Crucial role of hematopoietic *JAK2* V617F in the development of aortic aneurysms

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ABSTRACT

JAK2 V617F is the most frequent driver mutation in myeloproliferative neoplasms (MPN) and is associated with vascular complications. However, the impact of hematopoietic *JAK2* V617F on aortic aneurysms (AA) remains unknown. Our cross-sectional study indicated that nine (23%) of 39 MPN patients with *JAK2* V617F exhibited the presence of AA. In order to clarify whether the hematopoietic *JAK2* V617F contributes to the AA, we applied bone marrow transplantation (BMT) with the donor cells from *Jak2* V617F transgenic (JAK2[V617F]) mice or control wild-type (WT) mice into lethally irradiated apolipoprotein E-deficient mice. Five weeks after BMT, the JAK2[V617F]-BMT mice and WT-BMT mice were subjected to continuous angiotensin II infusion to induce AA formation. Four weeks after angiotensin II infusion, the abdominal aorta diameter in the JAK2[V617F]-BMT mice was significantly enlarged compared to that in the WT-BMT mice. Additionally, the abdominal AA-free survival rate was significantly lower in the JAK2[V617F]-BMT mice. Hematopoietic JAK2 V617F accelerated aortic elastic lamina degradation as well as activation of matrix metalloproteinase (MMP)-2 and MMP-9 in the abdominal aorta. The numbers of infiltrated macrophages were significantly upregulated in the abdominal aorta of the JAK2[V617F]-BMT mice accompanied by STAT3 phosphorylation. The accumulation of BM-derived hematopoietic cells carrying JAK2 V617F in the abdominal aorta was confirmed by use of the reporter green fluorescent protein-transgene. BM-derived macrophages carrying JAK2 V617F showed increases in mRNA expression levels of *Mmp2*, *Mmp9*, and *Mmp13*. Ruxolitinib decreased the abdominal aorta diameter and the incidence of abdominal AA in the *JAK2*[V617F]-BMT mice. Our findings provide a novel feature of vascular complications of AA in MPN with *JAK2* V617F.

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Introduction

Myeloproliferative neoplasms (MPN) including polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) are characterized by chronic proliferation of mature myeloid cells and extramedullary hematopoiesis. The prevalence of MPN is reported to be 0.4 to 2.8 per 100,000 persons and the incident rates are 3.1 to 10.9 per one million person-years, which are increasing with age.[1,2] Major causes of morbidity and mortality in MPN patients are represented by vascular complications, progression to myelofibrosis, and transformation to acute leukemia.[3] To date, vascular disorders in MPN are known as arterial and venous thrombosis and advanced atherosclerosis.[3-5] Among the MPN, *JAK2* V617F is the most frequent driver mutation, which is observed in over 95% of PV patients as well as in 50–60% of ET and PMF patients.[6] Several murine studies

have shown that expression of *Jak2* V617F confers cytokine-independent growth of the myeloid cells, resulting in a variety of MPN phenotypes, like PV, ET, or PMF depending on the animal model.[7,8] By contrast, the etiological link between hematopoietic *JAK2* V617F and vascular disorders has not fully been elucidated *in vivo*.

Aortic aneurysms (AA) are a life-threatening aortic disease, characterized by dilatation of the aorta.[9] There are increasing numbers of patients with AA, with a prevalence in western countries of 1–5% in the adult population aged over 65 years old.[10,11] Although small sized AA remain mostly asymptomatic, AA are slowly progressive, and large AA often lead to aortic rupture and/or sudden death when their diameters are >6.0 cm in thoracic AA (TAA) and >5.0 cm in abdominal AA (AAA). It has been reported that the incidence of rupture of AA is 21.3 per 100,000 people in the general population with a mortality of 85-90%.[12] Thus, it is important to stratify the risk of AA rupture, and to discover a novel mechanism for the formation and progression of AA. It is known that circulating inflammatory cells play important roles in the development of AA, accompanied by the secretion of various inflammatory factors such as cytokines and chemokines.[13,14] In this term, inflammatory AAA which characterizes an unusually thickened aneurysm wall and dense adhesions of adjacent intra-abdominal structures represent different features from atherosclerotic AAA.[9] Although bone marrow (BM) progenitor cells contribute to the tissue inflammation and pathogenesis of cardiovascular diseases such as atherosclerotic diseases,[15,16] the causal role of BM-derived cells on the onset and progression of AA has not yet been fully explored. Moreover, the prevalence of AA has not been studied in MPN patients with *JAK2* V617F, who are more prone to vascular disorders than both the general population and MPN patients with driver mutations other than *JAK2* V617F.[17]

In the present study, we hypothesized that hematopoietic *JAK2* V617F might be causally associated with the development of AA. Strikingly, we demonstrated that the hematopoietic cells carrying JAK2 V617F promoted AAA progression in mice, as well as a common incidence of AA in MPN patients with *JAK2* V617F.

Methods

Detailed methods are provided in the *Online Supplementary Appendix*.

Patients

We enrolled 39 patients with *JAK2* V617F-positive MPN whose computed tomography data were available in a series of our former studies.[18,19] We evaluated the maximal diameter of the ascending aorta and abdominal aorta via computed tomography imaging. The protocol of the human studies was approved by the Institutional Ethics Committee of the Fukushima Medical University Hospital (approval ID, 29348 and 1242).

Animals

We used male *Jak2* V617F-expressing transgenic (JAK2[V617F]) mice.[7,19] Wild-type (WT) littermates were used as controls. Male apolipoprotein E-deficient (ApoE^{−/−}) mice on a C57BL/6J background were obtained from the Jackson Laboratory (JAX stock number, 002052, Bar Harbor, ME, USA).[20] CAG-EGFP reporter

mice with a C57BL/6J background were purchased from Japan SLC (Shizuoka, Japan). The JAK2[V617F] mice were crossed with the CAG-EGFP mice to generate JAK2[V617F]/CAG-EGFP double transgenic mice (JAK2[V617F]-GFP).[21] WT littermates were used as controls (WT-GFP). All animal studies were approved by the Fukushima Medical University Animal Research Committee (approval ID, 2019078).

Bone marrow transplantation

ApoE^{−/−} mice or WT mice aged between 8 and 12 weeks (body weight range, 20-30 g) were lethally irradiated (9.0 Gy) 24 hours before bone marrow transplantation (BMT).[19] Whole BM cells were harvested from the femurs and tibiae of the mice. BM cells (5.0×10^{6}) were injected into the ApoE^{−/−} recipient mice or the WT recipient mice via the tail vein.

Angiotensin II-induced aortic aneurysm model

Five weeks after BMT, the ApoE^{−/−} recipient mice or the WT recipient mice were implanted with an osmotic pump (ALZET micro-osmotic pump MODEL 1004, DURECT Co., Cupertino, CA, USA)[22] and angiotensin II (Ang II) (1900 ng/kg per min) or saline was continuously infused for 4 weeks.[23,24]

Futher methods

The details of blood sampling, chimeric analysis, ultrasound imaging and determination of aortic aneurysms, measurements of blood pressure and heart rate, histological analysis, measurement of total cholesterol and triglyceride, gelatin zymography, preparation for BM-derived macrophages, western blot analysis and reverse transcription-quantitative polymerase chain reaction are described in the *Online Supplementary Appendix*.

Treatment with a JAK1/2 inhibitor

For *in vitro* experiments, ruxolitinib (Novartis Pharmaceuticals, Basel, Switzerland) was used at a concentration of 250 nM for 24 hours prior to the RNA extraction. Dimethylsulfoxide was used as a control. For the *in vivo* study, ruxolitinib dissolved in 0.5% methylcellulose was administered to the mice orally at 60 mg/kg twice daily for 4 weeks.[25,26] We used 0.5% methylcellulose as a vehicle control.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). A value of *P*<0.05 was considered statistically significant.

