, 4] We collected the aneurysm tissues from mice that were treated with Cl-amidine or vehicle (n = 7 for each group). We measured mRNA expression levels of inflammation-related cytokines (ICAM-1 [intracellular adhesion molecule 1], IL-1β [interleukin-1 beta], MCP-1 [monocyte chemoattractant protein-1], TNF-α [tumor necrosis factor-α], CXCL-1 [chemokine (CXC motif) ligand-1], MIP-2 [macrophage inflammatory protein-2], and E-selectin. RNA was extracted using the RNeasy Mini Kit (Qiagen, Germantown, MD) and transcribed to cDNA using the QuantiTect reverse transcription kit (Qiagen). The mRNA expression levels were determined using SYBR Green technology (Applied Biosystems, Foster City, CA). Quantitative values were obtained from the threshold cycle value (Ct), and the data were analyzed by the $2^{-\Delta\Delta}$CT method. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression was quantified and used as an internal RNA control.

References

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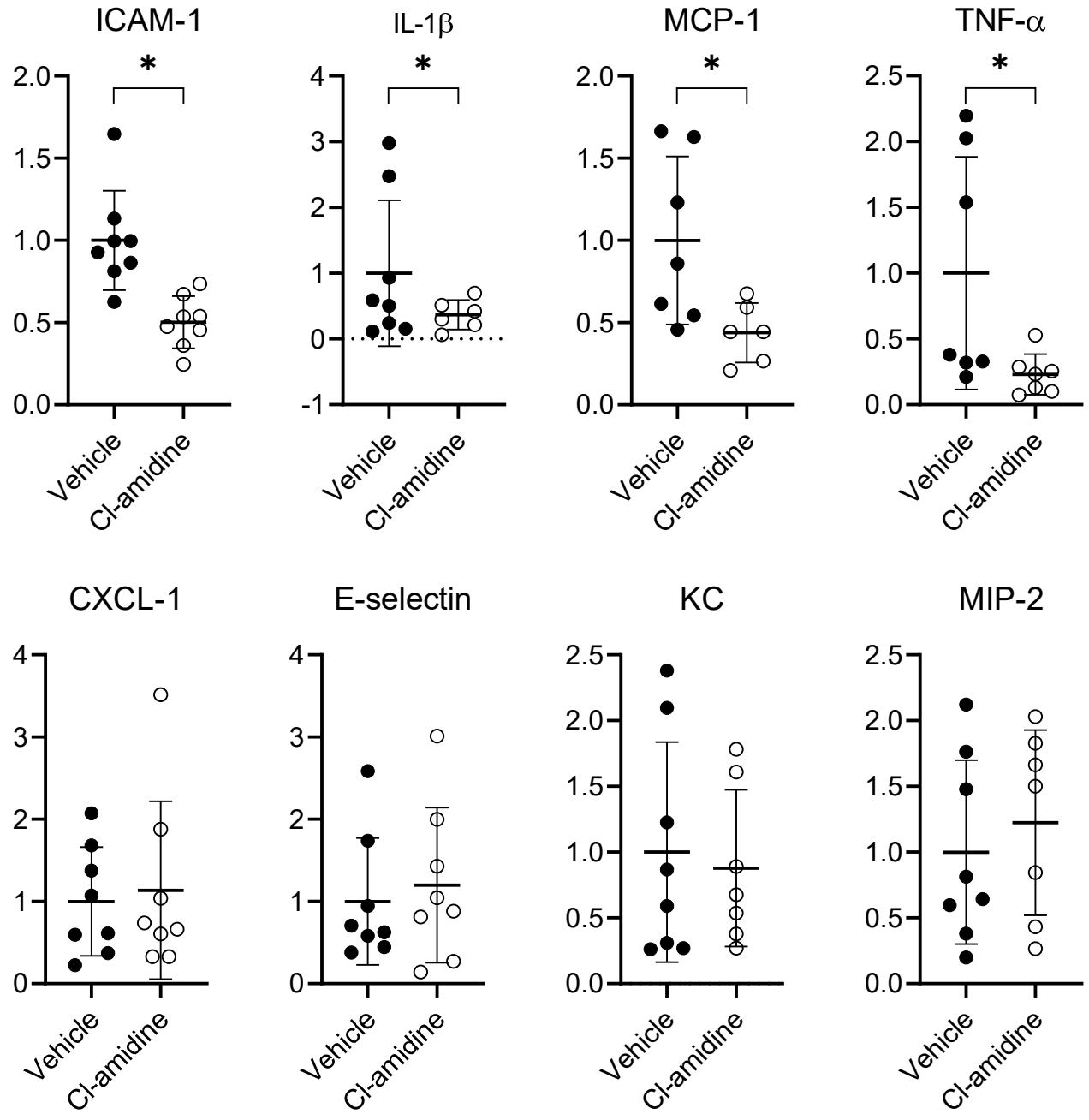

