Single-cell transcriptomics reveal cellular diversity of aortic valve and the immunomodulation by PPARγ during hyperlipidemia

Valvular inflammation triggered by hyperlipidemia has been considered as an important initial process of aortic valve disease; however, cellular and molecular evidence remains unclear. Here, we assess the relationship between plasma lipids and valvular inflammation, and identify association of low-density lipoprotein with increased valvular lipid and macrophage accumulation. Single-cell RNA sequencing analysis reveals the cellular heterogeneity of leukocytes, valvular interstitial cells, and valvular endothelial cells, and their phenotypic changes during hyperlipidemia leading to recruitment of monocyte-derived MHC-II hi macrophages. Interestingly, we find activated PPARγ pathway in Cd36+ valvular endothelial cells increased in hyperlipidemic mice, and the conservation of PPARγ activation in non-calcified human aortic valves. While the PPARγ inhibition promotes inflammation, PPARγ activation using pioglitazone reduces valvular inflammation in hyperlipidemic mice. These results show that low-density lipoprotein is the main lipoprotein accumulated in the aortic valve during hyperlipidemia, leading to early-stage aortic valve disease, and PPARγ activation protects the aortic valve against inflammation.

Aortic valve disease emerges as a worldwide health problem as the population ages. Unlike healthy, diseased aortic valve has a problem with its function of preventing blood from backflow. One of the most prevalent degenerative aortic valve diseases, calcific aortic valve disease (CAVD) is characterized by severe calcification of aortic valve leaflet, which gradually narrows the aortic orifice. This calcified lesion also causes a decrease in leaflet motion and obstruction of the left ventricular outflow tract, leading to aortic stenosis (AS). After the onset of AS symptoms, the mortality rate among those aged around 80 years is ~50% in 2 years and 80% in 5 years without surgical or transcatheter valve replacement. However, there is no non-invasive therapeutic modality yet to prevent or suppress the initiation and progression of disease. Thus, it is crucial to understand the molecular and cellular events in the early stage of aortic valve diseases to develop preventive therapeutics.

Inflammation is closely associated with the onset and progression of aortic valve disease. Various predisposing conditions including bicuspid valve, old age, male sex, hypertension, dyslipidemia, smoking, and obesity can induce the recruitment of leukocytes into aortic valve. The recruited leukocytes release pro-inflammatory cytokines...
like TNF, IL1β, and IL6, which provoked a phenotypic change of valvular interstitial cells (VICs) into proinflammatory and calcific phenotypes, leading to CAVD. Similar to atherosclerosis, hyperlipidemia is an important risk factor for aortic valve inflammation and is known to drive sclerotic changes in the aortic valve. Familial hypercholesterolemia due to the inheritance of defective alleles of genes related to cholesterol homeostasis, such as low-density lipoprotein (LDL) receptor (Ldlr), is associated with the prevalence of CAVD. Recently, proprotein convertase subtilisin/kexin type 9 (PCSK9), which increases blood LDL level by binding and preventing LDLR recycling, is also reported as a key regulator in the pre-calciﬁcation stage of aortic valve disease.

In the normal state, aortic valves contain various cell types, including VICs, valvular endothelial cells (VECs), and leukocytes including macrophages and dendritic cells (DCs). In the hyperlipidemic state, it is known that macrophages and T cells are accumulated in the lipid deposited region. But, the comprehensive immune cell proﬁling analysis is still lacking. In order to understand the initial disease mechanism, it is important to deﬁne the valvular cell components and dissect their phenotypic changes and cellular networks in hyperlipidemic mice. However, since the tissue is very small and composed of a few cells, it is challenging to analyze molecular and cellular events in murine aortic valves.

In this work, we ﬁrst conﬁrm the causal relationship between plasma lipids and valvular inﬂammation in Ldlr−/− and Apoe−/− mice having different plasma lipid proﬁles. We perform single-cell RNA sequencing (scRNA-seq) analysis of aortic valves from these mice. Our scRNA-seq analysis reveals the profound cellular heterogeneity of VICs, VECs, and leukocytes, and the increased pro-inﬂammatory gene expression in VECs and VICs during hyperlipidemia inducing the recruitment of monocyte-derived macrophages. Importantly, we ﬁnd that PPARγ pathway is activated in aortic valves from Ldlr−/− and Apoe−/− mice and in human aortic valves of pre-calcified stage but not in calciﬁed valves. When PPARγ is knocked down, VECs upregulate pro-inﬂammatory genes and cell adhesion molecules. While PPARγ inhibition promotes valvular inﬂammation, the activation of PPARγ protects the aortic valve from excessive inﬂammation during hyperlipidemia. Thus, we propose PPARγ as a key anti-inﬂammatory regulator in the pre-calcified stage of aortic valve disease.

**Results**

**Ldlr−/− mice have more prominent valvular lipid accumulation than do Apoe−/− mice**

To identify lesion formation in the aortic valve disease in hyperlipidemic conditions, we assessed lipid accumulation in the aortic valve, using the following two mouse models of hypercholesterolemia: Apoe−/− and Ldlr−/− mice (experimental group) and C57BL/6J mice (wild-type control group), fed either a chow or western diet (WD) for 10 weeks. We quantiﬁed valvular lipids and atherosclerotic lesions in the aortic sinuses by staining frozen tissue sections with Oil Red O staining. Whereas C57BL/6J mice showed no signiﬁcant difference in the valvular lipid content, Apoe−/− and Ldlr−/− mice that were fed with a WD had a higher valvular lipid deposition than did Apoe−/− and Ldlr−/− mice fed with a chow diet; however, Ldlr−/− mice showed a much higher increase in aortic valvular lipid accumulation than did Apoe−/− mice (Fig. 1a). In addition, whole-mount Oil Red O staining showed that lipid droplets mainly accumulated in the corpus arantri (C.A.) region of the aortic valve in hyperlipidemic mice (Apoe−/− and Ldlr−/− mice fed a WD), but this effect was not observed in the valve of normal mice (C57BL/6J mice fed a chow diet) (Supplementary Figs. 1 and 2a). We further compared serum levels of total cholesterol and LDL in WD-fed and chow diet-fed mice. LDL, but not total cholesterol, was higher in Ldlr−/− mice than in Apoe−/− mice (Fig. 1b). In addition, in WD-fed hyperlipidemic mice (Apoe−/− and Ldlr−/− mice), LDL levels positively correlated with aortic valvular lipid accumulation, whereas total cholesterol levels and atherosclerotic lesions in the aortic sinus did not correlate with aortic valvular lipid accumulation (Fig. 1c, d). Further examination into lipid accumulation and lipid proﬁles of WD-fed Apoe−/− and Ldlr−/− mice, at the 4-, 8-, or 12-weeks, revealed similar patterns: lipid accumulation was greater in Ldlr−/− than in Apoe−/− mice at every time point, and a positive correlation between valvular lipids and LDL was observed (Supplementary Fig. 2b–d).

