Adventitial CXCL1/G-CSF Expression in Response to Acute Aortic Dissection Triggers Local Neutrophil Recruitment and Activation Leading to Aortic Rupture

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Rationale: In-hospital outcomes are generally acceptable in patients with type B dissection; however, some patients present with undesirable complications, such as aortic expansion and rupture. Excessive inflammation is an independent predictor of adverse clinical outcomes.

Objective: We have investigated the underlying mechanisms of catastrophic complications after acute aortic dissection (AAD) in mice.

Methods and Results: When angiotensin II was administered in lysyl oxidase inhibitor–preconditioned mice, AAD emerged within 24 hours. The dissection was initiated at the proximal site of the descending thoracic aorta and propagated distally into an abdominal site. Dissection of the aorta caused dilatation, and ≈70% of the mice died of aortic rupture. AAD triggered CXCL1 and granulocyte-colony stimulating factor expression in the tunica adventitia of the dissected aorta, leading to elevation of circulating CXCL1/granulocyte-colony stimulating factor levels. Bone marrow CXCL12 was reduced. These chemokine changes facilitated neutrophil egress from bone marrow and infiltration into the aortic adventitia. Interference of CXCL1 function using an anti-CXCR2 antibody reduced neutrophil accumulation and limited aortic rupture post AAD. The tunica adventitia of the expanded dissected aorta demonstrated high levels of interleukin-6 (IL-6) expression. Neutrophils were the major sources of IL-6, and CXCR2 neutralization significantly reduced local and systemic levels of IL-6. Furthermore, disruption of IL-6 effectively suppressed dilatation and rupture of the dissected aorta without any influence on the incidence of AAD and neutrophil mobilization.

Conclusions: Adventitial CXCL1/granulocyte-colony stimulating factor expression in response to AAD triggers local neutrophil recruitment and activation. This leads to adventitial inflammation via IL-6 and results in aortic expansion and rupture. (Circ Res. 2015;116:612-623. DOI: 10.1161/CIRCRESAHA.116.304918.)

Key Words: angiotensin II ■ aortic dissection ■ chemokines ■ inflammation ■ interleukin-6 ■ neutrophils

An acute aortic dissection (AAD) is initiated by an intimal tear, with resultant propagation within the middle third of the medial layer of the aorta.1 To delineate treatment, the Stanford classification divides AAD into 2 types, type A and type B. Type A affects the ascending aorta, whereas type B does not. Type A AAD is more severe because of the higher mortality rate of 20% by 24 hours, 30% by 48 hours, 40% at 1 week, and 50% at 1 month.2 Thus, surgical repair is the first choice of treatment for patients with type A AAD to prevent life-threatening complications, including aortic rupture and cardiac tamponade. Although type B AAD is generally more benign and medical treatment for high blood pressure and intolerant pain can improve the patient’s clinical outcome, a substantial proportion of medically treated patients still encounter catastrophic events within 7 days, such as aortic expansion and subsequent aortic rupture, visceral ischemia, and lung oxygenation impairment.3 Thoracic endovascular repair with stent grafting is the emerging therapeutic strategy.
for patients with aortic dissection; however, the effect of stent grafting during acute and subacute type B AAD is still controversial. Thus, better understanding of the molecular and cellular mechanisms involved in the AAD-associated processes and the search for a novel therapeutic approach for patients with type B AAD outside of the currently used antihypertensive and analgesic strategies are matters of great importance.

Peak C-reactive protein is a strong predictor of adverse early and late complications in patients with type B AAD. Peak serum C-reactive protein concentration during hospitalization is positively correlated with the magnitude of aortic enlargement and oxygenation impairment, resulting in poor in-hospital prognosis for patients with distal AAD. Furthermore, an increased peak serum C-reactive protein level and fluorodeoxyglucose uptake in the dissected aorta, evidenced by positron emission tomography, predict worse long-term clinical outcomes. These data suggest that systemic activation of the inflammatory system after AAD plays a key role in the development of catastrophic complications. However, the underlying molecular and cellular mechanisms remain largely unknown because of the lack of suitable animal models.

Classically, long-term angiotensin II (Ang II) infusion is used to induce spontaneous AAD in apolipoprotein E−/− mice or aged mice. AAD is observed only by chance in these models; so, they are not applicable for the critical research needed to predict the onset of dissection and to investigate how inflammatory response initiates and progresses after AAD. We recently established a novel mouse model wherein AAD develops in 100% of cases within 24 hours after Ang II administration in mice. This model is dependent on preconditioning with the lysyl oxidase inhibitor β-aminopropionitrile monofumarate (BAPN) to create a pre-AAD status in immature mice. Collagen and elastin cross-links, which are critical for maintaining vessel wall integrity, are disrupted by BAPN administration, leading to the generation of a mechanically fragile aorta. This type of pre-AAD status is typically seen in human connective tissue diseases, such as Ehlers–Danlos syndrome, but also arises secondary to aging and atherosclerosis. As such, our mouse model would seem to recapitulate a similar state in which suitable triggers, such as Ang II, precipitate the transition from a pre-AAD status to AAD. Therefore, our mouse AAD model recapitulates key features of human aortic dissection and facilitates in vivo investigation of the natural disease course after its onset, although the acute catastrophic complication is rare in humans. In this study, we have investigated the molecular and cellular mechanisms involved in the systemic activation of the inflammatory system after AAD. Our aim is to find novel, potential therapeutic targets for preventing aortic enlargement and subsequent lethal rupture after AAD using our mouse model of AAD.

Methods

A detailed Methods section describing all procedures and protocols is available in the Online Data Supplement.

Results

Propagation of Dissection After the Onset of AAD

Time course examination of serial computed tomographic scan imaging, enhanced with a contrast agent, revealed that AAD invariably emerged in BAPN/Ang II–treated mice as early as several hours after Ang II infusion. It was initiated at the proximal site of the descending thoracic aorta and propagated distally into the abdominal aorta (Figure 1A). The dissected thoracic aorta increased in diameter with time, resulting in hematothorax and lethal rupture (Figure 1B). Approximately 70% of mice died of aortic rupture within 48 hours, whereas no BAPN-untreated mice died after Ang II infusion (Figure 1C).

