Deficiency of Interleukin-1 Receptor Antagonist Induces Aortic Valve Disease in BALB/c Mice

Kikuo Isoda, Taizo Matsuki, Harumi Kondo, Yoichiro Iwakura, Fumitaka Ohsuzu

Objective—Interleukin-1 receptor antagonist (IL-1Ra), one of the most important antiinflammatory cytokines, is crucial for homeostasis of the immune system. However, the role of IL-1Ra in aortic valve stenosis (AS) remains poorly understood.

Methods and Results—IL-1Ra–deficient (IL-1Ra<sup>−/−</sup>) mice on the BALB/c background showed increased aortic valve leaflet thickness compared to wild-type mice at the age of 12 weeks (P<0.001). We used peripheral T-cell transplantation to examine the role of T cells in the development of AS. T cells from IL-1Ra<sup>−/−</sup> but not from wild-type mice induced increased aortic valve thickness in nu/nu mice. Moreover, IL-1Ra<sup>−/−</sup> T cells produced much higher levels of tumor necrosis factor (TNF)-α in culture supernatants after anti-CD3 antibody stimulation compared to wild-type mice (P<0.001). Finally, we studied the role of TNF-α in the development of AS in IL-1Ra<sup>−/−</sup> mice by generating double-gene-deficient (TNF-α<sup>−/−</sup>/IL-1Ra<sup>−/−</sup>) mice. Interestingly, TNF-α<sup>−/−</sup>/IL-1Ra<sup>−/−</sup> mice did not have AS.

Conclusion—IL-1Ra deficiency in inflammatory cells induced aortic valve inflammation and TNF-α participates importantly in the development of AS in IL-1Ra<sup>−/−</sup> mice. (Arterioscler Thromb Vasc Biol. 2010;30:708-715.)

Key Words: aortic valve stenosis • cytokine • immune system • inflammation • transplantation

Degenerative aortic valve stenosis (AS) is the most common valvular disease in Western countries.1,2 Observations suggest a link between AS and inflammation.3,4 A previous study demonstrated that interleukin (IL)-1β participates in the pathogenesis of AS.4 Moreover, the IL-1 receptor antagonist (IL-1Ra) negatively regulates IL-1 signaling by binding and blocking the functional receptor without activation.5 Thus, IL-1Ra plays an antiinflammatory role in acute and chronic inflammation.6 Although we previously demonstrated that ∼50% of IL-1Ra–deficient (IL-1Ra<sup>−/−</sup>) mice on BALB/c background spontaneously developed aortitis,7 we also found almost all IL-1Ra<sup>−/−</sup> mice developed aortic valve lesions with or without aortitis. Thus, direct evidence implicating endogenous IL-1Ra in AS remained undetermined. The present study focused on the role of IL-1Ra in the development of AS in IL-1Ra<sup>−/−</sup> mice.

Materials and Methods

Animals

The generation of IL-1Ra<sup>−/−</sup> mice, which lack all 4 IL-1Ra isoforms, was described previously.10 We backcrossed mice to the BALB/c strain for 8 generations and performed IL-1Ra genotyping by analyzing polymerase chain reaction in tail DNA as described previously.8 Tumor necrosis factor (TNF)-α<sup>−/−</sup> mice (kindly provided by Dr K. Sekikawa, National Institute of Agrobiological Sciences, Tsukuba, Japan), were intercrossed with IL-1Ra<sup>−/−</sup> mice to generate double-deficient mice. Throughout the experiment, mice consumed a standard chow (CE7; CLEA) containing 4.1% crude fat and <0.02% cholesterol, which does not increase lipid levels in mice. To eliminate gender differences, we used only male mice. The studies were performed according to the protocols approved by the National Defense Medical College Board for Studies in Experimental Animals.

