Deficiency of Interleukin-1 Receptor Antagonist Promotes Spontaneous Femoral Artery Aneurysm Formation in Mice

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Femoral artery aneurysms (FAAs) are very rare, and their natural history is not well understood. In this study, we sought to analyze the pathogenesis of inflammatory FAAs in interleukin-1 receptor antagonist-deficient (*IL-1Ra*^{-/-}) B6 mice. Systolic arterial pressures and plasma lipid levels of *IL-1Ra*^{-/-} mice and wild-type (WT) mice did not differ significantly. However, *IL-1Ra*^{-/-} mice spontaneously developed fusiform FAAs. Real-time PCR of 9-month-old *IL-1Ra*^{-/-} mice revealed significantly increased mRNA levels of IL-1 β (6.6-fold), tumor necrosis factor- α (TNF- α) (12.4-fold), and matrix metalloproteinase-9 (6.0-fold) compared with WT mice. Histological analysis revealed numerous inflammatory cells around the FAAs in *IL-1Ra*^{-/-} mice, and elastin staining showed destruction of both the internal and external elastic lamina in *IL-1Ra*^{-/-} mice. Afterward, macrophage function was studied. After lipopolysaccharide (1 μ g/mL) stimulation, IL-1Ra-deficient macrophages produced much higher levels of TNF- α than those from WT mice. Finally, we performed bone marrow cell transplantation. FAAs with many inflammatory cells in the adventitia were detected in several WT mice that received bone marrow cells from *IL-1Ra*^{-/-} mice (44%), but not from WT mice (0%). Our study is the first to demonstrate that IL-1Ra deficiency in inflammatory cells disrupts immune system homeostasis and induces inflammatory FAAs in *IL-1Ra*^{-/-} B6 mice. We believe that these mice will provide much information about the natural history and management of FAAs. (*Am J Pathol* 2012, 180: 1254–1263; DOI: 10.1016/j.ajpath.2011.11.028)

Arterial aneurysms represent a complex degenerative disorder involving chronic arterial wall inflammation and destructive remodeling of structural connective tissue.¹ The relative balance of pro- and anti-inflammatory cytokines likely controls inflammation in the aneurysm wall.^{2,3} Previous studies demonstrated abundant expression of many cytokines, including IL-1 β , in the aneurysm wall.^{4,5} The IL-1 receptor antagonist (IL-1Ra) negatively regulates IL-1 signaling by binding and blocking the functional receptor without activating it.⁶ Thus, IL-1Ra plays an anti-inflammatory role in acute and chronic inflammation,⁷ but showed no direct evidence implicating endogenous IL-1Ra in aneurysm formation.

Femoral artery aneurysms typically occur at an older age,^{8,9} and those are often bilateral and may associate with multiple aneurysms. A previous report showed that 36% of patients with a femoral artery aneurysm also had a contralateral femoral artery aneurysm, and 69% had multiple aneurysms.¹⁰ Femoral artery aneurysms are very rare, and most studies investigating these aneurysms are published as case reports.^{8,9,11–13} Consequently, the natural history of femoral artery aneurysms is poorly understood.

We demonstrate here for the first time that IL-1Ra-deficient (*IL-1Ra*^{-/-}) mice^{14–16} develop spontaneous femoral artery aneurysms, and the mice represent the first animal model for femoral artery aneurysm. We believe these mice will provide much information about the natural history and management of femoral artery aneurysms.

Materials and Methods

Animals

The experimental protocol used in the study conforms with the Guide for the Care and Use of Laboratory Ani-

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IL-1Ra^{-/-} mice were generated by replacing all exons of the secreted form with the neo gene by homologous recombination as described.^{14–16} Throughout the experiment, the mice consumed a normal diet.

Blood Pressure Measurement

Systolic blood pressure was measured by the tail-cuff method (MK-2000; Muromachi Kikai, Tokyo, Japan) without anesthesia.

Plasma Lipid Measurements

After fasting the mice for 7 hours, plasma total cholesterol, and high-density lipoprotein cholesterol levels were measured by enzymatic assays as previously described by Hedrick et al.¹⁷

Leukocyte and Platelet Counts

Leukocyte and platelet counts were measured in blood using hematology analyzer Vet ABC Animal Blood Counter and ABC VET LMGE Reagent Pack (Horiba ABX, Kyoto, Japan).

Tissue Preparation and Histology

After measuring tail-cuff systolic blood pressure, male mice were euthanized with pentobarbital (dose: 100 mg/kg), and perfused with 0.9% NaCl, followed by 4% paraformaldehyde. Following perfusion, the femoral artery was harvested, fixed overnight in 4% paraformaldehyde, embedded in optimal cutting temperature compounds and sectioned (10 μ m thickness). Samples were routinely stained with H&E, Picrosirius red, Masson trichrome, or elastica van Gieson. Smooth muscle cells (SMCs) were visualized with α -smooth muscle actin staining (Roche, Indianapolis, IN), and apoptotic cells were visualized with cleaved caspase-3 antibody (Cell Signaling Technology, Danvers, MA). Granulocytes were visualized with monoclonal Ly-6G (BD Biosciences/Pharmingen, Franklin Lakes, NJ), mouse macrophages were visualized with monoclonal Mac-3 (BD Biosciences Pharmingen), and T cells were visualized with monoclonal CD4 (BD Biosciences Pharmingen). We also checked the expression of tumor necrosis factor- α (TNF- α) (Abcam, Cambridge, UK) and matrix metalloproteinase-9 (MMP-9) (Abcam). Endogenous peroxidase was blocked by incubation with 3% H₂O₂ in methanol for 5 minutes. Slides were incubated with normal swine serum (Vector Laboratories, Burlingame, CA) for 10 minutes and then with primary antibody overnight at 4°C. The secondary antibody was the biotinylated antibody (Dako, Glostrup, Denmark). We visualized the sections with a Vectastain ABC kit (Vector Laboratories), using diaminobenzidine as the substrate. The nuclei were counterstained with Mayer's hematoxylin solution.