Results

Prevalence of aortic aneurysms in patients with myeloproliferative neoplasms

In order to investigate the incidence of AA in association with the presence of *JAK2* V617F in the hematopoietic cells, we conducted a cross-sectional study that enrolled 39 MPN patients with the *JAK2* V617F mutation (mean age, 68 ± 12 years; female, 54%) in a series of our former studies[18,19] (Table 1). The allele burden of *JAK2* V617F was 51±30%. Computed tomography revealed the presence of AA in nine (23%) of the 39 *JAK2* V617F-positive MPN patients,[9,27] including two patients (22%) who underwent graft replacement surgery of the aorta due to progression of AA during the MPN course. When we divided the patients into two groups based on the presence of prior histories of thrombotic events as a crucial risk factor in the conventional prognostic system regarding the incidence of arterial and venous thrombotic

Table 1. Characteristics and prevalence of aortic aneurysms in JAK2 V617F-positive myeloproliferative neoplasm patients.

	All patients (n = 39)	Thrombosis (+) (n = 10)	Thrombosis (-) (n = 29)	P
Age, years	68 ± 12	73 ± 9	66 ± 13	0.118
Female, n. (%)	21 (54)	6 (60)	15 (52)	0.468
MPN diagnosis				
Polycythemia vera, n. (%)	16 (41)	2 (20)	14 (48)	0.141
Essential thrombocythemia, n. (%)	12 (31)	5 (50)	7 (24)	0.130
Primary myelofibrosis, n. (%)	11 (28)	3 (30)	8 (28)	0.591
Laboratory data				
White blood cell count, ×10^9/L	13.5 ± 9.4	10.6 ± 5.5	14.5 ± 10.4	0.270
Red blood cell count, ×10^{12}/L	4.7 ± 1.5	4.6 ± 1.3	4.7 ± 1.6	0.889
Hemoglobin concentration, g/dL	14.0 ± 3.9	12.7 ± 3.0	14.5 ± 4.0	0.213
Platelet count, ×10^9/L	509 ± 427	500 ± 270	512 ± 473	0.939
JAK2 V617F allele burden, %	51 ± 30	43 ± 33	53 ± 29	0.332
Comorbidities				
Hypertension, n. (%)	17 (44)	5 (50)	12 (41)	0.456
Diabetes mellitus, n. (%)	6 (15)	1 (10)	5 (17)	0.510
Dyslipidemia, n. (%)	7 (18)	5 (50)	2 (7)	0.007
Smoking history, n. (%)	13 (33)	4 (40)	9 (31)	0.440
Thrombosis history				
Arterial thrombosis, n. (%)	7 (18)	7 (70)	0 (0)	<0.001
Venous thrombosis, n. (%)	3 (8)	3 (30)	0 (0)	0.013
Cytoreductive treatment				
Hydroxyurea, n. (%)	17 (44)	1 (10)	16 (55)	0.014
Ruxolitinib, n. (%)	1 (3)	0 (0)	1 (3)	0.744
Presence of aortic aneurysms	9 (23)	5 (50)	4 (14)	0.032
Thoracic aortic aneurysm, n. (%)	7 (18)	4 (40)	3 (10)	0.057
Abdominal aortic aneurysm, n. (%)	2 (5)	1 (10)	1 (3)	0.452

events in PV and ET,[28,29] the prevalence of AA was significantly higher in the JAK 2V617F-positive MPN patients with a history of thrombosis than in those without (Table 1 and *Online Supplementary Table S1*). Next, we examined the expression levels of genes known to be related to AA, such as MMP and cytokines,[30] in circulating leukocytes. The *MMP9*, which is independently associated with aortic wall thickness and aortic luminal diameter during the process of AA,[31] as well as *TGFB3* and in interleukin 8 (*IL-8*) were significantly increased in *JAK2* V617F-positive MPN patients compared to the age- and sex-matched control subjects (*Online Supplementary Figure S1* and *Online Supplementary Table S2*). These findings suggest that incidences of AA are not rare in *JAK2* V617F-positive MPN patients, and that the presence of *JAK2* V617F in leukocytes may lead to increases in the expression levels of genes involved in the pathogenesis of AA.

Hematopoietic JAK2 V617F promotes abdominal aortic aneurysms formation in angiotensin II-infused ApoE$^{-/-}$ mice

In order to clarify whether or not hematopoietic *Jak2* V617F could indeed play a mechanistic role in the development of AA, we applied the BMT strategy using the donor BM cells from the JAK2^{V617F} mice[7] into the lethally irradiated ApoE$^{-/-}$ mice[24] (JAK2^{V617F}-BMT mice) (Figure 1A). The irradiated ApoE$^{-/-}$ recipient mice transplanted with donor BM cells from WT littermates (WT-BMT mice) were used as controls. Five weeks after BMT, the donor cells were similarly engrafted as shown in the chimeric analysis of the peripheral blood cells between the WT-BMT mice and JAK2^{V617F}-BMT mice (Figure 1B).

