Inflammatory and immune checkpoint markers are associated with the severity of aortic stenosis

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ABSTRACT

Objective: Aortic stenosis (AS) is a disease characterized by narrowing of the aortic valve (AV) orifice. In relation to this disease, the purpose of this study was to elucidate the relationships among factors such as expression of programmed cell death-1 ligand (PD-L1, which is the ligand of PD-1 protein; together, they play a central role in the inhibition of T lymphocyte function), clinicopathologic characteristics, infiltrating immune cells, and disease severity.

Methods: We performed immunohistochemical analysis on the surgically-resected AVs of 53 patients with AS. We used the resultant data to identify relationships among PD-L1 expression, disease severity, and the infiltration of immune cells including cluster of differentiation (CD8)-positive T lymphocytes, cluster of differentiation 163 (CD163)-positive macrophages, and forkhead box protein 3 (FOXP3)-positive regulatory T lymphocytes (Tregs).

Results: PD-L1 expression in resected AVs was significantly associated with being nonsmoker, valve calcification, and the infiltration of CD8-positive T cells and CD163-positive macrophages. Disease severity and valve calcification were significantly associated with low infiltration of FOXP3-positive Tregs and high infiltration of CD8-positive T cells and CD163-positive macrophages. Moreover, calcified AVs with high PD-L1 expression showed active inflammation without FOXP3-positive Tregs but with high levels of CD8-positive T lymphocytes and CD163-positive macrophages.

Conclusions: Immune cell infiltration in the AVs and expression of the immune checkpoint protein PD-L1 were associated with the calcification of AS and disease severity. (JTCVS Open 2021;5:1-12)

CENTRAL MESSAGE

Immune cell-related inflammation and an immune checkpoint protein are associated with AS severity.

PERSPECTIVE

For the first time, we have shown that PD-L1 expression and active inflammation, characterized by high infiltration of CD8-positive T lymphocytes and CD163-positive macrophages and less infiltration of FOXP3-positive Tregs, is associated with disease severity and valve calcification in patients with AS. These findings begin to uncover the mechanisms of this disease.

See Commentaries on pages 13 and 15.
transcatheter aortic valve implantation, a less-invasive process, are considered effective treatments for patients with AS. However, these interventions and prostheses have several limitations, such as procedure-related complications, postoperative anticoagulation therapy, and reoperation. Therefore, to improve the quality of life of patients with AS and to prevent disease progression, it is essential to elucidate the fundamental mechanisms underlying the disease.

Active inflammation due to increased infiltration of T lymphocytes, macrophages, and inflammation-induced osteoblast-like valvular interstitial cells (VICs) is known to worsen the disease condition of AS and induce aortic valve (AV) calcification, which leads to irreversible degeneration via mechanisms similar to those of osteogenesis.\(^3\)\(^4\) In relation to T lymphocytes, the programmed cell death-ligand 1 (PD-1) protein and its ligand (PD-L1) have been reported to play a central role in the inhibition of T-cell receptor-mediated lymphocyte proliferation and cytokine secretory function.\(^5\)\(^6\) However, active inflammation is regulated by both the PD-1/PD-L1 axis as well as several immune cells, including forhead box protein 3 (FOXP3)–positive regulatory T lymphocytes (Tregs) and macrophages.\(^7\)\(^8\)

Taken together, these previous findings strongly suggest that relationships exist among immune checkpoint proteins, active inflammation related to T cells/macrophages, and the severity of AS (ie, stenosis and calcification in the AV). Therefore, we hypothesized that the expression of the immune checkpoint protein PD-L1 and Treg infiltration may act as anti-inflammatory factors that might regulate inflammation via inflammatory immune cells, including T lymphocytes and macrophages, and therefore, modulate the AS disease state. However, few studies have investigated whether the expression of the immune checkpoint protein PD-L1 in resected AVs is associated with clinicopathologic factors, valve-infiltrating immune cells, and AV conditions in clinical patients with AS. In our study, we performed an immunohistochemical analysis of the surgically-resected AVs of 53 patients with AS to examine the association of pro- and anti-inflammatory components with the AS disease state.