Morphometry

We removed femoral artery specimens from mice at 7, 8, and 9 months of age and used 20 equally spaced cross sections in all mice for quantification. The images were digitized to perform morphometric analysis using ECLIPS LV100 microscopy (Nikon, Tokyo, Japan), and we measured the maximal vessel area (the area within the media/adventitial border) and lumen area using NIS-Elements (Nikon). The intimal + medial area was calculated by subtracting the lumen area from the vessel area. Cleaved caspase-3-positive nuclei were quantified, and apoptotic indexes (apoptotic nuclei/total nuclei $\times$ 100) were calculated. α -SMA-positive areas were calculated in the cross sections of the media of femoral arteries and expressed as a percentage of the medial area. MMP-9- and TNF- α -positive areas were calculated in the longitudinal sections of femoral arteries and expressed as a percentage of the total area. For quantification of the positive area, captured photomicrographs were transferred into an image analysis system (ImagePro Plus 5.1; Media Cybernetics, Bethesda, MD). A color-threshold mask for immunostaining was established to detect reddish brown (positive) staining, and the same threshold was applied to all specimens. The percentage area with positive color for each section was recorded.

Analysis of Gene Expression by Real-Time Quantitative PCR

Real-time PCR was performed as described before.¹⁶ RNA was prepared from the pooled samples using TRI Reagent (Sigma-Aldrich, St. Louis, MO). Complementary DNA was synthesized using reverse transcriptase from 200 ng of total RNA according to the manufacturer's protocol (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA). The following oligonucleotide primer pairs were used: IL-1 α sense, 5'-CAAACCTGATGAAGCTCGTCA-3'; antisense, 5'-TCTC-TTGAGCGCTCACGAA-3'; IL-1 β sense, 5'-TGGTGTG-TGACGTTCCCAT-3'; antisense, 5'-CAGCACGAGGC-TTTTTGTG-3'; MCP-1 sense, 5'-GCCCAGCACCAG-CACCAG-3'; antisense, 5'-GGCATCACAGTCCGAGTC-ACAC-3'; MMP-9 sense, 5'-TGAGTGAGTTGGACT-CTCGG-3'; antisense, 5'-CTCTCTACTGGGCGTTAGGG-3'; TNF- α sense, 5'-TCCCAGGTTCTCTCAAGGGA-3'; antisense, 5'-GGTGAGGAGCACGTAGTCGG-'; urokinase-type plasminogen activator (u-PA) sense, 5'-GCTC-CTATAATCCTGGAGAGATGAA-3'; antisense, 5'-AC-CTGTCTTTTCAGCTTCTCCCTCC-3'; 18S rRNA sense, 5'-AGTCGGAGGTTCCAAGACGAT-3'; antisense, 5'-GCGGGTCATGGGAATAACG-3'. These primers were mixed with the Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan). The ABI PRISM 7900HT machine (Applied Biosystems) was used for real-time PCR by denaturation at 95°C for 10 minutes, followed by 40 amplification cycles (15 seconds at 95°C and 30 seconds at 60°C). Each mRNA expression level was corrected by 18S mRNA level. Data were analyzed using RQ Manager 1.2 (Applied Biosystems).

Table 1. Characteristics of Wild-Type and *IL-1Ra*^{-/-} Mice at 7 and 9 Months of Age

	7 months			9 months		
	WT (n = 6)	<i>Ra</i> ^{-/-} (n = 6)	P value	WT (n = 6)	<i>Ra</i> ^{-/-} (n = 6)	P value
BW (g)	31.0 ± 1.2	27.1 ± 0.8	0.02	31.8 ± 1.0	27.5 ± 0.7	0.01
SBP (mm Hg)	92.2 ± 3.9	81.8 ± 4.6	0.12	96.3 ± 8.3	90.0 ± 9.7	0.63
T-chol (mg/dL)	99.5 ± 4.3	86.5 ± 4.4	0.61	101.7 ± 4.3	101.5 ± 3.9	0.97
HDL (mg/dL)	79.5 ± 5.5	63.8 ± 2.8	0.66	61.8 ± 3.7	56.0 ± 2.2	0.21
TG (mg/dL)	40.6 ± 2.0	42.2 ± 2.9	0.67	50.3 ± 4.1	63.7 ± 5.8	0.09
Leukocytes	41.5 ± 2.6	38.7 ± 2.5	0.44	41.0 ± 1.9	43.5 ± 3.7	0.56
Platelets	90.0 ± 3.8	92.6 ± 2.4	0.58	83.6 ± 6.7	110.7 ± 11.7	0.07

The results are shown as the mean ± SEM.

BW, body weight; HDL, high-density lipoprotein; SBP, systolic blood pressure; T-chol, total cholesterol; TG, triglyceride.