To identify whether the difference in valvular lipid accumulation between Apoe−/− and Ldlr−/− mice originated from differences in scavenger receptor expression levels, we examined the average expression levels of scavenger receptors and the level of each scavenger receptor (Msr1, Scarb1, Cfd, and Cfd2) associated with LDL, via transcriptomic analysis of the whole aortic valve tissue, using ﬁve different experimental groups (C57BL/6J chow mouse, Apoe−/− chow and WD mice, and Ldlr−/− chow and WD mice). We observed no signiﬁcant differences among the groups (Supplementary Fig. 3).

**Ldlr−/− mice have a higher percentage of valvular leukocytes and associated subsets than do Apoe−/− mice, in hyperlipidemic conditions**

To understand the inﬂammatory process in the aortic valve under conditions of hyperlipidemia, we performed a ﬂow cytometric analysis of aortic valvular cells, using C57BL/6J, Apoe−/−, and Ldlr−/− mice fed either a chow or WD (Fig. 2a, b and Supplementary Fig. 4). Apoe−/− and Ldlr−/− mice fed with a WD showed a proportional increase in leukocytes and a decrease in VICs compared to Apoe−/− and Ldlr−/− mice fed with a chow diet, whereas C57BL/6J mice showed no difference between WD-fed and chow-fed mice (Fig. 2a, b and Supplementary Fig. 5a). Similar to trends in lipid accumulation, Ldlr−/− mice showed a more prominent increase in total leukocytes than did Apoe−/− mice. Among leukocyte subsets, macrophages were signiﬁcantly increased in both Apoe−/− and Ldlr−/− mice (Fig. 2a, b). Macrophages were classiﬁed into two different subsets: MHC-II⁺CD11c⁺CD206⁺ and MHC-II⁺CD11c⁻CD206⁺.
IIloCD11c−CD206+ macrophages (Supplementary Fig. 4). Between the two subsets, only MHC-II hiCD11c+CD206- macrophages were significantly increased in WD-fed Apoe−/− and Ldlr−/− mice (Fig. 2b).

Moreover, among the leukocyte subsets, MHC-II hiCD11c+CD206- macrophage percentage positively correlated with LDL levels, but not with total cholesterol levels (Fig. 2c and Supplementary Fig. 5b, c).

High blood LDL level induced by hepatic PCSK9 overexpression increases the accumulation of lipids and macrophages in the aortic valve
To confirm the importance of blood LDL levels in the progression of aortic valve lesion formation, we induced the overexpression of gain-of-function PCSK9 mutant using adeno-associated virus (AAV).
serotype 8 (PCSK9-AAV). Injection of PCSK9-AAV induces a great elevation of blood LDL levels in mice. Using Oil Red O staining, we found that PCSK9-AAV-injected Apoe−/− mice (24 weeks of WD) had more severe lipid accumulation, about 5.7-fold higher percentage, than control Apoe−/− mice. In addition, the overexpression of PCSK9 increased valve thickness in Apoe−/− mice and elevated plasma LDL levels (Fig. 2d–f). Furthermore, we checked the correlation between valvular lipid accumulation and each blood lipid profile, and only LDL presented a positive correlation with valvular lipid accumulation (Fig. 2g).

To verify the relationship between LDL levels and valvular inflammation, we performed immunostaining for CD68 and vimentin with lipid staining using BODIPY 493/503. Macrophages were localized in the lipid-accumulated C.A. region of the aortic valve and appeared as foamy cells containing many lipid droplets. Adjacent to the macrophages, vimentin+CD68− VICs also showed a foamy phenotype (Fig. 2h).

Next, we quantified the number of macrophages per aortic valve and found that macrophages were increased in PCSK9-AAV-infected mice, suggesting that the elevation of serum LDL levels worsened the aortic valvular inflammation (Fig. 2i). Altogether, these findings indicate that an increase in LDL enhances lipid accumulation and inflammation of the aortic valve, leading to the aortic valve sclerosis progression.

Ezetimibe-induced lipid reduction ameliorates lipid accumulation and inflammation in the aortic valve

We assessed whether lipid-lowering treatment would alleviate lesion formations, caused by hyperlipidemia, during the early stages of aortic valve disease. To achieve this, we utilized Ldlr−/− mice, which were suitable to identify the change of valvular lesion, because they showed high levels of valvular lipid accumulation and inflammation when fed WD (Figs. 1a and 2a, b). Ldlr−/− mice were fed with WD containing a lipid-lowering drug (ezetimibe) and compared valvular lipid accumulation and immune cell proportions to control WD-fed Ldlr−/− mice (Fig. 2j–m). Ezetimibe clearly decreased total cholesterol and LDL levels in the blood of Ldlr−/− mice fed with an ezetimibe-based WD, and also decreased valvular lipid accumulation as well (Fig. 2j, k). Similarly, ezetimibe treatment significantly reduced the percentage of immune cells (leukocytes, macrophages, and MHC-II+CD11c+CD206− macrophages) in aortic valves (Figs. 2l, m and Supplementary Fig. 5d). Collectively, we identified that lipid-lowering treatment was effective in reducing lipid accumulation and inflammation in hyperlipidemia-induced aortic valve disease.