Aortic Dissection Promotes Rapid Mobilization of Neutrophils to the Aortic Tunica Adventitia

Histological analysis of the expanded dissected aorta from BAPN/Ang II–treated mice demonstrated massive inflammatory cell accumulation in the tunica adventitia (Figure 2A). Such inflammatory cell accumulation in the tunica adventitia was not observed in the nondissected aorta from BAPN-untreated/Ang II–treated mice (Figure 2A). Immunohistochemical analysis revealed that Ly6B.2+ neutrophils dominated the tunica adventitia of dissected aortas, whereas Mac3+ macrophages and CD3+ T cells were relatively minor populations (Figure 2B). Flow cytometric analysis to investigate the temporal dynamics of inflammatory cell accumulation in the dissected aorta of BAPN/Ang II–treated mice revealed that CD45+CD11b+Ly6G+ neutrophils increased as early as 24 hours after Ang II infusion, followed by CD45+CD11b+ F4/80+ macrophage and CD45+CD11b−CD3+ T cell infiltration (Figure 2C and 2D). Neutrophils were the predominant cell type infiltrating the dissected aorta by 48 hours after Ang II infusion. This inflammatory cell accumulation was not observed in the nondissected aorta from BAPN-untreated/Ang II–treated mice. Of note, these temporal and spatial characteristics of the leukocyte accumulation pattern were consistent with previously reported human AAD pathology.

Interleukin-6–Producing Neutrophils Accumulate to High Levels at the Edge of the Rupture Site

We next examined the temporal changes of proinflammatory cytokine gene expression in the dissected aorta. The gene expression levels of interleukin-6 (IL-6), IL-1β, and tumor necrosis factor-α in the dissected aorta from BAPN/Ang II–treated mice increased, peaked at 24 hours after Ang II infusion, and decreased thereafter (Figure 3A–3C). In contrast, Ang II infusion did not augment the aortic gene expression of these proinflammatory cytokines in the nondissected aorta from BAPN-untreated mice (Figure 3A–3C). The serum level of IL-6 increased in BAPN/Ang II–treated mice, peaking at 24 to 48 hours after Ang II infusion (Figure 3D).
Immunofluorescent analysis revealed that high levels of IL-6 were primarily observed in the tunica adventitia of the expanded dissected aorta from BAPN/Ang II–treated mice, whereas the nondissected aorta from BAPN-untreated/Ang II–treated mice showed negligible expression (Figure 3E-3H). Notably, IL-6+ neutrophils were highly accumulated at the edge of the rupture site (Figure 4D). Intracellular staining of a single-cell suspension from a dissected aorta demonstrated that the majority of IL-6–producing CD45+ leukocytes were Ly6G+ neutrophils (Figure 4E). Further immunofluorescence analysis revealed that smooth muscle actin (SMA+) myofibroblasts and F4/80+ CD206+ macrophages were the main sources of CXCL1 (Figure 4F). In parallel with temporal dynamics of gene expression in the dissected aorta, serum CXCL1 and G-CSF concentrations rapidly increased and peaked at 24 hours after Ang II infusion in BAPN-treated mice, whereas serum CXCL2 concentrations did not increase in response to AAD (Figure 4G-4I). In contrast, Ang II increased vascular cell adhesion molecule-1 expression in the aorta regardless of the presence or absence of BAPN preconditioning (Online Figure IA and IB).

**AAD Facilitates Mobilization of Neutrophils From Bone Marrow**

The percentage of peripheral blood CD11b+ Ly6G+ neutrophils in BAPN-treated mice was elevated at 24 hours and peaked at 48 hours after Ang II infusion (Figure 5A and 5B). It was higher than that in BAPN-untreated/Ang II–treated mice at any time. On the other hand, temporal changes in the percentage of blood Ly6Cint and Ly6Cinh monocytes were comparable between BAPN/Ang II–treated mice and BAPN-untreated/Ang II–treated mice (Figure 5C and 5D).

Bone marrow (BM) serves as a reservoir for neutrophils that can be rapidly mobilized in response to CXCL1, CXCL2, and G-CSF released from injured peripheral tissues. To examine the BM environment in response to AAD, flow cytometric analysis of BM cells was conducted. Interestingly, CD45+...
CD11b+ Ly6G+ mature neutrophils were transiently reduced at 24 hours after Ang II administration into BAPN-treated mice (Figure 7A and 7B). This result indicated rapid neutrophil egress from the BM after the onset of AAD. Previous studies demonstrated that neutrophil release from the BM to the blood depends on the balance between the CXCL1/2-CXCR2 and CXCL12-CXCR4 axes. The CXCR2 ligands, CXCL1 and CXCL2, promote neutrophil egress, whereas the function of the CXCR4 ligand, CXCL12, retains neutrophils in the BM. G-CSF stimulates proliferation of myeloid precursors and promotes neutrophil release from the BM by reducing the BM fluid levels of CXCL12 produced by BM stromal cells. Notably, the fluorescent intensity of CXCR2 was significantly increased in BM CD45+ CD11b+ Ly6G+ neutrophils in response to AAD (Figure 6C–6E). In contrast, the protein concentration of CXCL12 in BM fluids was significantly reduced in response to AAD (Figure 6F). These results indicated that the BM milieu was changed by aortic injury in favor of neutrophil egress from the BM. Moreover, an adoptive transfer strategy using 2 different mouse strains with a congenic marker (CD45.1+ versus CD45.2+) confirmed that intravenously transferred BM-derived neutrophils accumulated in the tunica adventitia of the dissected aorta of recipient mice (Figure 6G and Online Figure IIA–IIC), supporting our hypothesis that BM-derived neutrophils infiltrate the dissected aorta via peripheral circulation.