The JAK2^{V617F}-BMT mice displayed significantly higher white blood cell counts and spleen weights compared to the WT-BMT mice, indicating a hematological feature of MPN-like phenotype in the JAK2^{V617F}-BMT mice (Figure 1C and *Online Supplementary Figure S2*). Next, the WT-BMT mice and JAK2^{V617F}-BMT mice were continuously infused with Ang II with 1900 ng/kg/min for 4 weeks, as a higher dose of Ang II is required to induce the AA formation when the irradiated ApoE$^{-/-}$ recipient mice are used.[24] At 2 and 4 weeks following Ang II infusion, systolic, mean, and diastolic blood pressures were all significantly increased compared to those of the saline groups, but there were no differences in these parameters between the WT-BMT mice and JAK2^{V617F}-BMT mice (Figure 1D; *Online Supplementary Figure S3A* and *S3B*). No significant difference was observed regarding the heart rate between the WT-BMT mice and JAK2^{V617F}-BMT mice after Ang II infusion (*Online Supplementary Figure S3C*). Total cholesterol and triglyceride concentrations were decreased in the JAK2^{V617F}-BMT mice compared to WT-BMT mice (*Online Supplementary Table S3*), in agreement with the findings that MPN patients with *JAK2* V617F often display low lipid profiles that correlate with advanced disease.[32,33] Chimerism was not significantly different between the WT-BMT mice and JAK2^{V617F}-BMT mice 4 weeks after saline or Ang II infusion (*Online Supplementary Figure S3D*). We monitored both the thoracic aorta and suprarenal abdominal aorta by ultrasonography. The presence of TAA and AAA was defined as an increase by ≥50% in aortic diameter in comparison to the baseline. We did not observe any significant differences in the thoracic aorta diameter or the incidence of
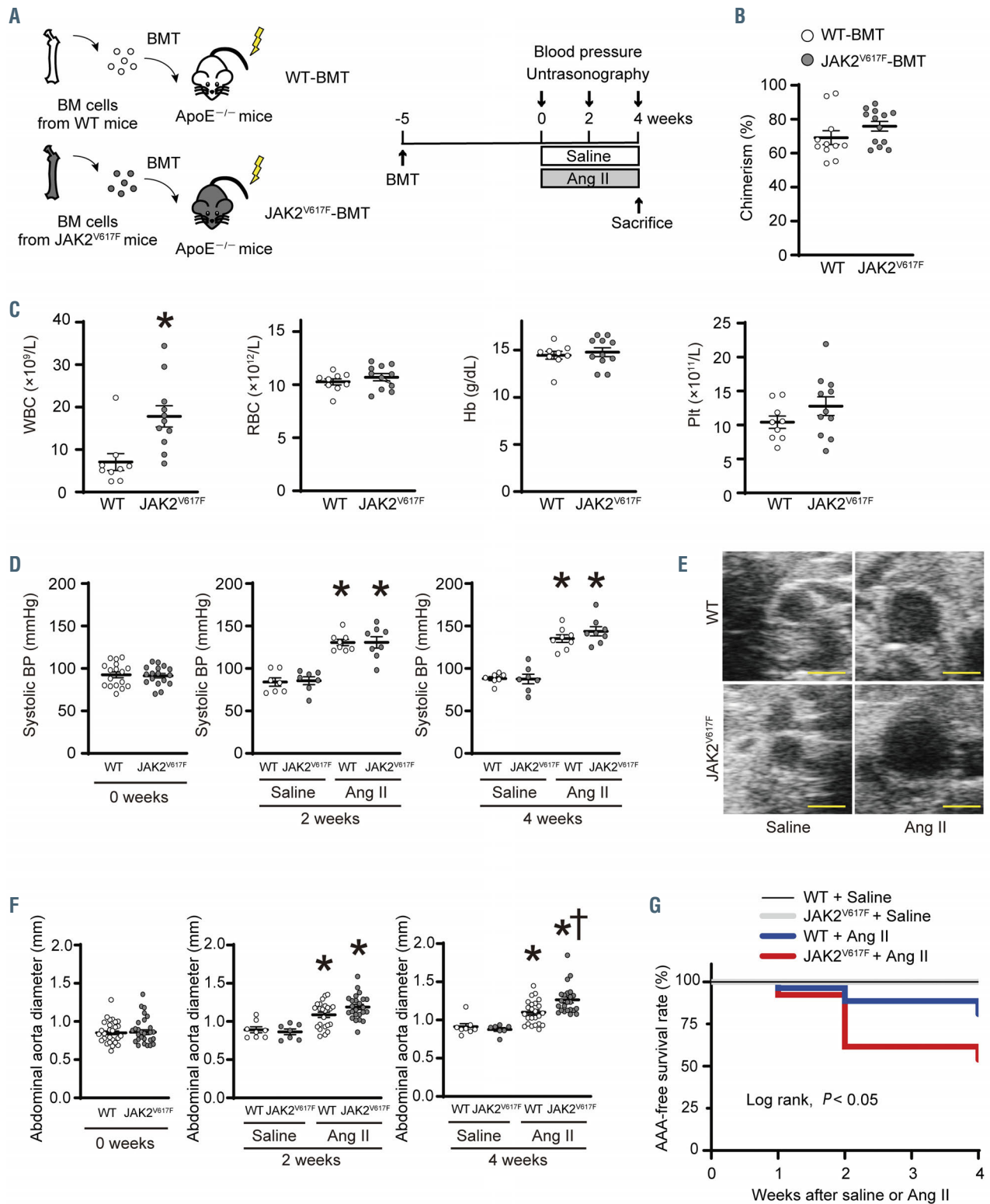
Figure 1. Hematopoietic JAK2 V617F contributes to abdominal aortic aneurysm formation. (A) Schematic diagram of the experimental design of bone marrow (BM) transplantation (BMT). BM cells from control wild-type (WT) mice or JAK2[V617F] mice were injected into the lethally irradiated ApoE^{−/−} recipient mice. Five weeks after BMT, the ApoE^{−/−} recipient mice transplanted with WT BM cells (WT-BMT mice) or JAK2[V617F] BM cells (JAK2[V617F]-BMT mice) were subjected to saline or angiotensin II (Ang II, 1900 ng/kg per min) infusion for 4 weeks. (B) Chimerism of donor cells in the peripheral leukocytes (n=11–13) and (C) blood cell counts (n=9–11) in the WT-BMT mice and JAK2[V617F]-BMT mice at 5 weeks after BMT. *P<0.05 *versus* the WT-BMT mice by the unpaired Student's *t*-test. (D) Systolic blood pressure (BP) at the baseline (n=17 each), 2 weeks (n =7–10) and 4 weeks (n=7–8) after saline or Ang II infusion. (E) Representative ultrasound images of abdominal aorta under saline or Ang II infusion for 4 weeks. Scale bars, 1.0 mm. (F) Abdominal aorta diameter at the baseline (n= 33–34), 2 weeks (n=7–24) and 4 weeks (n=7–24) after saline or Ang II infusion. (G) Abdominal aortic aneurysm (AAA)-free survival rate in the WT-BMT mice and JAK2[V617F]-BMT mice after saline or Ang II infusion (n=7–26) by log-rank test. All data are presented as mean ± standard error of the mean. *P<0.05 *vs*. the corresponding saline-infused mice and [†]P<0.05 *vs*. the corresponding WT-BMT mice by one-way ANOVA with Tukey *post-hoc* analysis. WT, ApoE^{−/−}: mice transplanted with bone marrow cells from wild-type mice; JAK2[V617F], ApoE^{−/−}: mice transplanted with bone marrow cells from JAK2[V617F] mice; Ang II: angiotensin II; WBC: white blood cell count; RBC: red blood cell count; Hb: hemoglobin concentration; Plt: platelet count.

TAA between the WT-BMT mice and JAK2[V617F]-BMT mice after Ang II infusion (*Online Supplementary Figure S4*). In contrast, although abdominal aorta diameters in both WT-BMT mice and JAK2[V617F]-BMT mice were significantly increased at 2 and 4 weeks after Ang II infusion compared to the corresponding saline-infused groups, the abdominal aorta diameter in the JAK2[V617F]-BMT mice was significantly enlarged compared to that of WT-BMT mice at 4 weeks after Ang II infusion (Figure 1E and 1F). The incidence of AAA was 0% in both the saline-infused WT-BMT mice and JAK2[V617F]-BMT mice, while it was 18.5% of the WT-BMT mice (5 of 27) and 46.1% of the JAK2[V617F]-BMT mice (12 of 26) developed AAA after Ang II infusion. Accordingly, Kaplan-Meier analysis demonstrated that the AAA-free survival rate was significantly lower in the JAK2[V617F]-BMT mice than in WT-BMT mice (Figure 1G). No death was observed in either saline-infused WT-BMT mice or JAK2[V617F]-BMT mice, while 3.7% of the Ang II-infused WT-BMT mice (1 of 27) and 7.6% of the Ang II-infused JAK2[V617F]-BMT mice (2 of 26) died from AAA rupture during the study period. Although continuous Ang II infusion significantly increased heart weight in both the WT-BMT and the JAK2[V617F]-BMT mice, there was no significant difference between the two groups (*Online Supplementary Figure S2B*). When we used mice with a WT background as recipients, as opposed to the ApoE^{-/-} recipient mice, the WT recipients transplanted with JAK2[V617F] BM cells did not exhibit AA formation (*Online Supplementary Figure S5*). These findings indicate that JAK2 V617F-mediated AAA development is required for an ApoE-deficient background experimentally, which is consistent with the previous work that the incidence of AAA in WT mice was much lower than in hyperlipidemic mice.[34] Taken together, these data suggest that hematopoietic JAK2 V617F promoted the formation of AAA in response to continuous Ang II infusion independently of the levels of elevated blood pressure.

Hematopoietic JAK2 V617F induces aortic elastic lamina degradation and rupture accompanied by activation of matrix metalloproteinases

In the morphometric analysis of aortas, a local enlargement of the abdominal aorta with a blood clot was observed in the Ang II-infused JAK2[V617F]-BMT mice (Figure 2A). Hematoxylin-eosin staining indicated thickening of the arterial wall as well as inflammatory cell infiltration accompanied by thrombus formation in the JAK2[V617F]-BMT mice after Ang II infusion (Figure 2B). Elastica-Masson staining revealed that discontinuity and breakage of elastin fibers in the aortic wall were detected in the Ang II-infused JAK2[V617F]-BMT mice (Figure 2B). The degree of elastin disruption in the abdominal aortic medial layers was more severe in the JAK2[V617F]-BMT mice than in WT-BMT mice after Ang II infusion based on the quantitative analysis by grading scores[35] (Figure 2C). Activation of MMP, particularly MMP-2 and MMP-9, is required to produce AAA.[36] Gelatin zymography assay using homogenates from the abdominal aorta demonstrated that either MMP-2 activity or pro MMP-9 expression levels were not different between saline-infused WT-BMT and JAK2[V617F]-BMT mice while both MMP-2 activity and pro MMP-9 expression were significantly elevated in the JAK2[V617F]-BMT mice compared to WT-BMT mice after Ang II infusion (Figure 2D and 2E). These data suggest that hematopoietic JAK2 V617F contributes to elastin disruption accompanied by activation of MMP, resulting in enlargement of the abdominal aorta and AAA rupture.

Inflammatory responses are accelerated in the abdominal aortas in JAK2[V617F]-bone marrow transplantation mice with activation of STAT3

We next examined inflammatory responses in the AAA formation and progression.[37] Immunohistochemical analysis showed that there were significant increases in CD45^{+} leukocytes, CD68^{+} macrophages, Ly6B.2^{+} neutrophils, and TER119^{+} erythrocytes in the abdominal arterial walls in the JAK2[V617F]-BMT mice compared to WT-BMT mice after Ang II infusion (Figure 3A and 3B; *Online Supplementary Figure S6*). The phosphorylation levels of STAT3 in the abdominal aorta were not significantly different between saline-infused WT-BMT mice and JAK2[V617F]-BMT mice, but the levels in the JAK2[V617F]-BMT mice were higher than those in the WT-BMT mice in response to Ang II (Figure 4A). For the assessment of the inflammatory mediators related to AAA development, mRNA expression levels of *Ccl6*, an important macrophage chemokine, as well as *Tgfb1*, which is involved in collagen and elastin production, were increased in the abdominal aorta of the JAK2[V617F]-BMT mice in comparison to the levels in the WT-BMT mice after Ang II infusion. These results suggest that macrophages and neutrophils carrying JAK2 V617F induce inflammatory responses in abdominal aortic walls in response to Ang II.