scRNA-seq analysis reveals cellular heterogeneity in aortic valves from normal and hyperlipidemic mice

To explore hyperlipidemia-associated cellular dynamics in aortic valves, we sorted total live single cells from the aortic valves of C57BL/6j (wild type), Ldlr−/−, and Apoe−/− mice and performed single-cell transcriptome analysis using a droplet-based 10x Genomics Chromium system (Supplementary Figs. 1b and 6). After quality filtering, we classified 6574 cells into 12 cell clusters using unsupervised cell clustering projected in a two-dimensional space through uniform manifold approximation and projection (UMAP) (Fig. 3a). Each cell cluster was assigned into three distinct cell lineages based on established canonical marker gene expression: VICs with stromal cells, VECs, and leukocytes (macrophages, DCs, T cells, and B cells) (Fig. 3b and Supplementary Data 1). All cell lineages were present in both normal and hyperlipidemic aortic valves, showing differences in cell distribution (Fig. 3c).VICs were the main cell population in aortic valves of C57BL/6j, and macrophages were the most abundant leukocytes in the aortic valves. Importantly the leukocytes, such as macrophages, DCs, and T cells were markedly increased in Ldlr−/− mice compared with C57BL/6j and Apoe−/− mice. Overall, the cellular proportion of scRNA-seq analysis sufficiently recapitulated the flow cytometric analysis (Fig. 2a–c and Supplementary Fig. 5a–c). The results indicated a tight association between macrophages and the pathogenesis of aortic valve disease. The extensive single-cell profiling enabled us to investigate cellular and molecular changes associated with hyperlipidemia in the aortic valve.

The aortic valve contains two major macrophage populations, and monocyte-derived MHC-II+ macrophages accumulate during hyperlipidemia

Based on the expression levels of H2-Ab1 (encoding a subunit of MHC-II), Itgax (encoding CD11c), and Mrc1 (encoding CD206), valvular macrophages were subdivided into the following two main populations: H2-Ab1+Itgax− and Mrc1+ macrophages (Fig. 3a, d), similar to the results of flow cytometric analyses (Fig. 2a, b and Supplementary Fig. 4). Using whole-mount immunofluorescence with MHC-II and CD206, we found that MHC-II+ and CD206+ macrophages were spatially separated from each other. MHC-II+ cells were mainly infiltrated into the fibrosa side of the C.A. region of the hyperlipidemic aortic valve, whereas CD206+ macrophages were lined in the ventricularis side of the area near the annulus (Fig. 3e, Supplementary Fig. 7 and Supplementary Movies 4–6).

To assess whether the MHC-II+CD11c+CD206+ macrophages are monocyte-derived, we induced hyperlipidemia by PCSK9-AAV infection and WD feeding to Ccr2−/− or Ccr2+ mice. Although there was no difference in LDL levels of Ccr2−/− and Ccr2+ mice, MHC-II+CD11c+CD206+ macrophages were substantially decreased in Ccr2−/− mice, indicating that the valvular MHC-II+ macrophages mainly originated from blood monocytes (Fig. 3f, g).

MHC-II+ valvular macrophages express pro-inflammatory genes whereas Lyve1+ macrophages show anti-inflammatory gene profiles

We re-classified leukocyte clusters to understand their dynamics and role in valvular inflammation. Sub-clustering of 3160 leukocytes identified them as macrophages (Macs), monocyte-derived dendritic cells
(moDCs), conventional type 1 dendritic cells (cDC1s), CD8+ T cells, CD4+ T cells, and B cells (Fig. 4a). A total of 11 clusters were assigned to 6 leukocyte lineages along with the expression of known canonical markers (Fig. 4b). The Ldr−/− mice were enriched in leukocytes, with a 50% increase in cell numbers compared with those in Apoe−/− and C57BL/6J mice (Fig. 4c). The relative proportion of clusters within immune cells showed a similar distribution at this resolution. Notably, macrophages (LEU_C0, C1, C4, C5, and C7) were the predominant leukocyte type in the aortic valve under all conditions. Each macrophage sub-cluster showed distinctive cellular pathways in functional categories (Supplementary Data 1), suggesting phenotypic and functional heterogeneity. LEU_C0 expressed Crts, Vcam1, and genes related
to MHC-II (Cd74, H2-Eb1, and H2-Aa), and enriched with a lysosome pathway. LEU_C1 showed Cd9, Actg1, and Cd14 as top genes, and upregulated with pathways (glycolysis, gluconeogenesis, and pentose phosphate pathway) related to glucose metabolism. LEU_C2 expressed marker genes of the M2-like macrophage such as F4/80, Ly6e, Mrc1, and Cd63, and enriched with the endocytosis, and the complement and coagulation cascade pathways. LEU_C4 was identified as the proliferating macrophage, showing a high level of genes (Mki67, Ccna2, and Cdk1) and pathways (DNA replication and spliceosome) associated with cell cycles. LEU_C5 contained the expression of genes related to Cdk1 and showed heterogeneous subsets of VICs. The following four VIC types were compared with non-injected Apoe−/− mice (WD for 24 weeks). Representative Oil Red O stain images. Scale bar: 150 μm (top), 50 μm (bottom) (d), quantification of valvular lipid deposition and thickness of aortic valve (n = 5) (e), total cholesterol and LDL levels in blood plasma (n = 9 total; 5 from Apoe−/−; 4 from Apoe−/−+PCSK9-AAV) (f), immunostaining of CD68 (red) and Vimentin (white) along with the lipid staining using BODIPY 493/503 (green). Scale bar: 150 μm (top), 50 μm (bottom) (h), and quantification of CD68 + macrophages accumulated in the aortic valve (n = 5) (i).

