

# Adventitial matrix metalloproteinase production and distribution of immunoglobulin G4-related abdominal aortic aneurysms

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## ABSTRACT

**Objective:** IgG4-related diseases are systemic inflammatory fibrous lesions characterized by elevated serum IgG4 and infiltration of IgG4-positive plasmacytes. They can manifest in vascular lesions as frequently formed aneurysms with prominent thickening of the adventitia (IgG4-related abdominal aortic aneurysm; IgG4-AAA). Matrix metalloproteinases (MMPs) degrade the extracellular matrix, mainly in the tunica media, resulting in destruction of aortic structures to cause enlargement of the aneurysm. However, the expression of adventitial MMPs in IgG4-AAAs is poorly understood.

**Methods:** MMPs and MMPs-presenting cells in the adventitia of IgG4-AAAs ( $n = 19$ ) of human surgical specimens were evaluated by immunohistochemistry and dual messenger RNA in situ hybridization. The results were compared with those from control groups of non-IgG4-related inflammatory AAA ( $n = 18$ ), atherosclerotic AAA (aAAA;  $n = 11$ ), and autopsy cases ( $n = 11$ ). Preoperative serum MMPs levels of these groups were compared with the histologic data.

**Results:** Expression of MMP-9, MMP-2, and MMP-14 at the protein and messenger RNA levels in the adventitia was significantly higher in IgG4-AAAs than in controls. Other MMPs were scarce. The total number of MMP-9-positive cells was positively correlated with the diameter of the aneurysm ( $R = 0.461$ ;  $P = .031$ ), the adventitial thickness ( $R = 0.688$ ;  $P < .001$ ), and the number of IgG4-positive cells ( $R = 0.764$ ;  $P < .001$ ). Within lymphoid follicles, MMP-9-presenting cells were predominantly detected in large follicular dendritic cells, followed by histiocytes, fibroblasts, and plasmacytic dendritic cells. Outside lymphoid follicles, fibroblasts, and histiocytes mainly expressed MMP-9, and tissue dendritic cells also produced MMP-9. The levels of MMP-9 derived from follicular dendritic cells and histiocytes and plasmacytic dendritic cells outside lymphoid follicles were significantly higher in IgG4-AAA group than in other groups. Expression of adventitial MMP-2 and MMP-14 by histiocytes and fibroblasts was predominantly detected outside lymphoid follicles. Serum MMP-9 levels were significantly higher in IgG4-AAAs (835 ng/mL) than in controls, and correlated with serum IgG4 levels and the total numbers of adventitial MMP-9-positive cells, whereas serum MMP-2 levels did not differ among the three aneurysmal groups.

**Conclusions:** MMP-9 production in adventitial immune cells concerning lymphoid follicles was characteristic of IgG4-AAAs and might work in its activity with aneurysmal dilatation and adventitial thickening. Expressions of adventitial MMP-2 and MMP-14 were detected in histiocytes and fibroblasts outside lymphoid follicles, and were less concerned with the activity of IgG4-AAAs. (*JVS—Vascular Science* 2020;1:151-65.)

**Clinical relevance:** This retrospective multicenter study analyzed adventitial matrix metalloproteinases (MMPs) production in 19 patients with IgG4-related abdominal aortic aneurysms (AAAs) and 40 control cases. Adventitial MMP-9 production by various kinds of immune cells was increased in patients with IgG4-related AAAs and concerned with IgG4-AAA activity to cause aneurysmal progression and adventitial fibrosis, compared with aAAA. Serum MMP-9 levels reflected histologic MMP-9. Adventitial MMP-2 and MMP-14 were less concerned with IgG4-AAA activity. Thus, for IgG4-AAA patients, monitoring serum MMP-9 level might be the exacerbating factors related to adverse events during the treatment course.

**Keywords:** IgG4-related disease; IgG4-related aortic aneurysm; Matrix metalloproteinase; Adventitial thickening; Artery tertiary lymphoid organs; Follicular dendritic cells; Histiocytes

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IgG4-related diseases are systemic inflammatory fibrous and tumorous lesions characterized by elevated serum IgG4 and pathologically characterized by massive lymphoplasmacytic infiltration that are rich in IgG4<sup>+</sup> plasma cells, storiform fibrosis, and obliterative phlebitis.<sup>1</sup> They can manifest in vascular lesions as frequently formed inflammatory abdominal aortic aneurysms (IAAA), which is a special aneurysm type characterized by marked adventitial fibrous thickening and accounts for 5%-10% of all AAAs.<sup>2-5</sup> Approximately 50% of IAAA cases are IgG4-related AAA (IgG4-AAA).<sup>2-5</sup> The general clinical features of IgG4-AAA are similar to those of other IgG4-related diseases, namely, male predominance, advanced patient age, and frequent involvement of multiple other organs.<sup>1</sup>

Although IgG4-related diseases in other organs progress slowly with restricted clinical symptoms owing to pressure on the neighbor organs, IgG4-AAA can result in acute fatal complications, such as aortic dissection and ruptured aneurysm. Although IgG4-related diseases respond well to steroid treatment, setting the dose and duration of steroid treatment for IgG4-AAA is difficult because steroid medications cause fragility of the aortic wall, resulting in concern for severe vascular complications.<sup>6</sup> Regardless of the etiology, surgery is recommended for aneurysm patients, either conventional open surgical repair or endovascular aortic repair. In comparison with atherosclerotic AAA (aAAA), several postoperative problems, such as dilation of the aneurysm and exacerbation of periarteritis, frequently occur in IgG4-AAA.<sup>7-10</sup> Therefore, for IgG4-AAA, it is necessary to clarify the exacerbating factors related to adverse events during the treatment course, in comparison with aAAA.

Matrix metalloproteinases (MMPs) are a large (28 members) family of zinc-dependent endopeptidases classified according to their substrate as collagenases, gelatinases, stromelysins, and membrane-bound MMPs.<sup>11</sup> The collagenases include MMP-1, -8, -13, and -18, which digest fibrillar collagen types I, II, III; and the gelatinases include MMP-2 and -9, which digest denatured collagen. MMPs directly degrade the elastic and collagenous fibers of the tunica media, causing fragility and destruction of the aortic or arterial wall. They are attracting attention as factors in the progression and/or prognosis of aortic and arterial aneurysms. MMPs are also involved in the migration and proliferation of immune cells<sup>12</sup> and have bidirectional activation mechanism between inflammatory cytokines.<sup>13,14</sup> Previous studies of the interrelationship between cytokines and MMPs in aAAA have focused predominantly on the tunica media.<sup>15</sup> In contrast, there are few reports of the association between cytokines and MMPs in the adventitia.<sup>16-18</sup> A recent report showed that various immune cells in the thicker adventitia of IgG4-AAA are associated with activated cytokine status.<sup>19</sup> We hypothesized that, compared with aAAA, the adventitia of IgG4-AAA might have a specific

## ARTICLE HIGHLIGHTS

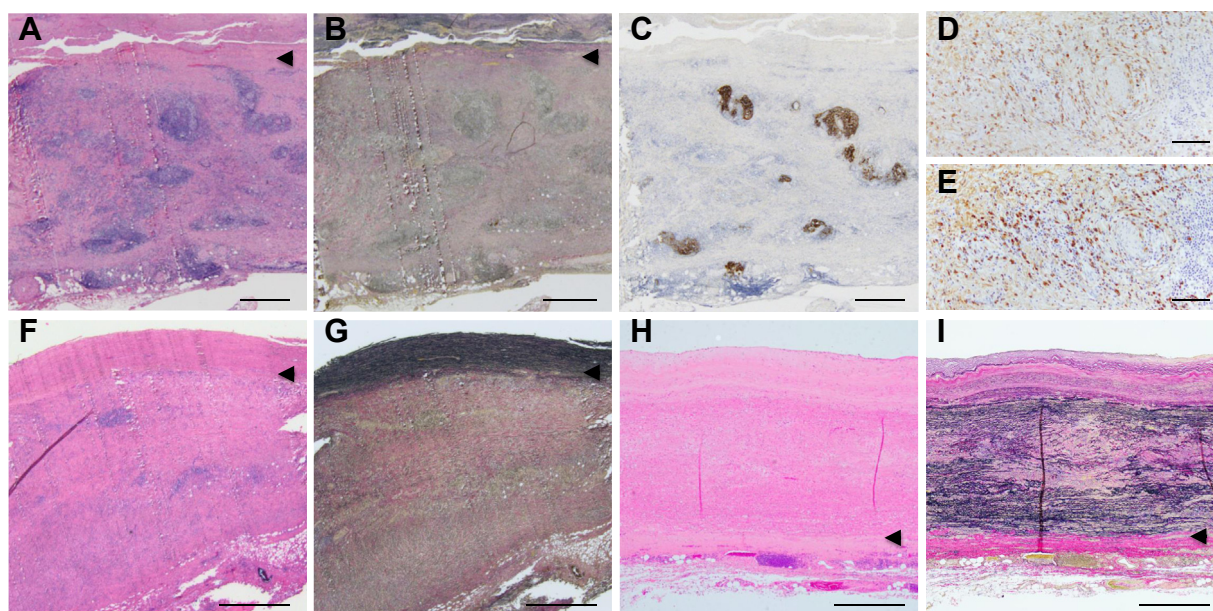
- **Type of Research:** Multicenter retrospective cohort study
- **Key Findings:** Adventitial matrix metalloproteinase (MMP)-9 production by various kinds of immune cells was increased in patients with IgG4-related abdominal aortic aneurysms (AAAs) and concerned with IgG4-AAA activity to cause aneurysmal progression and adventitial fibrosis, compared with atherosclerotic AAA. Serum MMP-9 levels reflected histologic MMP-9.
- **Take Home Message:** Adventitial MMP-9 expression is characteristically increased in patients with IgG4-related diseases and is concerned with IgG4-AAA activity to cause aneurysmal progression and adventitial fibrosis, compared with atherosclerotic AAA.