Characterization of bone marrow-derived hematopoietic cells carrying JAK2 V617F in the abdominal aorta by use of GFP-transgene

In order to further characterize the specific contribution of BM-derived circulating cells in the AAA formation in the Ang II-infused JAK2[V617F]-BMT mice, we generated double transgenic mice by crossing the JAK2[V617F] mice with CAG-EGFP mice (JAK2[V617F]-GFP).[21] Then, we transplanted BM cells derived from JAK2[V617F]-GFP mice or control WT-GFP mice into lethally irradiated ApoE^{-/-} mice. After BMT followed by Ang II infusion for 4 weeks, immunostaining showed that more GFP+ cells accumulated in the aorta of the JAK2[V617F]-GFP-BMT mice compared to WT-GFP BMT mice (Figure 5A). In the JAK2[V617F]-GFP-BMT mice, most GFP^{+} cells expressed CD45, CD68, or Ly6B.2 in the abdominal aorta (Figure 5B). These data indicate that the accumulated inflammatory cells carrying JAK2 V617F are mobilized from BM, migrated, and engrafted into the aortic walls in response to Ang II stimulation.

Impact of bone marrow-derived JAK2[V617F] macrophages on the genesis of matrix metalloproteinase

We next cultured mononuclear cells isolated from the BM in the presence of granulocyte-macrophage colony-stimulating factor to elucidate the effect of JAK2[V617F] macrophages on MMP. The BM cells were mostly differentiated into macrophages with proportions of CD68^{+} cells of over 80% both in the WT and JAK2[V617F] BM-derived cells (Figure 6A). The mRNA expression levels of *Mmp2* and *Mmp9* were significantly increased in the JAK2[V617F] BM-derived macrophages compared to the WT BM-derived macrophages (Figure 6B). Additionally, the expression levels of *Mmp13*, an activator of MMP-9,[38] in the JAK2[V617F] BM-
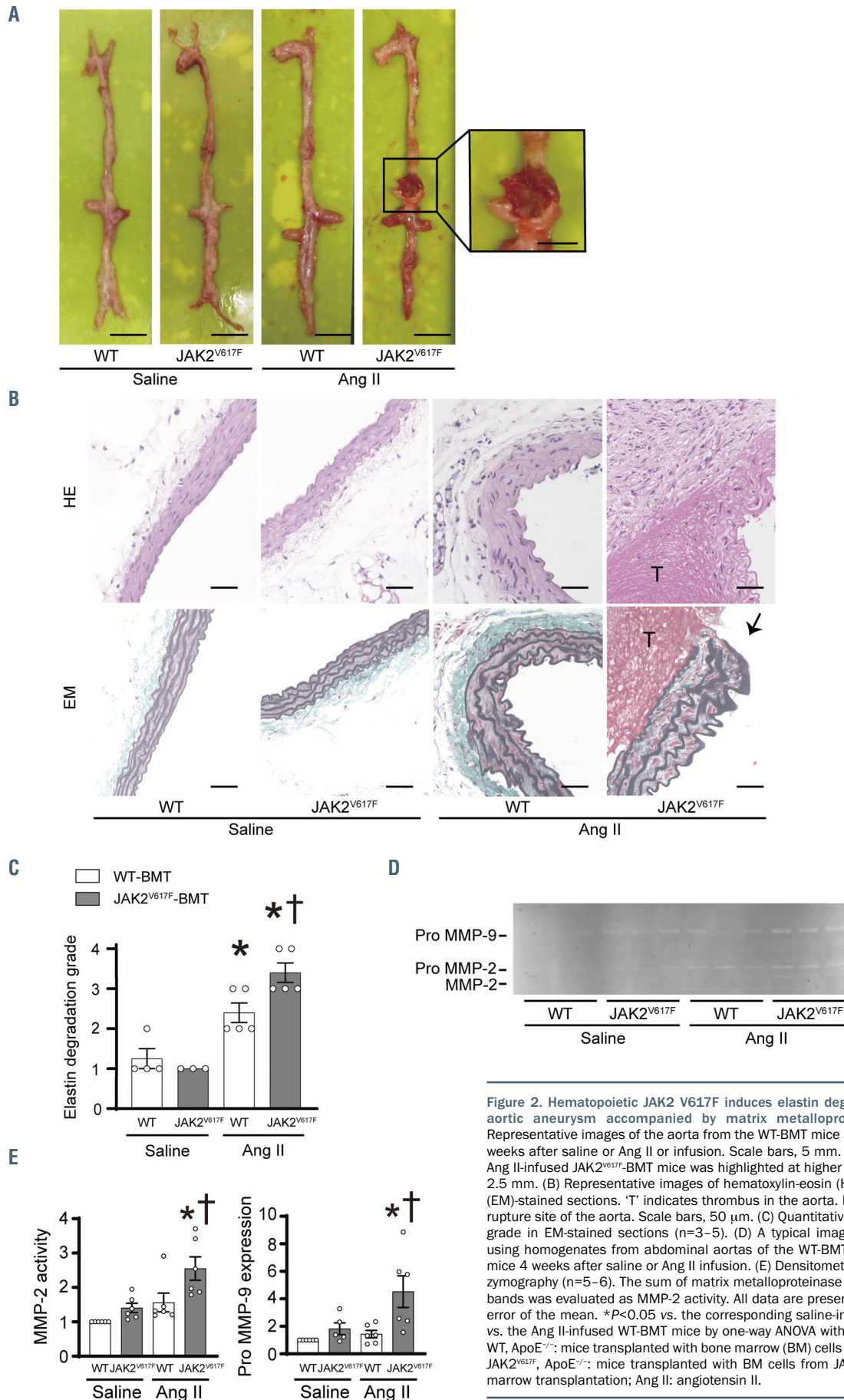
Figure 2. Hematopoietic JAK 2 V617F induces elastin degradation and rupture of aortic aneurysm accompanied by matrix metalloproteinase activation. (A) Representative images of the aorta from the WT-BMT mice and JAK2V617F-BMT mice 4 weeks after saline or Ang II or infusion. Scale bars, 5 mm. The boxed area from the Ang II-infused JAK2V617F-BMT mice was highlighted at higher magnification. Scale bar, 2.5 mm. (B) Representative images of hematoxylin-eosin (HE)- and Elastica-Masson (EM)-stained sections. 'T' indicates thrombus in the aorta. Black arrow indicates the rupture site of the aorta. Scale bars, 50 μm. (C) Quantitative analysis of degradation grade in EM-stained sections (n=3–5). (D) A typical image of gelatin zymography using homogenates from abdominal aortas of the WT-BMT mice and JAK2V617F-BMT mice 4 weeks after saline or Ang II infusion. (E) Densitometric analysis of the gelatin zymography (n=5–6). The sum of matrix metalloproteinase (MMP)-2 and pro MMP-2 bands was evaluated as MMP-2 activity. All data are presented as mean ± standard error of the mean. *$P<0.05$ vs. the corresponding saline-infused mice and †$P<0.05$ vs. the Ang II-infused WT-BMT mice by one-way ANOVA with Tukey post-hoc analysis. WT, ApoE$^{-/-}$: mice transplanted with bone marrow (BM) cells from wild-type (WT) mice; JAK2V617F, ApoE$^{-/-}$: mice transplanted with BM cells from JAK2V617F mice; BMT: bone-marrow transplantation; Ang II: angiotensin II.

derived macrophages were higher than those in the WT BM-derived macrophages (Figure 6B). Ruxolitinib, a selective JAK1/2 inhibitor, significantly decreased both *Mmp2* and *Mmp9* expression levels in the JAK2[V617F] macrophages (Figure 6C). These data suggest that BM-derived macrophages carrying JAK2 V617F play important roles in the genesis of MMP.