**VECs contain three major populations and Cd36+ VECs are markedly increased in hyperlipidemic condition**

Sub-clustering of the 536 VECs revealed six clusters (Fig. 6a). Fewer VECs than MHC-II and leukocytes were recovered from the aortic valve. Nonetheless, distinct Cd36+ VECs (VEC_C1) were identified in both the Apoe−/− and Ldlr−/− groups (Fig. 6b, c). Other cell clusters were assigned to vascular endothelial cell types, including Fgfr3+ ECs (VEC_C0), Prox1+ ECs (VEC_C2), Pdpn+ ECs (VEC_C3), and Edn1+ ECs (VEC_C5) (Fig. 6a–c, Supplementary Data 1). The clusters (VEC_C0, C1, and C2) populated more than 80% of the total VECs (Fig. 6c). In total, VECs in Apoe−/− and Ldlr−/− mice showed upregulation of pro-inflammatory genes and over-enrichment in functional gene sets related to monocyte chemotaxis when compared with the C57BL/6j group, suggesting that VECs are also related to valvular inflammation (Fig. 6d, e).

Fgfr3+ VECs (VEC_C0) and Prox1+ VECs (VEC_C2) were the main EC populations in the C57BL/6j mice. However, Cd36+ VECs (VEC_C1) were markedly increased in Apoe−/− and Ldlr−/− mice (Fig. 6a, c). In addition, combined analyses of VECs with public scRNA-seq for aortic ECs formed distinct clusters of Cd36+ ECs from normal ECs (Supplementary Fig. 9). Using RNA in situ hybridization and immunofluorescence, we validated three major VEC clusters. Among these, Fgfr3+ VECs (VEC_C0) and Prox1+ VECs (VEC_C2) exhibited distinct spatial patterns. Fgfr3+ VECs were regionally confined to the ventricularis side while Prox1+ VECs were only positioned on the fibrosa side (Fig. 6f). Unlike the two clusters, Cd36+ VECs (VEC_C1) did not have a specific spatial pattern. Consistent with the scRNA-seq data, more Cd36+ VECs were observed in the diseased valve (Ldlr−/−) than in the normal valve (C57BL/6j) (Fig. 6c, d). Previously, Kalluri et al. demonstrated that aortic ECs were classified into three subtypes—EC1, EC2, and EC3. We found that the transcriptional characteristics of the three ECs were comparable to those of the VEC clusters (Fig. 6g). Cd36+ VECs expressed EC2-associated gene signatures, whereas Fgfr3+ was similar to EC1. Pdpn+ EC showed higher expression of the EC3-related genes. Fgfr3+ VECs showed significantly upregulated extracellular matrix organization and integrin signaling, consistent with EC1. We confirmed the enrichment of a Cd36+ VEC population with lipid transport and

**VCs showed increased expression of genes related to myofibroblast activation and calcification than those of normal mice (C57BL/6j)**

To further validate the cell clusters in relation to valvular pathology, we performed scRNA-seq in WD-fed (for 10 weeks) Ldlr−/− mice (n = 4). Representative lipid stain images. Scale bar: 150 μm (top), 20μm (bottom) (i) and quantification of valvular lipid deposition with blood lipid profiles (k). LEU_C1 expressed marker genes of foamy macrophage and enriched with pathways (glycolysis, gluconeogenesis, and pentose phosphate pathway) related to glucose metabolism. LEU_C2 expressed marker genes of the M2-like macrophage such as F4/80, Ly6e, Mrc1, and Cd63, and enriched with the endocytosis, and the complement and coagulation cascade pathways. LEU_C4 was identified as the proliferating macrophage, showing a high level of genes (Mki67, Ccna2, and Cdk1) and pathways (DNA replication and spliceosome) associated with cell cycles. LEU_C5 contained the expression of genes related to Cdk1 and showed heterogeneous subsets of VICs. The following four VIC types were compared with non-injected Apoe−/− mice (WD for 24 weeks). Representative Oil Red O stain images. Scale bar: 150 μm (top), 50 μm (bottom) (d), quantification of valvular lipid deposition and thickness of aortic valve (n = 5) (e), total cholesterol and LDL levels in blood plasma (n = 4 for Apoe−/−+PCSK9-AAV) (f), correlation between valvular lipid deposition and each lipid profile (total cholesterol and LDL) (n = 9 total; 5 from Apoe−/−; 4 from Apoe−/−+PCSK9-AAV) (g), immunostaining of CD68 (red) and Vimentin (white) along with the lipid staining using BODIPY 493/503 (green). Scale bar: 150 μm (top), 50 μm (bottom) (h), and quantification of CD68 + macrophages accumulated in the aortic valve (n = 5) (i).

**Lipid-lowering effects of ezetimibe on lipid accumulation in the aortic valves of WD-fed (for 10 weeks) Ldlr−/− mice, through flow cytometry (n = 5).** Representative plot of leukocytes (I) and percentages of each cell population in single cells (M). WD: western diet. For (b), (e), (f), (i), (k), and (m), two-sided Mann-Whitney test was used. For (c) and (g), the Spearman correlation test was used. Data are presented as mean ± SD.
angiogenesis, represented by the upregulation of genes in lipoprotein handling and the angiogenic tip cell gene set. Interestingly, EC2 type, Cd36+ VECs (VEC_CI) exhibited enhanced gene expression associated with PPARγ signaling pathway including Cd36 and Fabp4 (Fig. 6b, g and Supplementary Data 1). Cluster-specific pathway analysis also showed enhanced PPAR signaling in Cd36+ VECs (Fig. 6h).