**METHODS**

**Patients and Samples**

We selected a total of 53 consecutive patients (25 male and 28 female) diagnosed with AS, each of whom undergone surgical resection and valve implantation in Gunma University Hospital between 2010 and 2017, and were included in this retrospective study. The median age of the patients was 73 years (range, 56-87 years). Patients with traumatic aortic injury, infectious aortic disease, or other connective tissue disorders were excluded from this study. The diagnosis of valve dysfunction, disease severity, and morphology was based on echocardiographic evaluations. The majority of the patients were diagnosed with senile AS (n = 24; 45.3%) and tricuspid AV morphology (n = 50, 94.3%). Postoperative complications, such as infection, atrial fibrillation, and hospital mortality, were evaluated throughout the hospital stay of patients with AS. Other characteristics of the patients are summarized in Table 1. This study conformed to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board for Clinical Research at the Gunma University Hospital (Maebashi, Gunma, Japan; approval number: HS2020-014).

**Immunohistochemical Staining and Histopathology**

Paraﬃn-embedded blocks of all surgically resected specimens were cut into 4-μm thick sections and mounted on glass slides. Sections were deparaffinized using xylene and then dehydrated in alcohol. Endogenous peroxidase was inhibited using 0.3% H₂O₂/methanol for 30 minutes at room temperature. After rehydration through a graded series of ethanol treatments, antigen retrieval was performed in Immunosaver (Nishin EM, Tokyo, Japan) at 98°C to 100°C for 45 minutes, and PD-L1 was retrieved using Universal HIER antigen retrieval reagent (Abcam, ab208572) at 120°C for 20 minutes in an autoclave. Nonspecific binding sites were blocked by incubation with Protein Block Serum-Free (Dako, Carpinteria, Calif) for 30 minutes. Subsequently, the sections were incubated at 4°C overnight with primary antibodies against PD-L1 (28-8 Rabbit mAb, 1:200 dilution; Abcam), cluster of differentiation 8 (CD8; 9F3, Mouse mAb, 1:400 dilution; Abcam), FOXP3 (Mouse mAb, 1:80 dilution; Abcam), and cluster of differentiation 163 (CD163; D6U1J, Rabbit mAb, 1:200 dilution; Abcam), cluster of differentiation 8 (CD8; 9F3, Mouse mAb, 1:400 dilution; Abcam), FOXP3 (Mouse mAb, 1:80 dilution; Abcam), and cluster of differentiation 163 (CD163; D6U1J, Rabbit mAb, 1:200 dilution; Cell Signaling Technology). For the PD-L1 sections, the Rabbit Specific IHC Polymer Detection Kit HRP/DAB (ab209101; Abcam) was used for the secondary antibody. In the other sections, the Histo-Simple Stain MAX-PO (Multi) Kit (Nichirei, Tokyo, Japan) was used for the secondary antibody. The chromogen 3,3′-diaminobenzidine tetrahydrochloride was also applied as a 0.02% solution; the solution contained 0.005% H₂O₂ in a 50 mM ammonium acetate–citrate acid buffer (pH 6.0). Finally, nuclear counterstaining was performed using Mayer’s haematoxylin solution.

For histopathologic analyses, von Kossa and hematoxylin and eosin staining were each performed. To summarize, 4-μm-thick formalin-fixed, paraffin-embedded sections were deparaffinized in xylene and rehydrated using an ethanol series. They were then incubated with a 5% silver nitrate solution while being exposed to a 60-watt incandescent bulb for 1 hour. Subsequently, they were incubated with a 5% sodium thiosulfate solution for 3 minutes at room temperature. Similarly, sections incubated in the dark were used as negative controls. Excess silver precipitation was rinsed away with distilled water and then nuclear red fast solution (NFS125; ScyTek Laboratories, West Logan, Utah) was used for counter staining.

