Granulocyte macrophage colony-stimulating factor
is required for aortic dissection/intramural hematoma

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Aortic dissection and intramural hematoma comprise an aortopathy involving separation of the aortic wall. Underlying mechanisms of the condition remain unclear. Here we show that granulocyte macrophage colony-stimulating factor (GM-CSF) is a triggering molecule for this condition. Transcription factor Krüppel-like factor 6 (KLF6)-myeloid-specific conditional deficient mice exhibit this aortic phenotype when subjected to aortic inflammation. Mechanistically, KLF6 down-regulates expression and secretion of GM-CSF. Administration of neutralizing antibody against GM-CSF prevents the condition in these mice. Conversely, administration of GM-CSF in combination with aortic inflammation to wild-type mice is sufficient to induce the phenotype suggesting the general nature of effects. Moreover, patients with this condition show highly increased circulating levels of GM-CSF, which is also locally expressed in the dissected aorta. GM-CSF is therefore a key regulatory molecule causative of this aortopathy, and modulation of this cytokine might be an exploitable treatment strategy for the condition.
Aortic dissection and intramural hematoma comprise a potentially life-threatening aortopathy involving separation of the aortic wall\textsuperscript{1-6}. The two conditions are distinguished by a tear in the aortic intima as present in the former classical form of aortic dissection which results in blood flow into the aortic wall and is absent in the latter form of intramural hematoma with bleeding confined within the aortic wall. This aortopathy is presently understood to be a continuum with the latter a variant and precursory condition of the former\textsuperscript{7-9}. Advancements in the understanding of genetic underpinnings (e.g. \textit{ACTA2})\textsuperscript{10,11}, clinical/epidemiological aspects (e.g. IRAD)\textsuperscript{4,5} as well as biochemical approaches (e.g. smooth muscle biomarkers)\textsuperscript{5,6} have been made in the last decade, but underlying mechanisms have remained obscure owing much to lack of a reliable animal model.

Recent advancements in understanding mechanisms of aortic disease have stemmed from hallmark studies in the genetically fragile Marfan aorta which have shown that TGFβ and its downstream intracellular kinase signaling pathways play a central role in the pathogenesis\textsuperscript{12-14}. In contrast, an inflammatory pathway is thought to be a major component of aortic conditions in the atherosclerotic/ degenerative aorta seen in the typical elderly patient\textsuperscript{15,16}. Commonalities and differences in mechanisms as well as relative contributions of underlying processes in these different aortic
conditions have only begun to be unraveled. In the present study, we sought to address the underlying mechanisms of aortic dissection/intramural hematoma, and to understand the triggering mechanism of the condition.

Krüppel-like factor 6 (KLF6) is a transcription factor that has been shown to be robustly expressed in macrophages\textsuperscript{17}, and to regulate inflammatory fibrotic diseases of multiple organs including the liver\textsuperscript{18} and kidney\textsuperscript{19}. In the present study, we hypothesized that this factor might regulate pathogenic mechanisms underlying aortic disease, and observed that mice deficient for KLF6 in macrophages when subjected to aortic inflammation manifest aortic dissection/intramural hematoma.

Intriguingly, we find that the inflammatory cytokine, granulocyte macrophage colony-stimulating factor (GM-CSF)\textsuperscript{20}, plays a central role in onset of this condition. Administration of neutralizing antibody against GM-CSF prevents the condition in these mice. Conversely, administration of the cytokine in combination with aortic inflammation to wild-type mice is sufficient to induce the condition suggesting general effects. Clinically, patients with aortic dissection show elevated circulating levels of the cytokine, which is also expressed in the dissected aorta. GM-CSF is therefore a key regulatory molecule causative of aortic dissection/intramural hematoma.
Results

Aortic aneurysm with inflammation in *Klf6* heterozygous mice. We initially found that mice heterozygously depleted for *Klf6* manifest a phenotype of exacerbated aortic aneurysm (defined as greater than 50% increase in external aortic diameter with conserved aortic wall)\(^{21,22}\) when subjected to aortic inflammation [two weeks-infusion of angiotensin II (AngII) with local application of calcium chloride (CaCl\(_2\))].

Histological findings showed enlargement of the aortic lumen with fragile aortic wall and further fibrotic tissue deposition compounded with marked infiltration of macrophages (Mac3-positive cells) (Fig. 1a-e). Mechanistically, increased expression of *matrix metalloprotease-9* (*MMP9*, as a marker of vascular remodeling)\(^{23}\), *F4/80* (as a marker of macrophages)\(^{24,25}\) and *IL-6* (as a marker of inflammation)\(^{16,26-30}\) were seen in the aorta (Fig. 1f).

As marked infiltration of immune cells was seen in the diseased aorta of these mice, macrophages were depleted using clodronate, which abrogated the aortic phenotype with near absence of macrophage infiltration (Fig. 1g, h). Thus, immune cells including macrophages were important for aortic remodeling in this model.

*Klf6°/°;LysM Cre* mice exhibit aortic dissection/hematoma. As the aortic condition
in Klf6-deficient mice appeared to involve a dysregulated inflammatory response by
macrophages, myeloid-specific Klf6-deficient mice (Klf6^0/0;LysM Cre mice) were
further generated which showed specific reduction of KLF6 expression in the myeloid
lineage by 70% as compared to control mice.