**Serum Levels of IL-6 and Neutrophil Chemoattractants Increase After the Onset of Acute Type B Aortic Dissection in Human Patients**

We examined the temporal dynamics of change in serum IL-6, CXCL8 (which corresponds to murine CXCL1), and
G-CSF in patients with uncomplicated acute type B aortic dissection. The protocol for human blood sampling and patient characteristics are described in the Methods section in the Online Data Supplement and Online Table I. Serum IL-6 concentrations in patients with AAD consistently increased after the onset of AAD (Online Figure IIIB and IIIC). Consistent with the results in our mouse model of AAD, immunofluorescent analysis of human aortic autopsy samples obtained from patients with AAD demonstrated enhanced accumulation of IL-6--producing neutrophils in the tunica adventitia at the edge of the rupture site (Online Figure IIID). Collectively, these human data suggest the existence of a common pathway leading to aortic expansion and subsequent rupture.

Figure 3. Acute aortic dissection triggers local and systemic inflammatory responses by neutrophils. A–C, Time course of interleukin (IL)-6 (A), IL-1β (B), and tumor necrosis factor (TNF)–α (C) mRNA expression in the aorta of angiotensin II (Ang II)–infused vehicle- and β-aminopropionitrile monofumarate (BAPN)–treated mice. D, Temporal change of serum IL-6 concentration in BAPN-treated mice before and after Ang II infusion (n=4–5 per group). *P<0.05 and **P<0.01 vs baseline of the group. E, Hematoxylin and eosin (HE, top) or Elastica van Gieson (EVG, bottom) stained sections of ruptured aorta. Arrows indicate rupture sites. Scale bar, 500 μm. F, Immunofluorescence staining of neutrophils and IL-6 using serial sections from E. Scale bar, 500 μm. G, Higher magnification of the rectangle in F. Cells stained yellow indicate IL-6–expressing neutrophils. H, Immunofluorescent-staining of neutrophils and IL-6 in the nondissected aorta from BAPN-untreated/Ang II–treated mice. I, Intracellular staining of IL-6 in single cells from a dissected aorta. Adv indicates tunica adventitia.
Neutrophil Influx Occurs as a Result of Aortic Dissection

To provide direct evidence that the dissection and not the combined BAPN/Ang II treatment causes neutrophilia, we analyzed the neutrophil behavior at earlier time points in mice with and without aortic dissection. BAPN/Ang II–treated mice were analyzed at 6 and 12 hours after Ang II infusion. CXCL1 and G-CSF gene expressions were induced in the dissected aorta but not in the nondissected aorta (Figure 7A and 7B). The percentage of blood neutrophils increased in mice with aortic dissection but not in mice without aortic dissection (Figure 7C and 7D). Furthermore, immunohistochemical analysis revealed neutrophil accumulation in the tunica adventitia of mice with aortic dissection but not in mice without aortic dissection (Figure 7E and 7F). These data indicate that neutrophil mobilization from the BM to the blood and subsequent neutrophil accumulation in the aortic adventitia occur as a result of the onset of aortic dissection.

CXCR2 Neutralization Reduces Neutrophil Mobilization and Limits Aortic Rupture After AAD

To determine whether neutrophil mobilization from BM plays a causative role in lethal aortic rupture in our mouse model of AAD, BAPN/Ang II–treated mice were administered intraperitoneally with either a neutralizing anti-CXCR2 antibody or control IgG. CXCR2 neutralization did not affect AAD incidence and systolic blood pressure at 48 hours after Ang II infusion (Online Table II). The number of neutrophils in blood and aorta was significantly suppressed in the dissected mice treated with neutralizing anti-CXCR2 antibody (Figure 8A). Flow cytometric analysis showed that almost all CXCR2high circulating blood cells during the progression of AAD were Ly6G+ CD11b+ neutrophils (Figure 8B). The incidence of death was significantly reduced in BAPN/Ang II–treated mice receiving neutralizing anti-CXCR2 antibody (Figure 8C). Contrast-enhanced computed tomographic scanning at 48 hours after Ang II infusion revealed that both dissection length and maximum length of
IL-6–Mediated Adventitial Inflammation Plays a Causative Role in Aortic Expansion and Rupture After the Onset of AAD

The tunica adventitia of the expanded dissected aorta from BAPN/Ang II–treated mice displayed high levels of IL-6 expression, whereas the nondissected aorta from BAPN-untreated/Ang II–treated mice showed negligible expression (Figure 3E–3H). The majority of IL-6–producing leukocytes in the dissected aorta were Ly6G+ neutrophils (Figure 3I). Strong expression of IL-6 on neutrophils was observed at the edge of the rupture site in mice and humans with AAD (Figure 3E–3G and Online Figure IIIID). Of note, aortic IL-6 gene expression and serum IL-6 concentrations were significantly attenuated in BAPN/Ang II–treated mice with CXCR2 neutralization compared with control IgG treatment (Figure 8F and 8G). These results prompted us to speculate that blockade of neutrophil migration from BM by neutralizing an anti-CXCR2 antibody prevents progression of aortic dissection and lethal aortic rupture, at least in part via the inhibition of IL-6–mediated adventitial inflammation.

To determine whether IL-6 plays a causative role in the lethal complications after AAD onset, IL-6−/− mice were subjected to BAPN and Ang II treatment. Importantly, AAD developed successfully in BAPN-treated IL-6−/− mice with an incidence of 100% within 24 hours after Ang II infusion, and systolic blood pressure was comparable between BAPN/Ang II–treated littermate controls and BAPN/Ang II–treated IL-6−/− mice (Online Table II). There were no significant differences between BAPN/Ang II–treated IL-6−/− mice and BAPN/Ang II–treated littermate controls in the degree of peripheral neutrophilia or percentage of accumulated neutrophils in the dissected aorta (Online VA and VB). Nevertheless, the magnitude of aortic dilatation and the incidence of death were significantly reduced in BAPN/Ang II–treated IL-6−/− mice compared with BAPN/Ang II–treated littermate controls (Figure 8H and 8I).

Discussion

Several previous studies, including our own,12 have attempted to elucidate the mechanisms initiating AAD using a mouse model of aortic dissection and rupture.11,20,21 In BAPN/Ang II–treated mice, neutrophils infiltrate the aortic intima, invariably triggering aortic dissection via metalloproteinase-9 production.12 The sequence of events leading to aortic dissection in this model may well reflect those that underly a similar aortic pathology in humans. Indeed, serum Ang II concentrations are significantly higher in patients with AAD compared with patients with acute myocardial infarction, and metalloproteinase-9–producing neutrophils have been shown to infiltrate the intima on tearing in the inner layer of the aorta. To the best of our knowledge, this study is the first to examine aortic behavior after AAD onset and has revealed as yet unrecognized cellular and molecular pathways leading to aortic expansion and subsequent rupture.