Tissue Preparation and Histology

After measuring tail-cuff systolic blood, mice were euthanized with pentobarbital and perfused with 0.9% NaCl, followed by 4% paraformaldehyde. After perfusion, the aortas and hearts were harvested, fixed overnight in 4% paraformaldehyde, embedded in OCT compounds (Tissue-Tek; Sakura Finetechical), and sectioned (10-μm thickness). All samples were routinely stained with hematoxylin and eosin and Masson trichrome. TNF-α was visualized with polyclonal TNF-α (Abcam), mouse macrophages/monocytes were visualized with clone MOMA-2 (BioSource International), and myofibroblast cells were visualized with α-smooth muscle cell actin staining (Roche). We visualized sections using a Vectastain ABC kit (Vector Laboratories) with DAB as the substrate.
Quantification of Aortic Valve Leaflet Thickness

We prepared aortic sinus sections as described previously. The thickness of the aortic valve leaflet was measured using National Institutes of Health Image 1.55 (public domain software). The values reported represent the mean thickness of 3 leaflets from each animal.

Plasma Lipid Measurements

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

Echocardiography

To examine valve and heart function, we performed transthoracic echocardiography using a Sonos 5500-U (Phillips) equipped with 12-MHz and 13-MHz imaging transducers. After anesthetizing the mice with 2,2,2-tribromoethanol (250 mg/g intraperitoneal), we shaved their chests and attached ECG leads to each limb using needle electrodes. We imaged the mice in a shallow, left lateral, decubitus position, obtaining short-axis and long-axis views of the left ventricle (LV), which were obtained by slight angulation and rotation of the transducer. Two-dimensional, targeted, M-mode studies were generally taken from the short axis (at the level of the largest LV diameter). We measured intraventricular septum thickness, end-diastolic LV internal diameter, end-systolic LV internal diameter, and LV posterior wall thickness, and we calculated fractional shortening percentage as (end-diastolic LV internal diameter)/(end-systolic LV internal diameter×100). Color flow Doppler measurements identified areas of increased (aliased) velocities in the outflow tract from angulated parasternal long-axis views; we quantified these areas using pulsed-wave or continuous-wave Doppler. As closely as possible, we attempted to align the ultrasound beam parallel to the direction of flow and to record the highest velocities.
To elucidate the role of T cells in AS development, we performed T-cell transplantation. Cells prepared from the spleen and lymph nodes of IL-1Ra\(^{-/-}\)/H11002 mice (male; age, 6 to 8 weeks) and wild-type (WT; male; age, 6 to 8 weeks) mice were treated with hemolysis buffer (17 mmol/L Tris-HCl and 140 mmol/L NH\(_4\)Cl; pH 7.2) to remove red blood cells, and then they were washed and passed through a nylon wool column. We then passed cells treated with antimouse B220 and anti-Mac-1 magnetic beads (Miltenyi Biotec) through a MACS column (Miltenyi Biotec) to obtain T cells. We resuspended the purified T cells in 0.2 mL phosphate-buffered saline (2\(\times\)10\(^7\) cells/mouse) and transplanted them intravenously into BALB/c–nu/nu mice (male; age, 6 weeks). We analyzed AS development in recipient mice 12 weeks later.

For bone marrow (BM) cell transplantation, we removed BM cells from femurs, tibias, and pelves of IL-1Ra\(^{-/-}\)/H11002 (male; age, 5–6 weeks) and WT (male; age, 5–6 weeks) mice and treated them with hemolysis buffer. To remove T cells, we treated the BM cells with antimouse Thy1.2 magnetic beads and passed the cells through a MACS column (Miltenyi Biotec) to obtain T cells. We resuspended the purified T cells in 0.2 mL phosphate-buffered saline (2\(\times\)10\(^7\) cells/mouse) and transplanted them intravenously into BALB/c–nu/nu mice (male; age, 6 weeks). We analyzed AS development in recipient mice 12 weeks later.

### T-Cell Activation Study

Purified T cells (2\(\times\)10\(^7\) cells) were plated on a 96-well plate coated with 10 \(\mu\)g/mL of antimouse CD3 mAb (145-2C11; BD Biosciences–Pharmingen) in a final volume of 200 \(\mu\)L RPMI 1640 plus 10% fetal calf serum and cultured for 48 hours before collecting culture supernatants; TNF-\(\alpha\) levels in culture were measured by enzyme-linked immunosorbent assay, as described.