Macrophages and T-Cell Activation Study

T cells were prepared from the spleen and lymph nodes of *IL-1Ra*^{-/-} (6 to 8 week old) and WT (6 to 8 week old) mice, and then the cells were treated with hemolysis buffer [17 mmol/L Tris-HCl and 140 mmol/L NH₄Cl (pH 7.2)] to remove red blood cells, washed, and passed through a nylon wool column. Then anti-mouse B220 and anti-Mac-1 magnetic bead (Miltenyi Biotec, Bergisch Gladbach, Germany)-treated cells were passed through a MACS column (Miltenyi Biotec) to obtain T cells. Purified T cells (2 × 10⁵ cells) were plated on a 96-well plate coated with 10 μg/mL of anti-mouse CD3 mAb (145-2C11; BD Biosciences/Pharmingen) in a final volume of 200 μL of Roswell Park Memorial Institute (RPMI) 1640 plus 10% fetal calf serum and cultured for 48 hours.

Peritoneal macrophages were recovered from mice 4 days after intraperitoneal injection of 2 mL of 2% thioglycollate broth (Nissui Pharmaceutical Co., Tokyo, Japan), and those (2 × 10⁵ cells) were plated on a 96-well plate and stimulated with 1 μg/mL lipopolysaccharide for 18 hours. Culture supernatant was then collected. We measured TNF-α levels in culture using enzyme-linked immunosorbent assay as described before.¹⁸

In Vitro Migration Assay

Migration of mouse peritoneal macrophages was measured in a modified Boyden chamber migration assay using the Chemicon QCM 96-well (5 μm) Cell Migration Assay kit (Millipore, Billerica, MA). A total of 1 × 10⁵ cells from WT or *IL-1Ra*^{-/-} mice were loaded into the migration chamber, and medium containing 10 ng/mL MCP-1 was placed in the lower chamber. After allowing cell migration for 12 hours, cells were removed from the upper side of the membrane, and migratory cells on the lower side of the membrane were collected. These cells were lysed and detected by the patented CyQuant GR dye.

Bone Marrow Cell Transplantation

To elucidate the role of bone marrow (BM) cells in the development of femoral artery aneurysms, BM cell transplantation (BMT) was performed as described previ-

ously.¹⁸ For BM cell transplantation, BM cells were taken from femurs, tibias, and pelvises of *IL-1Ra*^{-/-} (5 to 6 week old) and WT (5 to 6 week old) mice and were treated with hemolysis buffer. Purified BM cells (10⁷ cells/mouse) in 0.2 mL of phosphate-buffered saline (PBS) were transplanted intravenously into lethally irradiated (750 rad) recipient mice at 6 weeks of age.

Statistical Analysis

The results are shown as mean ± SEM. Statistical analysis was performed by unpaired *t*-test or analysis of variance. A value of *P* < 0.05 was regarded as a significant difference. All tests were performed using SPSS software, version 14.0 (IBM, Armonk, NY).

Results

IL-1Ra^{-/-} Mice Spontaneously Develop Fusiform Femoral Artery Aneurysms

Phenotypic analysis of *IL-1Ra*^{-/-} mice backcrossed to the C57BL/6J background for eight generations revealed that the body weight of 7-month-old *IL-1Ra*^{-/-} male mice was lower than that of the WT mice (*n* = 6 for each group) (Table 1). We observed no significant differences in systolic blood pressure, plasma total cholesterol levels, or high-density lipoprotein cholesterol levels, triglyceride levels, leukocyte counts, or platelet counts between groups (*n* = 6 for each group) (Table 1). In addition, femoral artery diameter was similar in *IL-1Ra*^{-/-} (*n* = 12) and WT mice (*n* = 12). However, *IL-1Ra*^{-/-} mice (*n* = 12) spontaneously developed fusiform femoral artery aneurysms (Figure 1A) after 8 months of age, and both maximal vessel area and intimal + medial area of femoral arteries in such mice were significantly larger compared with WT mice (*n* = 12) after 8 months of age (Figure 1B). We observed numerous inflammatory cells around the fusiform femoral artery aneurysm in *IL-1Ra*^{-/-} mice, and the lumen was larger in *IL-1Ra*^{-/-} mice compared with WT mice (Figure 1A). The maximal vessel area and intima + medial area of aneurysms in 9-month-old *IL-1Ra*^{-/-} mice (*n* = 12) were significantly larger compared with 8-month-old *IL-1Ra*^{-/-} mice (Figure 1B). No WT mice developed femoral artery aneurysms during our experimental period (Figure 1B). We checked characteristics in

Figure 1. *IL-1Ra*^{-/-} mice spontaneously developed femoral artery aneurysms. **A:** Macroscopic appearance of femoral arteries of *IL-1Ra*^{-/-} (right panel) and WT mice (left panel) at 8 months of age (upper panels). Femoral artery sections were stained with hematoxylin and eosin (middle panels) or Masson's trichrome (lower panels). Scale bar = 200 μm . **B:** Femoral artery specimens were taken from *IL-1Ra*^{-/-} and WT mice at 7 months ($n = 12$ for each group), 8 months ($n = 12$ for each group), or 9 months ($n = 12$ for each group). We analyzed maximal vessel area (left) as well as intima + medial area (right). ** $P < 0.01$, *** $P < 0.001$.

both groups at 9 months of age ($n = 6$ for each group) (Table 1). Triglyceride levels and platelet counts tended to increased in *IL-1Ra*^{-/-} mice compared to WT mice, but did not reached statistical significance (Table 1).