Klf6^0/0;LysM Cre mice subjected to aortic inflammation showed a similar
phenotype of exacerbated abdominal aortic aneurysm to that seen in heterozygous
knockout mice (Fig. 2a, b), but intriguingly, further showed supra-renal aortic
dissection/intramural hematoma as defined as separation of the intra-aortic wall with
hematoma formation accompanied by intimal tear for dissection⁴ (Fig 2c-f). Mice that
died were from aortic rupture most likely secondary to aortic dissection/intramural
hematoma (Fig. 2g). This lesion also showed fibrotic tissue deposition with
infiltration of Mac3-positive macrophages (Fig 3a, b), thus confirming that the aortic
phenotype in KLF6-deficiency was associated with perturbation of the inflammatory
response.

Mechanistically, Klf6^0/0;LysM Cre mice showed elevated expression of IL-6 in the
aortic lesion (Fig. 3d) and elevated circulating levels (Fig. 3c). Further, macrophages
obtained from bone marrow of Klf6^0/0;LysM Cre mice exhibited increased IL-6
expression (Fig. 3e). Differences in expression were not seen in other major
pro-inflammatory cytokines such as \textit{IL-1β}, \textit{MCP-1} or \textit{TNFα} between macrophages from \textit{Klf6^{flo/flo}} and \textit{Klf6^{flo/flo};LysM} mice (Fig. 3e). Immune cells in the diseased aorta of these mice were characterized by flow cytometry analysis which showed a markedly increased population of \textit{CD11b}^+\textit{Ly6C}^{hi} inflammatory monocytes and this increase was also seen in the peripheral blood (Fig. 3f). Granulocytes (e.g. neutrophils;\textit{Ly6G}^+ cells) were not affected in number or (sub-) population under basal conditions or in the setting of \textit{CaCl}_2 and \textit{AngII} infusion (Supplementary Table 1 and Supplementary Fig. 1a, c) nor was the functional activity of neutrophils as examined by inflammatory cytokine expression such as \textit{IL-8} or \textit{TNFα}^31 (Supplementary Fig. 1e) or that of population of dendritic cells (Lineage'\textit{CD11c}^+ cells) (Supplementary Fig. 1b, d) affected under these conditions. Taken together, expansion of inflammatory monocytes in the aorta and circulation was selectively associated with the present experimental model and conditions.

\textit{TGFβ}, a central molecule in the pathogenesis of Marfan aortopathy\textsuperscript{12,14,32-34}, and its downstream signaling pathways (canonical pSmad-2\textsuperscript{35} and non-canonical pERK1/2\textsuperscript{12}) were not affected in either \textit{Klf6^{flo/flo};LysM Cre} mice or heterozygous knockout mice while pSTAT3, a downstream signaling pathway of IL-6, was activated in both mice (Fig. 4a-d), suggesting that the \textit{TGFβ}-mediated pathway was not critically
involved in the underlying phenotype, and that the IL-6/STAT3 pathway is.

**GM-CSF is a downstream target of KLF6.** Delineation of target molecules and mechanisms of regulation of immune cells was next addressed using RNA profiling array analysis. Remarkably, GM-CSF levels showed the greatest increase in macrophages derived from bone marrow of $Klf6^{0\beta};LysM\ Cre$ mice in response to AngII stimulation (3.89-fold) as compared to control macrophages (Fig. 5a). Macrophages obtained from aorta of $Klf6^{0\beta};LysM\ Cre$ mice showed markedly increased expression of GM-CSF under experimental conditions of CaCl$_2$ application and AngII infusion (Fig. 5b), and in macrophages derived from bone marrow of these mice (Supplementary Fig. 2a). Expression of GM-CSF in the aorta was elevated from three days after treatment (before onset of aortic dissection) of $Klf6^{0\beta};LysM\ Cre$ mice (Fig. 5c). Whether deletion of KLF6 in macrophages affects secretion of GM-CSF and further systemic circulating levels was next asked. Macrophages and GM-CSF co-localized in the aorta of $Klf6^{0\beta};LysM\ Cre$ mice, and GM-CSF was markedly produced by macrophages in response to pro-inflammatory stimuli (Fig. 5d and Supplementary Fig. 2b). Circulating levels of GM-CSF were at least 73.3-fold higher in $Klf6$-deleted mice (Fig. 5e). It therefore seems that a markedly increased response in GM-CSF is a hallmark
feature of the aorta in \( Klf6^{fl/fl};LysM \) Cre mice.

We next sought to understand mechanisms underlying regulation of GM-CSF expression and secretion by KLF6. Over-expression of \( KLF6 \) significantly attenuated GM-CSF expression induced by pro-inflammatory stimuli in macrophages (Supplementary Fig. 2c). Transcriptionally, several KLF-binding sites were present in the promoter region of GM-CSF to which KLF6 was recruited by agonistic stimuli treatment in macrophages (Supplementary Fig. 2d). These results demonstrated that, mechanistically, GM-CSF is a direct target of KLF6 and that KLF6 represses expression of GM-CSF.

**GM-CSF manipulation regulates aortic dissection/hematoma.** To next test the requirement of GM-CSF in aortic dissection in these mice, the actions of GM-CSF were blocked using neutralizing antibody which abrogated aortic dissection/intramural hematoma (Fig. 6a, b) as well as expression of GM-CSF receptor \( \alpha \), MMP9, F4/80 and \( IL-6 \) (Fig. 6d) in addition to serum levels of IL-6 (Fig. 6c). GM-CSF was therefore required for the aortic phenotype in \( Klf6^{fl/fl};LysM \) Cre mice.