Inhibition of JAK1/2 with ruxolitinib prevents hematopoietic JAK2 V617F-induced abdominal aortic aneurysm formation

We investigated whether inhibition of JAK1/2 could attenuate hematopoietic JAK2 V617F-induced AAA formation (Figure 7A). Ruxolitinib treatment significantly decreased white blood cell counts and spleen weights in
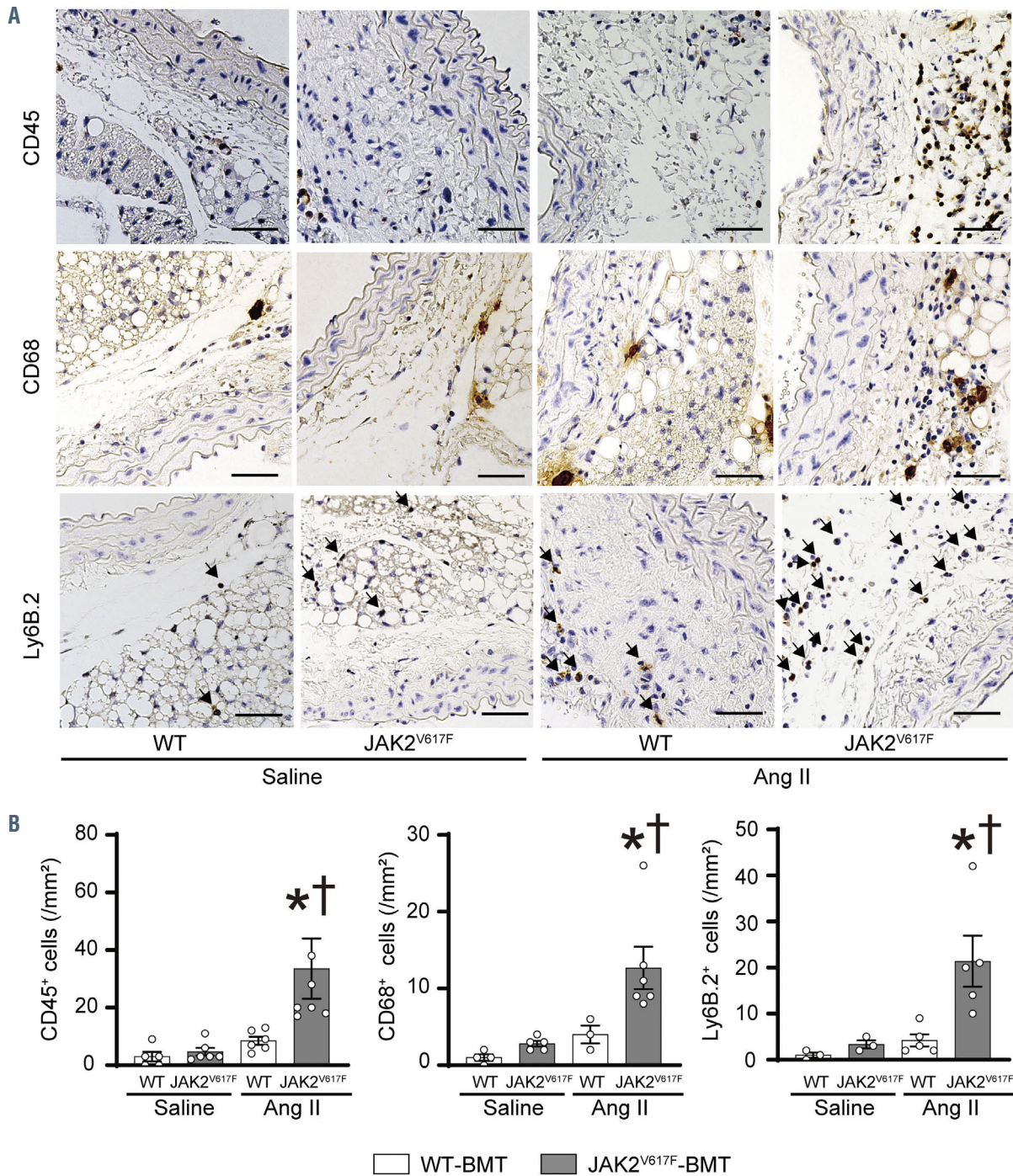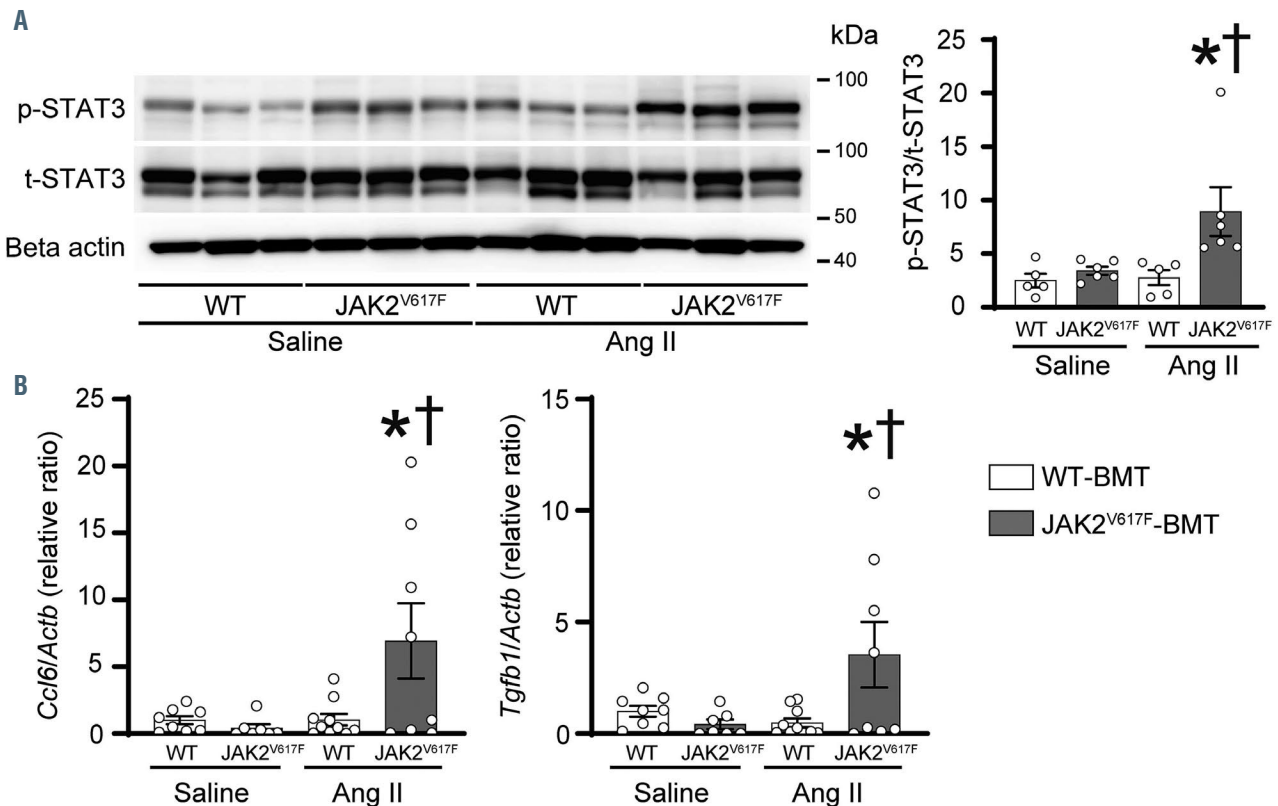

Figure 3. Hematopoietic JAK2 V617F increases inflammatory cell infiltration in the abdominal aorta. (A) Representative immunohistochemical images of the aorta stained by anti-CD45, CD68, and Ly6B.2 antibodies in the WT-BMT mice and JAK2[V617F]-BMT mice 4 weeks after saline or angiotensin II infusion. Arrows indicate Ly6B.2-positive cells. Scale bars, 50 μm. (B) Quantitative analyses of the CD45^{+} (n=5–6), CD68^{+} (n=3–5), and Ly6B.2^{+} (n= 3–5) cells in the aortic sections. All data are presented as mean ± standard error of the mean. *P<0.05 *vs.* the corresponding saline-infused mice and [†]P <0.05 *vs.* the Ang II-infused WT-BMT mice by one-way ANOVA with Tukey *post-hoc* analysis. WT, ApoE^{−/−}: mice transplanted with bone marrow (BM) cells from wild-type (WT) mice; JAK2[V617F], ApoE^{−/−}: mice transplanted with BM cells from JAK2[V617F] mice; BMT: BM transplantation; Ang II: angiotensin II.