### Plasma Cytokine Levels

Proinflammatory cytokine levels in the plasma from 8-week-old male IL-1Ra\(^{-/-}\)/H11002 and WT mice were measured by enzyme-linked immunosorbent assay.\(^4\) Hamster antimouse IL-1\(\beta\) monoclonal antibody and polyclonal goat antimouse TNF-\(\alpha\) antibody (all from Genzyme) were used as capture antibodies. Polyclonal rabbit antimouse IL-1\(\beta\) and polyclonal biotinylated goat antimouse TNF-\(\alpha\) antibodies (all from Genzyme) were used as secondary antibodies. Detection was performed with horseradish peroxidase-conjugated goat antirabbit IgG and horseradish peroxidase–streptavidin (Zymed). TMB substrate was purchased from Dako. IL-6 levels were measured with the OptEIASet mouse IL-6 kit (BD Pharmingen). All assays were performed in duplicate.

### Statistical Analysis

The results are shown as the mean±SE. Two groups were compared using Student \(t\) test. A value of \(P<0.05\) was regarded as a significant difference.

### Results

We backcrossed IL-1Ra\(^{-/-}\)/H11002 mice to the BALB/c background for 8 generations. We observed no significant differences in body weight, systolic blood pressure, plasma total cholesterol levels, or high-density lipoprotein chole-
terol levels at age 12 weeks (Figure 1A). However, 53% (16/30) of IL-1Ra−/−/H11002 mice on the BALB/c background developed aortitis spontaneously, and we found degenerative elastic lamina in the media of the aorta with the infiltration of many inflammatory cells (Figure 1B), similar to a previous report.7 Interestingly, all IL-1Ra−/−/H11002 mice showed increased thickness of the aortic valve leaflet compared to WT mice at the age of 12 weeks (2.8-fold; P<0.001; n=5; Figure 1C). IL-1Ra−/− mice also displayed higher transvalvular velocities than WT mice at age 40 weeks (181.8±22.6 vs 94.1±6.2 cm/s; P<0.01; n=6; Figure 2A), suggesting IL-1Ra−/− mice had mild AS. B-mode echocardiograms showed neither subaortic stenosis nor LV outflow obstruction (Figure 2B) but revealed a highly echoic area that lacked shadowing at the site of proximalis. We also observed chondrocyte-like cells at that site (Figure 2B). Because the aortic valve participates crucially in cardiac function, we also took M-mode echocardiograms of the LV of IL-1Ra−/− mice. The thickness of the interventricular septum wall and the LV posterior wall increased notably in IL-1Ra−/− mice. In contrast, LV end-diastolic and end-systolic dimensions and fractional shortening remained unchanged (Table). The heart-to-body weight ratio also increased significantly in IL-1Ra−/− mice (6.07±0.17 vs 4.84±0.08 mg/g; P<0.001; n=6). Furthermore, the tissue sections from IL-1Ra−/− mice showed increased thickness of the LV free wall (Figure 2C). Histological analysis displayed no obvious myofibrillar disarray or prominent interstitial infiltrating cells in tissue sections from IL-1Ra−/− mice (Figure 2C). These findings suggest that pressure overload by AS may induce LV hypertrophy.

Table. Echocardiographic Parameters in IL-1Ra−/− and WT Mice

<table>
<thead>
<tr>
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<th>WT (n=6)</th>
<th>IL-1Ra−/− (n=6)</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>LVD-ED, mm</td>
<td>1.92±0.41</td>
<td>2.15±0.43</td>
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<tr>
<td>LVD-ES, mm</td>
<td>0.80±0.21</td>
<td>0.82±0.25</td>
<td>0.57</td>
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<tr>
<td>FS, %</td>
<td>57±4</td>
<td>61±6</td>
<td>0.15</td>
</tr>
<tr>
<td>IVS-ED, mm</td>
<td>0.75±0.12</td>
<td>1.21±0.24</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PW-ED, mm</td>
<td>0.76±0.12</td>
<td>1.15±0.16</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

ED indicates end-diastole; ES, end-systole; FS, fractional shortening; IVS, interventricular septum thickness; LVD, left ventricular lumen diameter; PW, posterior wall thickness.

Values are mean±SEM. All mice were 40 weeks old.