**PPARγ pathway is activated in Cd36+ VECs**

The upregulation of the lipoprotein handling pathway in Cd36+ VECs was further supported by regulatory network inference, using Single-cell Regulatory Network Inference and Clustering (SCENIC) analysis27. The transcription factors activated in Cd36+ VECs were related to lipid metabolisms such as Nrf2 (encoding LXRα) and Ppard (encoding PPARγ) (Supplementary Fig. 10). In particular, the PPARγ signaling pathway was significantly associated with Cd36+ VECs in the gene expression level. We highlighted the activation score and expression level of PPARγ regulon (a gene module composed of the transcription factor and its direct target genes). As expected, the PPARγ regulon was highly activated in Cd36+ VECs (Fig. 7a, b and Supplementary Fig. 11). Next, we classified aortic valvular cells into two classes (PPARγ high and low groups) according to the gene...
expression level of PPARγ regulon. Most of the valvular cells in PPARγ high group were VECs, accounting for a high proportion in Cd36+ VECs (VEC_C1) (Supplementary Fig. 12a). To identify genes associated with the expression level of PPARγ regulon, we selected differentially expressed genes specific for PPARγ high group compared to the low group in aortic valvular cells and VECs, respectively. Cells with high expression of PPARγ regulon showed significant upregulation of Cd36, Fabp4, and Gpihbp1, which belong to PPARγ dependent genes (Supplementary Fig. 12b and Supplementary Data 3). Interestingly, they also represented upregulation of Cxcl12, which is known as a monocyte chemoattract and involved in the polarization of macrophage to an anti-inflammatory state28–31. We also evaluated the average expression level of PPARγ target genes (49 genes, http://www.ppargene.org/)32 in VECs, VICs, and macrophages. Only VECs in hyperlipidemic (Apoe−/− and Ldlr−/−) mice were increased when compared to control (C57BL/6J) mice (Supplementary Fig. 12c, d). Notably, even in C57BL/6J mice, the average expression level of PPARγ target genes was higher in VECs than in VICs and macrophages.
PPARγ activity is conserved in non-calciﬁed human aortic valves
To identify whether PPARγ-activated VECs are conserved in the human aortic valve, we re-analyzed samples comparing human aortic valves (Supplementary Fig. 13). Notably, PPARγ-dependent genes were enriched in VECs and calciﬁed aortic valves (Fig. 7e). There was no signiﬁcant difference in CD36 expression of VECs between non-calciﬁed and calciﬁed aortic valves, and the overall CD36 expression was only barely detectable (Supplementary Fig. 14). Next, to elucidate the VEC-speciﬁc function of PPARγ, we isolated VECs from human aortic valves (Supplementary Fig. 15a, b), transfected siRNA to PPARγ in human aortic VECs (Supplementary Fig. 15c), and performed RNA-seq analyses comparing PPARγ knockdowns versus negative controls—with or without the stimulation of oxLDL (Fig. 7f, g and Supplementary Data 4). We found that PPARγ knockdown upregulated pro-inﬂammatory genes and cell adhesion molecules, such as Ccl2, Cxcl11, Il6, C5ar1, Icam1, and C4d in VECs (Fig. 7f, g). Moreover, immunohistochemistry (IHC) of PPARγ on human aortic valve tissues conﬁrmed that PPARγ-activated valvular cells, especially VECs were also conserved in the human aortic valve and that PPARγ proteins were more abundant in non-calciﬁed samples than in calciﬁed samples (Fig. 7h, i, and Supplementary Table 1). Interestingly, the percentage of PPARγ+ VECs was positively correlated with plasma total cholesterol and LDL in non-calciﬁed aortic valves (Fig. 7j) and Supplementary Table 1. Collectively, these results suggest that PPARγ pathway in VECs is activated by increased plasma LDL. The PPARγ pathway in VECs appears to possess anti-inﬂammatory properties.

PPARγ activation protects the aortic valve against hyperlipidemia-induced inﬂammation
To identify the role of PPARγ in aortic valvular inﬂammation, we ﬁrst performed a monocyte adhesion assay in the ex vivo aortic valve culture system. Treatment of PPARγ antagonist (T0070907) with oxLDL enhanced the attachment of monocytes on valves (Fig. 8a). We next administered the PPARγ antagonist (T0070907) or vehicle daily to Ldlr−/− mice fed the WD. The proportion of leukocytes was increased in the T0070907-treated group than in the vehicle-treated group. In particular, monocyte-derived MHC-II+CD11c+CD206+ macrophages and DCs were signiﬁcantly increased in the T0070907-treated group than in the vehicle-treated group, while MHC-II+CD11c+CD206+ macrophages and T cell showed no differences (Fig. 8b, c). Conversely, when we administered the PPARγ agonist pioglitazone to hyperlipidemic mice, the percentage of leukocytes in aortic valve was markedly decreased compared to the control group. While there was no proportional change of MHC-II+CD11c+CD206+ macrophages, DCs and T cell, the monocyte-derived MHC-II+CD11c+CD206+ macrophages were signiﬁcantly decreased by the administration of pioglitazone (Fig. 8d). The treatment of T0070907 or pioglitazone did not affect the level of total cholesterol and LDL (Supplementary Fig. 16a, b). Also, PPARγ activation by pioglitazone did not change the number of circulating monocytes and associated subsets in the blood (Supplementary Fig. 16c, d). Altogether, these results suggest that PPARγ activation during hyperlipidemia protects the aortic valve against excessive inﬂammation (Fig. 8e).

Discussion
AS is a progressive disease with its early asymptomatic stage longer than its symptomatic stage. In the context of AS prevention and considering the ﬁndings of statin trials, it is important to elucidate the mechanism of the early stage of AS progression, especially the mechanism of disease initiation. Here, we provided strong evidence that LDL cholesterol is the predisposing factor in the initiation of valvular inﬂammation leading to AS, demonstrated by a dramatic decrease in valvular lipid accumulation and inﬂammation following lipid-lowering treatment. Our data are supported by the recent clinical study on the association of LDL with AS. Thus we postulate that lipid-lowering therapy with statins or PCSK9 inhibitors may be useful in preventing the initiation of valvular inﬂammation.