We performed histological examination of the maximal size of femoral arteries from *IL-1Ra*^{-/-} mice at 7, 8, 9, and 10 months of age (Figure 2A). At 7 months of age, the femoral artery media consisted predominantly of SMCs (brown layer) and few connective tissues (red fibers), and lacked intimal hyperplasia. By contrast, 8-month-old *IL-1Ra*^{-/-} mice showed increased medial thickness and elevated SMCs. Furthermore, the aneurismal arterial wall of 9-month-old *IL-1Ra*^{-/-} mice developed intimal hyperplasia, resulting in more pronounced thickness. Moreover, elastin staining revealed destruction of the internal and external elastic lamina. After 10 months of age, the intimal + medial area of aneurismal femoral arteries plateaued in *IL-1Ra*^{-/-} mice, and histological analysis showed acellular layers in the media of aneurismal femoral arteries (Figure 2A). In contrast, nonaneurismal femoral arteries in *IL-1Ra*^{-/-} mice retained their normal structures, and we observed no inflammatory cells in the adventitia (Figure 2A). At 10 months, immunostaining revealed that the α -SMA-positive area in the media of *IL-1Ra*^{-/-} mice ($n = 6$) was significantly decreased compared with WT mice ($n = 6$) ($P < 0.0001$) (Figure 2B). Importantly, at 9 months, the cleaved caspase-3-positive apoptotic index was significantly increased in the media of *IL-1Ra*^{-/-} mice ($n = 6$) compared with that of WT mice ($n = 6$) ($P < 0.0001$) (Figure 2C).

The longitudinal sections displayed the entire developmental progression of aneurysms from normal tissue to aneurysm formation (Figure 3B). We observed many inflammatory cells in the adventitia at the initiation site of neointima formation, and also detected MMP-9 protein

expression in the media and neointima, and several inflammatory cells at the initiation site (Figure 3B). As shown Figure 3C, the percent positive area of MMP-9 in *IL-1Ra*^{-/-} mice ($n = 6$) was much larger than in WT mice ($n = 6$). Furthermore, we observed TNF- α protein expression in neointimal cells and in the many inflammatory cells found in the adventitia at the site, and quantitative analysis also revealed TNF- α expression increased significantly in 9-month-old *IL-1Ra*^{-/-} ($n = 6$) compared to 9-month-old WT mice ($n = 6$) (Figure 3, B and C). These changes (ie, accumulation of inflammatory cells in the adventitia, and expression of TNF- α and MMP-9) might contribute to aneurysm formation. Indeed, the changes did not occur in the normal femoral arteries of WT mice (Figure 3A). The normal femoral artery is a muscular artery, consisting mainly of SMCs in the media and little collagen (Figure 3A). Many collagens were detected, mainly in the adventitia. By contrast, we detected collagen in the neointima of femoral artery aneurysm, and polarization microscopy showed the collagen present as a green color (Figure 3B).

The detection rates of both bilateral femoral artery aneurysms (16.6% at 8 months, 50% at 9 months, and 58.2% at 10 months) and multiple aneurysms with other peripheral artery aneurysms (ie, deep femoral artery aneurysm: 0% at 8 months, 33.3% at 9 months, and 33.3% at 10 months; popliteal artery aneurysm: 0% at 8 months, 33.3% at 9 months, and 50% at 10 months) increased with age. Interestingly, longitudinal sections of aneurysm in 9-month-old *IL-1Ra*^{-/-} mice showed many inflammatory cells, predominantly in the adventitia at the aneurysm initiation site (Figure 4). Immunostaining revealed that the majority of inflammatory cells were granulocytes. Moreover, we detected several

Figure 2. Time-dependent changes of histological findings of fusiform femoral artery aneurysms. **A:** Histological sections of femoral arteries of *IL-1Ra*^{-/-} mice at 7 months (upper left), 8 months (8M, upper middle), 9 months (9M, upper right), and 10 months (10M, lower panels). The sections were stained with elastica van Gieson (upper panels) or hematoxylin and eosin (lower panels). Scale bar = 100 μm. Black arrows show the internal elastic lamina, and blue arrows show the external elastic lamina. **B:** Microscopic appearance for α-SMA staining of femoral arteries from WT (upper left) and *IL-1Ra*^{-/-} mice (upper right) at 10 months (10M) of age. Scale bar = 100 μm. Lower graph shows quantification of α-SMA-positive area in the media of 10-month-old *IL-1Ra*^{-/-} (n = 6) and WT mice (n = 6). ****P < 0.0001. **C:** Microscopic appearance for cleaved caspase-3 staining of femoral arteries from WT (upper left) and *IL-1Ra*^{-/-} mice (upper right) at 9 months (9M) of age. Scale bar = 100 μm. Lower graph shows cleaved caspase-3-positive apoptotic indexes in the media of 9-month-old *IL-1Ra*^{-/-} (n = 6) and WT mice (n = 6). ****P < 0.0001.

macrophages (Figure 4) and a few T cells (data not shown) at the same site.