**Assessment of AV Calcification and Inflammation Status**

We evaluated the inflammatory infiltrate status in the AV using the quantification method described by Sakata and colleagues with slight
CD163- and FOXP3-positive cell infiltration was defined as negative or
positive according to the absence or presence of positive cells. Valve

calcifications were assessed by von Kossa staining; we microscopically
examined the overall area of the stained section and identified the positively
and negatively stained regions in the tissue based on the presence of silver
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Multiple Immunofluorescence Staining

Multiple immunofluorescence staining was achieved using a PerkinElmer Opal Kit (NEL810001KT; PerkinElmer, Hopkinton, Mass); staining was optimized and performed according to manufacturer’s instructions. Deparaffinization, antigen retrieval, peroxidase blocking, and blocking were performed as described previously. Sections were incubated with primary antibodies against PD-L1, CD8, CD163, FOXP3, α-smooth muscle actin (α-SMA) (Mouse mAb, 1:600 dilution; Sigma, St Louis, Mo), and cluster of differentiation 31 (CD31; JC70A, Mouse mAb, 1:40 dilution; Dako). Respective stainings were visualized as follows: PD-L1 with Opal 520 fluorophore, α-SMA and CD163 with Opal 570 fluorophore, FOXP3 with coumarin-tyramide, and CD31 and CD8 with Cyanine 5. All sections were then counterstained with hematoxylin and examined under an all-in-one BZX-710 fluorescence microscope (Keyence, Tokyo, Japan).

Statistical Analysis

The JMP Pro 14.0 software package (SAS Institute Inc, Cary, NC) was used to perform all statistical analyses. All continuous variables were assessed for normality. Normal and categorical variables are expressed as mean (standard deviation) and n (%), respectively, and non-normal variables are expressed as median (25%-75%) in the tables. The Student t test, Wilcoxon test, χ² test, and Fisher exact test were used to analyze the associations described in the present study.

RESULTS

PD-L1 Expression, Immune Cells, and Calcification in Resected AVs

PD-L1 expression, valve calcification, and immune cell infiltrations, including CD8-positive T lymphocytes, CD163-positive macrophages, and FOXP3-positive Tregs, were detected in the AV specimens, and representative results are shown in Figure 1. PD-L1 expression was detected in the stromal area of the resected AVs. No positive staining was detected in the disease-free regions of the AV (Figure 3).

Clinical Significance of PD-L1 Expression in Resected AVs

From all AV specimens, 34 (64.2%) and 19 (35.8%) specimens were assigned to the low and high stromal PD-L1 expression groups, respectively (Table 2; Figure 2). Of the patients’ assessed clinicopathologic factors (Tables 1 and 2), a high expression of stromal PD-L1 was significantly associated with being a nonsmoker (P = .0493), showing valve calcification (P = .002), and having a history of cerebral infarction (P = .02). No significant correlation was found between PD-L1 expression and postoperative infections. In this study cohort, 2 (3.7%) surgical-site infections occurred in the low PD-L1 expression group.

Association of PD-L1 Expression With Immune Cell Infiltration in Resected AVs

PD-L1 expression was significantly associated with high infiltration of CD8-positive T lymphocytes (P = .0439) and CD163-positive macrophages (P = .0175) (Table 3; Figure 2).

Association of AS Severity With Active Inflammation Related to T Lymphocytes and Macrophages

As shown in Table 4, the ejection fractions of patients did not show any association with infiltration of immune cells expressing CD8, CD163, or FOXP3. However, high mean
pressure gradient levels (≥40 mm Hg) in patients were significantly associated with high infiltration of CD8-positive T lymphocytes \( (P = .0005) \) and FOXP3-positive Tregs \( (P = .0469) \). Moreover, the existence of AV calcification as an irreversible degeneration was significantly related to high infiltrations of CD8-positive lymphocytes \( (P = .0439) \) and CD163-positive macrophages \( (P = .0028) \), and to the lack of FOXP3 Tregs \( (P = .0165) \), in resected AVs.