We further investigated whether GM-CSF is sufficient to induce the aortopathy. Administration of GM-CSF in wild-type mice subjected to aortic inflammation
(CaCl₂+AngII) caused aortic dissection/intramural hematoma confirming the generality of the role of GM-CSF in the pathogenesis of the condition. Mice died from aortic rupture due to the aortic lesion and showed pathological features of the condition (e.g. fragile aorta, intimal tear with hematoma) (Fig. 6e-h, j). However, aortic dissection/intramural hematoma did not develop by administration of GM-CSF alone, even with abnormally increased circulating levels (at least 180.9 fold) of GM-CSF (Supplementary Fig. 3a, b). As AngII, CaCl₂ or GM-CSF alone was not sufficient to induce the condition, it seems that combination of aortic inflammation with GM-CSF infusion is necessary for the phenotype (Supplementary Fig. 4a, b). Consistent with this, circulating levels of GM-CSF in mice were only markedly elevated when treated with combination of measures as compared to each alone (Fig. 6i). Note that these elevated levels were comparable to those in Klf6<sup>fl/fl</sup>;LysM<sup>Cre</sup> mice suggesting that highly elevated levels of GM-CSF are required but not sufficient to cause aortic dissection/intramural hematoma (Fig. 5e).

Finally, whether manipulation of GM-CSF affects the number of peripheral leukocytes was examined. With GM-CSF administration, the number of circulating lymphocytes did not change in either the early phase (5 days) or developed phase (14 days) of the model (Supplementary Table 2 and 3). With respect to neutrophils, the
number in peripheral blood was markedly increased in the early phase but no difference was observed at 14 days of GM-CSF administration. This was similarly seen in the group in which GM-CSF alone was administered which did not result in the aortic phenotype. While these changes might be due to acute effects by exogenous GM-CSF treatment, this alone had no bearing on the phenotype. Moreover, the number of circulating granulocytes and lymphocytes was not affected when GM-CSF was depleted by neutralizing antibody (Supplementary Table 4). Based on these results, manipulation of GM-CSF did not affect the number of circulating leukocytes in the present model, at least during the observation period (14 days).

**Up-regulation of GM-CSF in patients with aortic dissection.** To confirm the clinical relevance of our findings, circulating levels of GM-CSF were measured in sera of patients with acute aortic dissection which showed marked increases in contrast to patients with coronary artery disease, aortic aneurysm or healthy volunteers which showed markedly lower if not negligible levels (Fig. 7a). Furthermore, inflammatory infiltration (CD68+ monocytes/macrophage) and GM-CSF expression were up-regulated and co-localized in dissected aorta (Fig. 7b). Thus, GM-CSF is associated with aortic acute dissection not only in mice but also in human conditions.
Discussion

The present findings show that GM-CSF is a key regulatory molecule causative of aortic dissection/intramural hematoma in a murine model of the condition and to also be associated with the condition in humans. In mice, modulation of GM-CSF by neutralizing antibody or exogenous administration respectively prevented or induced onset of this phenotype. In humans, elevated serum GM-CSF levels and expression of the cytokine in aortic tissue were seen in patients with aortic dissection.

GM-CSF was a central component of the aortic dissection/intramural hematoma phenotype in our murine model. Previous studies had suggested a limited role of GM-CSF in the pathogenesis of aortic disease\textsuperscript{36-39}. For example, mice that lack Smad3 manifested a phenotype of aortic aneurysm formation\textsuperscript{39} and GM-CSF was shown to play a pivotal role in the pathogenesis; however, it was assumed that because smad3 is a downstream target of TGF\(\beta\), which is a central molecule associated in Marfan aortopathy, that the pathogenic mechanism was limited to this genetic aortopathy. Our findings show that activation of the GM-CSF pathway in a manner independent of the TGF\(\beta\)-SMAD pathway is sufficient to trigger this condition in a model of inflammation and degenerative aorta (calcium chloride treatment causes stiffening of the aorta to mimic the condition as seen in atherosclerotic human aortas\textsuperscript{40}) as reflective of aortic
dissection/intramural hematoma seen in the elderly adult in humans and should be
differentiated from the genetic aortopathy in young patients with Marfan syndrome.

GM-CSF tissue expression had also been shown to be increased in a patient presenting
with aortic dissection in Cogan’s disease\textsuperscript{41}, an apparently auto-immune condition which
is characterized by recurrent corneal inflammation\textsuperscript{42} that was thought to be an isolated
finding.

Effects on other non-macrophage myeloid cells were investigated which showed
that dendritic cells (CD11c\textsuperscript{+}MHCII\textsuperscript{+} cells) were increased in the diseased aorta but not
in the circulation under KLF6-deficient conditions, and lack of effects on neutrophils
(Ly6G\textsuperscript{+} cells) either in the circulation or in the aortic tissue (Supplementary Fig. 1 and
Supplementary Fig. 5). The contributory role of non-macrophage myeloid cells (e.g.
dendritic cells) needs to be further investigated.