Enhanced mRNA Expression of Cytokines, Chemokines, and the Regulation Genes of Extracellular Matrix in Femoral Aneurysms in *IL-1Ra*^{-/-} Mice

Using femoral arteries of 7- and 9-month-old WT (n = 6 for each group) and 9-month-old *IL-1Ra*^{-/-} mice (n = 6 for each group), we analyzed mRNA expression of genes involved in cytokines and chemokines, and the selected genes that regulate extracellular matrix formation. Notably, we used the sites of femoral artery aneurysms and nonaneurysmal arteries to analyze the arteries of 9-month-old *IL-1Ra*^{-/-} mice, and normal arteries of 9-month-old WT mice. mRNA levels did not differ significantly between WT and *IL-1Ra*^{-/-} mice before aneurysm formation (at 7 months of age) (data not shown). By contrast, real-time PCR of aneurysms in 9-month-old *IL-1Ra*^{-/-} mice revealed significantly increased mRNA levels of IL-1α (6.4- and 3.8-fold, P < 0.05), IL-1β (7.0- and 4.1-fold, P < 0.05) and TNF-α (13.3- and 8.9-fold, P < 0.05) compared with arteries in WT mice and nonaneurysm arteries in *IL-1Ra*^{-/-} mice (Figure 5). Furthermore, MCP-1 mRNA levels increased significantly in aneurysms in *IL-1Ra*^{-/-} mice (8.2- and 5.4-fold, P < 0.05) compared with normal arteries in both WT and *IL-1Ra*^{-/-} mice (Figure 5). In the selected genes regulating extracellular matrix formation, mRNA levels of u-PA (7.5- and 5.6-fold, P < 0.05) and MMP-9 (6.0- and 4.7-fold, P < 0.01) were significantly higher in aneurysms of *IL-1Ra*^{-/-} mice compared with normal arteries in both WT and *IL-1Ra*^{-/-} mice (Figure 5). Therefore, IL-1Ra deficiency may induce chronic medial and adventitial inflammation and aneurysm development, possibly due to enhanced mRNA expression of cytokines and chemokines, and the regulation genes of extracellular matrix. We also investigated mRNA expression in the ascending aorta, aortic arch, descending aorta, and abdominal aorta of both groups. Only MMP-9 mRNA levels in the abdominal aorta in *IL-1Ra*^{-/-} mice were significantly higher than those in WT mice (P < 0.05); other genes did not differ significantly between groups. Very few *IL-1Ra*^{-/-} mice (2.4%, 2 of 83), and no WT mice developed abdominal aortic aneurysms. Neither group developed thoracic aortic aneurysms. Our results suggest that *IL-1Ra*^{-/-} mice changed mRNA expression and developed aneurysm in femoral artery specifically.

Spontaneous Development of Saccular, Dissecting, and Thrombosed Large Femoral Artery Aneurysms in *IL-1Ra*^{-/-} Mice

Interestingly, we frequently observed saccular aneurysms, which appear like a small blister on the side of the femoral artery (Figure 6, A and B), with fusiform aneurysms in 10-month-old *IL-1Ra*^{-/-} mice (75%, 9 of 12). The detection rate for saccular aneurysms on the femoral

Figure 3. Histological findings of fusiform femoral artery aneurysms. **A:** The longitudinal sample from a 9-month-old WT mouse was stained with hematoxylin and eosin (**upper left**). **Boxed area** is shown **below** and in **right panels**. **Middle left panel** shows Picrosirius Red staining without polarization. **Lower left panel** showed Picrosirius Red staining viewed under linearity polarized light in the same section as in the **upper panel**. The serial sections were stained with MMP-9 (**upper right**) or TNF-α (**lower right**). Scale bar = 100 μm. **B:** The longitudinal femoral artery aneurysm sample from a 9-month-old *IL-1Ra*^{-/-} mouse was stained with hematoxylin and eosin (**upper left**). **Boxed area** is shown **below** and in **right panels**. **Middle left panel** shows Picrosirius Red staining without polarization. **Lower left panel** showed Picrosirius Red staining viewed under linearity polarized light in the same section as in the **upper panel**. The serial sections were stained for MMP-9 (**upper right**) or TNF-α (**lower right**). Scale bar = 200 μm. **C:** Quantification of MMP-9 and TNF-α expression in the femoral arteries of 9-month-old *IL-1Ra*^{-/-} (*n* = 6) and WT mice (*n* = 6). ****P* < 0.001.

artery increased with age (0% at 8 months, 25% at 9 months, and 75% at 10 months).

Although the femoral artery aneurysms of several 9-month-old *IL-1Ra*^{-/-} mice (6.4%, 6 of 93) were red (Figure 6C), the majority were white (Figure 1A). Following perfusion, we harvested, fixed, and sectioned the aneurysms that showed as red. Remarkably, intimal and medial thickening increased in the aneurysms that showed red, and dissection occurred between the intima and media (Figure 6D). Elastin staining revealed frag-

mentation of the internal and external elastic lamina (Figure 6D) in *IL-1Ra*^{-/-} mice.

Furthermore, several thrombosed large aneurysms of femoral and/or popliteal arteries were detected in 10-month-old *IL-1Ra*^{-/-} mice (24%, 7 of 29) (Figure 6E). However, we did not find any ruptured large aneurysms.

Activation of *IL-1Ra*^{-/-} Macrophages and T Cells Is Excessive Compared with WT Mice

We next studied macrophage and T-cell function. Following lipopolysaccharide (1 μg/mL) stimulation, *IL-1Ra*-deficient macrophages from 6- to 8-week-old *IL-1Ra*^{-/-} mice produced much higher levels of TNF-α compared with 6- to 8-week-old WT mice (*n* = 6) (Figure 7A). Furthermore, TNF-α production of T cells in 6- to 8-week-old *IL-1Ra*^{-/-} mice stimulated with anti-CD3 antibody was higher compared with 6- to 8-week-old WT mice (*n* = 6) (Figure 7A). Our results suggest excessive activation of *IL-1Ra*-deficient macrophages and T cells compared with WT mice, even at the same levels of stimulation.