**Correlations Between PD-L1 Expression, T Lymphocytes, Macrophages, and Tregs in Resected AVs**

Calcified AVs with high expression of PD-L1 showed active inflammation without FOXP3-positive Tregs but with high infiltration of CD8-positive T lymphocytes and CD163-positive macrophages (Figure 4). In contrast, non-calcified AVs with low expression of PD-L1 showed high positivity for Treg cells but low infiltration of CD8-positive T lymphocytes and CD163-positive macrophages (Figure 4).

**DISCUSSION**

In this study, we demonstrated that the expression of stromal PD-L1 in resected AV tissues from patients with AS was significantly associated with being a nonsmoker, a history of cerebral infarction, valve calcification, and the infiltration of immune cells, including CD8-positive T cells and CD163-positive macrophages. Moreover, disease severity and valve calcification were significantly related to the low infiltration of FOXP3-positive Tregs and high infiltration of CD8-positive T cells and CD163-positive macrophages. Thus, active inflammation with high levels of CD8-positive T cells and CD163-positive macrophages but without FOXP3 Tregs may have induced the valve calcification and PD-L1 expression we observed in resected AV specimens.

Smoking is a known risk factor for cardiovascular diseases.10 Wasén and colleagues11 have previously reported that smoking inhibits T-cell activation and soluble PD-L1 expression in patients with rheumatoid arthritis, whereas T-cell activation can induce PD-L1 expression.12 In this study, a high PD-L1 expression in the resected AS samples...
was associated with being a nonsmoker and the female sex (Table 2). Interestingly, the female patients in our Japanese cohort were significantly associated with nonsmoking status \((P = .003, \text{data not shown})\). These observations suggest that the high PD-L1 expression in the resected samples from female patients with AS may be induced by T-cell activation attributed to being a nonsmoker. To date, researchers have attempted to elucidate the molecular pathologic mechanism of AS by investigating the roles of immune cell infiltration and resident cells, such as VICs and valvular endothelial cells (VECs), in the disease’s progression.\cite{12,13,14,15} These cells have been the focus of research because macrophages and T lymphocytes are major contributors to inflammation, whereas VICs function in calcification and fibrosis during

FIGURE 3. Representative images showing immune cells and immune checkpoint protein expression in disease-free regions of the AVs. Images were taken from two different samples, and the panels of all staining markers are shown at \(\times 200\) magnification. Scale bar: 50 \(\mu\)m. AV, aortic valve; A. side, aortic side; V. side, ventricular side; HE, hematoxylin and eosin; PD-L1, programmed death ligand 1; CD8, cluster of differentiation 8; CD163, cluster of differentiation 163; FOXP3, forkhead box protein 3.
disease progression. In one example, Oba and colleagues found a greater distribution of CD163-positive macrophages in calcified AVs than that in noncalcified AVs and showed that the infiltrating CD163-positive macrophages were expressing high levels of bone morphogenetic protein 2. Infiltrated macrophages are also known to be important for VIC activation, inflammatory cytokine secretion, and calcification. Consistent with these previous findings, we showed that high infiltration of CD163 macrophages was significantly correlated with AV calcification. In addition, we showed that PD-L1 expression was correlated with CD163 macrophage infiltration in the calcified AVs. These findings suggest that CD163-positive macrophages play a pivotal role in PD-L1 expression and calcification in active inflammation-mediated AS.