Previously, two research groups performed scRNA-seq analysis on the aortic valve. One group focused on the development mechanism of cardiac valves, and the other group analyzed single-cell transcriptome of the human CAVD. Meanwhile, we performed scRNA-seq analysis primarily focusing on the early inﬂammatory stage in hyperlipidemia. Our scRNA-seq revealed that monocyte-derived MHC-II+CD11c+CD206+ macrophage is the main leukocyte in aortic valvular inﬂammation by hyperlipidemia. In previous research, there have been some reports of the accumulation of MHC-II+ macrophage in the aortic valve with Notch1 haploinsufﬁciency, or monocyte-derived macrophages that invoke inﬂammation in the mitral valve with Marfan syndrome. It might be worthwhile to compare the phenotypic similarity/dissimilarity among those inﬂltrated macrophages with the identical surface marker but in different kinds of aortic valve diseases. Another main valvular macrophage population was MHC-II+CD206+Lyve1- macrophages, which showed an anti-inﬂammatory gene expression proﬁle compared to MHC-II+CD11c+CD206+ macrophages. Some of the MHC-II+CD206+Lyve1- macrophages might have originated from endothelial-derived resident macrophages, which have recently been reported to play a role in extracellular matrix (ECM) regulation for tissue remodeling during aortic valve development. Also, according to a previous study of resident macrophages in the aorta, CD206Lyve1- aortic macrophages regulate the ECM to maintain the arterial tone. Therefore, MHC-II+CD206Lyve1- resident macrophages in the aortic valve may have a similar function of ECM regulation for tissue homeostasis.
Notably, Cd36+ VEC was enriched with the PPARγ pathway gene expression, and its proportion was markedly increased in hyperlipidemic mice. Cd36+ VECs expressed high levels of Cd36 (encoding CD36) and Scarb1 (encoding SR-BI). These two scavenger receptors (CD36 and SR-BI) participate in LDL uptake; thus, VECs may transport LDL from the blood into the tissue via CD36- and SR-BI-related mechanisms. Although the distribution of CD36 or SR-BI is widespread (in other words, not confined to ECs only), direct exposure of ECs to blood functions to take circulating lipoproteins from the blood and provides them to peripheral tissues. Therefore, the expression of CD36 and SR-BI in VECs might be crucial for lipid accumulation in the aortic valve during early-stage aortic valve disease.
supported by our data, which showed that CD36 and SR-BI blockages decreased LDL accumulation in the aortic valve. Meanwhile, a previous report showed that stenotic aortic valves expressed lower levels of CD36 than did normal aortic valves. We speculate that these differences may be attributed to distinct tissue microenvironments of the aortic valve in the early versus late stages of aortic valve disease.

Tissue-accumulated LDL can become oxLDL, which may trigger the inflammatory responses; however, oxLDL also acts as an endogenous ligand that activates PPARγ. PPARγ is a transcription factor with pleiotropic functions. PPARγ is associated with glucose homeostasis, adipocyte differentiation, and lipid metabolism. PPARγ activation upregulates the expression of liver X receptor α (LXRα). oxLDL, another important transcription factor involved in lipid metabolism, is expressed in the VECs of hyperlipidemic pigs, and this gene is missing in mice. In our scRNA-seq analysis, VECs that were high in PPARγ expressed notable levels of CD36, Scarb1, Lpl, and Gpihbp1, implicating the lipoprotein-specific function of these genes in the hyperlipidemic state.

Interestingly, PPARγ-activated VECs are conserved in the human aortic valve, especially in the pre-calciﬁed valvular state. In our RNA-seq analysis of human aortic VECs, knockdown of PPARγ increased the expression of pro-inﬂammatory genes (CXCL1, CCL2, CXCL2, IL6, CXCL8, CXCL16, and PTGDS) and cell adhesion molecules (ICAM1, ICAM2, VCAM1, PECAM1, and MCAM), showing the anti-inﬂammatory role of PPARγ in a VEC-speciﬁc manner. Previously, it has been reported that PPARγ is expressed in the VECs of hyperlipidemic pigs, and pioglitazone attenuated valvular lipid deposition and inﬂammation in hyperlipidemic mice. It is also known that the activation of PPARγ induces anti-inﬂammatory functions in vascular ECs. The mechanisms underlying the anti-inﬂammatory effects of PPARγ may be attributed to the negative regulation of NF-κB activation by PPARγ.

Two molecular mechanisms of the negative regulation of NF-κB by PPARγ have been reported previously. First, PPARγ functions as an E3 ubiquitin ligase for p65 (also known as RELA, a subunit of NF-κB). Through the binding and ubiquitination of p65, PPARγ can induce p65 degradation via proteasomes. Second, PPARγ can undergo sumoylation, where sumoylated PPARγ has an afﬁnity for binding to the nuclear receptor coactivator 3 (NCoR3-histone deacetylase 3 (HDAC3) complex. The NCoR3-HDAC3 complex interferes with NF-κB by binding to its target genes. The sumoylated PPARγ, bound to the NCoR3-HDAC3 complex, represses the ubiquitination and degradation of this complex, thereby eliciting transrepression of NF-κB.

In this study, we found that PPARγ inhibition aggravates valvular inﬂammation by inﬁltrated macrophages, and PPARγ agonist pioglitazone effectively mitigates aortic valvular inﬂammation. These results indicate that VECs exert anti-inﬂammatory feedback function via upregulation of the PPARγ pathway in the early stage of the disease. We suggest pioglitazone as the drug candidate for inhibiting aortic valvular inﬂammation via PPARγ activation. Since pioglitazone is currently prescribed for the treatment of diabetes mellitus, further observational study of the effect of pioglitazone on aortic valve diseases such as CAVD, might be clinically meaningful.

In our results, ezetimibe reduced valvular lipid accumulation and inﬂammation via a lipid-lowering effect, and pioglitazone showed an anti-inﬂammatory effect on the aortic valve by PPARγ activation, without the change in blood total cholesterol and LDL levels. Previous studies showed the synergic beneﬁcial effects of co-administration of lipid-lowering drugs and pioglitazone in patients with cardiovascular disease including atherosclerosis. Therefore, it is needed to investigate the effect of co-treatment of lipid-lowering drugs and PPARγ agonists in the early aortic valve disease.