MMPs expression pattern to cause aortic wall fragility and remodeling of fibrous adventitia. However, MMP production in IAAA has been studied very rarely.<sup>20</sup> The role of MMPs of IgG4-AAA, particularly in the adventitia, has not been examined yet.

In this study, using the resected aortic tissue obtained by a surgery or an autopsy, we histologically examined which MMPs would be expressed at protein and RNA levels and what sort of cells would be producing them in IgG4-AAAs. Additionally, we estimated serum MMPs levels in IgG4-AAAs and control groups to study the association between the serum levels and histologic expression of MMPs.

## METHODS

**Case selection.** Among the patients undergoing surgery for aneurysm at Kanazawa University Hospital and Kanazawa Medical Center between January 2006 and December 2016, we selected 37 cases of IAAA, according to the histologic thick dense adventitia with severe sclerosing inflammation and fibrosis, appearing more than 2 mm thick. Overall 19 cases of IgG4-AAAs were selected from IAAA patients, according to the comprehensive histologic diagnostic criteria for IgG4-related diseases as follows<sup>21</sup>: (1) a dense lymphoplasmacytic inflammatory infiltrate with increased numbers of IgG4<sup>+</sup> plasma cells (>50/high-power fields [HPFs; 10 × eyepiece, 40 × lens] and IgG4/IgG ratio of >50%) (Fig 1, A-E), (2) storiform pattern of fibrosis, and (3) obliterative vasculitis. About 50% cases of IgG4-AAA had been included in our previous report.<sup>19</sup> The 18 cases of rest IAAAs were classified into non-IgG4-related IAAAs (non-IgG4-IAAA; Fig 1, F and G). No significant differentiation of the radiologic findings was observed between IgG4-AAAs and non-IgG4-AAAs.<sup>22</sup> aAAA patients had aneurysms with severe atherosclerosis in the intima and media and were



**Fig 1.** Histopathologic features of IgG4-related abdominal aortic aneurysm (AAA) (**A–E**), non-IgG4-related inflammatory AAA (**F and G**), and atherosclerotic AAA (aAAA) (**H and I**). Adventitial fibrous thickening are detected in IgG4-related AAAs (**A and B**) and non-IgG4-related inflammatory AAAs (**F and G**), while thin adventitia are seen in aAAAs (**H and I**). **A, F and H**, Hematoxylin and eosin stain. **B, G and I**, Elastica van Gieson stain. **A, F and G**, Original magnification  $\times 12.5$ ; scale bar = 1000  $\mu\text{m}$ . **H and I**, Original magnification  $\times 40$ ; scale bar = 400  $\mu\text{m}$ ). In addition, many lymphoid follicles and IgG4-immunopositive cells are seen in IgG4-related AAAs. **C**, Network of follicular dendritic cells (FDCs) indicated by immunostaining of CD21 show many lymphoid follicles in the adventitia (arterial tertiary lymphoid organs [ATLOs]). **C**, Original magnification  $\times 12.5$ ; scale bar = 1000  $\mu\text{m}$ . **D**, IgG-immunopositive cells. **E**, IgG4-immunopositive cells. **D and E**, Original magnification  $\times 100$ ; scale bar = 100  $\mu\text{m}$ .

sequentially resected between January 2006 and December 2006. The aAAA adventitia was less than 1 mm thick with minimal inflammatory cell infiltration (Fig 1, H and I). Normal control aortas (no or slight atherosclerotic abdominal aorta without dilatation) were obtained from consecutive autopsies performed at Kanazawa Medical Center between January and August 2012 (Table). In the three control groups, IgG4<sup>+</sup> plasma cells were scarce, and serum IgG4 was within the normal range. No patient had ruptured aneurysm and a medical history of any special type of vasculitis (such as giant cell aortitis, Takayasu aortitis, or antineutrophil cytoplasmic antibody-related vasculitis) or pathologic neutrophil aggregation and granulomas.

**Data collection.** Based on the patients' electronic medical records, we retrospectively recorded clinical features, including age, sex, clinical outcome, complications, medication, and laboratory findings such as serum IgG4, C-reactive protein (CRP), and white blood cell (WBC) count. Aneurysm diameters were measured on semiannual computed tomography images with a slice thickness of 5 mm.

**Pathologic measurements and immunohistochemistry.** Adventitial thickness from the lowest elastic fiber of the tunica media to the lowest part of the adventitia was calculated on Elastica van Gieson-stained sections using microscopy.

Immunohistochemical examination was performed using IgG antibodies (DakoCytomation, Glostrup, Denmark; polyclonal,  $\times 1000$ ), IgG4 (Nichirei Bioscience, Tokyo, Japan; clone HP6025,  $\times 2$ ), MMP-1 (R&D Systems, Minneapolis, Minn; clone 366665,  $\times 100$ ), MMP-2 (R&D Systems; clone 36006,  $\times 100$ ), MMP-3 (R&D Systems; clone 10D6,  $\times 100$ ), MMP-8 (R&D Systems; clone 100608,  $\times 100$ ), MMP-9 (R&D Systems; clone 4H3,  $\times 100$ ), MMP-10 (R&D Systems; clone 110304,  $\times 100$ ), MMP-12 (R&D Systems; clone 82902,  $\times 100$ ), MMP-13 (R&D Systems; clone 87512,  $\times 100$ ), MMP-14/MT-MMP (R&D Systems; clone 5H2,  $\times 100$ ), CD21 (Leica Biosystems, Wetzlar, Germany; clone 2G9,  $\times 100$ ), CD163 (Leica Biosystems; clone 10D6,  $\times 400$ ), CD1a (Abcam, Cambridge, UK; clone EP3622,  $\times 20$ ), and CD123 (Leica Biosystems; clone BR4MS,  $\times 400$ ). CD21 revealed the network of follicular dendritic cells (FDCs) in lymphoid follicles. CD163 is an M2-type macrophage marker. CD1a and CD123 were selected to detect the presence of tissue dendritic cells (tDCs) and plasmacytic dendritic cells (pDCs), respectively. The immunoreactivities of all antibodies were confirmed using positive and negative controls. For each antibody, the number of immunopositive cells in prominently inflamed areas was counted in five different HPFs; the average number of immunopositive cells per HPF was also calculated.<sup>2,3,19</sup> Double immunostaining of combined MMPs and immune cells was performed;

**Table.** Clinicopathologic features of IgG4-related abdominal aortic aneurysm (AAA), non-IgG4-related inflammatory AAA (*non-IgG4-IAAA*), atherosclerotic AAA (*aAAA*), and normal aorta obtained by autopsy without aneurysms and atherosclerosis

	IgG4-AAA	Non-IgG4-IAAA	aAAA	Autopsy	P value	
	(19 cases)	(18 cases)	(11 cases)	(11 cases)	IgG4-AAA vs non-IgG4-IAAA	non-IgG4-IAAA vs aAAA
<b>Clinical features</b>						
Age, years	68.3 (63-78)	77.2 (73-81)	77.1 (65-87)	72.3 (68-76)	.270	.082
Sex, male/female	17/2	13/5	9/2	8/3	.889	.998
Aortic diameter, mm	48 (36-87)	54 (50-57)	50 (36-70)	25 (22-30)	.702	.998
Other aneurysms	13 (68.4%)	8 (61.5%)	4 (36.4%)	0	.681	.213
Other IgG4-related disease	5 (26.3%)	0	0	0	.001	NP
Serum IgG4, mg/dL	185 (50-559)	55 (13-97)	43 (12-111)	37 (12-80)	.001	.468
Serum IgG4/IgG ratio, %	7.3 (2-43)	2.2 (0-7)	2.8 (1-8)	2.1 (1-7.2)	.001	.743
Serum IgE, IU/mL	355 (28-2179)	28 (20-35)	68 (21-544)	49 (19-411)	.003	.131
CRP, mg/dL	0.7 (0.1-6.0)	0.9 (0.1-1.5)	0.3 (0-1.6)	0.1 (0-0.7)	.118	.059
WBC, $\times 10^3$ /mm	6.9 (4.7-15.4)	6.9 (5.7-8.2)	5.8 (4.0-7.1)	5.2 (4.0-9.1)	.801	.091
<b>Pathologic features</b>						
Adventitial thickness, mm	3.8 (2.0-9.1)	3.1 (2.1-6.3)	0.5 (0.1-1.1)	0.1 (0.1-0.2)	.509	.001
IgG4 <sup>+</sup> cells, /HPF	104 (58-145)	20 (0-71)	7 (0-37)	2 (0-12)	.001	.789
IgG4/IgG ratio, %	80 (55-94)	2 (8-36)	9 (0-39)	4 (0-19)	.001	.656
Storiform fibrosis	12 (63.1%)	2 (11.1%)	0	0	.003	.696
Obstructive phlebitis	14 (73.7%)	1 (5.6%)	0	0	.001	.811
Perineural infiltration	11 (57.9%)	2 (11.1%)	1 (9.0%)	0	.008	.649
ATLOs, /LPF	8.2 (2.2-18.2)	3.2 (0-8.5)	2.9 (0-6.8)	0.1 (0-1)	.068	.891
tDCs, /HPF	14.4 (4.2-25.6)	6.1 (2.1-12.2)	5.2 (0.9-13.2)	8 (2.1-13.5)	.001	.821
pDCs, /HPF	10.3 (3.1-20.5)	3.2 (0-12.1)	0.9 (0-2.3)	0.5 (0-2.5)	.001	.067
aAAA, Atherosclerotic abdominal aortic aneurysm; ATLOs, arterial tertiary lymphoid organs; Autopsy, normal aorta obtained by autopsy without aneurysms and atherosclerosis; CRP, C-reactive protein; HPF, high-power field; IgG4-AAA, IgG4-related abdominal aortic aneurysm; LPF, low-power field; non-IgG4-IAAA, non-IgG4-related inflammatory abdominal aortic aneurysm; pDCs, plasmacytic dendritic cells; tDCs, tissue dendritic cells; WBC, white blood count.						