Figure 3. Effect of IL-1Ra deficiency on aortic valve thickening and inflammation. Aortic valves were taken from WT and IL-1Ra−/− (Ra−/−) mice at 4, 8, or 16 weeks of age. Valve sections were prepared for histology and stained with (A) HE, (B) Masson trichrome (MT), or (C) macrophages. D, Schema of histological findings. E indicates endothelial cell; I, interstitial cell; M, monocyte/macrophage. E, Aortic valves of WT and Ra−/− mice at age 40 weeks. Valve sections were stained with HE, MT, or α-smooth muscle cell actin (α-SMA). F, The sections of proximalis in Ra−/− mice at age 43 weeks were stained with HE (left panel) or MT (right panel). Bar, 50 μm.
We performed histological examination of aortic valves from IL-1Ra−/− and WT mice at 4, 8, and 16 weeks of age (Figure 3). Aortic valve leaflets from WT mice were thin and composed predominantly of fibroblast-like cells covered by endothelial cells. Furthermore, histology demonstrated a time-dependent increase in valve thickness and loose connective tissue, as well as increased numbers of interstitial cells in the spongiosa lacking macrophages. In contrast, 4-week-old IL-1Ra−/− mice showed thickened leaflets and edema (Figure 3A–D), inflammatory cell infiltration, increased numbers of interstitial cells in the spongiosa, and edema (Figure 3A–D), whereas the late phase showed markedly increased cellularity and collagen staining in blue Masson trichrome (Figure 3E). Furthermore, we detected macrophage infiltration, increased interstitial cell counts, and edema, resulting in more pronounced thickness (Figure 3A–D). The early phase revealed valve thickening attributable to inflammatory cell infiltration, increased numbers of interstitial cells in the spongiosa, and edema (Figure 3A–D), whereas the late phase showed markedly increased cellularity and collagen staining in blue Masson trichrome (Figure 3E). Furthermore, we detected α-smooth muscle cell actin-positive cells in ventricular layer cells and in the collagen cleft of the spongiosa of IL-1Ra−/− mice (Figure 3E). Regarding calcification, we observed chondrocyte-like cells at the proximalis of several 40-week-old IL-1Ra−/− mice (Figure 2B), and we detected calcified lesions in 43-week-old IL-1Ra−/− mice (Figure 3F).

To test the hypothesis that IL-1Ra deficiency in hematopoietic cell types participates crucially in AS development, we performed BM cell transplantation, injecting BM from IL-1Ra−/− or WT mice into previously lethally irradiated recipient WT mice. We observed increased aortic valve thickness in WT mice receiving BM cells from IL-1Ra−/− mice, but not from WT mice (3.3-fold; **P<0.001; n=10; Figure 4A). We next used peripheral T-cell transplantation to examine the role of T cells in AS development. We intravenously transplanted purified T cells from IL-1Ra−/− or WT mice into BALB/c-nu/nu mice. T cells transplanted from WT mice induced mild swelling of the aortic valve leaflet in nu/nu mice (Figure 4B). In contrast, T cells from IL-1Ra−/− mice induced greater aortic valve thickness in nu/nu mice (2.9-fold; **P<0.001; n=10; Figure 4B).

We then checked T-cell function. After stimulation with anti-CD3 antibody, IL-1Ra−/− T cells produced much higher levels of TNF-α in culture supernatants compared to WT mice (129.9±13.1 vs 40.2±7.8 pg/mL; **P<0.001; n=3). These results suggest that IL-1Ra−/− T cells are excessively activated even by physiological level of IL-1. Figure 5A shows plasma levels of proinflammatory cytokines. In addition, TNF-α protein levels were higher in IL-Ra−/− mice compared to WT mice (P<0.01; n=6), whereas the levels of IL-1β and IL-6 were normal compared to WT mice.

Finally, we studied the role of TNF-α in AS development in IL-1Ra−/− mice by generating double-gene-deficient (TNF-α−/−/IL-1Ra−/−) mice. Interestingly, aortic valve thickness did not increased in TNF-α−/−/IL-1Ra−/− mice (0.45-fold; **P<0.01; n=6; Figure 5B). Furthermore, we localized the TNF-α protein in the aortic valves of both mice. Immunohistochemical analysis of TNF-α−/−/IL-1Ra−/− mice revealed that the cytokine localized predominantly in inflammatory cells. As expected, we detected no TNF-α protein in TNF-α−/−/IL-1Ra−/− mice (Figure 5C). Thus, our results show that TNF-α participates importantly in AS development in IL-1Ra−/− mice.