In our mouse model of AAD, dissection was usually initiated at the proximal site of the descending thoracic aorta and propagated distally into the abdominal aorta for 24 to 48 hours. The dissected aorta caused dilatation, and ≈70% of the mice...
ultimately died of aortic rupture. We found that the aortic expansion and rupture were associated with massive neutrophil accumulation in the tunica adventitia of the dissected aorta. Such neutrophil accumulation was not observed in nondissected aorta from BAPN-untreated/Ang II–treated and even from BAPN-treated/Ang II–treated mice, indicating that the adventitial neutrophil infiltration occurred as a consequence of AAD.

An increased local expression of CXCL1 and G-CSF in adventitial cells, such as activated myofibroblasts and resident macrophages, preceded the recruitment of neutrophils into the postdissected aorta and induced systemic changes. These included an increased concentration of circulating CXCL1 and G-CSF and decreased concentration of BM CXCL12, which altered BM milieu in favor of neutrophil release from the BM to peripheral circulation. Unlike CXCL1, serum CXCL2 concentration was not augmented after AAD despite a significant increase in CXCL2 gene expression in the dissected aorta, suggesting distinct functions of CXCL1 and CXCL2 in post-AAD inflammatory processes similar to those in other acute inflammatory conditions. In several acute and chronic inflammatory disease models, blockade of CXCL1 receptor, CXCR2, can substantially attenuate tissue damage via inhibition of neutrophil recruitment. We demonstrated that administration of the CXCR2-neutralizing antibody effectively suppressed the expansion and rupture of the dissected aorta via preventing neutrophil egress from BM and neutrophil infiltration into the tunica adventitia. Together, these findings indicated that chemokine signaling is a key factor for neutrophil mobilization from the BM to the tunica adventitia of the dissected aorta. We previously reported that neutrophil depletion by injection of antigranulocyte-differentiation antigen-1 antibody significantly decreased the incidence of AAD in our mouse model. In contrast, we showed in this study that CXCR2 neutralization did not affect the prevalence of AAD. We attribute this conflicting result to the following reasons.

Figure 6. Neutrophil mobilization from the bone marrow (BM) on acute aortic dissection. A, Representative flow cytometric dot plots for CD11b and Ly6G in CD45+ BM cells harvested from vehicle- and β-aminopropionitrile monofumarate (BAPN)–treated mice before and 24 hours after angiotensin II (Ang II) infusion. B, Time course of the percentage of BM neutrophils in BAPN-treated mice before and after Ang II infusion (n=3–4 per group). C, Representative flow cytometric dot plots for Ly6G and CXCR2 in CD45+ CD11b+ BM cells obtained from BAPN-treated mice before and 24 hours after Ang II infusion. Dotted frames indicate BM neutrophils, and solid frames indicate CXCR2+ BM neutrophils. The number next to each solid frame corresponds to the percentage of total CXCR2+ cells in BM neutrophils. D, The percentage of CXCR2+ cells in BM CD45+ CD11b+ Ly6G+ neutrophils in indicated groups (n=4 per group). E, Comparison of mean fluorescent intensity (MFI) for CXCR2 in CD45+ CD11b+ Ly6G+ BM neutrophils from the indicated groups (n=4 per group). F, CXCL12 concentration in BM fluid from the indicated groups (n=3–4 per group). G, Schematic diagram of the experimental protocol showing that BM-derived neutrophils harvested from CD45.2+ nondissected donor mice were administered intravenously into CD45.1+ BAPN–treated mice that had received Ang II for 24 hours and flow cytometric dot plots of single living cells from dissected aorta of recipient CD45.1+ animals showing CD45.2 and Ly6G expression 3 hours after the cell transfer. Rectangle indicates donor-derived neutrophils in the dissected aorta. *P<0.05 vs baseline (B) and **P<0.05 vs other groups (D–F).
First, unlike antigranulocyte-differentiation antigen-1 antibody, the CXCR2-neutralizing antibody never decreased the peripheral blood neutrophil count below the normal range. Second, attachment of neutrophils along the aortic intima, which triggers the onset of AAD, might be independent of CXCR2 signaling.

Although the G-CSF responses were more consistent than the CXCL1 responses (Figure 4A and 4C; Figure 7A), the antibody against G-CSF had a marginal protective effect in terms of the incidence of aortic rupture, despite the fact that neutrophil egress from BM and neutrophil infiltration in the tunica adventitia were suppressed to the same degree as after CXCR2 neutralization. CXCR2 is almost exclusively expressed by neutrophils, whereas G-CSFR is expressed by both hematopoietic and nonhematopoietic cells. In the setting of acute myocardial infarction, administration of G-CSF has beneficial effects on early ventricular function by prevention of cardiomyocytes and endothelial cell apoptotic death in the infarcted heart. This is conferred by G-CSF signaling in the G-CSFR-expressing cardiomyocytes and endothelial cells. These findings suggest that G-CSFR signaling in cells other than infiltrating neutrophils may play a protective role in the dissected aorta.