Migration Activity of *IL-1Ra*^{-/-} Macrophages Is Increased Compared with that of WT Macrophages

As shown in Table 1, total counts of leukocytes were similar between WT and *IL-1Ra*^{-/-} mice at both 7 and 9 months of age, suggesting proliferation of peripheral leukocytes were not increased in *IL-1Ra*^{-/-} mice compared

Figure 4. Immunostaining findings of fusiform aneurysms. The longitudinal sample from a 9-month-old *IL-1Ra*^{-/-} mouse was stained with hematoxylin and eosin (**upper left**). Scale bar = 500 μm. **Boxed area** is shown in **upper right panel**. Scale bar = 50 μm. Immunostaining for macrophages (**lower left**) and granulocytes (**lower right**) was performed. Scale bar = 50 μm. **Red arrows** indicate positive cells (macrophages).

Figure 5. Enhanced mRNA expression of cytokines, chemokines, and the regulation genes of extracellular matrix in femoral aneurysms in *IL-1Ra*^{-/-} mice. mRNA levels of the indicated genes in the femoral arteries of 9-month old of WT (*n* = 6), nonaneurysmal artery in *IL-1Ra*^{-/-} (*n* = 6), and aneurysmal artery in *IL-1Ra*^{-/-} mice (*n* = 6) were quantified using real-time PCR. Degree of change in gene expression is based on the 7-month baseline expression level of WT mice. **P* < 0.05 WT versus *Ra*^{-/-} (+); ***P* < 0.01 WT versus *Ra*^{-/-} (+); ****P* < 0.001 WT versus *Ra*^{-/-} (+); †*P* < 0.05 *Ra*^{-/-} (-) versus *Ra*^{-/-} (+); ††*P* < 0.01 *Ra*^{-/-} (-) versus *Ra*^{-/-} (+); †††*P* < 0.001 *Ra*^{-/-} (-) versus *Ra*^{-/-} (+).

with WT mice. Therefore, further experiments determined the chemotactic response of WT and *IL-1Ra*^{-/-} macrophages to MCP-1. The migration activity of *IL-1Ra*^{-/-} macrophages (*n* = 6) were 4.2-fold higher than that of WT macrophages (*n* = 6) (Figure 7B). These findings indicated that the increased presence of inflammatory cells in lesions of *IL-1Ra*^{-/-} mice could result from increased recruitment of inflammatory cells.

IL-1Ra Deficiency in Hematopoietic Cell Types Participates Crucially in the Development of Femoral Artery Aneurysm

To test the hypothesis that *IL-1Ra* deficiency in hematopoietic cell types contributes importantly to the development of femoral artery aneurysm, we performed BMT. After injecting BM from *IL-1Ra*^{-/-} or WT mice into lethally irradiated WT mice, we detected femoral artery aneurysms with increased intimal + medial area and many inflammatory cells in the adventitia of several WT mice (55%, 6 of 11) that received BM cells from *IL-1Ra*^{-/-} mice, but not from WT mice (0%, 0 of 10) (Figure 8A) at 8 months post-BMT. Quantitative analyses also showed

Figure 6. Histological findings of saccular dissecting and thrombosed femoral artery aneurysms in *IL-1Ra*^{-/-} mice. **A:** The macroscopic appearance of the femoral artery showed saccular aneurysm (black arrows) in a 10-month-old *IL-1Ra*^{-/-} mouse. **B:** The longitudinal section of saccular aneurysm in a 10-month-old *IL-1Ra*^{-/-} mouse was stained with elastica van Gieson. Scale bar = 1 mm. **C:** The macroscopic appearance of the femoral artery showed as red (black arrows) in a 9-month-old *IL-1Ra*^{-/-} mouse. **D:** The longitudinal sample from a 9-month-old *IL-1Ra*^{-/-} mouse was stained with elastica van Gieson (scale bar = 200 μm). **E:** The cross section of a thrombosed large aneurysm in a 10-month-old *IL-1Ra*^{-/-} mouse was stained with hematoxylin and eosin. Scale bar = 2 mm.

Figure 7. Functions of macrophages and T cells from WT and *IL-1Ra*^{-/-} mice. **A:** Activation of *IL-1Ra*^{-/-} macrophages (Mac) and T cells is excessive compared with WT mice. Peritoneal macrophages (2×10^5 cells) were plated on a 96-well plate and stimulated with 1 μg/mL lipopolysaccharide for 18 hours (left). Purified T cells (2×10^5 cells) were plated on a 96-well plate coated with anti-mouse CD3 antibody and cultured for 48 hours (right). TNF-α levels in the cultured supernatants were measured by enzyme-linked immunosorbent assay (*n* = 6). ***P* < 0.01. **B:** Deficiency of *IL-1Ra* increased MCP-1-mediated macrophage migration. Data are shown as mean ± SEM (*n* = 6). ***P* < 0.01.