Discussion

Our study demonstrates that IL-1Ra−/− mice on BALB/c background spontaneously developed aortic valve disease...
and inflammation. Furthermore, we suggest that IL-1Ra deficiency in macrophages and T cells accelerates aortic valve inflammation, and that TNF-α participates importantly in the development of AS in IL-1Ra−/− mice.

Histological analysis revealed macrophage adhesion on the aortic side of the valve and further showed inflammatory cell infiltration in the valve leaflet in 4-week-old IL-1Ra−/− but not WT mice. High shear forces on the leaflet result in increased cell damage, and an earlier study revealed disruption of endothelium on the aortic side of the leaflet in early AS lesions. Inflammatory cells then infiltrate into the valve leaflet at sites of endothelial disruption. Adhesion molecules, eg, intracellular cell adhesion molecule-1 and vascular cell adhesion molecule-1, may facilitate the recruitment of inflammatory cells into valves. We showed previously that IL-1Ra deficiency induced increased mRNA levels of both intracellular cell adhesion molecule-1 and vascular cell adhesion molecule-1 in the aorta. These changes likely contribute to enhanced accumulation of inflammatory cells in the aortic valve of IL-1Ra−/− mice. Although the same level of endothelial injury might occur in WT mice, adherent macrophages in WT mice were not so active as IL-1Ra−/− macrophages and did not infiltrate into the valves. Thus, endothelial cells might be cured easily in WT mice. We detected no macrophages in the aortic valves of WT mice during our 16-weeks study.

Almost all IL-1Ra−/− mice had aortic valve lesions develop, but only about 50% of IL-1Ra−/− mice had aortitis develop. There may be several reasons for the different rates between them. First, our preliminary data showed that although IL-1Ra−/− mice on a C57BL/6J background had mild aortic valve swelling, they did not have aortitis, suggesting that autoimmunity may induce aortitis in this mouse model. Second, the degree of inflammation differed among individual mice and several mice had aortitis, eg, apolipoprotein E-deficient mice infused with angiotensin II (angiotensin II induces atherosclerotic lesions in all of them, but it occurs with aortic aneurysms in some of them). Clarifying the reasons why the rates of aortic valve disease and aortitis differ will require further investigation.

Histological analysis also showed slightly thickened leaflets with edema in the spongiosa in 4-week-old mice and greater thickening with increased interstitial cells in 8-week-old mice. Furthermore, the aortic valves of 16-week-old IL-1Ra−/− mice showed elevated interstitial cell counts and increased accumulation of extracellular matrix components, resulting in greater thickness. Macrophage-derived proteolytic enzymes may contribute to edema in the spongiosa, leading to structural morphological abnormalities. Aikawa et al reported that inflammation induced remodeling associated with valvular dysfunction, as determined by MRI in vivo. The same group also reported that myofibroblast-like cells respond to various stimuli by activation and sequential phenotypic differentiation, and they participate importantly in
the development of valvular disease.\textsuperscript{21,22} Our histological findings might be compatible with their observations.

An earlier study revealed an abundant accumulation of macrophages and T cells in the stenotic valve.\textsuperscript{16} We observed many macrophages (Figure 2C) and several T cells (data not shown) in an early AS lesion in IL-1Ra\textsuperscript{−/−} mice. Our previous study detected IL-1Ra protein in the many inflammatory cells in WT mice but not in IL-1Ra\textsuperscript{−/−}.\textsuperscript{9} Because those findings suggested that IL-1Ra deficiency in inflammatory cells may promote AS, we performed BM cell transplantation. We observed increased aortic valve thickness in WT mice receiving BM cells from IL-1Ra\textsuperscript{−/−} mice but not from WT mice. Furthermore, T-cell transplantation from IL-1Ra\textsuperscript{−/−} mice induced AS in recipient nu/nu mice. These results demonstrate that IL-1Ra deficiency in inflammatory cells induces inflammation and AS.