Data presented here highlight the importance of aortic tunica adventitia in the post-AAD inflammatory response. Although a traditional inside-out mechanism was considered central to the progress of various vascular diseases, in which inflammatory cells migrate into the tissue through the injured intima and accelerate vascular injury, accumulating evidence suggests the outside-in theory is more important to initiate and advance inflammatory reactions, at least in several disease models. A coordinated link between tunica intima and adventitia in AAD might be similar to that in the coronary artery after balloon angioplasty. In this setting, inflammatory cues, such as intimal minor dissection, elastic lamina tear or stretching after intravascular ballooning, trigger transient expression of an adventitial neutrophil chemotactic and promote neutrophil accumulation in the tunica adventitia. This eventually

Figure 7. Neutrophil influx occurs as a result of aortic dissection. A and B, CXCL1 and granulocyte-colony stimulating factor (G-CSF) mRNA expression in β-aminopropionitrile monofumarate (BAPN)-preconditioned mice with and without acute aortic dissection (AAD) 6 (A) and 12 (B) hours after angiotensin II (Ang II) infusion. C and D, Percentage of blood neutrophils in BAPN-preconditioning mice with and without AAD 6 (C) and 12 (D) hours after Ang II infusion. *P<0.05. E and F, Immunohistochemical images for neutrophils in the aorta from BAPN-preconditioning mice with and without AAD 6 (E) and 12 (F) hours after Ang II infusion. Adv indicates tunica adventitia; and PL, pseudolumen.
promotes vascular inflammation toward the intima and thereby neointimal formation. We speculate that aortic wall stretching associated with pseudolumen formation activates the conversion of fibroblasts into smooth muscle actin (SMA⁺) myofibroblasts and stimulates F4/80⁺ CD206⁺ resident macrophages in the tunica adventitia. This results in strong CXCL1 production that leads to neutrophil accumulation. Indeed, it was previously demonstrated that myofibroblasts converted...
from resident fibroblasts in response to tissue injury were one of the major sources of chemokines. Moreover, resident macrophages are recognized as sentinels; they sense tissue damage and promote neutrophil mobilization by production of neutrophil-recruiting chemokines, such as CXCL1. These data suggest that these cell populations are potent initiators of the adventitial inflammatory response after AAD.

IL-6 is secreted at high levels in human aortic aneurysm disease and may be associated with risk of aneurysm rupture. A recent publication showed that increased IL-6 production contributes to aortic extracellular matrix degeneration and expansion in an aortic aneurysm setting, independent of monocyte recruitment and subsequent differentiation of macrophages. Previous animal studies have demonstrated that long-term Ang II promoted IL-6 expression in the aorta and triggered AAD. In contrast, IL-6 was not required for AAD induction in our mouse model. This discrepancy may be because of difference in the animal model and that sustained administration of the lysyl oxidase inhibitor, BAPN, is sufficient to induce medial degeneration even in the absence of IL-6. We showed that IL-6 was markedly expressed in the tunica adventitia of the expanded dissected aorta and that neutrophils were major producers of IL-6 in the dissected aorta. Notably, strong production of IL-6 by adventitial neutrophils was observed at the edge of the rupture site in mice and humans with AAD. Furthermore, inhibition of neutrophil infiltration with CXCR2 neutralization significantly reduced local and systemic IL-6 levels. Intriguingly, disruption of IL-6 effectively suppressed dilatation and rupture of the dissected aorta without any influence on the incidence of AAD. Although IL-6 can drive emergency myelopoiesis under other inflammatory disease conditions, the dissected aorta lacking IL-6 showed only a modest decrease in neutrophil infiltration compared with the control dissected aorta, suggesting that IL-6 plays a dispensable role in neutrophil recruitment to the aorta during AAD progression.

Together, these data indicate that neutrophil-derived IL-6 is a potent enhancer of the post-AAD adventitial inflammation that leads to aortic rupture, and thus, an IL-6 inhibitor could potentially have a therapeutic effect in patients with AAD.

The limitations of this study include the low patient numbers and the lack of complicated patients with type B aortic dissection. The human data only suggest that the response to dissection may be similar to that in the murine model and not that there is a common pathway leading to subsequent rupture. A prospective study is needed that focuses on serum levels of CXCL8, G-CSF, and IL-6 in early and late complications of type B aortic dissection. We investigated the mechanism behind the acute catastrophic aortic dilatation and rupture in a murine AAD model, which is rare in humans. Moreover, the effect of end-organ damage on the pathological changes in the aorta was ignored.

In conclusions, this study showed that dissection per se induced neutrophil chemokine expression in the aortic tunica adventitia, possibly by mechanical injury and stretching followed by pseudolumen formation. Subsequent systemic changes in chemokine-dependent signaling caused neutrophilia and massive neutrophil accumulation in the dissected aorta, thereby leading to aortic expansion and rupture via IL-6 production (summarized in Online Figure VII). Importantly, temporal and spatial dynamics of inflammatory cytokine and chemokine elevation, as well as leukocyte recruitment, were consistent between rodents and humans. Our study provides a new mechanistic insight into neutrophil-mediated adventitial inflammation after AAD and suggests novel therapeutic approaches for patients with AAD.

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Disclosures
None.

References

What Is Known?

- Although type B acute aortic dissection (AAD) is generally benign, a substantial proportion of medically treated patients still encounter catastrophic events, such as aortic enlargement and subsequent aortic rupture.
- Elevation of C-reactive protein, an indicator of inflammation, is a strong predictor of an adverse outcome in patients with type B AAD.
- The molecular and cellular mechanisms involved in AAD-associated inflammatory processes remain unexamined.

What New Information Does This Article Contribute?

- Adventitial CXCL1/granulocyte-colony stimulating factor production in response to AAD triggers neutrophil mobilization from the bone marrow and subsequent neutrophil accumulation in the aortic tunica adventitia in mice.
- CXCR2 neutralization reduces neutrophil mobilization and limits aortic enlargement and rupture after AAD.
- Interleukin-6, predominantly produced by neutrophils, plays a causative role in aortic enlargement and rupture after the onset of AAD.