MMPs (MMP-2, MMP-9, and MMP-14) were blue in the cytoplasm to the membrane, and immune cells (CD34, CD123, CD163, CD1a, and CD21) were brown in the cytoplasm.

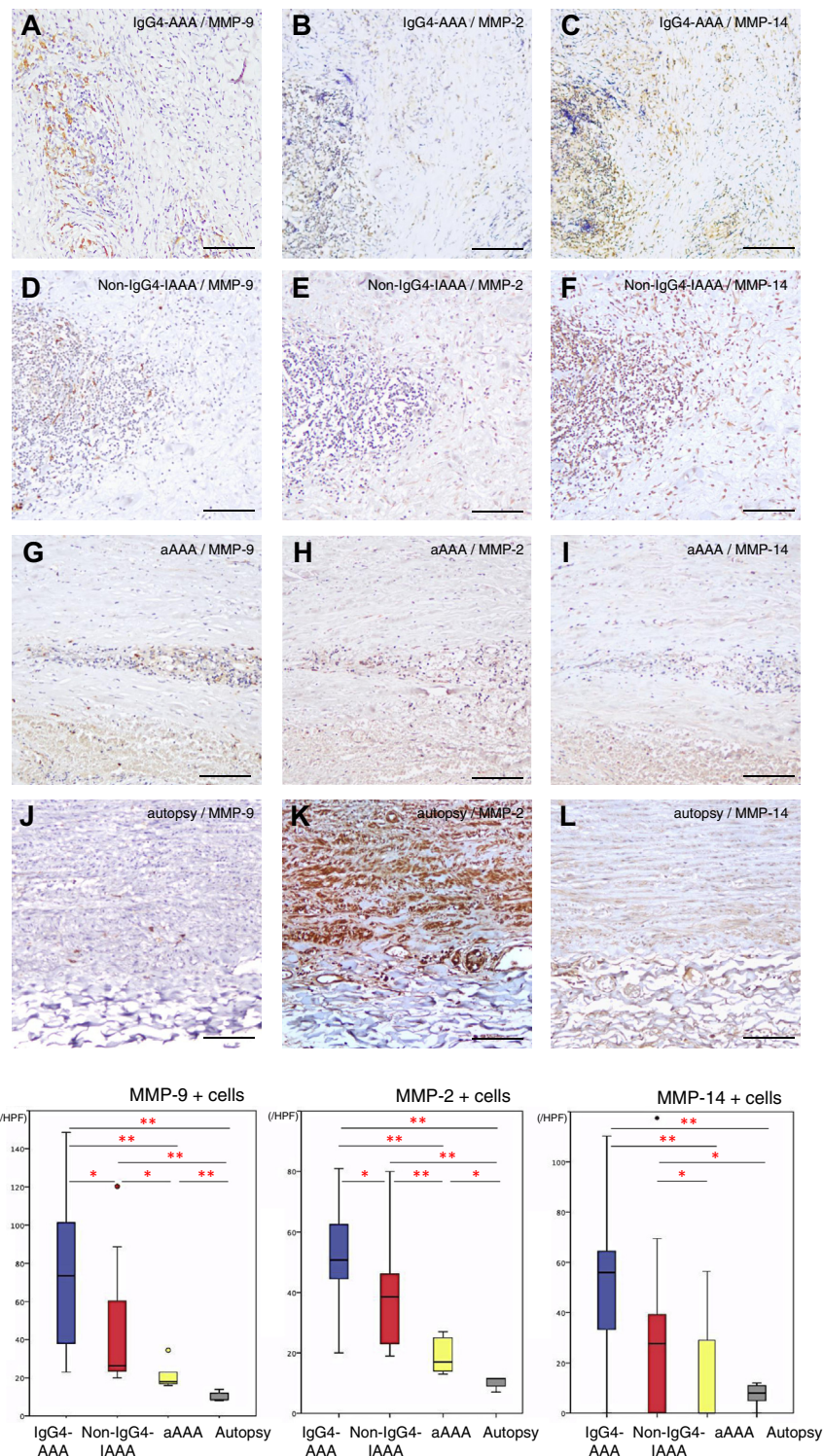
**In situ hybridization to detect expression of MMP messenger RNA.** RNAscope 2-plex chromogenic duplex assay for simultaneous detection of two RNA species (RNAscope, Advanced Cell Diagnostics, Hayward, Calif) was used to detect MMP-producing cells at the messenger RNA level.<sup>23</sup> We used the target probes as a combination of horseradish peroxidase-linked probes for the MMPs (MMP-2 [311751-C1], MMP-9 [311331-C1], and MMP-14 [311225-C1]) and Fast Red-linked probes for cell profiling (CD34 [560821-C2], CD123 [606401-C2], CD163 [56091-C2], and CD21 [560928-C2]).

**Measurement of serum MMPs.** Preoperative serum MMPs could be measured in nine cases of IgG4-AAA, seven cases of non-IgG4-IAAA, six cases of aAAA, and seven autopsy cases. MMP-2 (Quantikine enzyme-linked

immunosorbent assay [ELISA], Human MMP-2 Immunoassay; R&D Systems; normal range, 200-300 ng/mL), MMP-9 (Quantikine ELISA, Human MMP-9 Immunoassay, R&D Systems; normal range, <100 ng/mL),<sup>24</sup> and MMP-14 (Human MMP DuoSet ELISA, Human Total MMP-14 DuoSet ELISA, R&D Systems; normal range undetermined) were measured by ELISA.

**Statistical methods.** A Steel-Dwass analysis was used to compare continuous variables. A  $\chi^2$  test of variance was used to compare the four groups. Spearman correlation coefficients were used to test associations between continuous variables. The threshold of statistical significance was a *P* value of less than .05. Analyses were performed using BellCurve for Excel (Social Survey Research Information Corporation, Tokyo, Japan) and SPSS (version 20; IBM Corporation, Armonk, NY).

**Ethics.** The study was approved by the Human Investigation Review Committee of Kanazawa Medical Center (No. 24-63 and 26-17) and Kanazawa University Hospital



**Fig 2.** Immunohistochemical analysis of matrix metalloproteinases (MMPs) in the adventitia of IgG4-related abdominal aortic aneurysms (*IgG4-AAA*) and control groups. (Top) Immunopositive cells were stained with antibodies to MMP-9, MMP-2, and MMP-14; original magnification  $\times 100$ ; scale bar = 20  $\mu\text{m}$ . MMP-9 are seen star-like large cells distributed inside arterial tertiary lymphoid organs (ATLOs) and ovoid to spindle cells outside of ATLOs, and MMP-2 and MMP-14 are present in spindle cells mainly outside of ATLOs. (Bottom) Box-and-whisker diagrams for immunohistochemical analysis of MMP-9, MMP-2, and MMP-14 in the adventitia and control groups. The horizontal line in the middle of each box indicates the median; the top and bottom borders of the box mark the 75th and 25th percentiles, respectively, and the whiskers above and below the box indicate the 1.5 interquartile ranges. The numbers of MMPs were counted per high-power field (HPF). Statistically significant differences between groups are indicated as  $*P < .05$ ,  $**P < .001$ . *IgG4-AAA*,  $n = 19$ . Non-*IgG4*-related inflammatory AAA (*non-IgG4-AAA*),  $n = 18$ . Atherosclerotic AAA (*aAAA*),  $n = 11$ . Autopsy (normal aorta obtained by autopsy),  $n = 11$ . The numbers of each MMP are significantly higher in *IgG4-AAA* than in the control groups.

(No. 495-1) and conformed to the principles outlined in the Declaration of Helsinki. The patients provided informed consent before the study.

## RESULTS

### Histologic findings of the adventitia of each type of aneurysm

Considerable adventitial fibrous thickening were observed in both IgG4-AAAs (median, 3.8 mm; Fig 1, A and B) and non-IgG4-IAAAs (median, 3.1 mm; Fig 1, F and G), whereas thin adventitia were observed in aAAAs (median, 0.5 mm; Fig 1, H and I; Table). In addition, there were many lymphoid follicles (arterial tertiary lymphoid organs [ATLOs]) indicated by network of FDCs (Fig 1, C), and many IgG4-immunopositive cells with high IgG4/IgG ratio (Fig 1, D and E) in the adventitia of IgG4-AAAs.

### Total number of MMP<sup>+</sup> cells in the adventitia

In all four groups, MMP-2<sup>+</sup>, MMP-9<sup>+</sup>, and MMP-14<sup>+</sup> cells were detected (Fig 2). The other MMPs were scarce in all four groups, and there were no significant differences among them.