IL-1Ra\textsuperscript{−/−} deficient T cells produced much higher levels of TNF-α compared to those from WT mice after the same stimulation. Furthermore, TNF-α deficiency completely suppressed AS development in IL-1Ra\textsuperscript{−/−} mice, suggesting that TNF-α participates importantly in AS development in IL-1Ra\textsuperscript{−/−} mice. These novel findings might be supported by previous reports that showed the abundant presence of TNF-α in areas of inflammatory cells infiltration in stenotic aortic valves, and TNF-α may stimulate valvular myofibroblasts to proliferate and express matrix metalloproteinases, thus actively regulating matrix remodeling in AS.\textsuperscript{23,24} More recently, Al-Aly et al\textsuperscript{25} reported that TNF-α directly induced Mxs2-Wnt signaling in aortic myofibroblasts, contributing to cardiovascular calcification. Dkk1, an antagonist of paracrine Wnt signals, inhibits TNF-α induction of an early osteogenic gene downstream of Mxs2-Wnt activation in aortic myofibroblasts.\textsuperscript{25} Thus, TNF-α likely promotes calcific AS. These findings might provide the basis for new therapies for AS.

A possible mechanism that may explain the deleterious effects of activated IL-1Ra−/− deficient inflammatory cells includes the possibility that such cells attach to easily disrupted endothelial cells and then actively infiltrate the aortic valve, where they secrete higher levels of TNF-α compared to WT mice, thus inducing edema of interstitial cells in the spongiosa. Inflammation increases fibroblasts, changing the phenotype of myofibroblast-like cells and accumulated extracellular matrix components, resulting in thicker and stiffer valve leaflets. These histological changes are associated with the progression of AS. Differences in transvalvular velocities between IL-1Ra\textsuperscript{−/−} and WT mice reached statistical significance (90.3±7.7 vs 81±2.3 cm/s; P<0.05; n=6) at the age of 12 weeks of age, when both increased aortic valve thickness and interstitial cells progressed somewhat in IL-1Ra\textsuperscript{−/−} mice, but those of IL-1Ra\textsuperscript{−/−} mice increased slowly after 12 weeks. However, those of IL-1Ra\textsuperscript{−/−} mice increased more quickly after 30 weeks when accumulation of extracellular matrix components in the spongiosa progressed and maximized at approximately 40 weeks on initiation of the highly echoic points at the proximalis (Figure 2A,B). After that, transvalvular velocities reached their plateau in IL-1Ra\textsuperscript{−/−} mice.

**Clinical Implication**

Epidemiological studies have identified several atherosclerotic risk factors for the progression and outcome of AS, including age, smoking, high cholesterol levels, low high-density lipoprotein levels, and hypertension.\textsuperscript{2,26,27} To our best knowledge, the present study is the first to demonstrate a direct association between inflammation and AS development without the potential influence of the common and clinically relevant confounding factors mentioned. Interestingly, our preliminary study showed that pitavastatin did not prevent AS in IL-1Ra\textsuperscript{−/−} mice, although many experimental\textsuperscript{28–30} and clinical\textsuperscript{31–33} studies have reported that statin drugs prevented AS. Our findings suggest that statins may not be effective in preventing AS development induced predominantly by inflammation, not atherosclerosis.

The histological changes in our mice closely resemble the findings of rheumatic valve disease, ie, inflammatory cells induce local cytokine release (such as TNF-α) in rheumatic valve tissue, and inflammation leads to aortic valve thickening associated with cellular infiltration, edema, and increased valve interstitial cells.\textsuperscript{34} We also detected calcified lesions at the proximalis. However, we observed no calcified lesions in the leaflets, and the AS did not become more severe. These points differ from rheumatic valve disease. Determining why these differences occur will require further study.

**Conclusion**

We show here that IL-1Ra\textsuperscript{−/−} mice on the BALB/c background spontaneously develop aortic valve disease. We further suggest that IL-1Ra-deficiency in inflammatory cells induces aortic valve inflammation, and TNF-α participates importantly in the development of AS in IL-1Ra\textsuperscript{−/−} mice.

**Acknowledgments**

The authors thank the members of their animal institute for assistance in animal care.

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**Disclosures**

None.

**References**


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Correction

In the Original Contribution “Deficiency of Interleukin-1 Receptor Antagonist Induces Aortic Valve Disease in BALB/c Mice” by Isoda et al, which appeared in the April 2010 issue of the journal Arterioscler Thromb Vasc Biol. 2010;30:708–715; DOI: 10.1161/ATVBAHA.109.201749), the publisher omitted several important corrections from the final, published version:

1. Page 708, 2nd line of abstract: the word “degenerative” should be deleted, for the correct version to read: “However, the role of IL-1Ra in aortic valve stenosis (AS) remains poorly understood.”