Novelty and Significance

Type B AAD spares the ascending aorta and is optimally managed by medical therapy in the absence of complications. However, patients with enhanced inflammation sometimes present with aortic enlargement, thereby facing undesirable outcomes. Thus, a better understanding of the molecular and cellular mechanisms involved in AAD-associated inflammatory processes and the requirement for a novel therapeutic approach for patients with type B AAD are unmet clinical needs. This study showed that dissection per se induced neutrophil-chemoattractant chemokine expression in the aortic tunica adventitia, possibly by mechanical injury and stretching followed by pseudolumen formation. Subsequent systemic changes in chemokine-dependent signaling caused neutrophilia and massive neutrophil accumulation in the dissected aorta, thereby leading to aortic enlargement and rupture via interleukin-6 production. Importantly, temporal and spatial dynamics of inflammatory cytokine and chemokine elevation, as well as leukocyte recruitment, were consistent between rodents and humans. Our study provides a new mechanistic insight into neutrophil-mediated adventitial inflammation after AAD and implicates CXCR2- or interleukin-6 neutralization as novel therapeutic strategies to prevent large-artery complications, including aneurysm formation and rupture, in patients with type B AAD.
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Supplemental Methods

Mice and In Vivo Experimental Strategies
All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the Keio University School of Medicine. Wild-type C57BL/6 mice (CD45.2⁺) were purchased from CLEA Japan (Tokyo, Japan), and B6.SJL congenic mice on a C57BL/6 background (CD45.1⁺) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Interleukin (IL)-6⁻/⁻ mice¹ on a C57BL/6 background (CD45.2⁺) were kindly provided by RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. We created AAD in the indicated mice as previously described.² In brief, after giving β-aminopropionitrile monofumarate (BAPN) at 1 g/kg/day (Sigma-Aldrich, St. Louis, MO, USA) via a gastric tube to 3-week-old immature male mice for 3-4 weeks, 1 µg/kg/min Ang II was continuously infused subcutaneously for 72 hours using a micro-osmotic mini pump (Alzet, Cupertino, CA, USA). To prevent neutrophil mobilization from the bone marrow (BM) and subsequent neutrophil recruitment to injured sites in response to aortic dissection, either rat anti–mouse neutralizing monoclonal antibody for CXCR2 (R&D systems, Minneapolis, MN, USA) or granulocyte-colony stimulating factor (G-CSF, R&D systems) was administered to BAPN-treated mice. Anti-CXCR2 antibody was administered intraperitoneally at a dose of 20 μg/mouse 1 hour prior to, and 24 hours after, the Ang II infusion. Anti-G-CSF antibody was administered intraperitoneally at a dose of 50 μg/mouse every day for 2 days commenced 24 hours before Ang II infusion. In these neutralizing experiments, appropriate rat isotype antibodies were used as controls.

Contrast-Enhanced Computed Tomography (CT) Scan Imaging
CT imaging, enhanced using a contrast agent, was used to interrogate the location, length, and diameter of the dissected aorta in indicated animals. Immediately after intravenously injecting a dose of 70 µl/mouse of ExTron nano 12000 (Miltenyi Biotec, Sunnyvale, CA, USA) to deeply anesthetized mice, CT imaging was obtained using a preclinical Micro-CT II (Rigaku Co., Tokyo, Japan)³ with respiratory synchronization at the indicated time points. Maximum length of minor axis on the transverse image was measured was evaluated using OsiriX Imaging Software (OsiriX).

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)
Total RNA was isolated by the acid-phenol extraction method in the presence of chaotropic salts (Trizol, Invitrogen, Carlsbad, CA) and subsequent isopropanol-ethanol precipitation, as described previously.⁴ Reverse transcription was performed using a Super-Script First-Strand Synthesis System (Invitrogen) in accordance with the manufacturer’s protocol. Quantitative RT-PCR was performed
using the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). Predesigned gene-specific primer and probe sets (Taqman Gene Expression Assays, Applied Biosystems) were used. Data were normalized using the expression levels of mouse 18S ribosomal RNA.

**Human Blood Sampling**

Between August 2013 and May 2014, 5 consecutive patients admitted to our institute with newly diagnosed, uncomplicated acute type B aortic dissection, and 7 consecutive patients with chronic non-ruptured thoracic aortic aneurysm indicated for elective surgical repair, were registered in the study. Patient characteristics are shown in Online Table I. All patients were free from connective tissue diseases such as Marfan syndrome, Ehlers-Danlos syndrome, and aortitis based on their clinical history and physical examination. All the uncomplicated acute type B aortic dissection patients were managed with adequate pain relief and aggressive blood pressure control using a β-blocker and vasodilators, with a target systolic blood pressure of 100 to 120 mmHg. There were no signs of complications during the time course of the study. A blood sample from uncomplicated acute type B aortic dissection patients was collected within 1 hour of arrival at the hospital emergency room, followed by daily blood sampling until discharge. Blood samples from patients with chronic non-ruptured thoracic aortic aneurysm were collected before surgical operation. Human samples were collected in the Keio University Hospital. Signed informed consent for sample use in these experiments was obtained from all subjects. This study of human samples was approved by the ethics committee of the Keio University School of Medicine.

**Morphological Assessment**

The murine aorta was carefully excised, fixed in formalin, embedded in paraffin, and cut into 5-µm-thick sections. Sections were stained with hematoxylin and eosin (HE) or Elastica van Gieson (EVG) to assess gross morphology. Adjacent sections were used for additional immunohistochemical staining and immunofluorescence. Aortic samples were obtained from autopsied patients who died of aortic dissection. These sections were stained with HE or EVG and used for additional immunohistological evaluations. All histological analyses on human samples were approved by the research ethics board at the Keio University School of Medicine.

**Immunohistochemical Staining and Immunofluorescence**

Endogenous peroxidase activity was inhibited prior to sections being incubated with primary anti-Ly6B.2, anti-CD3 (Serotec, Oxford, UK), anti-Mac3 (BD Bioscience, San Jose, NJ, USA), or anti-CXCL1 (Abcam, Cambridge, MA, USA), antibodies at 4°C, overnight. After the incubation, a Vectastain ABC elite Kit (Vector Laboratories, Burlingame, CA, USA) was used according to the
manufacturer’s instruction. Following visualization with 3,3’-diaminobenzidine, the sections were finally counterstained with hematoxylin. For detecting neutrophil accumulation in human dissected aortas, anti-neutrophil elastase (Dako, Glostrup, Denmark) was used. The number of each leukocyte subset was assessed by counting the total cell number in at least 20 randomly chosen fields in the indicated samples. For immunofluorescence, serial paraffinized and frozen sections were blocked using Block Ace (DS Pharma Biomedical Co. Ltd., Osaka, Japan) at 37°C for 30 min, and then incubated with purified anti-Ly6B.2, anti-F4/80 (Serotec), purified anti-CD206 (BioLegend, San Diego, CA, USA), purified anti-IL-6, purified anti-CD45.1 (Abcam), purified anti-G-CSF (Santa Cruz Biotech, Santa Cruz, CA, USA), purified anti-G-CSF receptor (G-CSFR, Santa Cruz Biotech), and purified anti-smooth muscle actin (SMA, Sigma) at 4°C overnight followed by incubation with the appropriate secondary antibodies (Molecular Probes, Carlsbad, CA, USA) for 30 min at room temperature. Nuclei were identified with DAPI. Negative controls underwent the same procedure without the primary antibody. The sections were finally photographed under a confocal laser-scanning microscope (TCS-SP5, Leica).