the Ang II-infused JAK2[V617F]-BMT mice (Figure 7B; *Online Supplementary Figure 7A*), but did not affect the levels of blood pressures or heart rate (Figure 7C; *Online Supplementary Figure S7B* to *D*). Total cholesterol and triglyceride concentrations were increased in the ruxolitinib-treated JAK2[V617F]-BMT mice compared to the vehicle-treated JAK2[V617F]-BMT mice, as previously reported in MPN patients (*Online Supplementary Table S5*). Importantly, abdominal aorta diameters were significantly decreased in the ruxolitinib-treated JAK2[V617F]-BMT mice compared to the vehicle-treated JAK2[V617F]-BMT mice at both 2 and 4 weeks after Ang II infusion (Figure 7D and 7E). The incidence of AAA was 64% of the vehicle-treated JAK2[V617F]-BMT mice (7 of 11) while 18% of the ruxolitinib-treated JAK2[V617F]-BMT mice (2 of 11). Accordingly, the Kaplan-Meier analysis demonstrated that the AAA-free survival rate was significantly higher in the ruxolitinib-treated JAK2[V617F]-BMT mice than in the vehicle-treated JAK2[V617F]-BMT mice (Figure 7F). Ruxolitinib decreased MMP-2 activity and pro MMP-9 expression in the abdominal aorta in Ang II-infused JAK2[V617F]-BMT mice (Figure 7G). In contrast, thoracic aorta diameters or incidence of TAA were not different between the ruxolitinib- and vehicle-treated JAK2[V617F]-BMT mice (*Online Supplementary Figure S7E* and *S7F*). Taken together, these findings strongly suggest that hematopoietic JAK2 V617F plays a causal role in the onset and development of AAA.

Discussion

The present study is the first to demonstrate that hematopoietic cells carrying JAK2 V617F are causally associated with AAA formation in mice. In response to Ang II stimulation, the infiltrated CD68+ macrophages with JAK2 V617F in the arterial walls may have contributed to the genesis and activation of matrix metalloproteinase, resulting in increases of elastin degradation in the abdominal aorta and the development of AAA. Our findings provide a novel feature of vascular complication of AA in MPN patients due to the constitutive activation of the hematopoietic JAK-STAT pathway.

To date, vascular complications of MPN patients have been reported mainly in the arterial and venous thrombotic events from clinical and murine studies.[3,39] History of thrombosis and age >60 years are the highest risk factors for thrombosis in MPN patients.[40] Interestingly, *JAK2* V617F-positive MPN patients with a history of thrombosis more frequently showed AA, suggesting that history of thrombosis may be a risk factor for AA as well as thrombosis, whereas age was not different between *JAK2* V617F-positive MPN patients with AA and those without AA. Leukocytosis is another factor that increases the risks of thrombosis in MPN patients whereas the roles of increased number or functional alteration of platelets have been controversial in cases of thrombosis in MPN,[3,41] indicating that abnormal MPN-clone-derived leukocytes

Figure 4. Hematopoietic JAK2 V617F increases STAT3 phosphorylation and cytokine expression in response to angiotensin II infusion in the abdominal aorta. (A) Western blot analysis on the STAT3 in the abdominal aorta. Aorta extracts from the WT-BMT mice or JAK2[V617F]-BMT mice were immunoblotted with the indicated antibodies. Phosphorylated STAT3 (p-STAT3) to total STAT3 (t-STAT3) ratios are shown in the bar graphs. The average value for the saline-infused WT-BMT mice was set to 1 (n=5–6). β actin was used as the loading control. (B) Relative mRNA expression levels of *Ccl6* and *Tgfb1* in the aorta. *Actb* was used for normalization. The average value for saline-infused WT-BMT mice was set to 1 (n=7–10). All data are presented as mean ± standard error of the mean. *$P<0.05$ *vs.* the corresponding saline-infused mice and †$P<0.05$ *vs.* the Ang II-infused WT-BMT mice by one-way ANOVA with Tukey *post-hoc* analysis. WT, ApoE^{-/-}: mice transplanted with bone marrow (BM) cells from wild-type (WT) mice; JAK2[V617F], ApoE^{-/-}: mice transplanted with BM cells from JAK2[V617F] mice; BMT: BM transplantation; Ang II: angiotensin II.
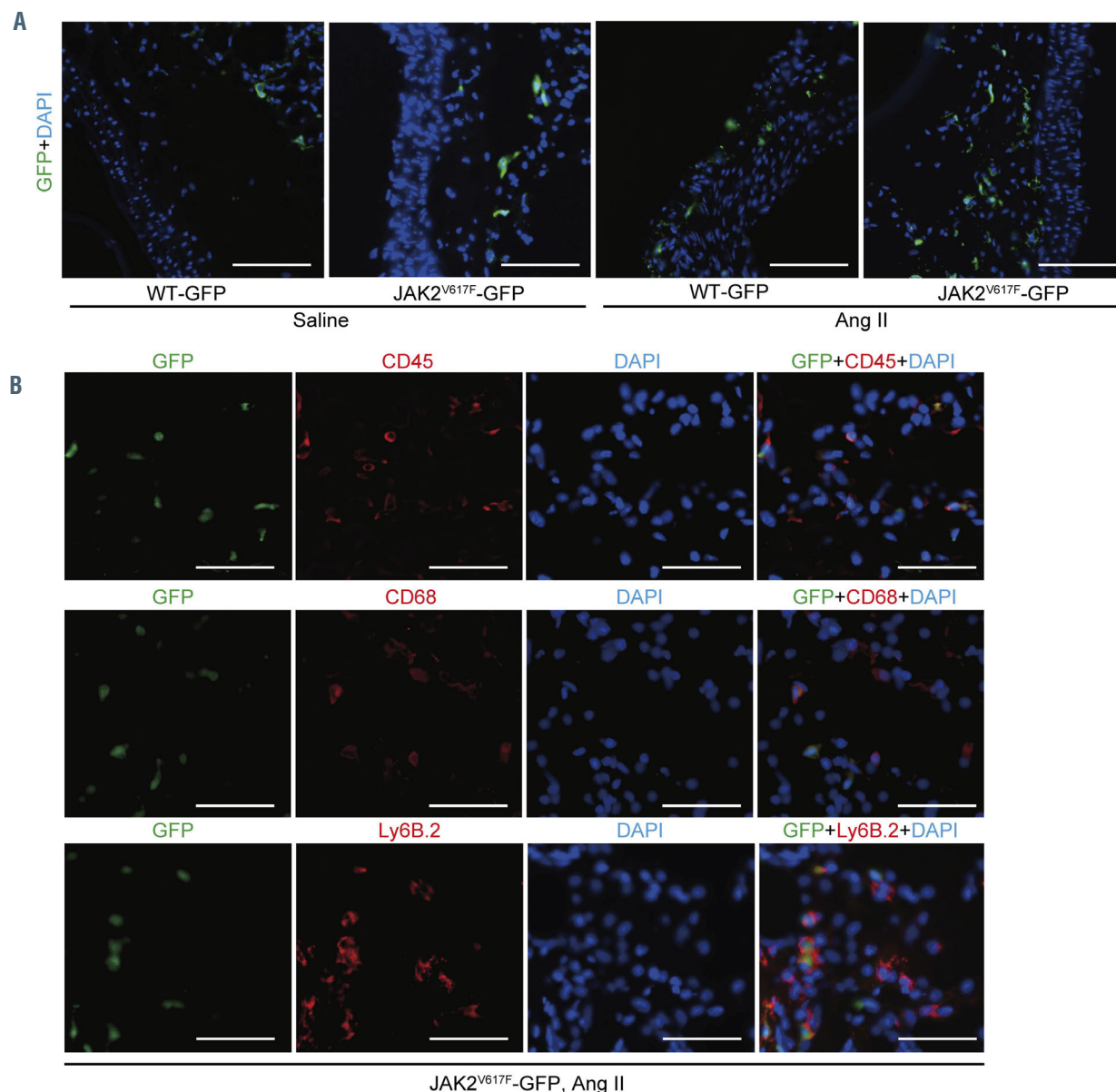
Figure 5. Characterization of bone marrow-derived JAK2 V617F hematopoietic cells in the abdominal aorta by use of GFP-transgene. (A) The lethally irradiated ApoE^{−/−} mice were transplanted with bone marrow (BM) cells from the WT/CAG-EGFP (WT-GFP) mice or JAK2[V617F]/CAG-EGFP (JAK2[V617F]-GFP) double transgenic mice. The ApoE^{−/−} recipient mice were subjected to saline or angiotensin II (Ang II) infusion for 4 weeks. The abdominal aortas were stained with an anti-GFP (green) antibody and DAPI (blue). Scale bars, 100 μm. (B) Representative immunofluorescence images of the aorta sections stained with anti-CD45 (red), anti-CD68 (red), or anti-Ly6B.2 (red) and anti-GFP (green) antibodies and DAPI (blue) in ApoE^{−/−} mice transplanted with JAK[2V617F]-GFP BM cells. Scale bars, 50 μm. Ang II: angiotensin II; GFP: green fluorescent protein; DAPI: 4′,6-diamidino-2-phenylindole; WT: wild-type.