Macrophage colony-stimulating factor (M-CSF) has been also suggested to be
an important regulator of vascular remodeling\textsuperscript{43,44}. Although the precise molecular
mechanisms of the actions of M-CSF are still unclear, different actions as compared to
GM-CSF are envisioned given different expression patterns in the vascular wall.
Whereas M-CSF is constitutively expressed under physiological conditions in
endothelial cells, fibroblasts, macrophages and smooth muscle cells, GM-CSF, by
contrast, is expressed only in minute amounts in these cells under basal conditions but
instead is induced by inflammatory stimuli (e.g. TNF)\(^45\) or oxidized-low density
lipoprotein (LDL) cholesterol stimulation\(^46\). In murine and human lesions, M-CSF is
detected both in healthy arteries and in atherosclerotic lesions associated with
macrophage and foam cell content, and is correlated with plaque progression in the
latter. By contrast, only minute levels of GM-CSF are seen in smooth muscle cells and
endothelial cells of healthy human arteries but are elevated upon atherosclerotic
development and macrophage accumulation\(^47\). Based on these observations,
collectively, while M-CSF is a constitutively expressed cytokine in the vasculature,
GM-CSF is markedly induced in diseased vessels to regulate pathological conditions
including the described aortopathy.

On the experimental model, most previous studies have used AngII infusion alone
as an intervention to induce a dissection phenotype\(^16,48\). However, the limitation of this
procedure for mechanistic investigations including on inflammation was the low
reproducibility (less than 30%), need for long-term infusion of AngII (more than 4
weeks) and incidence/expression of phenotype only in aged mice (over 7 to 10 months
age) with specific genetic background (\textit{apoE}~\(^/-\) or \textit{ldl receptor}~\(^/-\) mice). Most
noteworthy is that the present model could induce aortic dissection/intramural
hematoma within 2 weeks with high reproducibility (at least 70%) even in young
wild-type mice. Mechanistically, this model might involve hemodynamic stress on the
supra-renal dissection site due to loss of Windkessel effect49 because of increased
stiffening in the infra-renal aorta (e.g. downward shift of pressure-diameter curve after
CaCl\textsubscript{2} application with continuous AngII infusion)\textsuperscript{40} that showed aneurysmal formation
which when exposed to inflammatory effects of GM-CSF triggered
dissection/intramural hematoma formation in the weak and fragile supra-renal aorta.
As aortic aneurysm is commonly co-present in patients with dissection\textsuperscript{4}, the described
animal model and findings closely resemble the condition seen in patients.

Taken together, our findings suggest that GM-CSF is a central regulator of aortic
dissection/intramural hematoma in the atherosclerotic and inflammatory aorta which is
typically seen in the elderly patient with this condition, and may serve as a potential
target for diagnostic and therapeutic exploitation (e.g. aortic stabilization using
GM-CSF antagonists) as well as a diagnostic biomarker.

**Methods**

**Mice.** Heterozygous Klf6\textsuperscript{+/-} mice (C57BL/6) were originally generated by Tarocchi et
al.\textsuperscript{50}. To generate macrophage-specific Klf6-knockout mice, Klf6\textsuperscript{0/0} mice
(C57BL/6;129Sv) were cross-bred with *LysM Cre* mice (C57BL/6, Jackson laboratory)\(^5\). Only male mice 10- to 13-weeks of age and C57BL/6 as wild-type mouse (CLEA Japan) were used. All experimental protocols were approved by the Ethics Committee for Animal Experimentation at the Graduate School of Medicine, the University of Tokyo and conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Department of Medicine, the University of Tokyo.

**Murine aortic dissection/intramural hematoma model.** To induce aortic dissection/intramural hematoma, peri-aortic application of CaCl\(_2\) was done to the abdominal aorta, followed by two-week infusion of AngII (2,000 ng kg\(^{-1}\) min\(^{-1}\))\(^4\). In detail, mice were anesthetized and underwent laparotomy at 10- to 13-weeks of age. The abdominal aorta between the renal arteries and bifurcation of the iliac arteries was isolated from the surrounding retroperitoneal structure, and 0.5 M CaCl\(_2\) was applied to the external surface of the infrarenal aorta. NaCl (0.9%) was substituted for CaCl\(_2\) in sham control mice. The aorta was rinsed with 0.9% sterile saline after 15 min and the incision was closed.

**Macrophage depletion and manipulation of GM-CSF.** Wild-type mice were
injected intraperitoneally with 110 mg kg\(^{-1}\) of clodronate liposomes or equal volume of PBS liposomes 2 days prior and 7 days after induction of aortic dissection.

Neutralizing antibody against GM-CSF (300 \(\mu\)g, R&D systems) or control anti-rat IgG antibody (Equitech Bio) was administered every other day by intraperitoneal injection.

Recombinant murine GM-CSF (10, 50, 100 \(\mu\)g kg\(^{-1}\) day\(^{-1}\), PeproTech) was administered for two weeks or four weeks after induction of aortic dissection.

**Histological analysis and immunohistochemistry.** Aortas from mice were embedded in paraffin then 5-\(\mu\)m-thick serial sections were prepared for Elastic Van Gienson (EVG) and hematoxylin/eosin (HE) staining. Digital images of EVG-stained aortas with reference scale were used for absolute measurement of diameter. Human aortic tissue was obtained from patients undergoing surgical aortic repair with informed consent under a protocol approved by the University of Tokyo Hospital Research Ethics Committee. Paraffin-embedded sections were taken from the aorta for EVG staining and immunohistochemistry. For immunohistochemistry, after deparaffinization and blocking, serial sections were incubated with the following antibodies; Mac-3 (dilution 1:200; rat; BD Pharmingen) or F4/80 (1:100; rat; Serotec) for macrophages in mice and CD68 (1:50; mouse; DAKO) in humans, and GM-CSF (1:100, rabbit; Abcam for mouse
and 1:50; rabbit; Acris for humans) or p-STAT3 (1:200; rabbit; Cell Signaling
Technology), then followed by biotinylated secondary antibodies (1:200; DAKO). For
detection, anti-streptavidin-conjugated AlexFluor 488 or AlexFluor 594 (1:200;
Invitrogen) was used. The nuclei were stained with 4’, 6-diamidino-2-phenylindole
(1:5,000; Sigma-Aldrich) after the final series of washes.