However, our study has several limitations. First, hyperlipidemic mouse models used in this study (Apoe−/− and Ldlr−/− mice) are less efﬁcient to progress into aortic stenosis, although these models are sufﬁcient to induce the early-stage lesion of aortic valve disease showing lipid accumulation and inﬂammation. It was reported that Ldlr−/− mice fed WD for 16 weeks only produced a scant amount of valvular calcification, and Apoe−/− mice fed WD for 20 weeks upregulated osteoblastic protein expression on the aortic valve but did not present microscopic valvular calcification. In the case of the induction of severe calcification that may lead to aortic stenosis, utilizing a more suitable mouse model for valvular calcification such as Notch1−/− mice could be more appropriate. Second, this study did not analyze the effect of lipoprotein(a) (Lp(a)), the LDL-like particle in humans. Assembly of Lp(a) needs apolipoprotein(a), coded by the gene named LPA, and this gene is missing in mice. Lp(a) works as a carrier of oxidized phospholipid (OxPL) in human blood plasma, and it is reported that the Lp(a) and OxPL are associated with the incidence of AS by acceleration of aortic valvular inﬂammation and calcification. Further investigation is required using Lp(a)-transgenic mice to dissect the role of Lp(a) in the early stage of aortic valve disease.

In summary, we demonstrated that lipid accumulation and inﬂammation of the aortic valve disease in the early stage is determined by LDL rather than total cholesterol, and identiﬁed the single-cell-based cellular characteristics among the normal and hyperlipidemic mouse models. Hyperlipidemic models showed a higher ratio of valvular leukocytes than the normal model, and leukocytes in the aortic valve were mainly monocye-derived MHC-II+ macrophages. These monocye-derived MHC-II+ macrophages were recruited by pro-inﬂammatory cytokines and chemokines, produced by VICS and VECs. Meanwhile, activated PPARγ exerted a protective role in the early inﬂammatory phase of aortic valve disease induced by hyperlipidemia, suggesting PPARγ as a putative drug target for aortic valve disease.

Methods

Human samples

The experimental protocols for the human study were reviewed and approved by the Institutional Review Board (IRB) of Yonsei Severance Hospital (Seoul, Korea, IRB No. 4-2018-0813), Seoul National University Hospital (Seoul, Korea, IRB No. 1104-122-360) and the Catholic Hospital (Seoul, Korea, IRB No. 4-2018-0813), Seoul National University Hospital (Seoul, Korea, IRB No. 1104-122-360) and the Catholic
Supplementary Table 1 for the clinical information of patients. For human aortic VEC isolation, human aortic valves from two patients were provided by The Catholic University of Korea, Uijeongbu St. Mary’s Hospital (Uijeongbu, Korea, IRB No. UC19TIDE0142). Patient information was as follows: patient #1 (age 73, male) was diagnosed with aortic aneurysm and underwent Bentall operation with aortic arch repair. Patient #2 (age 80, female) was diagnosed with severe aortic stenosis and underwent aortic valve replacement. The clinical information of patients included age, gender, diagnosis, and treatment outcomes.
replacement, and patient #2 (age 76, male) was diagnosed with aortic aneurysm and aortic stenosis caused by a bicuspid aortic valve and underwent both aortic valve and ascending aorta replacement.

Animal experiments

B6.129S7-Ldrtm1Hkrj (#:002207, Ldr−/−), B6.129P2- Apoe1null/J (#:002052, Apoe−/−), and B6.129S4-Ccr2tm1Jfc (#:004999, Ccr2−/−) mice were obtained from The Jackson Laboratory. Male C57BL/6 mice were purchased from SLC (Japan) or DBL (Korea). All mice were housed in the animal facility of Hanyang University under specific pathogen-free conditions in a 12-h light/12-h dark cycle with controlled temperature (20–24 °C) and humidity (40–60%), and supplied with a normal chow diet. A week after the injection, the serum lipid profile was measured, and the WD diet was continued for 5 weeks.

For the analysis of blood monocytes, peripheral blood was collected from euthanized mice and stained with PE anti-CD11c, FITC anti-CD11b, and PE/Cyanine 7 anti-F4/80. The cells were then intracellularly stained with Alexa Fluor 488 anti-CD206 antibody for 30 min. The stained cells were washed, resuspended in 2% fetal bovine serum, and permeabilized with 0.5% saponin. The treated cells were recorded using the BD FACSCanto II Flow Cytometer.

VIC isolation and culture conditions

C57BL/6J mice (2- to 4-month-old) were sacrificed in a CO2 chamber, and the aortic valve and mitral valve were collected in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich, #D6429). The aortic valve and mitral valve were minced for a minute. For culture, tissue was digested for 30 minutes with 1000 U/mL collagenase II and 90 U/mL DNase I in Ca2+/Mg2+ DPBS at 37 °C. Cell suspensions were centrifuged at 635 × g for 5 min, and the obtained pellets were used for the experiments, as shown in Figs. 2d–1, 2f, g, h, and i, respectively. After the injection, the mice were switched from a chow diet to WD. A week after the injection, the serum lipid profile (total cholesterol, LDL, triglyceride, and HDL levels) was measured, and the WD diet was continued for 24 weeks (experiments using Apoe−/− or Ccr2−/− mice) or 6 weeks (pioglitazone administration experiment) in only the mice with elevated serum total cholesterol and LDL levels.