Figure 8. IL-1Ra deficiency in hematopoietic cell types participates in the development of femoral artery aneurysm. **A:** Bone marrow (BM) of *IL-1Ra*^{-/-} (middle, Ra^{-/-} > WT) or WT (left, WT > WT) mice was injected in lethally irradiated recipient WT mice. Furthermore, BM of WT mice was injected in lethally irradiated recipient *IL-1Ra*^{-/-} mice (right, WT > Ra^{-/-}). We detected femoral artery aneurysms in several WT mice receiving BM cells from *IL-1Ra*^{-/-} mice [upper middle (scale bar = 500 μ m) and lower middle (scale bar = 200 μ m)], but not WT mice [upper left (scale bar = 500 μ m) and lower left (scale bar = 200 μ m)]. Moreover, BM of WT mice significantly suppressed the development of femoral artery aneurysms in *IL-1Ra*^{-/-} mice [upper right (scale bar = 500 μ m) and lower right (scale bar = 200 μ m)]. **(B)** Femoral artery specimens were taken from WT mice receiving BM cells from *IL-1Ra*^{-/-} mice ($n = 11$) or WT mice ($n = 10$), and *IL-1Ra*^{-/-} mice receiving BM cells from WT mice ($n = 7$) at 8 months after bone marrow cell transplantation. We analyzed maximal vessel area (upper left), maximal lumen area (upper right), and intima + medial area (lower left). * $P < 0.05$ Ra^{-/-} > WT versus WT > WT; † $P < 0.05$ Ra^{-/-} > WT versus WT > Ra^{-/-}.

maximal vessel area, maximal lumen area, and intima + medial area were significantly larger in WT mice receiving BM cells from *IL-1Ra*^{-/-} compared with WT mice receiving BM cells from WT mice at 8 months post-BMT (Figure 8B). In contrast, BM of WT mice significantly suppressed the development of femoral artery aneurysm in *IL-1Ra*^{-/-} mice at 8 months post-BMT (14%, 1/7) (Figure 8A), and quantitative analyses revealed maximal vessel area, maximal lumen area, and intima + medial area were significantly smaller in *IL-1Ra*^{-/-} mice receiving BM cells from WT mice compared with WT mice receiving BM cells from *IL-1Ra*^{-/-} mice at 8 months post-BMT (Figure 8B). Thus, IL-1Ra-deficient hematopoietic cells induce femoral artery aneurysm.

Discussion

Femoral artery aneurysms are rare, and their natural history is poorly understood. Occurring most frequently in elderly persons,^{8,9} such aneurysms are often bilateral and may associate with multiple aneurysms. A previous report showed that 36% of patients with a femoral artery aneurysm also had a contralateral femoral artery aneurysm, and 69% had multiple aneurysms.¹⁰ Our study detected femoral artery aneurysms in *IL-1Ra*^{-/-} mice older than 8 months of age. Among 10-month-old *IL-1Ra*^{-/-} mice, 58% (7 of 12) had bilateral femoral artery aneurysms and 50% (6 of 12) had popliteal artery aneurysms. These findings are very similar to results previously reported in humans.^{8–10}

Yamamoto et al⁹ reported that femoral artery aneurysms with a background of arteriomegaly defined as diffuse arterial enlargement involving several arterial segments, might represent a subgroup of patients at risk for multiple femoral aneurysms. In the results reported here, 67% (8 of 12) of 8-month-old *IL-1Ra*^{-/-} mice demon-

strated diffuse long fusiform femoral artery aneurysms; almost all mice with such lesions developed multiple aneurysms. Thus, if diffuse long fusiform lesions were described as arteriomegaly, our results might support Yamamoto and colleagues' suggestions. Interestingly, such dilating diathesis has not been observed in patients with abdominal aortic aneurysm (AAA).¹¹ In our study, very few *IL-1Ra*^{-/-} mice developed AAA (2.4%, 2 of 83), suggesting that other specific factors—genetic or other—may participate in the development of femoral artery aneurysm.

Aneurysms in peripheral arteries may associate with etiological factors such as infectious arteritis, noninfectious immunological or inflammatory arteritis, and connective tissue diseases, but atherosclerotic aneurysms occur frequently.^{12,13} However, the notion that atherosclerotic changes in the aneurismal wall cause or result from arterial aneurysm remains controversial. The current study showed no significant differences in plasma total cholesterol levels, triglyceride levels, or high-density lipoprotein cholesterol levels between *IL-1Ra*^{-/-} and WT mice at 7 and 9 months of age. Interestingly, a comparison between 7-month-old and 9-month-old mice in the same strain revealed that increased total cholesterol levels ($P = 0.03$) with an increase in the levels of triglyceride ($P = 0.007$) were detected in *IL-1Ra*^{-/-} mice, but were not significant in WT mice. Increased triglyceride levels might induce the development of aneurysm, but we thought that chronic inflammation in *IL-1Ra*^{-/-} mice should increase triglyceride levels. Furthermore, we detected no atherosclerotic occlusive lesions in arteries. Our results suggest that femoral artery aneurysm may associate mainly with inflammation, not atherosclerotic changes. However, atherosclerosis involves an ongoing inflammatory response,¹⁹ and we believe atherosclero-

sis-induced inflammation likely contributes to femoral artery aneurysm.

The normal femoral artery is a muscular artery, characterized mainly by SMCs in the media and little collagen. In contrast, Sirius Red staining revealed collagen in the neointima of femoral artery aneurysm, and polarization microscopy showed the collagen presented as green, suggesting several collagen type IIIs might be included in it.²⁰ These findings might be supported by a previous report that showed collagen, especially type III, synthesis increases during the early stages of aneurysm formation.²¹ Furthermore, we found high collagen content at the initiation site of aneurysm formation. In early stages of aneurysm, interstitial collagen in the adventitia provides tensile strength to the arterial wall. In later stages, collagen degeneration exceeds its synthesis, ultimately favoring the enlargement of aneurysm.²² We also detected several elastic laminae at the initiation site of aneurysm formation (data not shown). By contrast, we observed only two elastic laminae (internal and external) at the normal site. However, elastic lamina degradation exceeds its synthesis in later stages of aneurysm formation (accompanied by excessive degradation of other extracellular matrix macromolecules), ultimately favoring arterial wall dissection. Increased turnover and loss of elastin fibers caused by increased MMP expression probably underlie femoral artery dilation and dissection.