The role and importance of FOXP3-positive Tregs in AS has not previously been elucidated. Two aspects must be addressed regarding the increased Treg positivity in noncalcified AS. Tregs play a protective role by secreting immunosuppressive cytokines or competing with effector T lymphocytes in several cardiovascular diseases, such as atherosclerosis. This may be related to the pathophysiologic mechanism of AS. Interestingly, we found that the infiltration of Foxp3-positive Treg cells in the resected samples of patients with AS was negatively associated with calcification and disease severity. In contrast to our findings, Shimoni and colleagues have reported that Treg cells circulate at high levels in patients with AS and that these high levels of circulating Tregs are related to the progression of valve narrowing. However, we found that the infiltration of Treg cells in the AV of patients with AS was negatively associated with calcification and disease severity. Therefore, further studies will be required to clarify the characteristics and potential differences of circulating Tregs in the blood and in the local lesions of AS, either with or without calcification or valve narrowing. In addition, a therapeutic strategy targeting Tregs has been suggested as a promising approach for improving the survival of patients with advanced cancer. Based on our findings and the protective effect of Tregs in cardiovascular disease, careless administration of Treg inhibitors to patients with AS with advanced cancer may have a potential for cardiovascular disease progression; this is the same as that occurring with immune checkpoint inhibitor (ICI) treatment, resulting in cardiovascular side effects.

### Table 2. Characteristics of patients with AS according to low or high PD-L1 expression in resected AVs

| Factors                        | PD-L1 |           |           |           |           |           |
|--------------------------------|-------|-----------|-----------|-----------|-----------|-----------|
|                                | Low, n = 34 (%) | High, n = 19 (%) | P value | Low, n = 37 (%) | High, n = 16 (%) | P value |
| Age, ≥65 y                     | 30 (88.2%)     | 18 (94.8%)     | .418     | 27 (79.4%)     | 7 (36.8%)     | .002*    |
| Sex, female                    | 15 (44.1%)     | 13 (68.4%)     | .086     | 14 (41.2%)     | 3 (15.8%)     | .049*    |
| Smoking, yes                   | 14 (41.2%)     | 3 (15.8%)      | .498     | 26 (76.5%)     | 16 (84.2%)    | .177     |
| Ejection fraction, ≥50%        | 23 (67.7%)     | 16 (84.2%)     | .149     | 34 (100.0%)    | 18 (94.8%)    | .149     |
| Mean pressure gradient, ≥40 mm Hg | 34 (100.0%)  | 18 (94.8%)     | .149     | 23 (67.7%)     | 16 (84.2%)    | .177     |
| Aortic valve area, ≤1.0 cm²    | 7 (20.6%)      | 12 (63.2%)     | .002*    | 7 (20.6%)      | 12 (63.2%)    | .002*    |

**PD-L1**, Programmed death ligand 1. *Statistically significant at P < .05.

### Table 3. Relationships among PD-L1 expression and immune cell infiltrations in the AVs of patients with AS

| Factors | PD-L1 | CD8 | CD163 | FOXP3 |
|---------|-------|-----|-------|-------|
|         | Low, n = 34 (%) | High, n = 19 (%) | Low, n = 37 (%) | High, n = 16 (%) | Low, n = 36 (%) | High, n = 17 (%) | P value |
| CD8     |       |     |       |       |       |       |       |
| Low     | 27 (79.4%)     | 10 (52.6%)     | –     | –     | –     | –     | –     |
| High    | 7 (20.6%)      | 9 (47.4%)      | –     | –     | –     | –     | –     |
| CD163   |       |     |       |       |       |       |       |
| Negative | 27 (79.4%) | 9 (47.4%) | 28 (75.7%) | 8 (50.0%) | 33 (91.7%) | 14 (82.4%) | .3323 |
| Positive | 7 (20.6%) | 10 (52.6%) | 9 (24.3%) | 8 (50.0%) | 3 (8.3%) | 3 (17.6%) | –     |
| FOXP3   |       |     |       |       |       |       |       |
| Negative | 32 (94.1%) | 15 (79.0%) | 32 (86.5%) | 15 (93.8%) | 32 (86.5%) | 15 (93.8%) | .1025 |
| Positive | 2 (5.9%) | 4 (21.0%) | 5 (13.5%) | 1 (6.2%) | 3 (4.8%) | 4 (21.0%) | –     |