**MMP-9<sup>+</sup> cells.** A large number of MMP-9<sup>+</sup> cells were found both outside and inside ATLOs. Inside ATLOs, MMP-9<sup>+</sup> cells were composed of large, star-shaped cells and polygonal to spindle-shaped cells (Fig 2, left). Outside ATLOs, MMP-9<sup>+</sup> cells were mainly spindle cells and scattered small polygonal cells. Many MMP-9<sup>+</sup> spindle cells surrounded the ATLOs. The median total number of MMP-9<sup>+</sup> cells was highest in IgG4-AAA (70.5/HPF; Fig 2, A) followed by non-IgG4-IAAA (41.1/HPF; Fig 2, D), aAAA (22.7/HPF; Fig 2, G), and autopsy cases (7.4/HPF; Fig 2, J), with significant differences among all groups.

**MMP-2<sup>+</sup> cells.** MMP-2<sup>+</sup> cells were distributed mainly outside ATLOs and were rarely seen inside ATLOs. They were mostly composed of short-to-long spindle cells and several polygonal cells (Fig 2, middle). The median total number of MMP-2<sup>+</sup> cells was highest in IgG4-AAA (54.6/HPF; Fig 2, B) followed by non-IgG4-IAAA (34.6/HPF; Fig 2, E), aAAA (17.6/HPF; Fig 2, H), and autopsy cases (11.5/HPF; Fig 2, K). The number of MMP-2<sup>+</sup> cells differed significantly among all groups.

**MMP-14<sup>+</sup> cells.** MMP-14<sup>+</sup> cells were predominantly detected outside ATLOs and consisted primarily of short-to-long spindle cells (Fig 2, right). The median total number of MMP-14<sup>+</sup> cells was higher in IgG4-AAA (58.6/HPF; Fig 2, C) followed by non-IgG4-IAAA (49.4/HPF; Fig 2, F), aAAA (36.3/HPF; Fig 2, I), and autopsy cases (8.8/HPF; Fig 2, L). No significant difference was observed in the total number of MMP-14<sup>+</sup> cells, between IgG4-AAA and non-IgG4-IAAA.

**Relationships between the total number of MMPs<sup>+</sup> cells and clinicopathologic parameters.** The total number of MMP-9<sup>+</sup> cells was positively correlated with the number of IgG4<sup>+</sup> cells ( $R = 0.764$ ;  $P < .001$ ), aneurysmal diameter ( $R = 0.461$ ;  $P = .031$ ), and adventitial thickness

( $R = 0.688$ ;  $P < .001$ ; Fig 3, top). The total number of MMP-2<sup>+</sup> cells was positively correlated with the number of IgG4<sup>+</sup> cells ( $R = 0.744$ ;  $P < .001$ ) and adventitial thickness ( $R = 0.720$ ;  $P < .001$ ) and was not correlated with aneurysmal diameter ( $R = 0.354$ ;  $P = .071$ ; Fig 3, middle). In contrast, the total number of MMP-14<sup>+</sup> cells was not correlated with aneurysmal diameter, adventitial thickness, or the number of IgG4<sup>+</sup> cells (Fig 3, bottom).

### MMP-producing cells in the adventitia

#### Shapes and distributions of MMPs-producing cells.

Based on the morphology of MMP<sup>+</sup> cells, histochemically, we assumed that these MMPs were produced in fibroblasts and histiocytes (CD163<sup>+</sup>), tDCs (CD1a<sup>+</sup>), pDCs (CD123<sup>+</sup>), and FDCs (CD21<sup>+</sup>). Dual in situ hybridization supported the double presence of messenger RNA of MMPs and immune cells.

Many MMP-9<sup>+</sup> cells were distributed inside ATLOs (Fig 4); large star-shaped MMP-9<sup>+</sup>CD21<sup>+</sup> cells were uniformly distributed inside ATLOs (Fig 4, A and E). Polygonal shaped MMP-9<sup>+</sup>CD163<sup>+</sup> cells were found mainly in the periphery of the ATLOs (Fig 4, B and F). Inside ATLOs, MMP-9<sup>+</sup>CD123<sup>+</sup> cells and MMP-9<sup>+</sup>CD34<sup>+</sup> cells were spindle to polygonal shaped and uniformly distributed (Fig 4, C, D and G). Outside ATLOs, separately distributed MMP-9<sup>+</sup> spindle to polygonal cells showed double-immunopositivity with CD163, CD1a, CD34, and CD123 (Fig 5, A-D).

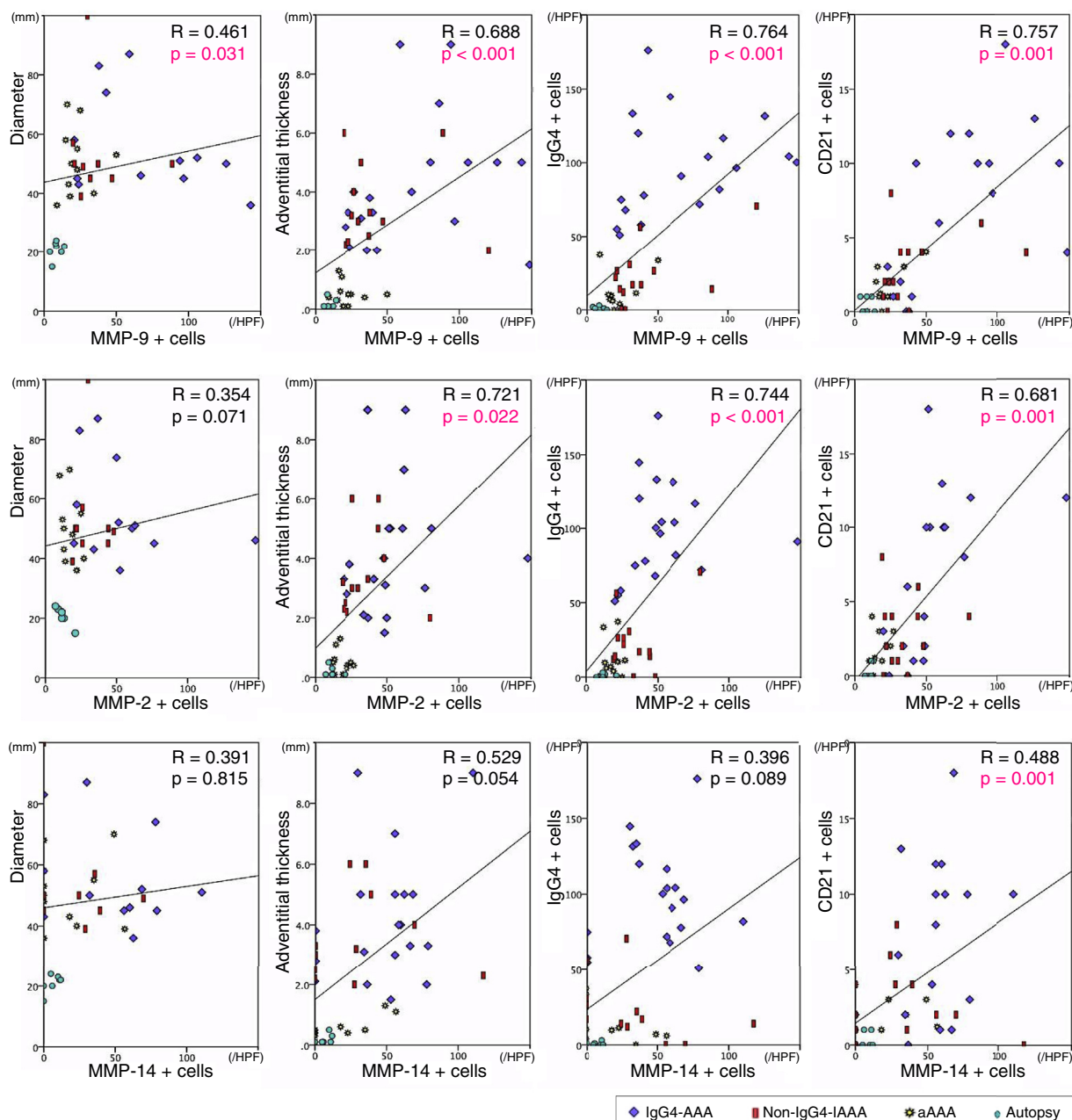
Outside ATLOs, MMP-2<sup>+</sup> spindle to polygonal cells expressed double positivity with CD163 and CD34 (Fig 6, A and B) and MMP-14<sup>+</sup> spindle to polygonal cells also showed double-positivity with CD163 and CD34 (Fig 6, C and D).

#### MMPs-producing cells in each group.

**MMP-9-producing cells.** MMP-9 producing cells inside ATLOs. The total number of MMP-9<sup>+</sup>CD21<sup>+</sup> cells was highest in IgG4-AAA (median, 11.9/HPF), with significant differences from the other three groups, while there were no significant differences between among three groups as non-IgG4-IAAA, aAAA, and autopsy cases (median, 1.9/HPF, 0.5/HPF, and 0.1/HPF, respectively; Fig 7, A).

The median number of MMP-9<sup>+</sup>CD163<sup>+</sup> cells inside ATLOs was highest in IgG4-AAA (9.7/HPF), with significant differences among all groups. The numbers of MMP-9<sup>+</sup>CD123<sup>+</sup> cells and MMP-9<sup>+</sup>CD34<sup>+</sup> cells inside ATLOs were significantly higher in IgG4-AAA (median, 3.5/HPF and 6.1/HPF, respectively) than in the other three groups, and were low and similar to those seen in the three control groups.