Figure S1. Pharmacological inhibition of NETs formation decreased mRNA expression of pro-inflammatory cytokines in cerebral arteries. Cl-amidine-treated mice had significantly decreased mRNA expression of ICAM-1, IL-1β, MCP-1 and TNF-α as compared to vehicle-treated controls. No significant difference was detected on the mRNA expression of CXCL-1, E-selectin, KC and MIP-2. Data are expressed as means ± standard deviation, the Mann-Whitney test, $* P < 0.05$.

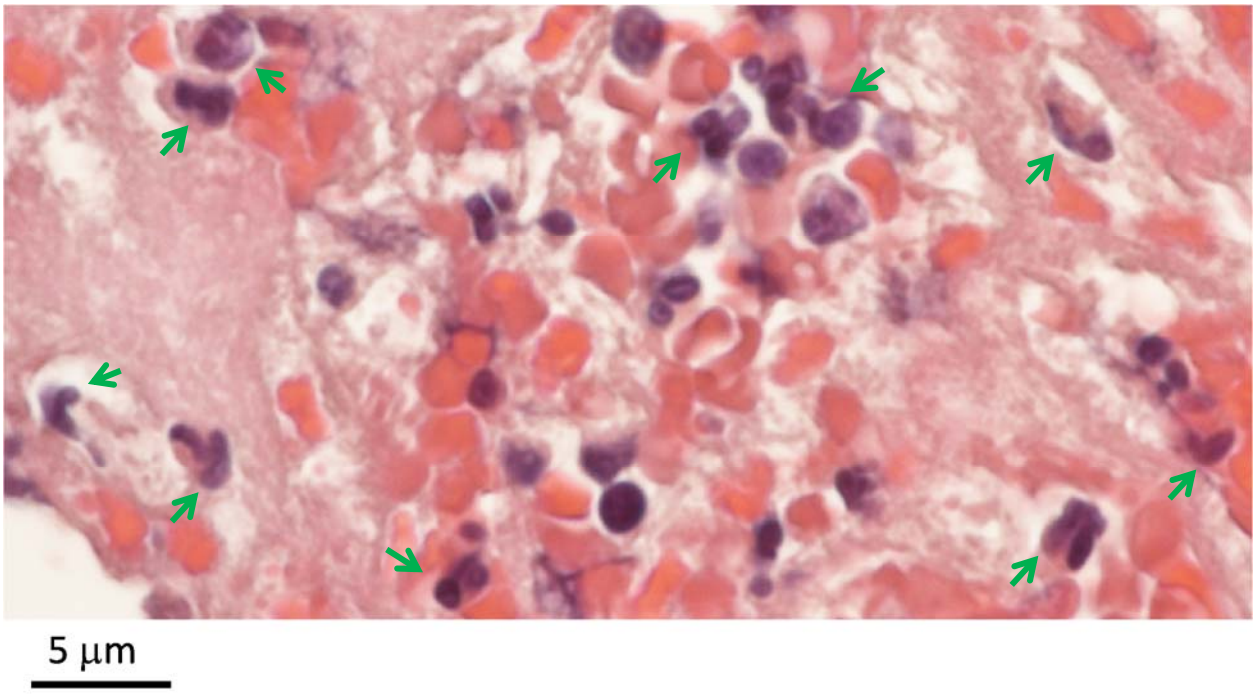

Figure S2. Aggregation of polymorphonuclear cells in artery wall from an unruptured human aneurysm. The image is from the same area marked in the left panel-upper image in Figure 1A (hematoxylin and eosin staining). The area was positively stained by H3Cit (NETs marker), and largely overlapping with MPO staining (neutrophils marker), but not with CD68 (macrophages marker, See Figure 1A). Polymorphonuclear cells (presumably neutrophils) are indicated with green arrows.