2. Page 709, first line of “Echocardiography” section: the words “and heart” should be inserted between valve and function, for the correct version to read: “To examine the valve and heart function, we performed transthoracic echocardiography using a Sonos 550-U (Philips) equipped with 12-MHz and 15-MHz imaging transducers.”

3. Page 709, 1st line of 2nd column: the words “(male; age, 40 weeks) should have been deleted, for the correct version to read: “After anesthetizing the mice with 2,2,2-tribromoethanol (250 mg/g intraperitoneal), we shaved their chests and attached ECG leads to each limb using needle electrodes.”

4. Page 709, 2nd line of Figure 1 legend: the word “degenerative” should have been deleted and “aortic valve disease” should be replaced with the abbreviation “AS,” for the correct version to read: “Our study demonstrates that IL-1Ra/−/ mice on BALB/c background spontaneously developed aortic valve disease and inflammation.”

5. Page 711, 1st line of Table footnote: a hyphen should be inserted between “end” and “diastole” and “end” and “systole,” for the correct version to read: “ED indicates end-diastole; ES, end-systole . . .”

6. Page 712, 3rd line of 2nd column: the word “Finally” should begin a new paragraph.

7. Page 712, 2nd line of “Discussion” section: the word “degenerative” should be deleted, for the correct version to read: “Almost all IL-1Ra/−/ mice on a C57BL/6J background had mild aortic valve swelling, they did not have aortitis . . .”

8. Page 713, last line of 1st column: the sentence beginning with “In contrast . . .” should be a new paragraph, in addition, the words “In contrast” should be replaced with the sentence “Almost all IL-1Ra/−/ mice had aortic valve lesions develop, but about only 50% of IL-1Ra/−/ mice had aortitis develop. There may be several reasons for the different rates between them. First . . .” The correct version should read: Almost all IL-1Ra/−/ mice had aortic valve lesions develop, but about only 50% of IL-1Ra/−/ mice had aortitis develop. There may be several reasons for the different rates between them. First, our preliminary data showed that although IL-1Ra/−/ mice on a C57BL/6J background had mild aortic valve swelling, they did not have aortitis . . .”

9. Page 713, 4th line of 2nd column: the word “in addition,” should be replaced with “Second,” for the correct version to read: “Second, the degree of inflammation differed among individual mice and several mice had aortitis . . .”


11. Page 713, Figure 5 legend, 1st line, the word “upper” should be deleted, 2nd line, the words “upper right” should be replaced with the word “middle” and the words “lower left” should be replaced with the word “right,” last line, the word “(left)” should be replaced with the word “(right)” for the correct version to read: “A, Plasma levels of IL-1β (left), TNF-α (middle), and IL-6 (right) in both IL-1Ra/−/ and WT mice (n=6; **P<0.01). B, Macroscopic appearance of the aortic valve of IL-1Ra/−/ (TNF−/+/Ra−/−; upper left) and double-gene-deficient (TNF−/−/Ra−/−; lower left; MT staining; bar, 200 μm). We analyzed mean aortic valve leaflet thickness and the mean thickness of WT mice at 12 weeks old was used as a reference against that of TNF−/−/Ra−/− and TNF−/−/Ra−/− mice (***P<0.001; n=6; right panel). C, Representative photomicrographs depict immunohistochemical staining for TNF-α in aortic valve of TNF−/−/IL-1Ra−/− (left) and TNF−/−/IL-1Ra−/− (right) mice (8 weeks old).”

12. Page 714, 7th line of 1st column: the word “inflammatory cells” should be inserted between “deficiency” and “and” for the correct version to read: “Because those findings
suggested that IL-1Ra deficiency in inflammatory cells may promote AS, we performed BM cell transplantation.”

13. Page 714, conclusion section, the current conclusion section should be the last paragraph of the “Discussion” section, the following should have been inserted as the “Conclusion” section: “We show here that IL-1Ra−/− mice on the BALB/c background spontaneously develop aortic valve disease. We further suggest that IL-1Ra-deficiency in inflammatory cells induces aortic valve inflammation, and TNF-α participates importantly in the development of AS in IL-1Ra−/− mice.”

The online version of the article has been corrected.

The publisher sincerely regrets the errors.

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