**MMP-9 producing cells outside ATLOs.** Outside ATLOs, the median number of MMP-9<sup>+</sup>CD163<sup>+</sup> cells was highest in IgG4-AAA (13.4/HPF), followed by non-IgG4-IAAA (6.3/HPF), aAAA (6.0/HPF), and autopsy cases (0.6/HPF), with significant differences between IgG4-AAA and non-IgG4-IAAA ( $P < .001$ ; Fig 7, B). The numbers of MMP-9<sup>+</sup>CD34<sup>+</sup> cells were almost equal in the three

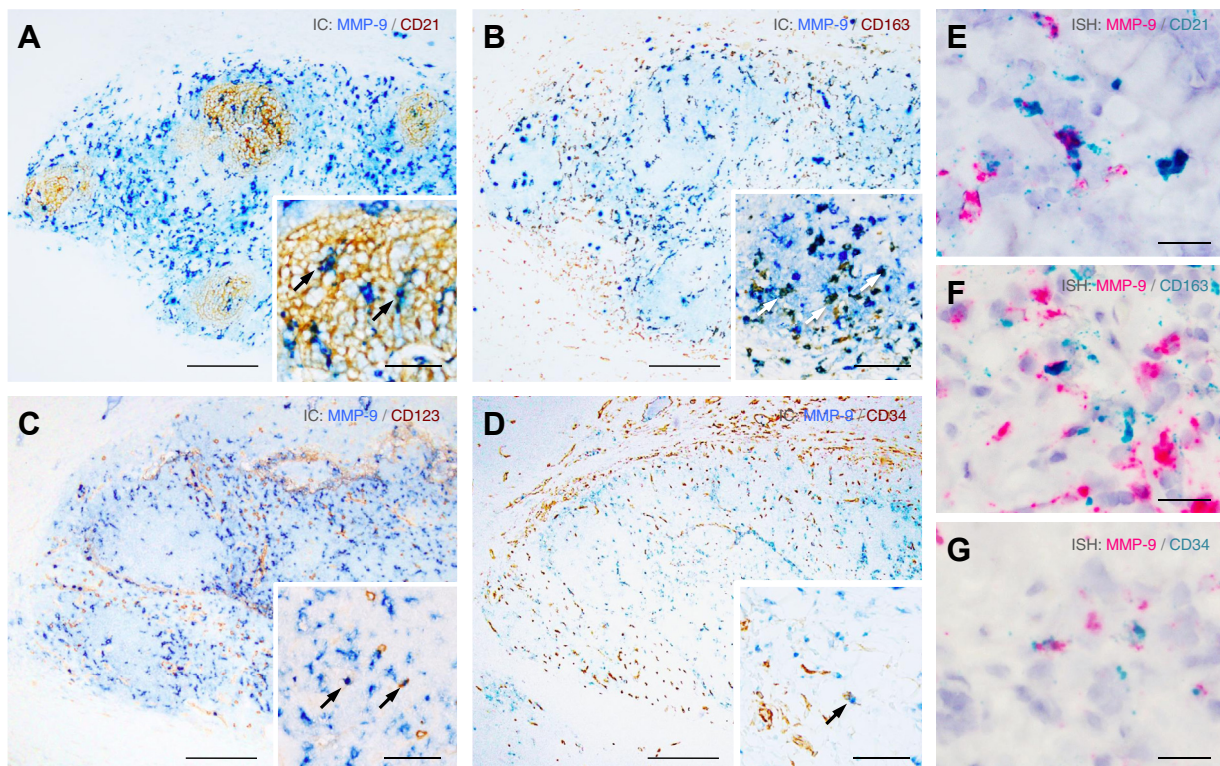


**Fig 3.** Relationships between the total counts of matrix metalloproteinases (*MMPs*; *top*, MMP-9; *middle*, MMP-2; *bottom*, MMP-14) and important parameters of IgG4-related abdominal aortic aneurysms (*IgG4-AAA*): aortic diameter, pathologic adventitial thickness, total counts of IgG4-immunopositive cells, and total counts of CD21-immunopositive cells (follicular dendritic cells [FDCs]) among all 59 cases. Rhombuses show cases of IgG4-AAA ( $n = 19$ ), rectangles show cases of non-IgG4-related inflammatory AAA (*non-IgG4-AAA*;  $n = 18$ ), asterisks show cases of atherosclerotic AAA (*aAAA*;  $n = 11$ ), and circles show cases of normal aorta obtained by autopsy ( $n = 11$ ).

aneurysmal groups (about 4-6/HPF) and were fewer in the autopsy group (median, 1.2/HPF). The median number of MMP-9<sup>+</sup>CD1a<sup>+</sup> cells was slightly higher in IgG4-AAA (5.3/HPF) than in non-IgG4-IAAA and aAAA (2.5/HPF and 1.8/HPF, respectively). MMP-9<sup>+</sup>CD123<sup>+</sup> cells were sparsely detected in IgG4-AAA (median, 3.2/HPF) and were very scarce in the other three groups (<0.1/HPF).

**MMP-2-producing cells.** The median number of MMP-2<sup>+</sup>CD163<sup>+</sup> cells was highest in IgG4-AAA (19.6/HPF), followed by non-IgG4-IAAA (11.2/HPF), aAAA (6.6/HPF), and autopsy (0.8/HPF), with significant differences among all groups (Fig 7, C). The numbers of MMP-2<sup>+</sup>CD34<sup>+</sup> cells did not significantly differ among all groups.

**MMP-14-producing cells.** The median numbers of MMP-14<sup>+</sup>CD163<sup>+</sup> cells of IgG4-AAA and non-IgG4-IAAA



**Fig 4.** Matrix metalloproteinase-9 (*MMP-9*)-presenting cells distributed inside lymphoid follicles in the adventitia of IgG4-related abdominal aortic aneurysms (IgG4-AAA) using double immunohistochemistry (*IC*) and dual messenger RNA (mRNA) in situ hybridization (*ISH*). **A–D**, Immunopositivity of *MMP-9* (blue) and CD21 (**A**), CD163 (**B**), CD123 (**C**), and CD34 (**D**) (brown). Original magnification  $\times 100$ ; scale bar = 400  $\mu\text{m}$ ; inset, original magnification  $\times 400$ ; scale bar = 20  $\mu\text{m}$ . **E–G**, Dual presence of mRNA of *MMP-9* (red) and CD21 (**E**), CD163 (**F**), and CD34 (**G**) (green) Original magnification  $\times 1000$ , scale bar = 10  $\mu\text{m}$ .

were almost the same (23.9/HPF and 21.4/HPF, respectively) and were few in the autopsy group (median, 4.0/HPF), with a significant difference from the other three groups (Fig 7, D). The median numbers of  $\text{MMP-14}^+\text{CD34}^+$  cells were about the same among IgG4-AAA, non-IgG4-IAAA, and aAAA (7.3/HPF, 8.7/HPF, and 11.9/HPF, respectively) and were few in the autopsy group (2.8/HPF), with significant differences among the other three groups.

#### Preoperative serum MMP levels

**Preoperative serum MMP levels in each group.** The median serum *MMP-9* level was highest in IgG4-AAA (835 ng/mL), followed by non-IgG4-IAAA (576 ng/mL) and aAAA (307 ng/mL; Fig 8, top left). The difference in serum *MMP-9* levels between IgG4-AAA and non-IgG4-IAAA did not reach significance ( $P = .059$ ). The median serum *MMP-9* level in the autopsy group (81 ng/mL) was significantly lower than that in the other three aneurysmal groups.

The median serum *MMP-2* levels were did not differ among the three groups IgG4-AAA, non-IgG4-IAAA, and aAAA (256, 301, and 345 ng/mL, respectively; Fig 8, top right), whereas the median serum *MMP-2* level of IgG4-AAA was within the normal limit. The median

serum *MMP-2* level of the autopsy group (120 ng/mL) was significantly lower than that of the other three groups. Serum *MMP-14* levels were very low in almost all cases.