Cell Isolation
At the indicated time points, mice were deeply anesthetized and intracardially perfused with ice-cold phosphate buffered saline (PBS) to remove blood cells before euthanasia. The murine aorta was dissected, minced with fine scissors, and enzymatically digested with a cocktail of type II collagenase (Worthington Biochemical Corporation, Likewood, NJ, USA), elastase (Worthington Biochemical Corporation), and DNase I (Sigma) for 1 hour at 37°C with gentle agitation. After digestion and subsequent red blood cell lysis using ACK lysis buffer solution (0.15 M NH₄Cl, 1.0 mM KHCO₃, 0.1 mM Na₂EDTA [pH 7.2]), the tissue was triturated and passed through a 70-μm-pore cell strainer. Peripheral blood and BM cells were prepared as previously described³.

Flow Cytometry
Single cell suspensions harvested from aorta, BM, and peripheral blood were analyzed by flow cytometry. To block nonspecific binding of antibodies to Fcy receptors, isolated cells were first incubated with anti-CD16/32 antibody (BD Bioscience) at 4°C for 5 min. Subsequently, the cells were stained with a mixture of the following antibodies at 4°C for 25 min: anti-Ly6G-PE, anti-CD45-PE, anti-CD45-FITC, anti-CD45.1-PE, anti-Ly6C-APC, anti-CD3-APC (BD Bioscience), anti-Ly6G-APC, anti-CD11b-FITC, anti-CD45.2-APC, anti-CXCR2-APC (BioLegend), anti-CD11b-PerCP-Cy5.5, and anti-F4/80-APC (eBioscience, San Diego, CA, USA). Dead cells were identified with 7-amino-actinomycin D (Sigma-Aldrich, St. Louis, MO, USA). For intracellular staining of IL-6, cell suspensions were incubated in the presence of GolgiStop (BD Bioscience) for 2 hours at 37°C, 5% CO₂ and then fixed and permeabilized with BD Cytofix/Cytoperm (BD Bioscience) according to the
manufacturer’s instruction. Flow cytometry was performed on a FACSCalibur (BD Bioscience) and the data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

**Adoptive Transfer of Bone Marrow-Derived Neutrophils**
To obtain BM-derived neutrophils, a BM cell suspension was incubated with biotin-conjugated anti-Ly6G antibody (BioLegend) at 4°C for 10 min, followed by incubation with streptavidin-conjugated microbeads (Miltenyi Biotec, Sunnyvale, CA, USA) at 4°C for 15 min. Ly6G⁺ neutrophils were positively collected by magnetic sorting with AutoMACS (Miltenyi Biotec). The purity of cells was generally >95%. The isolated cells were suspended in 200 μl of PBS and transferred intravenously into BAPN-treated mice 24 hours after the Ang II infusion.

**Serum Analysis**
Serum CXCR2 ligand (i.e., CXCL1 and CXCL2 for mice and CXCL8 for human), G-CSF, IL-6, and tumor necrosis factor-α concentrations were quantified in the indicated mice and humans using Quantikine ELISA kits (R&D Systems) according to the manufacturer’s instructions.

**Blood Pressure Measurement**
Blood pressure was monitored in indicated mice with the noninvasive tail cuff CODA System (Kent Scientific Co., Torrington, CO, USA). Temporal change in systolic blood pressure in the WT mice treated with control, BAPN alone, Ang II alone, BAPN + Ang II was shown in Online Figure VI.

**Statistical Analyses**
All data were expressed as mean ± SEM. Comparisons between groups were performed using the Wilcoxon rank sum test. Comparisons of more than two groups used the Kruskal-Wallis test followed by Bonferroni’s post hoc test. Dissection length was analyzed by Fisher’s exact test. Survival distributions were estimated using the Kaplan-Meier method and compared by the log-rank test. A $P$ value of < 0.05 was considered significant. All statistical analyses were performed with GraphPad Prism 5.0 (Graph Pad Prism Software Inc, San Diego, CA, USA) and SPSS 15.0 for Windows (SPSS, Inc, Chicago, IL, USA).
Supplemental References


## Online Table I. Patient Characteristics

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<th>TAA (n = 7)</th>
<th>AAD (n = 5)</th>
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<tr>
<td>Age (years)</td>
<td>75.4 ± 2.6</td>
<td>62.8 ± 6.2</td>
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<tr>
<td>Male gender, n (%)</td>
<td>5 (71)</td>
<td>3 (60)</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>4 (57)</td>
<td>3 (60)</td>
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<tr>
<td>Diabetes Mellitus, n (%)</td>
<td>1 (14)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Dyslipidemia, n (%)</td>
<td>2 (29)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>History of smoking, n (%)</td>
<td>7 (100)</td>
<td>4 (80)</td>
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<tr>
<td>Elapsed time from onset, min</td>
<td>N/A</td>
<td>96.6 ± 28.6</td>
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<td>Medication after admission</td>
<td></td>
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<tr>
<td>Beta-blocker, n (%)</td>
<td>2 (29)</td>
<td>5 (100)</td>
</tr>
<tr>
<td>Calcium-channel blocker, n (%)</td>
<td>6 (86)</td>
<td>5 (100)</td>
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<tr>
<td>ACE-I/ARB, n (%)</td>
<td>5 (71)</td>
<td>3 (60)</td>
</tr>
<tr>
<td>Statin, n (%)</td>
<td>3 (43)</td>
<td>3 (60)</td>
</tr>
<tr>
<td>Analgesic agent, n (%)</td>
<td>0 (0)</td>
<td>5 (100)</td>
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TAA, Thoracic aortic aneurysm; AAD, Acute aortic dissection; N/A, not applicable; ACE-I, Angiotensin converting enzyme-inhibitor; ARB, Angiotensin receptor blocker.