contribute to vascular diseases. In our cohort, 23% of the patients with *JAK2* V617F-positive MPN exhibited the presence of AA. Given that the prevalence of AA is reported to be 1–5% in the general population above the age of 65 years,[10,11] *JAK2* V617F-positive MPN might be associated with a higher prevalence of AA. In combination with mouse studies, the presence of JAK2 V617F in leukocytes is likely to be a factor of AA development.

The pathogenetic feature of AA is mediated through the degenerative process involving extracellular matrix remodeling in all layers of the arterial wall. Particularly, local activation of inflammatory responses by immune cells plays an important role in the process. Leukocyte accumulation in inflammatory aneurysms provides addi-

tional evidence that inflammation represents variants along a spectrum of aneurysmal disease rather than distinct pathological entities of the inflammatory cells.[14] It has been reported that plasma inflammatory cytokine levels in *JAK2* V617F-positive MPN are significantly increased in the peripheral blood.[42,43] Similarly, we showed that circulating *JAK2* V617F leukocytes of MPN patients exhibited significant increases in the expression levels of the cytokines of *TGFB3* as well as the chemokines of *IL-8*. These findings are supported by previous reports that inflammatory macrophages and neutrophils contributed to AAA development.[44,45] It has been reported that macrophage NLRP3 inflammation activation in adventitia promotes inflammatory cytokine pro-
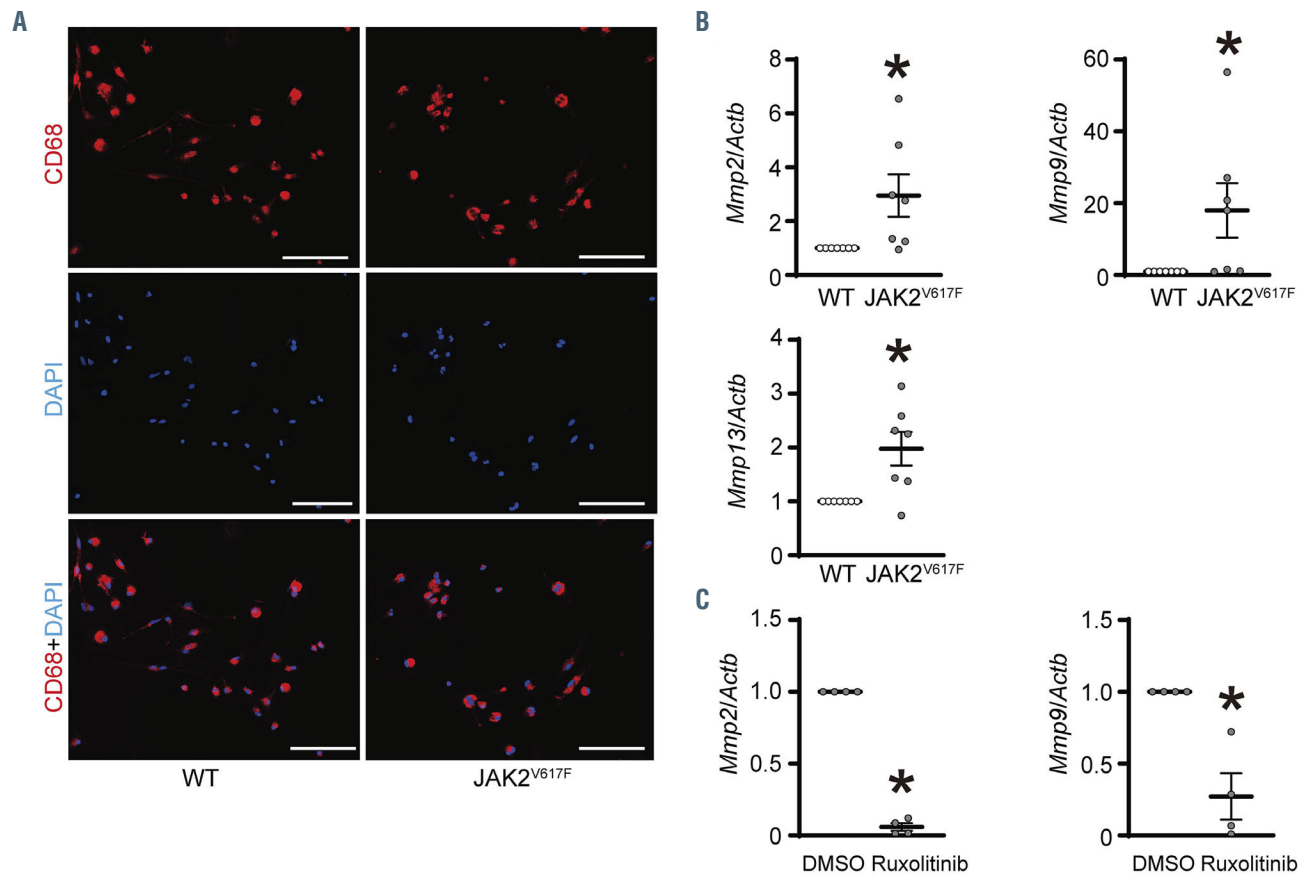
Figure 6. Matrix metalloproteinases are upregulated in bone marrow-derived macrophages with JAK2 V617F expression. (A) Mononuclear cells isolated from bone-marrow (BM) cells of wild-type (WT) mice or JAK2[V617F] mice were cultured in the presence of granulocyte-macrophage colony stimulating factor for 6 days. Representative immunofluorescence images of the cells stained with anti-CD68 (red) antibody and DAPI (blue). Scale bars, 100 μm. (B) Relative mRNA expression levels of *Mmp2*, *Mmp9*, and *Mmp13* in the cultured mononuclear cells (n=7 each). (C) *Mmp2* and *Mmp9* mRNA expressions with and without ruxolitinib pretreatment at 250 nM for 24 hours in the JAK2[V617F] mononuclear cells (n=4 each). Dimethylsulfoxide (DMSO) was used for control. Actb was used for normalization. The average value for the cultured mononuclear cells from WT mice or DMSO group was set to 1. All data are presented as mean ± standard error of the mean. *$P<0.05$ *vs.* the WT group or DMSO group by the unpaired Student's t-test. JAK2[V617F]: JAK2 V617F-expressing transgenic mice.

duction and MMP activation, and contributes to the development of AAA.[46] Recruitments of macrophages and neutrophils, and expression of pro-inflammatory cytokines may characterize the degenerative process during AA formation. We observed that the JAK2[V617F]-BMT mice did not display significant enlargement of the ascending aorta, and therefore it remains to be elucidated whether TAA develops in this model and/or some other condition.