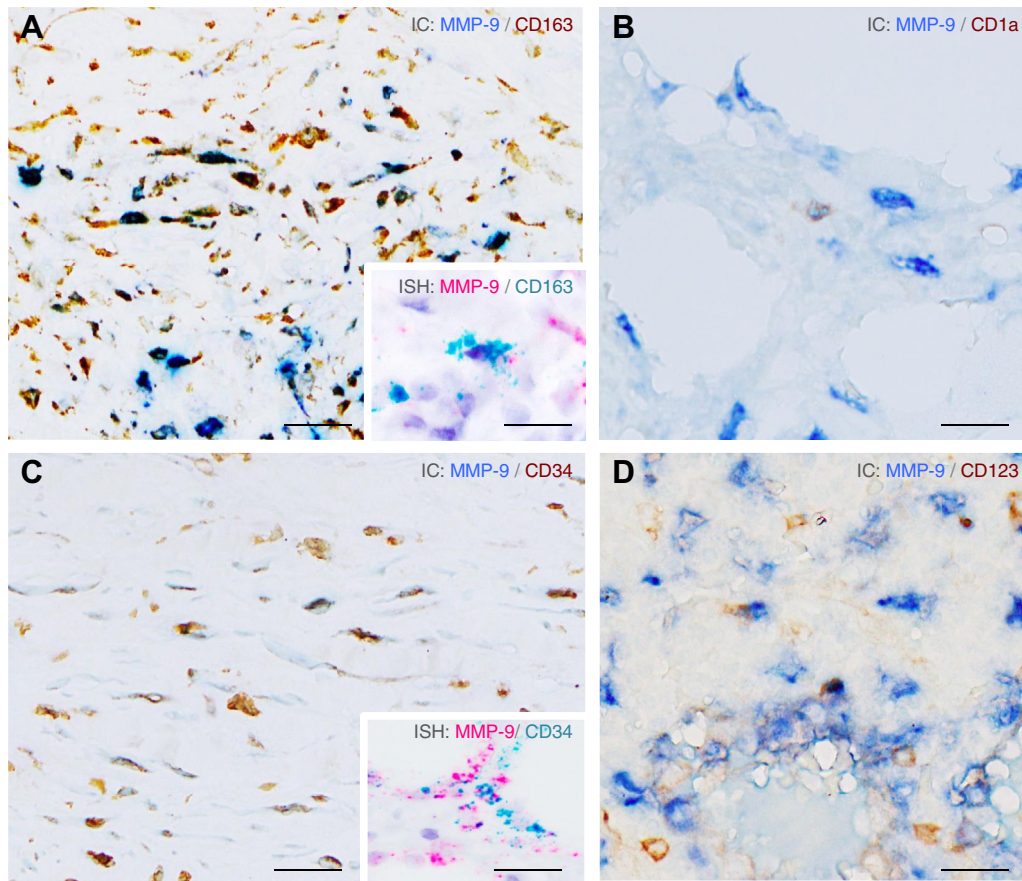
**Relationships between preoperative serum MMP levels and clinicopathologic findings.** There were positive correlations between serum *MMP-9* levels and the total number of  $\text{MMP-9}^+$  cells ( $R = 0.686$ ;  $P = .001$ ), aneurysmal diameter ( $R = 0.448$ ;  $P = .025$ ), adventitial thickness ( $R = 0.592$ ;  $P = .028$ ), and serum IgG4 ( $R = 0.492$ ;  $P = .008$ ), whereas no correlations were observed between serum *MMP-9* levels and CRP or WBC (Fig 8, middle).

There was no correlation between serum *MMP-2* levels and the total number of  $\text{MMP-2}^+$  cells. Serum *MMP-2* was positively correlated with aneurysmal diameter ( $R = 0.581$ ;  $P = .031$ ); however it was not correlated with adventitial thickness, serum IgG4, CRP, or WBC (Fig 8, bottom).

#### DISCUSSION

Previous studies of aAAA mainly focused on the tunica media, demonstrated expression of *MMP-1*, *MMP-2*, *MMP-3*, *MMP-8*, *MMP-9*, *MMP-13*, and *MMP-14* using immunohistochemistry and quantitative reverse-transcriptase polymerase chain reaction.<sup>25</sup> In particular,





**Fig 5.** Matrix metalloproteinase-9 (*MMP-9*)-presenting cells distributed outside of lymphoid follicles in the adventitia of IgG4-related AAAs (IgG4-AAA) using double immunohistochemistry (IC) and dual messenger RNA (mRNA) in situ hybridization (ISH). **A** and **C**, Immunopositivity of MMP9 (blue) and CD163 (**A**) and CD34 (**C**) (brown). Original magnification  $\times 200$ ; scale bar = 40  $\mu\text{m}$ ; inset, dual presence of mRNA of MMP9 (red) and CD163 and CD34 (green) Original magnification  $\times 1000$ ; scale bar = 10  $\mu\text{m}$ . **B** and **D**, Immunopositivity of MMP9 (blue) and CD1a (**B**) and CD123 (**D**) (brown). Original magnification  $\times 400$ ; scale bar = 20  $\mu\text{m}$ .

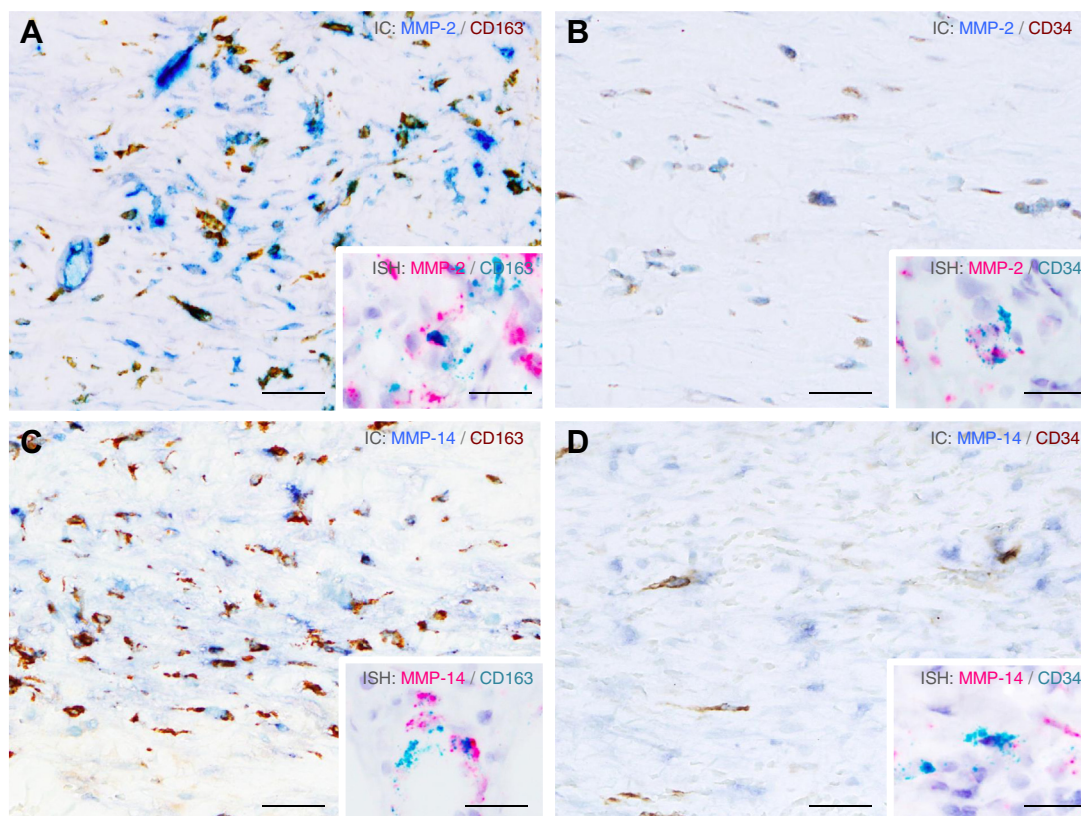
MMP-2 and MMP-9 were considered to generally play a key role in the pathogenesis and progression of an aneurysm.<sup>26,27</sup> The present study of the tunica adventitia found many MMP-2-, MMP-9-, and MMP-14-positive cells at the messenger RNA and protein levels, and other MMPs were scarce. Furthermore, the present study revealed that the adventitial total number of cells positive for MMP-2 and MMP-9 of IgG4-AAA was higher than that in other groups, and the variety and distribution of MMPs-producing cells was specific to IgG4-AAA.

TLOs are ectopic lymphoid structures that have morphologic features similar to secondary lymphoid organs that allow homing of naïve cells in the T-cell area, an interface between the T- and B-cell zones and the germinal center areas presenting FDCs.<sup>28</sup> TLOs are major active control sites for immune surveillance and innate immune responses. Many TLOs are well-known among the histopathologic attributes of IgG4-related diseases.<sup>1,21</sup> Recently, several reports have indicated that lymphoid follicular helper T cells in TLOs can induce the

differentiation of B cells into plasmablasts to produce specific IgG molecules, and should be involved in the pathogenesis of IgG4-related diseases.<sup>29</sup>

ATLOs have been described in autoimmunity, microbial infection, vasculitis, and atherosclerosis,<sup>30-32</sup> and they are also frequently found in IgG4-AAAs.<sup>2-4</sup> The normal adventitia contains resident macrophages, T cells, B cells, mast cells, and DCs.<sup>33,34</sup> These immune cells have different distributions inside and outside ATLOs. Consequently, we described MMP-producing cells, which were mainly immune cells, arranged in relation to ATLOs in this report.