Data are expressed as mean ± SEM.
Online Table II. Means and standard errors of systolic blood pressure in BAPN/Ang II-treated mice 48 hours after Ang II infusion

<table>
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<th>SBP (mmHg)</th>
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<td>Control IgG (n = 4)</td>
<td>121.5 ± 2.6</td>
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<td>anti-CXCR2 (n = 4)</td>
<td>123.2 ± 3.1</td>
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<td>anti-G-CSF (n = 4)</td>
<td>123.6 ± 4.4</td>
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<td>Control littermate (n = 4)</td>
<td>121.7 ± 2.2</td>
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<tr>
<td>IL-6&lt;sup&gt;−/−&lt;/sup&gt; (n = 4)</td>
<td>120.9 ± 2.9</td>
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SBP; Systolic blood pressure, BAPN; β-aminopropionitrile monofumarate, Ang II; Angiotensin II, G-CSF; Granulocyte-colony stimulating factor, IL; Interleukin.

Data are expressed as mean ± SEM. Systolic blood pressure of BAPN-treated Ang II-untreated mice was 90.2 ± 2.9 mmHg (n=4).
Supplemental Figure Legends

**Online Figure I. A and B,** Time course of mRNA expression of intracellular adhesion molecule (ICAM)-1 (A) and vascular cell adhesion molecule (VCAM)-1 (B) in the aorta of angiotensin (Ang) II-infused vehicle- and β-aminopropionitrile monofumarate (BAPN)-treated mice. Data were obtained from 6 to 10 independent experiments at each time point. **P < 0.01 vs. baseline of the group.

**Online Figure II. A,** Representative image of the dissected aorta from CD45.2+ mice which received intravenous adoptive transfer of CD45.1+ bone marrow-derived neutrophils. Asterisk indicates true lumen and PL indicates pseudolumen. Scale bar indicates 100 μm. **B,** High magnification view of a yellow rectangle in A, showing adoptively transferred CD45.1+ neutrophils in tunica adventitia of the dissected aorta. Scale bar indicates 50 μm. **C,** High magnification view of a red rectangle in A, showing adoptively transferred CD45.1+ neutrophils in pseudolumen of the dissected aorta. Scale bar indicates 50 μm.

**Online Figure III. A-C,** Serial change in serum interleukin (IL)-6 (A), CXCL8 (B), and granulocyte-colony stimulating factor (G-CSF) (C) levels in patients with acute aortic dissection (AAD, n = 5) compared to those with thoracic aortic aneurysm (TAA, n = 7). *P < 0.05 and **P < 0.01 vs. the data in patients with TAA. **D,** Representative image of double immunofluorescence for neutrophil elastase and IL-6 in an aortic autopsy sample from a patient with AAD. Scale bar indicates 50 μm.

**Online Figure IV.** Representative photograph of immunofluorescent staining for neutrophils (red) and G-CSF receptor (G-CSFR, green) in the tunica adventitia of a dissected aorta. White arrows indicate G-CSFR+ cells other than neutrophils.

**Online Figure V. A,** Representative flow cytometric dot plots for the dissected aorta and peripheral blood of angiotensin (Ang) II-infused β-aminopropionitrile monofumarate (BAPN)-treated interleukin (IL)-6+/− mice and littermate controls. Rectangles indicate CD11b+ Ly6G+ neutrophils. **B,** Percentage of blood and aorta neutrophils in Ang II/BAPN-treated IL-6+/− mice and littermate controls. (n = 4 per group).

**Online Figure VI.** Temporal change in systolic blood pressure in the WT mice treated with control, BAPN alone, Ang II alone, BAPN + Ang II (n = 4 per group). WT, wild type; BAPN, β-aminopropionitrile monofumarate; Ang II, angiotensin II.

**Online Figure VII. Schematic diagram illustrating the importance of tunica adventitia in post-acute aortic dissection (AAD) inflammatory response.**
1). AAD initiation; neutrophils that release matrix metalloproteinase (MMP)-9 attach along the aortic intima trigger the initiation of AAD.

2). Adventitial cell activation; dissection per se induced neutrophil chemoattractant expression in the tunica adventitia of the dissected aorta, possibly by mechanical injury and stretching followed by pseudolumen formation.

3). Adventitial neutrophil infiltration and aortic expansion; neutrophil accumulation and interleukin (IL)-6 production in the tunica adventitia caused vascular inflammation by the outside-in mechanism, leading to vascular wall fragility.

4). Aortic rupture

TL indicates true lumen; PL, pseudo lumen; Int, tunica intima; Med, tunica media; Adv, tunica adventitia; G-CSF, granulocyte-colony stimulating factor.
Online Figure I

A

**ICAM-1**

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B

**VCAM-1**

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** **
Online Figure II

A. Dissected aorta of CD45.2+ mice

B. Adventitia

C. Psuedolumen
Online Figure III

A

**Serum IL-6 (pg/ml)**

B

**Serum CXCL8 (pg/ml)**

C

**Serum G-CSF (pg/ml)**

D

DAPI  IL-6 Neutrophil
(in adventitia of human dissected aorta)
Online Figure IV
Online Figure V

A

CD11b

Ly6G

Control

IL-6-/-

Aorta

Blood

47.7

39.7

13.4

15.8

Control IL-6-/-

Aorta Blood

NS

NS

B

CD11b+ Ly6G+ Neutrophils (%)

Blood

Aorta

Control IL-6-/- Control IL-6-/-

NS

NS

CD11b+ Ly6G+ Neutrophils (%)

0

5

10

15

20

0

5

10

15

20

0

5

10

15

20
Online Figure VI

![Graph showing systolic blood pressure over time for different groups. Legend: WT, WT + AngII, BAPN, BAPN + AngII.](image)
Online Figure VII

1) AAD initiation

2) Adventitial cell activation

3) Adventitial neutrophil accumulation and aortic expansion

4) Aortic rupture

Rupture

Neutrophil
Macrophage
Myofibroblast

MMP-9
CXCL1
G-CSF
IL-6