**Cell preparation from aorta, spleen, bone marrow and blood.**  Aortas were minced
into 3- to 4-mm pieces and placed in 1 ml digestion solution containing collagenase
type II (1.25 mg ml⁻¹, Worthington) and porcine pancreatic elastase (50 μg ml⁻¹,
Worthington) in base solution of Accumax (Innovative Cell Technologies). Aortic
tissue was digested at room temperature with agitation for 1 h. After digestion, cells
were washed in FACS buffer (5% FCS in PBS) at 2,000 rpm for 5 min. Aortic
macrophages were isolated using CD11b microbeads according to the manufacturer’s
instructions (Miltenyi Biotec). Spleen was homogenized and passed through a cell
strainer to obtain single-cell suspensions. Bone marrow-derived cells were taken from
the femur and tibia of 5- to 6-week-old mice. Blood was collected in heparin-coated
vials and then 1.2% dextran was added for 45 min at room temperature. Counting of
peripheral leukocytes was done by automated hematology analyzer (XT-2,000i,
Neutrophils were isolated from bone marrow using a neutrophil isolation kit according to the manufacturer’s instructions (Miltenyi Biotec). From single-cell suspensions of spleen, bone marrow and blood, erythrocytes were lysed using ACK lysis buffer for 5, 3 and 2 min on ice, respectively. Cells were centrifuged at 2,000 rpm for 5 min to remove the ACK lysis buffer, then the single-cell suspensions were resuspended and washed in FACS buffer, followed by centrifugation at 2,000 rpm for 5 min.

**Cell cultures.** Bone marrow-derived cells were prepared from femur and tibia of *Klf6*^fl/fl^ mice or *Klf6*^fl/fl^;LysM Cre mice to assess the role of GM-CSF in macrophages. KLF6 overexpression was induced by retrovirus construct for KLF6 (pMXs-KLF6) in the presence of RetroNectin (5 μg/cm^2^, Takara Bio.).

**Flow cytometry.** Murine Fc receptors were blocked using antibodies against murine CD16/32 antigens (eBioscience) for 15 min on ice after which cells were washed and then resuspended in 100 μl FACS buffer. Fluorochrome-conjugated antibodies (all from BioLegend) for APC-CD11b[M1/70], PerCP-Cy5.5-Ly-6c[HK1.4], APC-Cy7-Ly6G[1A8] or APC-CD11c[N418] were added for 30-45 min at room
temperature. FITC-CD3e[145-2C11], FITC-Ly6G[RB6-8C5], FITC-CD11b[M1/70], FITC-CD45R/B220[RA3-6B2] and FITC-Ly76[Ter-119] (erythroid lineage marker) were used as lineage markers. Corresponding isotype control antibodies were added to samples at the same concentrations as the antibodies of interest. After incubation, samples were washed three times and analyzed by FACSverse (BD Pharmingen). Compensation was done using positive samples containing single color-stained aortic macrophages. Debris and dead cells, as defined by low forward scatter, were excluded from analysis. Data were analyzed with FlowJo (Tree Star).

**Chromatin immunoprecipitation.** ChIP analysis was performed using a Chromatin Immunoprecipitation Kit (Active Motif) according to the manufacturer’s instructions. Briefly, bone marrow-derived macrophages were stimulated with or without AngII (10 μM), TNFα (10 ng ml⁻¹) and IL-1β (20 ng ml⁻¹) for 3 h prior to crosslinking for 10 min with 1% formaldehyde. Chromatin was sheared by sonication to an average size of 200~1,000 base pairs (Covaris). Immunoprecipitation was performed using anti-KLF6 antibody (25 ng μl⁻¹, Santa Cruz Biotechnology) and rabbit IgG antibody (25 ng μl⁻¹, Santa Cruz Biotechnology). PCR amplification of the GM-CSF promoter region spanning KLF-binding elements was performed using the following primers: forward:
5’-AAGC CCTTCCAAGAAGCTGGC-3’ and reverse

5’-GGCCCCTCAAAGGAGAGG-3’. KLF6 recruitment was normalized by input DNA and compared to control group with KLF6 antibody.

**RNA isolation and quantitative real-time PCR.** Total RNA from cultured cells, aortic macrophages, bone marrow-derived neutrophils or murine aortic samples was extracted using either RNeasy minikit (Qiagen) or RNA later (Qiagen) according to the manufacturer’s instructions. 0.5-1 μg RNA was reverse-transcribed using Superscript III (Invitrogen) according to the manufacturer’s instructions. Real-time PCR reactions were performed using 2 μl of resulting cDNA per 20 μl reaction volume containing SYBR green I master (Roche). GAPDH was used as an internal control. Using bone marrow-derived macrophages with AngII (10 μM, 3h) stimulation, RT2 profiler PCR array (Qiagen) was performed with 84-related genes for the IL-6/STAT inflammatory pathway. PCR was performed on a LightCycler 480 Real-time PCR system (Roche) in accordance with the manufacturer’s recommended procedure. Real-time PCR primers are shown in Supplementary Table 5.