With regard to antigen-presenting cells, a few previous reports indicated that tDCs expressed the active form of MMP-9.<sup>35,36</sup> Additionally, we found that other antigen-presenting cells, such as pDCs and FDCs, could derive MMP-9 expression. Inside ATLOs, MMP9<sup>+</sup> cells were predominantly detected in FDCs, followed by histiocytes, fibroblasts, and pDCs. FDC networks were more numerous and larger in IgG4-AAA than in other groups, similar to a previous report of IAAA.<sup>37</sup> Moreover, the total number of



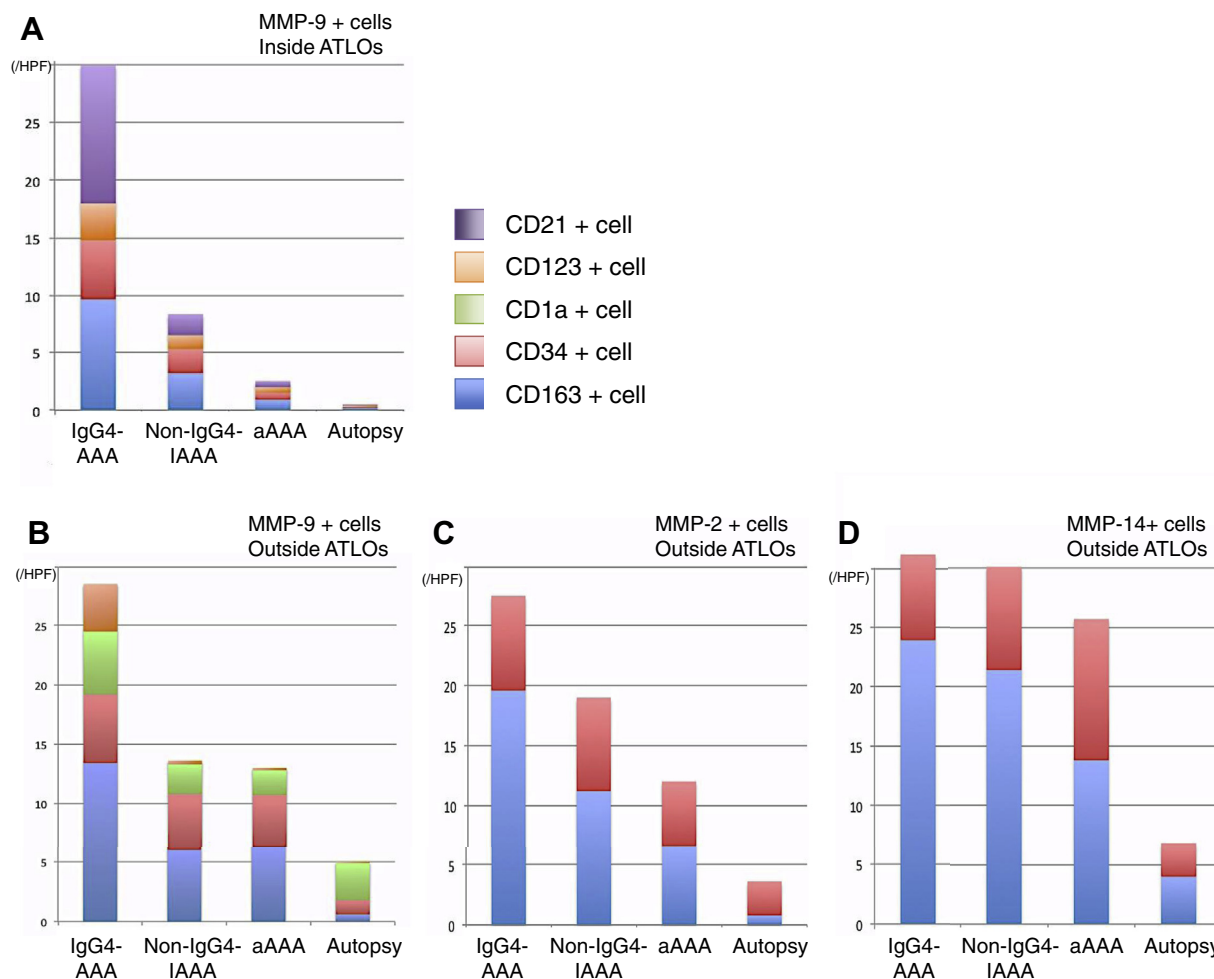
**Fig 6.** Matrix metalloproteinase-2 (*MMP-2*) and *MMP-14*-presenting cells distributed outside of lymphoid follicles in the adventitia of IgG4-related abdominal aortic aneurysms (IgG4-AAA) using double immunohistochemistry (*IC*) and dual messenger RNA (mRNA) in situ hybridization (*ISH*). **A** and **B**, Immunopositivity of *MMP-2* (blue) and CD163 (**A**), and CD34 (**B**). Original magnification  $\times 200$ ; scale bar = 40  $\mu\text{m}$ ; inset, dual presence of mRNA of *MMP-2* (red) and CD163, and CD34 (green). Original magnification  $\times 1000$ ; scale bar = 10  $\mu\text{m}$ . **C** and **D**, Immunopositivity of *MMP-14* (blue) and CD163 (**C**), and CD34 (**D**). Original magnification  $\times 200$ ; scale bar = 40  $\mu\text{m}$ ; inset, dual presence of mRNA of *MMP-14* (red) and CD163, and CD34 (green). Original magnification  $\times 1000$ ; scale bar = 10  $\mu\text{m}$ .

*MMP-9*<sup>+</sup> cells was correlated with the number of ATLOs. *MMP-9* production in FDCs and pDCs inside ATLOs is primary supplier of *MMP-9* in IgG4-AAA and one of the characteristic features of IgG4-AAA. Sakata et al<sup>4</sup> described several irregular shaped ATLOs containing differentially distributed FDCs in IgG4-AAA. *MMP-9* production inside ATLOs might be concerned with the shape and distribution of ATLOs itself in IgG4-AAA to affect the activity of local inflammation.

Outside ATLOs, fibroblasts and histiocytes primarily expressed *MMP-9* and, interestingly, pDCs also could produce *MMP-9*. With regard to aAAA, our results are largely consistent with previous studies.<sup>17,38</sup> In previous reports, *MMP-9*<sup>+</sup> cells in aAAA were primarily distributed in the dilated parts of the aneurysm, and therefore *MMP-9* was considered to function essentially in the formation and progression of aneurysms.<sup>20,39</sup> Although the numbers of *MMP-9*<sup>+</sup> fibroblasts and *MMP-9*<sup>+</sup> tDCs were almost equal among the three control groups, the numbers of *MMP-9*<sup>+</sup> histiocytes and *MMP-9*<sup>+</sup> pDCs were significantly higher in IgG4-AAA. In contrast, in the

autopsy cases, all kind of *MMP-9*<sup>+</sup> cells (histiocytes, fibroblasts, and pDCs) were low. Therefore, adventitial *MMP-9* production by histiocytes and fibroblasts would be rather associated with the beginning of aortic expansion from a normal-sized aorta to progression to aneurysm, similar to previous reports of aAAA.<sup>20,39</sup> Moreover, *MMP-9* derived from pDCs outside ATLOs was characteristically increased in IgG4-AAA. Recent reports indicated that, in IgG4-related diseases, many pDC infiltration and its relation to IgG4 production<sup>40</sup> and that activated pDCs could produce interferon alpha and IL-33 to play a pivotal role in the chronic fibroinflammatory response.<sup>41</sup> Further studies are needed to elucidate how *MMP-9* production by pDCs may contribute to the pathogenesis and/or progression of IgG4-AAA.

Several previous studies of aAAA have shown that *MMP-2*<sup>+</sup> cells are predominantly present in the smooth muscle cells of the intima and media in the nondilated parts of aneurysms, and therefore *MMP-2* was considered to be involved in elastic fiber disruption, although to a limited extent in aAAA.<sup>17,20</sup>



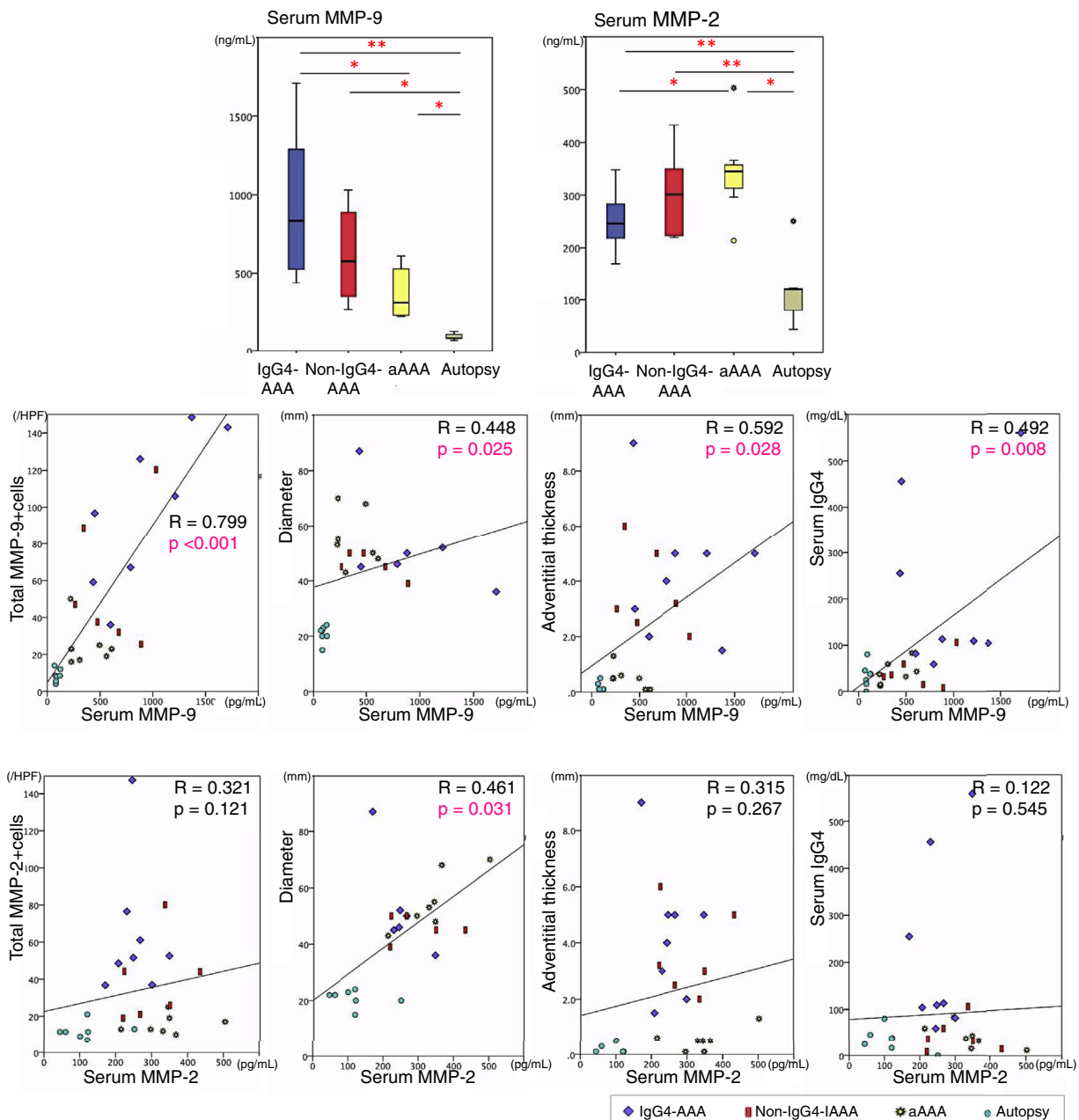
**Fig 7.** Cumulative bar chart of matrix metalloproteinase (MMP)-presenting cells in the adventitia of IgG4-related AAAs (*IgG4-AAA*) and control groups. **A**, MMP-9-presenting cells inside arterial tertiary lymphoid organs (ATLOs). **B**, MMP-9-presenting cells outside of ATLOs. **C**, MMP-2-presenting cells outside of ATLOs. **D**, MMP-14-presenting cells outside of ATLOs. The numbers of MMP-presenting cells were counted per high-power field (HPF). Median counts of the numbers of MMP-presenting cells are given. IgG4-AAA, n = 19; non-IgG4-related inflammatory AAA (*non-IgG4-AAA*), n = 18; atherosclerotic AAA (aAAA), n = 11; autopsy (normal aorta obtained by autopsy), n = 11.