It is known that matrix metalloproteases are closely involved in the pathogenesis of AA through elastin degradation.[47] Studies have reported that the JAK-STAT signaling pathway is one of the regulators of matrix metalloproteases,[48,49] and that macrophages are a significant source of matrix metalloproteinases.[47,50] Both MMP-9 and MMP-2 are required and work in concert to produce AAA.[36] We have demonstrated here that BM-derived macrophages with JAK2 V617F expression significantly upregulate *Mmp2*, *Mmp9*, and *Mmp13*. Likewise, substantially promoted macrophage infiltration was accompanied by increases in MMP-2 activity and pro MMP-9 expression in the abdominal aorta in the Ang II-stimulated JAK2[V617F]-BMT mice, suggesting that infiltration of JAK2 V617F-expressing macrophages with activation of matrix metalloproteinases plays causal roles in the patho-

genesis of AAA. In agreement, we found significantly higher mRNA levels of *MMP9* in the peripheral leukocytes, containing macrophage-progenitor monocytes, of patients with *JAK2* V617F-positive MPN compared to control subjects. MMP-9 plasma levels were independently associated with aortic wall thickness and aortic luminal diameter, but not with parameters of atherosclerosis.[31] Further studies are needed to clarify the MMP-related mechanism for progression to AA in MPN patients.

Currently, ruxolitinib is a clinically available JAK1/2 inhibitor for the treatment of patients with PMF and PV.[51] Ruxolitinib has been shown to improve long-term survival in patients with PMF and reduced thrombotic events in patients with PV.[52-54] Given our observations in the present study, ruxolitinib may be useful for preventing the onset and development of AAA in patients with MPN.

It has recently been reported that clonal expansion of hematopoietic cells with somatic mutations, termed as clonal hematopoiesis, is common in the seemingly healthy older general population, and significantly associated with atherosclerotic cardiovascular diseases.[55,56] Although *JAK2* V617F has been identified as one of the recurrent somatic mutations in individuals with clonal hematopoiesis, it remains unclear whether clonal hematopoiesis is involved in AA in addition to stenotic

atherosclerosis. However, given that our data from murine studies and clinical observations strongly indicate that hematopoietic JAK2 V617F plays crucial roles in the development of AA, we propose that *JAK2* V617F-positive clonal hematopoiesis may be also involved in the

formation and progression of AA regardless of the hematological phenotypes of MPN.[57]

The limitation of the present study is the small number of patients with MPN included while thrombosis and non-thrombosis categories were also small subsets for
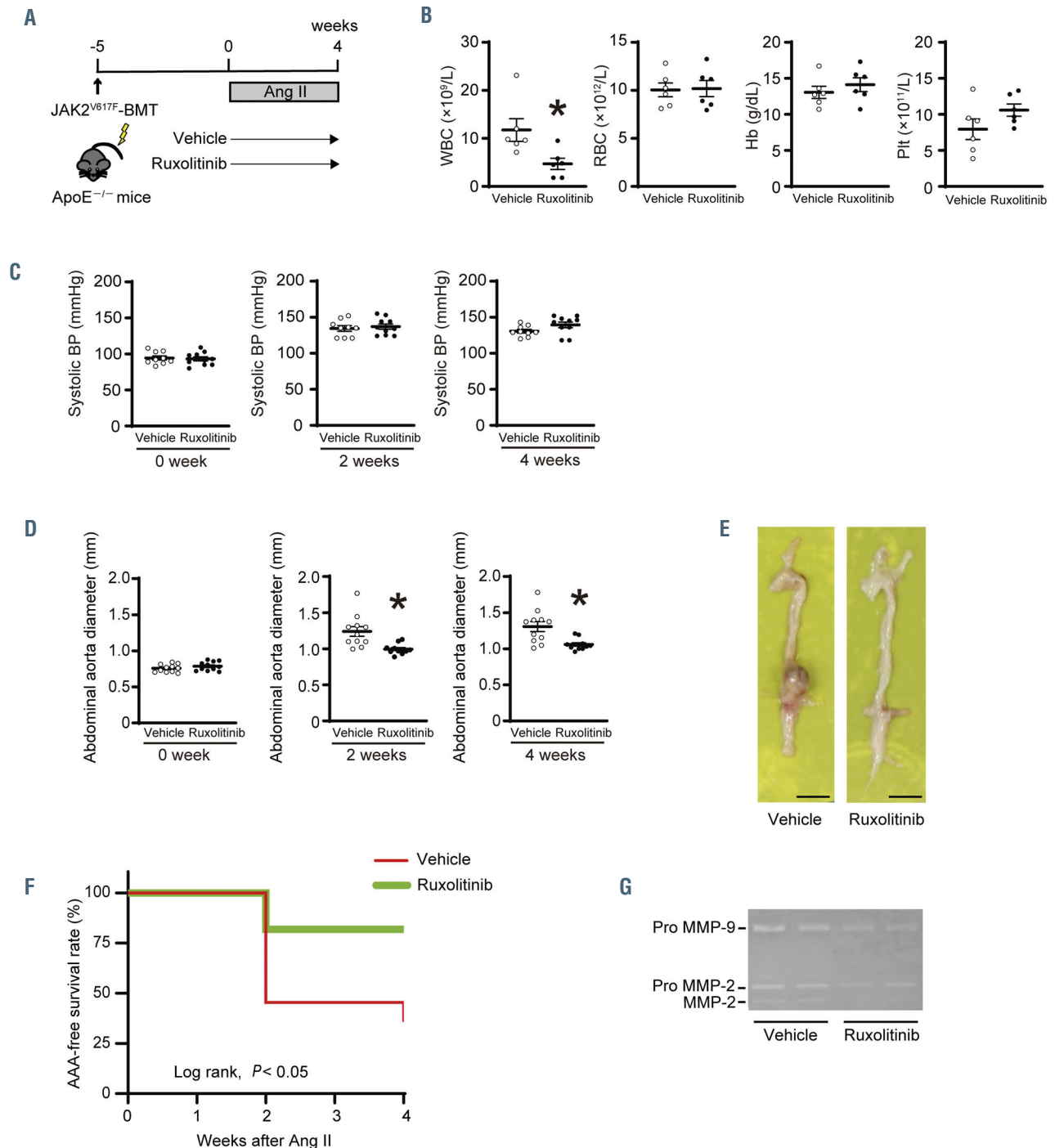
Figure 7. Ruxolitinib attenuates the formation of abdominal aortic aneurysms induced by hematopoietic JAK2 V617F. (A) Schematic diagram of the experimental design. The ApoE^{-/-} recipients transplanted with JAK2[V617F] bone marrow cells (JAK2[V617F]-BMT) were administered with vehicle (0.5% methylcellulose) or ruxolitinib was orally at 60 mg/kg twice daily for 4 weeks along with continuous angiotensin II (Ang II, 1900 ng/kg per min) infusion. (B) Blood cell counts at 4 weeks (n=6 each) and (C) systolic blood pressure (BP) at baseline (n=9–10), 2 weeks (n=9–10), and 4 weeks (n=9–10) in the vehicle- or ruxolitinib-treated JAK2[V617F]-BMT mice. (D) Abdominal aorta diameter at the baseline (n=11 each), 2 weeks (n=11 each), and 4 weeks (n=11 each). (E) Representative images from the aorta. Scale bars, 5 mm. (F) Abdominal aortic aneurysm (AAA)-free survival rate in the vehicle- or ruxolitinib-treated JAK2[V617F]-BMT mice (n=11 each) by log-rank test. (G) A typical image of gelatin zymography using homogenates from abdominal aortas. All data are presented as mean ± standard error of the mean. *$P<0.05$ *vs.* the vehicle group by the unpaired Student's *t*-test. WBC: white blood cell count; RBC: red blood cell count; Hb: hemoglobin concentration; Plt: platelet count; BP: blood pressure; MMP: matrix metalloproteinase.

comparison. Further studies are, thus, needed to validate our findings in larger cohorts.

In conclusion, AA are a possible vascular complication of MPN and hematopoietic JAK2 V617F may causally be involved in the development of AA through macrophage accumulation and MMP activation. The present study also provides novel mechanisms underlying the pathogenesis of AA. Hematopoietic JAK-STAT signaling may be a potential therapeutic target for the development of AA.

Contributions

TY and TM designed the research, performed experiments, analyzed the results, and wrote the manuscript; YK, KW, and KM performed the experiments and analyzed the results; KS and TI interpreted the results and supervised the study; SM and NK analyzed the results and interpreted the results, and supervised the study; KI designed and supervised the research, analyzed the data, and wrote the manuscript; YT designed and supervised the research, and approved the final version of the manuscript.

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