**Western blot analysis.** Mouse aortic specimens were homogenized with lysis buffer
(T-PER, Thermo Scientific) containing protease inhibitors complex (Roche) and phosphatase inhibitors (Roche). Protein concentration was assayed using BCA protein assay kit (Pierce), and five micrograms of the protein were resolved by 10% NuPAGE (Invitrogen) then transferred to polyvinylidene difluoride membrane. The blot was probed with primary antibodies; pSmad2 (dilution 1:400), pERK1/2 (1:3,000), pSTAT3 (1:3,000), Smad2 (1:1,000), ERK1/2 (1:3,000) or STAT3 (1:3,000) (all rabbit antibodies obtained from Cell Signaling Technology) and anti-GAPDH antibody (1:1,000, Ambion). Membranes were washed and incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology). Protein bands were detected by ECLplus (Thermo scientific) and GAPDH served as an internal control for protein loading.

**Enzyme-linked immunosorbent assay.** Plasma levels of IL-6, MCP-1 and GM-CSF in mice or in humans with or without aortic dissection/intramural hematoma were assayed with commercially available quantikine ELISA kits (R&D systems) according to the manufacturer’s instructions. Sera of healthy volunteers and of patients with aortic aneurysm, coronary artery disease or with aortic dissection were obtained with informed consent under a protocol approved by the University of Tokyo Hospital
Research Ethics Committee. Baseline characteristics of human subjects are shown in Supplementary Table 6.

**Statistical analyses.** All data are presented as means ± s.e.m. Statistical difference between two groups was determined with Student’s *t*-test (two-tailed) for parametric data or Mann-Whitney test for non-parametric data after testing for normality by F-test analysis. For data containing multiple time points, two group comparisons at the same time point were done. When comparing multiple groups, data were analyzed by the Kruskal-Wallis non-parametric one-way ANOVA with Dunn’s post test. Survival curves were created using the Kaplan-Meier method and compared by a log-rank test. Statistical power for mouse experiments was calculated using Biomath (biomath.info/power). All samples sizes were equal to or greater than the recommended minimum group size. All data were analyzed using Prism 6.0 (GraphPad Software). A *P* value of less than 0.05 was considered significant.
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Author contributions

B-K.S. conducted experiments and partially wrote the manuscript. D.S. discussed on results. S.T. conducted experiments. D.F. provided human aortic samples and analysis. K.A., H.A., M.A., I.M. and S.F. consulted on the project. I.K. discussed on results. R.N., T.S. planned and supervised the project. T.S. designed the study and wrote the manuscript. All authors discussed results and commented on the manuscript.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/
Competing financial interest statement: The authors declare no competing financial interests.
Figure legends

Figure 1. Aortic aneurysm and inflammation in *Klf6* heterozygous knockout mice.

(a) Representative aorta (infrarenal aorta:hash, suprarenal aorta:asterisk) induced by 2 weeks of AngII infusion with CaCl₂ application in wild-type (WT) littermates (n=11) and *Klf6* heterozygous knockout (*Klf6*⁺⁻, n=10) mice. Scale bar, 5 mm.

Histopathological analysis of infrarenal (b) and suprarenal aorta (c) by EVG (upper panels, a and c) and H&E staining (lower panels, b and d). Scale bar, 200 μm. (d) Quantification of infrarenal aortic diameters between wild-type (WT) littermates and *Klf6* heterozygous knockout (*K6⁺⁻*) mice before [(−), n=3] and after 2 weeks of AngII infusion with CaCl₂ application (CaCl₂+AngII, WT; n=10, *K6⁺⁻*; n=8). *P <0.05, Student’s *t*-test. (e) Immunofluorescent staining for macrophages (green;Mac3, blue;DAPI) in boxed area of EVG-stained aorta (b) of wild-type littermates and *Klf6* heterozygous knockout mice. Scale bar, 30 μm. (f) Expression of RNA levels of *MMP9, F4/80* and *IL-6* in aorta from wild-type (WT) littermates and *Klf6* heterozygous knockout (*K6⁺⁻*) mice before [(−), n=3] and after AngII infusion with CaCl₂ application (CaCl₂+AngII, n=5) as examined using real-time PCR and normalized by *GAPDH* mRNA. *P <0.05, Mann-Whitney test. (g) Inhibitory effect of clodronate-liposomes on aortic phenotype (n=4) compared with PBS-liposome administered mice (n=5) by
EVG (left panels, a and b); H&E (middle panels, c and d); and F4/80 staining (right panels, e and f, immunohistochemistry). Scale bar, 200 μm. (h) Quantification of infrarenal aortic diameters from clodronate-liposome- or PBS-liposome-administered mice (*P <0.05, Student’s t-test, n=4 or 5 mice per group). Results are from three independent experiments. All values are presented as means ± s.e.m.

**Figure 2. Myeloid Klf6 deficient-mice show aortic dissection/hematoma.** (a)

Representative aorta of Klf6<sup>0/0</sup> control mice (a) and Klf6<sup>0/0</sup>;LysM Cre mice (b) after 2 weeks of AngII infusion with CaCl2 application. Scale bar, 5 mm. (b) Quantification of infrarenal aortic diameters before [(−), n=3] and after 2 weeks of AngII infusion with CaCl2 application (CaCl2+AngII, n=5). Results are from three independent experiments. All values are presented as means ± s.e.m. *P <0.05, Student’s t-test.