**PD-L1**, Programmed death ligand 1; **CD8**, cluster of differentiation 8; **CD163**, cluster of differentiation 163; **FOXP3**, forkhead box protein 3. *Statistically significant at P < .05.
Previously, the origin of the PD-L1 protein in the AV had not been reported; in the present study, we detected PD-L1 expression in the stromal area of resected AVs. In cancer research, PD-L1 protein expression has been detected in the cells of tumor tissues including tumor cells, stromal fibroblasts, intratumoral endothelial cells, and infiltrating immune cells such as macrophages.\textsuperscript{23,24} In our additional analysis, we evaluated the potential co-expression of PD-L1, α-SMA (as a VIC marker), and CD31 (as a VEC marker); we found that stromal PD-L1 protein was not expressed on the cellular membrane of fibroblasts or VECs, but instead existed in the stromal area (Figure 5).

\begin{table}[h]
\centering
\caption{The associations among immune cell infiltration in resected AVs and disease severity in patients with AS}\\
\begin{tabular}{|l|c|c|c|c|c|c|c|}
\hline
Factors & \multicolumn{2}{c|}{CD8} & \multicolumn{2}{c|}{CD163} & \multicolumn{2}{c|}{FOXP3} \\
 & Low & High & Negative & Positive & Negative & Positive \\
\hline
EF, ≥50\% & 28 (75.6\%) & 14 (87.5\%) & .3121 & 27 (75.0\%) & 15 (88.2\%) & .2489 & 37 (78.7\%) & 5 (83.3\%) & .7884 \\
mPG, ≥40 & 23 (62.2\%) & 16 (100.0\%) & .0005\* & 24 (66.7\%) & 15 (88.2\%) & .0805 & 33 (70.2\%) & 6 (100.0\%) & .0469\* \\
AVA, ≤1.0 cm\textsuperscript{2} & 37 (100\%) & 15 (93.8\%) & .1183 & 35 (97.2\%) & 17 (100\%) & 46 (97.9\%) & 6 (100.0\%) & .6222 \\
Valve calcification & & & & & & & & \textsuperscript{2} & \textsuperscript{2} \\
Negative & 27 (73.0\%) & 7 (43.7\%) & .0439\* & 28 (77.8\%) & 6 (35.3\%) & .0028\* & 28 (59.6\%) & 6 (100\%) & .0165\* \\
Positive & 10 (27.0\%) & 9 (56.3\%) & 8 (22.2\%) & 11 (64.7\%) & 19 (40.4\%) & 0 & & & \textsuperscript{2} & \textsuperscript{2} \\
\hline
\end{tabular}
\end{table}

\textsuperscript{2}CD8, Cluster of differentiation 8; CD163, cluster of differentiation 163; FOXP3, forkhead box protein 3; EF, ejection fraction; mPG, mean pressure gradient; AVA, aortic valve area. \*Statistically significant at \( P \leq .05 \).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Multicolor immunofluorescence staining of immune cells and immune checkpoint protein markers in calcified and noncalcified AVs. Left panels show calcified AVs with staining of PD-L1 (green), CD8 (purple), CD163 (red), and FOXP3 (blue), as well as a merged image. Right panels show the same staining in noncalcified AVs. All images are shown at ×400 magnification. Scale bar: 50 μm. PD-L1, Programmed death ligand 1; CD8, cluster of differentiation 8; CD163, cluster of differentiation 163; FOXP3, forkhead box protein 3.}
\end{figure}
Interestingly, 2 types of PD-L1 exist: membrane PD-L1 and soluble PD-L1; high levels of soluble PD-L1 in the blood are associated with poor prognosis in patients with soft-tissue sarcoma, which suggests that soluble PD-L1 has an important function in combating local tumor immunity.\(^{25}\) Given these observations, stromal PD-L1 in resected AV specimens might be extracted from stromal cells including fibroblasts, VICs, and VECs to regulate inflammation in the AVs.