We detected that TNF- α protein expression was significantly higher in the femoral arteries in *IL-1Ra*^{-/-} mice compared to WT mice. A previous report showed that TNF- α can initiate apoptosis in many cell types.²³ Indeed, immunostaining revealed that the cleaved caspase-3-positive apoptotic index was significantly increased in the media of *IL-1Ra*^{-/-} mice compared with that of WT mice at 9 months of age ($P < 0.0001$). Furthermore, the α -SMA-positive area in the media of *IL-1Ra*^{-/-} mice was significantly decreased compared with WT mice ($P < 0.0001$). These processes induced by excessive inflammation in *IL-1Ra*^{-/-} mice should act in concert to progressively weaken the arterial wall, resulting in dilation and aneurysm formation.

Real-time PCR of aneurysms in 9-month-old *IL-1Ra*^{-/-} mice revealed significantly increased mRNA levels of cytokines and chemokines, and the regulation genes of extracellular matrix compared with both normal arteries in WT mice and nonaneurysmal arteries in *IL-1Ra*^{-/-} mice. Histological analysis showed that nonaneurysmal femoral arteries in *IL-1Ra*^{-/-} mice retained normal structures without any inflammatory cells in the adventitia. These data strengthen the results of real-time PCR, suggesting that altered mRNA might be a specific change for aneurysm formation. Taken together, increased femoral artery aneurysm formation in *IL-1Ra*^{-/-} mice results from greater local inflammation induced by cytokines, chemokines, and MMP.

IL-1Ra-deficient macrophages and T cells produced much higher levels of TNF- α compared to WT mice receiving the same stimulation. Our findings suggest that excessive activation occurs more frequently in IL-1Ra-deficient macrophages and T cells compared with those

from WT mice. Furthermore, we also showed that the migration activity of *IL-1Ra*^{-/-} macrophages were 4.2-fold higher than that of WT macrophages. Taken together, *IL-1Ra*^{-/-} inflammatory cells increased migration activity and production of proinflammatory cytokines around the femoral artery, thus inducing inflammatory femoral artery aneurysm.

Because our results also suggested that IL-1Ra deficiency in inflammatory cells might promote aneurysms, we performed BMT. We observed femoral artery aneurysms in several WT mice receiving BM cells from *IL-1Ra*^{-/-} mice (55%), but not WT mice (0%), demonstrating that IL-1Ra deficiency in inflammatory cells induces aneurysm. IL-1Ra protein expressed by endothelial cells¹⁵ and SMCs²⁴ may protect the vascular wall from inflammation induced by IL-1Ra-deficient inflammatory cells, possibly explaining why only 55% of WT mice receiving IL-1Ra-deficient BM cells developed femoral artery aneurysms. In contrast, BM of WT mice significantly suppressed the development of femoral artery aneurysm in *IL-1Ra*^{-/-} mice at 8 months post-BMT (14%, 1 of 7), suggesting active *IL-1Ra*^{-/-} inflammation might be needed to cause femoral artery aneurysm.

Most aneurysm lesion sites in the mouse models are abdominal aorta, and those lesions are evoked by genetically defined approaches and/or by chemical means. The genetic approaches are spontaneous and engineered mutations. Mice that lack the gene for apolipoprotein E (*ApoE*^{-/-}) develop atherosclerotic plaques in the aortic wall and showed subsequent fragmentation of the elastic lamellae in the media, and pseudomicroaneurysms.²⁵ The *ApoE*^{-/-} mouse provides an attractive model because the aneurysms are associated with atherosclerosis, but they have not yet been shown to produce progression aortic dilation.²⁵ Recently, many studies have shown infusion of angiotensin II into *ApoE*^{-/-} mice leads to the production of AAA.²⁵⁻²⁹ Delivery of angiotensin II leads to AAAs in the suprarenal region within the ~21- to 28-day infusion period.^{25,27,29} This study demonstrates that IL-1Ra deficiency in hematopoietic cells and T cells disrupts immune system homeostasis and may decrease tolerance for arterial wall components, resulting in the development of autoimmunity and inflammation. Consequently, inflammation induces spontaneous femoral artery aneurysms (but not AAA) in mice lacking interventions such as drugs or surgery.²⁵⁻²⁹ Thus, *IL-1Ra*^{-/-} mice provide easy observations as they age and diminish the mortality that typically follows surgical procedures. Furthermore, none of the small animal aneurysm models reproduces the complex intraluminal thrombosis that is a prominent feature of most human aneurysms.²⁶ Interestingly, our *IL-1Ra*^{-/-} mice demonstrated several thrombosed large aneurysms of femoral and/or popliteal arteries at 10 months of age (24%, 7 of 29). There is evidence to suggest that intraluminal thrombus affects aneurysm expansion in the human disease,³⁰ possibly by acting as a reservoir of proteolytic activity or impeding the oxygenation of the aortic wall.³¹ Taken together, because *IL-1Ra*^{-/-} mice contain the entire spectrum of lesions observed during inflammatory aneurysm, this mouse model likely will provide numerous opportu-

nities to study the pathogenesis and therapy of inflammatory aneurysm.

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