(c) Thoracic-abdominal aorta subjected to CaCl2 application and AngII infusion (infrarenal aorta:hash, suprarenal aorta:asterisk). Note that intramural thrombus formation is present in the suprarenal region. Scale bar, 5 mm. (d) Schematic illustration of the diseased aorta (TL: true lumen, FL: false lumen, H: hematoma). (e) Cross-sectional histological sections stained by Elastica van Gieson. a; cross section of the infrarenal abdominal aorta (CaCl2 application region). b; at the level of the renal
arteries. c; suprarenal level where the intima-medial layer shows a tear. d and e; suprarenal descending thoracic aorta beyond the intima-medial tear. Scale bar, 1 mm.

(f) High-magnification cross section at the suprarenal level (c). Intima-medial tear and false lumen/mural thrombus formation are present. Scale bar, 200 μm. (g) Survival curve between $Klf6^{fl/fl}$ control mice (n=19) and $Klf6^{fl/fl};LysM Cre$ mice (n=22) with CaCl$_2$ application and AngII infusion.

**Figure 3. Marked inflammation in aortic lesion of myeloid Klf6 deficient-mice.**

(a) Infiltrated macrophages were visualized by immunofluorescent staining (dotted line, green, Mac3) in aorta of $Klf6^{fl/fl};LysM Cre$ mice (right panels, c and d) compared to $Klf6^{fl/fl}$ mice (left panels, a and b). Scale bar, 100 μm. (b) Immunofluorescent staining for macrophages (b, green, Mac3), pSTAT3 (c, red) and nuclei (d, DAPI, blue) in diseased aorta (a) of $Klf6^{fl/fl};LysM Cre$ mice. Scale bar, 20 μm. (c) Plasma concentration of IL-6 in $Klf6^{fl/fl}$ mice (n=7) and $Klf6^{fl/fl};LysM Cre$ mice (n=9) after 2 weeks of AngII infusion with CaCl$_2$ application. *P <0.05, Student’s t-test  (d) Expression of RNA levels of IL-6 were examined in aorta from $Klf6^{fl/fl}$ mice and $Klf6^{fl/fl};LysM Cre$ mice before [(-), n=3] and after 2 weeks of AngII infusion with CaCl$_2$ application (CaCl$_2$+AngII, n=5) using real-time PCR and normalized by GAPDH
mRNA. (e) Expression of RNA levels of IL-6, CCR2, TNFα, IL-1β, iNOS and MCP-1 were examined in bone marrow-derived macrophages subjected to AngII stimulation (10 μM) for 3 h (n=3 mice per group). (f) Population of CD11b^Ly6C^hi^-cells in aorta, peripheral blood, spleen and bone marrow in Klf6^fl/fl and Klf6^fl/fl;LysM Cre mice after 2 weeks of AngII infusion with CaCl2 application. Results represent three independent experiments. All values are presented as means ± s.e.m. *P <0.05, Mann-Whitney test (d, e).

Figure 4. Involvement of TGFβ pathways in aortic dissection/hematoma.

Expression of mRNA levels of TGFβ1-related factors in aorta from Klf6^fl/fl and Klf6^fl/fl;LysM Cre mice (a), and in wild-type (WT) littermates and Klf6^+/- mice (c), using real-time PCR normalized by GAPDH mRNA. n=5 per group. All values are presented as mean ± s.e.m. Western blot analysis for pSmad2, Smad2, pERK1/2, ERK1/2, pSTAT3, STAT3 or GAPDH in aorta before (−) and after 2 weeks of AngII infusion with CaCl2 application (CaCl2+AngII) in Klf6^fl/fl and Klf6^fl/fl;LysM Cre mice (b) and wild-type (WT) littermates and Klf6^+/- mice (d). Results represent three independent experiments.
Figure 5. **GM-CSF is a direct target of KLF6 in macrophages.** (a) RT2 profiler PCR array analysis of genes related to IL-6/STAT3 inflammatory pathway between bone marrow (BM)-derived macrophages from $Klf6^0/0$ and $Klf6^0/0;LysM Cre$ mice with AngII stimulation (10 μM) for 3 h. Arrow indicates GM-CSF. List of genes that showed consistent changes between BM-derived macrophages from $Klf6^0/0$ and $Klf6^0/0;LysM Cre$ mice stimulated with AngII (10 μM) for 3 h. (b) mRNA expression of GM-CSF in aortic macrophages obtained from $Klf6^0/0$ [sham; (-), n=3; CaCl$_2$+AngII; n=3] and $Klf6^0/0;LysM Cre$ mice [sham; (-), n=3; CaCl$_2$+AngII; n=6]. N.D. indicates not detected. (c) mRNA expression of GM-CSF in aorta of $Klf6^0/0$ and $Klf6^0/0;LysM Cre$ mice at 0 (n=3), 3 (n=3), 7 (n=3) and 14 (n=4) days. (d) Immunohistochemistry for macrophages (red;F4/80, b, scale bar, 30 μm), GM-CSF (green, c) and nucleus (blue;DAPI, d) in aorta of $Klf6^0/0;LysM Cre$ mice with EVG stained infrarenal aorta (a, scale bar, 200 μm). (e) Plasma GM-CSF concentration between $Klf6^0/0$ (n=8) and $Klf6^0/0;LysM Cre$ mice (n=4) after 2 weeks of AngII infusion with CaCl$_2$ application. Results represent three independent experiments. All values are presented as means ± s.e.m. *P <0.05, Mann-Whitney test.
**Figure 6. GM-CSF is required for aortic dissection/intramural hematoma.**  
(a) Representative aortas of *Klf6^fl/fl*,*LysM Cre* mice with administration of anti-GM-CSF neutralizing antibody (b, anti-GM-CSF, n=8) or control IgG antibody (a, n=10) after 2 weeks of AngII infusion with CaCl₂ application. Scale bar, 5 mm. Quantification of infrarenal aortic diameters (b, anti-GM-SCF; n=7, anti-control IgG; n=9) and plasma concentration of IL-6 (c, n=5 or 6) between anti-GM-CSF antibody-administered and anti-control IgG-administered mice. *P <0.05, Student’s *t*-test.  
(d) Expression levels of RNA of *GM-CSFRα, MMP9, F4/80* and *IL-6* were examined in aorta of anti-GM-CSF antibody-administered mice or anti-control IgG administered mice using real-time PCR then normalized by *GAPDH* mRNA (n=5 mice per group).  
(e) Survival curve of mice with administration of recombinant GM-CSF (n=26) or PBS (n=19) with CaCl₂ application and AngII infusion in wild-type mice.  
(f) Representative aorta of wild-type mice with administration of recombinant GM-CSF (b) or PBS (a) with CaCl₂ application and AngII infusion (infrarenal aorta:hash, suprarenal aorta:asterisk) for 4 weeks. Scale bar, 5 mm.  
(g) Histopathological analysis of infrarenal aorta (upper panels, a and c, scale bar, 200 μm) and suprarenal aorta (lower panels, b and d, scale bar, 1 mm) by EVG staining.  
(h) Quantification of infrarenal aortic diameters between recombinant GM-CSF-administered mice or PBS-administered mice [sham;(-) n=3,
CaCl$_2$+AngII; n=5].  (i) Plasma GM-CSF concentration after 2 weeks infusion of recombinant GM-CSF or PBS with or without CaCl$_2$ application and AngII infusion (n=3~5 mice per group).  (j) Expression levels of RNA of $F4/80$ and $IL-6$ were examined in aorta from mice administered recombinant GM-CSF or PBS using real-time PCR then normalized with $GAPDH$ mRNA [sham;(-) n=3, CaCl$_2$+AngII n=5]. Results are from three independent experiments.  All values are presented as means ± s.e.m.  *P <0.05, Mann-Whitney test (d, h, j) and one-way ANOVA with Dunn’s post test (i).