In cancer treatment, ICIs are used to target PD-1 and PD-L1 and thereby improve patient survival.\(^{6}\) The adverse events of ICIs were initially thought to be mild compared with those of conventional cytotoxic anticancer drugs, which can cause hematologic toxicity, organ failure, hair loss, nausea, and vomiting.\(^{26,27}\) However, ICIs have now been reported to cause characteristic immune-related adverse events, associated with overactivated autoimmune inflammation against several organs, in patients with cancer.\(^{27}\) The unexpected ICI-induced activation of autoimmune responses against the pancreas, thyroid, and colon may lead to diabetes, dysregulation of thyroid function, and severe colitis (similar to inflammatory bowel disease), respectively.\(^{27}\) Interestingly, treatment with ICIs can also cause cardiovascular toxicity shown as ICI-associated myocarditis, myocardial infarction, and conduction disease related to cardiovascular mortality.\(^{28}\) thus, ICIs that target the PD-1/PD-L1 axis could induce T-cell-mediated inflammation related to myocarditis and coronary vasculitis without atherosclerosis.\(^{29}\) In the present study, we found that active inflammation caused by CD8-positive T lymphocytes and CD163-positive macrophages was associated with the severity of the pressure gradient and valve calcification in the AV, which is close to both the heart and the coronary arteries. Moreover, the cumulative 5-year incidence of sudden death in AS patients is reported to be 7.2\% (1.4\% per year), despite the existence of asymptomatic patients.\(^{30}\) Given the evidence of ICI-associated cardiovascular toxicity and immune cell infiltration in AVs, we suggest that activation of immune cell-related inflammation by ICIs may affect immune tolerance against not only organs such as the pancreas, thyroid, and colon but also cardiovascular tissues such as AVs.

To show the clinical importance of immune cells/PD-L1 expression in the resected AS samples, we performed an additional analysis of the preoperative comorbidity and complications, shown in Table 1. Interestingly, low CD8 infiltration was significantly associated with postoperative atrial fibrillation (data not shown, \(P = .0127\)). Therefore, our data suggested that an evaluation of immune infiltration, particularly infiltrated low CD8 cells, in resected samples
may be useful for identifying patients with high-risk AS, which may be useful for cardiovascular surgeons. In the future, we will evaluate the function of immune checkpoint proteins, including PD-L1, as anti-inflammatory tools to suppress the progression of AS. Moreover, we will investigate the relationship between cardiovascular disease progression and Treg inhibitors because in this study, Treg infiltration was significantly associated with a noncalcified AS status and patients with cancer with Treg inhibition are known to exhibit the induction of cardiovascular disorders.

Our study has several limitations. First, it is a retrospective single-institution study with a small sample size; these conditions may potentially bias our results. Large-scale clinical trials will therefore be needed to validate the relationships among PD-L1 expression, immune cell infiltration, and the severity of AS reported here. In addition, the relationship of immune checkpoint protein/immune cells and infectious heart diseases could not be shown because we excluded infectious heart diseases, such as aortic infections or endocarditis, in this study. Second, we discussed the potential adverse events caused by immunotherapy treatments for AS; however, none of the enrolled patients in our study had undergone immunotherapy to target the PD-1/PD-L1 axis, Tregs, or CD163. Finally, we did not functionally analyze the association between immune cell-induced inflammation, immune checkpoint protein (PD-L1) induction, and the progression of AS.

In conclusion, our data show that high PD-L1 expression is associated with CD8-positive T cells and CD163-positive macrophages in calcified AV samples (Figure 6 and Video 1), and that this active inflammation was related to PD-L1 expression, valve calcification, and disease severity in AS. These results suggest that immune cell-induced inflammation in the AVs, anti-inflammatory components-infiltrated

**FIGURE 6.** Inflammatory and immune checkpoint markers are associated with AS severity. High PD-L1 and active inflammation were associated with calcified AS. HE, Hematoxylin and eosin; PD-L1, programmed death ligand 1; CD8, cluster of differentiation 8; CD163, cluster of differentiation 163; FOXP3, forkhead box protein 3.
Inflammatory and immune checkpoint markers are associated with the severity of aortic stenosis

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Conflict of Interest Statement

The authors reported no conflicts of interest.

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