In the present study, the total number of adventitial MMP-2<sup>+</sup> cells did not correlate with aneurysmal diameter and adventitial MMP-2 expression was observed in histiocytes and fibroblasts mainly outside ATLOs. Interestingly, the number of adventitial MMP-2<sup>+</sup> histiocytes, but not that of MMP-2<sup>+</sup> fibroblasts, increased in IgG4-AAA.

Histiocytes are mainly classified in classically activated macrophages (M1-type macrophages) and alternatively activated macrophages (M2-type macrophages). M1-type macrophages tend to be more proinflammatory to produce inflammatory cytokines and M2-type macrophages are generally associated with a regenerative or anti-inflammatory response, tissue remodeling, and matrix deposition. Generally, aAAAs have an imbalance of M1-and M2-type macrophage, but are rather in a state of a predominance of M1 type in the intima to the media.

Previous studies have suggested that in IgG4-related disease, M2-type macrophages had supremacy and enhanced the T2 immune response and fibrosis via profibrotic factor production (IL-10 and chemokine ligand-18).<sup>42</sup> Similarly, a recent report indicated that adventitial M2-type macrophages, but fewer M1 types, characteristically increased in numbers and expressed type 2 cytokines and were concerned with the adventitial fibrosis of IgG4-AAA.<sup>19</sup> Our results suggested that many M2-type macrophages are distributed outside ATLOs might produce MMPs (MMP-9 and MMP-2) and cytokines within the same and/or neighbor cells to act as paracrine and/or autocrine signals in the adventitia of IgG4-AAAs to affect the activity of IgG4-related disease and the production of fibrosis.

MMP-14 expression was described in a few recent reports of thoracic aortic aneurysm<sup>25,43</sup> and has been rarely



**Fig 8.** Serum levels of matrix metalloproteinases (MMPs) of IgG4-related abdominal aortic aneurysms (IgG4-AAA) and control groups. (Top) Box-and-whisker diagrams for serum MMP-9 and MMP-2 of IgG4-AAA and control groups. The horizontal line in the middle of each box indicates the median; the top and bottom borders of the box mark the 75th and 25th percentiles, respectively, and the whiskers above and below the box indicate the 1.5 interquartile ranges. Statistically significant differences between groups are indicated as \* $P < .05$ , \*\* $P < .001$ . IgG4-AAA,  $n = 9$ ; non-IgG4-related inflammatory AAA (non-IgG4-AAA),  $n = 7$ ; atherosclerotic AAA (aAAA),  $n = 6$ ; autopsy (normal aorta obtained by autopsy)  $n = 7$ . (Middle, bottom) Relationships between serum MMPs (middle, MMP-9; bottom, MMP-2) and important parameters of IgG4-related AAAs (IgG4-AAA): total counts of each MMPs immunopositive cells, aortic diameter, pathologic adventitial thickness, and serum IgG4 levels among 29 cases. Rhombuses show cases of IgG4-AAA ( $n = 9$ ), rectangles show cases of non-IgG4-related inflammatory AAA ( $n = 7$ ), asterisks show cases of atherosclerotic AAA (aAAA;  $n = 6$ ), and circles show cases of normal aorta obtained by autopsy ( $n = 7$ ).

detected in aAAA.<sup>44</sup> In the present study, adventitial MMP14<sup>+</sup> cells were histiocytes and fibroblasts found only outside ATLOs. The total counts of MMP-14<sup>+</sup>

histiocytes and MMP-14<sup>+</sup> fibroblasts differed little among the aneurysm groups but were lower in autopsy cases. The total number of MMP-14<sup>+</sup> cells was not correlated

with aneurysmal diameter or the total number of IgG4<sup>+</sup> cells. Hence, it is speculated that MMP-14 production in the adventitia may be partially involved in the expansion of the aneurysmal wall from the normal aorta but not be associated with aneurysmal progression or IgG4-related disease activity.

MMPs have not only inhibitory but also stimulatory roles in fibrous proliferation.<sup>12</sup> MMP-2 could play fibrotic roles in the subsequent dysregulation of other MMPs,<sup>45</sup> and MMP-9 could activate latent transforming growth factor beta, which is a cytokine promoting fibrosis.<sup>46</sup> Inflammation and tissue remodeling in the adventitia are responsible for the structural alterations that characterize IAAA, including both IgG4-AAA and non-IgG4-IAAA. In our results, interestingly, the total numbers of adventitial MMP-9<sup>+</sup> and MMP-2<sup>+</sup> cells were correlated with adventitial thickening, and those were similarly higher in IAAA group of IgG4-AAA and non-IgG4-IAAA than others. The imbalance between MMPs and tissue inhibitors of metalloproteinases (TIMP)-1 and TIMP-2 also contributes to the remodeling process occurring in the vessel walls of large-vessel vasculitis.<sup>47</sup> Although further study of the expression of adventitial TIMPs would be necessary to identify the process of adventitial fibrosis in IgG4-AAA, we thought that greater production of MMPs might contribute to remodeling of the adventitia with growth or destruction of collagen fibers.

Several multiple logistic analyses of aAAA indicated that the serum MMP-9 level was an independent risk factor for the aneurysmal expansion.<sup>48</sup> These authors considered that serum MMP-9 levels might reflect local MMP-9 production in the aorta.<sup>48</sup> In our results, serum MMP-9 levels were correlated with the total number of adventitial MMP-9<sup>+</sup> cells. Serum MMP-9 of IgG4-AAA was higher than that of the aAAA and autopsy groups. Moreover, serum MMP-9 levels were positively correlated with serum IgG4 levels and the number of IgG4<sup>+</sup> cells. Our results suggested that increased serum MMP-9 in IgG4-AAA would reflect the local MMP-9 production in the adventitia to some extent. Thus, we considered that serum MMP-9 level might be a predictive marker for the activity of IgG4-AAA as well as for the progression of the aneurysm. In contrast, serum MMP-2 was similarly high in the three groups of aneurysms (IgG4-AAAs, non-IgG4-IAAAs, and AAAs) and was low in normal aorta without aneurysm. Serum MMP-2 levels were not positively correlated with the total numbers of MMP-2<sup>+</sup> cells in the adventitia and did not be associated with the activity of IgG4-AAA but had a statistically significant positive correlation with aneurysmal diameter. This would be because MMP-2 is mainly produced in the smooth muscle cells of the tunica media of the aneurysm more than in the histiocytes in the adventitia, and involved in the destruction of the tunica media to enlarge aneurysm.<sup>17,20</sup>

## CONCLUSIONS

In this study of the aortic adventitia, the distribution of various MMP-9-producing cells concerning ATLOs was unique, particularly in IgG4-AAA. MMP-9 derived from FDCs inside ATLOs and histiocytes and pDCs outside ATLOs was characteristically increased in IgG4-AAA and might be required and work in concert to IgG4-AAA. Expression of MMP-2 and MMP-14 was mainly detected in histiocytes and fibroblasts outside ATLOs. MMPs produced by fibroblasts were less concerned with activity of IgG4-AAA. The specific pattern of adventitial MMPs in IgG4-AAA may be a key factor inciting aneurysmal formation and progression and remodeling of the thick fibrous adventitia.

## AUTHOR CONTRIBUTIONS

Conception and design: SK, AK

Analysis and interpretation: SK, AK, FK, YM, YY, SO, HT

Data collection: SK, AK, FK, YM, YY, SO, HT

Writing the article: SK, AK, FK

Critical revision of the article: SK, AK, FK, YM, YY, SO, HT

Final approval of the article: SK, AK, FK, YM, YY, SO, HT

Statistical analysis: SK, FK, SO

Obtained funding: SK

Overall responsibility: SK

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