**Figure 7. Increased GM-CSF in patients with acute aortic dissection.**  (a) Plasma GM-CSF concentration in healthy volunteers (Healthy CTL, n=12) and patients with aortic aneurysm (AAA, n=3), coronary artery disease (CAD, n=11) or aortic dissection (n=10).  (b) Immunofluorescent staining for CD68 (red, c, scale bar, 50 μm), GM-CSF (green, d) and DAPI (blue, e) in descending dissected aorta (boxed area, a, scale bar, 2 mm) with EVG staining (b).  Results represent three independent experiments.
Figure 1

- **a** WT vs. Klf6+/−
- **b** WT vs. Klf6+/−
- **c** WT vs. Klf6+/−
- **d** Graph showing diameter (mm) comparison
- **e** Image of F4/80 expression
- **f** Graph showing fold expression to control for MMP9, F4/80, and IL-6
- **g** Images of PBS-liposome treatment
- **h** Graph showing diameter (mm) after PBS and Clodronate-liposome treatment
Figure 2

(a) Klf6fl/fl and Klf6fl/fl;LysM Cre

(b) Diameter (mm)

(Days)

Percent survival

Klf6fl/fl;LysM Cre

(c) Adventitia

(d) FL

(e) TL

(f) Medial tear

(g) Percent survival

Days

Suzuki
Figure 3

**a**  
Klf6<sup>fl/fl</sup>  

**b**  
Klf6<sup>fl/fl</sup>;LysM Cre

**c**

<table>
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<tr>
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<th>Fold expression to control</th>
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<tr>
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<td>(-) CaCl₂+AngII</td>
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**d**

IL-6

**e**

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**f**

Aorta  | Blood  | Spleen  | Bone Marrow

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<tr>
<th></th>
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<tr>
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Suzuki
Figure 4

(a) TGFβ1

Fold expression to control

(-) CaCl₂+AngII

(b) CaCl₂+AngII

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(c) TGFβ1

Fold expression to control

(-) CaCl₂+AngII

(d) CaCl₂+AngII

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Figure 5

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GM-CSF

Fold expression to control

Days

(-) CaCl$_2$+AngII

N.D

D

GM-CSF

Fold expression to control

(Days)

GM-CSF

Fold expression to control

Klf6$^{fl/fl}$;LysM Cre

Klf6$^{fl/fl}$
**Figure 6**

**a**

*Klf6<sup>fl/fl</sup>;LysM Cre*

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**b**

- **Diameter (mm)**

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**c**

- **IL-6 (pg/mL)**

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**d**

- **Fold expression to control**

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**e**

- **Percent survival**

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**f**

- **Diameter (mm)**

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**g**

- **GM-CSF**

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**h**

- **Diameter (mm)**

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**i**

- **GM-CSF (pg/mL)**

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**j**

- **Fold expression to control**

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*F<sub>p</sub><0.0001*
Figure 7

(a) Graph showing GM-CSF (pg/mL) levels in Healthy, AAA, and Aortic dissection groups.

(b) Images illustrating different stages or conditions.

(c) Image depicting cell structure or tissue.

(d) Image showing fluorescent staining or immunohistochemistry.

(e) Image possibly showing another staining or imaging technique.