In VEC clusters (VEC_C0, C1, and C2), the activity of PPARγ was increased in hyperlipidemic mice. A heatmap (left) and boxplot (right) of the average expression score of genes in EC1 and EC2-associated pathways in VEC_C0 and VEC_C1 (bottom), p, two-sided t-test p-value. h, Enrichment map of significant Kyoto Encyclopedia for Genes and Genomes (KEGG) gene sets for each cell cluster. Color represents the adjusted p-value (padj) and size represents the normalized enrichment score (NES), calculated by fgage R package. Image data are representative of three independent experiments unless otherwise stated. Data are presented as mean ± SD.
CD36 and SR-BI Inhibition
C57BL/6J mice (2- to 4-month-old) were sacrificed in a CO2 chamber, and the aortic valve attached to the aortic sinus was collected in DPBS containing 10 μM BLT-1 (SR-BI inhibitor, Sigma-Aldrich, #SML0059) or 200 μM SAB (CD36 inhibitor, Sigma-Aldrich, #SML0048) for 2 h on ice. The aortic valve was transferred into DMEM supplemented with 10% FBS and 50 μg/ml Primocin containing 50 μg/ml of DiI-LDL and 10 μM BLT-1 or 200 μM SAB and incubated for 48 h at 37 °C and 5% CO2. For flow cytometry, the tissue was digested for 30 min with 1000 U/ml collagenase II and 90 U/ml DNase I in Ca++/Mg++ DPBS at 37 °C.

Expression level of genes in PPARγ regulon

| Gene Set Activity (AUC) |
|------------------------|
| C57BL/6J Apoe−/− Ldlr−/− |
| 0                      |
| 1                      |
| 2                      |
| 3                      |
| 4                      |
| 5                      |
| 6                      |
| 7                      |
| 8                      |
| 9                      |

PPARγ (26 genes)

Expression level of genes in PPARγ regulon

| Pro-inflammatory genes |
|------------------------|
| C57BL/6J Apoe−/− Ldlr−/− |
| 0                      |
| 1                      |
| 2                      |
| 3                      |
| 4                      |
| 5                      |
| 6                      |
| 7                      |
| 8                      |
| 9                      |

Cell adhesion molecules

| Cell adhesion molecules |
|-------------------------|
| C57BL/6J Apoe−/− Ldlr−/− |
| 0                      |
| 1                      |
| 2                      |
| 3                      |
| 4                      |
| 5                      |
| 6                      |
| 7                      |
| 8                      |
| 9                      |

Valvular cells VECs

| Valvular cells VECs |
|---------------------|
| C57BL/6J Apoe−/− Ldlr−/− |
| 0                      |
| 1                      |
| 2                      |
| 3                      |
| 4                      |
| 5                      |
| 6                      |
| 7                      |
| 8                      |
| 9                      |

PPARγ percentage (%)

| PPARγ percentage (%) |
|-----------------------|
| C57BL/6J Apoe−/− Ldlr−/− |
| 0                      |
| 1                      |
| 2                      |
| 3                      |
| 4                      |
| 5                      |
| 6                      |
| 7                      |
| 8                      |
| 9                      |

Non-calcified1
Non-calcified2
Calcified1
Calcified2
Calcified3
Calcified4

Pro-inflammatory genes

| Pro-inflammatory genes |
|------------------------|
| C57BL/6J Apoe−/− Ldlr−/− |
| 0                      |
| 1                      |
| 2                      |
| 3                      |
| 4                      |
| 5                      |
| 6                      |
| 7                      |
| 8                      |
| 9                      |

Cell adhesion molecules

| Cell adhesion molecules |
|-------------------------|
| C57BL/6J Apoe−/− Ldlr−/− |
| 0                      |
| 1                      |
| 2                      |
| 3                      |
| 4                      |
| 5                      |
| 6                      |
| 7                      |
| 8                      |
| 9                      |

Valvular cells VECs

| Valvular cells VECs |
|---------------------|
| C57BL/6J Apoe−/− Ldlr−/− |
| 0                      |
| 1                      |
| 2                      |
| 3                      |
| 4                      |
| 5                      |
| 6                      |
| 7                      |
| 8                      |
| 9                      |

PPARγ percentage (%)

| PPARγ percentage (%) |
|-----------------------|
| C57BL/6J Apoe−/− Ldlr−/− |
| 0                      |
| 1                      |
| 2                      |
| 3                      |
| 4                      |
| 5                      |
| 6                      |
| 7                      |
| 8                      |
| 9                      |

Non-calcified1
Non-calcified2
Calcified1
Calcified2
Calcified3
Calcified4

Pro-inflammatory genes

| Pro-inflammatory genes |
|------------------------|
| C57BL/6J Apoe−/− Ldlr−/− |
| 0                      |
| 1                      |
| 2                      |
| 3                      |
| 4                      |
| 5                      |
| 6                      |
| 7                      |
| 8                      |
| 9                      |

Cell adhesion molecules

| Cell adhesion molecules |
|-------------------------|
| C57BL/6J Apoe−/− Ldlr−/− |
| 0                      |
| 1                      |
| 2                      |
| 3                      |
| 4                      |
| 5                      |
| 6                      |
| 7                      |
| 8                      |
| 9                      |

Valvular cells VECs

| Valvular cells VECs |
|---------------------|
| C57BL/6J Apoe−/− Ldlr−/− |
| 0                      |
| 1                      |
| 2                      |
| 3                      |
| 4                      |
| 5                      |
| 6                      |
| 7                      |
| 8                      |
| 9                      |

PPARγ percentage (%)

| PPARγ percentage (%) |
|-----------------------|
| C57BL/6J Apoe−/− Ldlr−/− |
| 0                      |
| 1                      |
| 2                      |
| 3                      |
| 4                      |
| 5                      |
| 6                      |
| 7                      |
| 8                      |
| 9                      |
Monocyte migration assay
To measure monocyte chemotaxis, VICs were incubated on a lower well of a transwell (Corning, #3388) in media (DMEM supplemented with 10% FBS and 50 μg/mL Primocin containing 50 μg/mL of LDL (Kalen Biomedical, #770200) or oxLDL (Kalen Biomedical, #770202) for 24 hours at 37°C, 5% CO₂, and isolated monocytes (5 × 10⁴ cells) using the manufacturer’s protocol [Mouse monocyte isolation kit (BM), Miltenyi Biotec, #130-100-629] were labeled with CFSE cell division tracker (BioLegend, #423801) and co-cultured with the aortic valve for an hour of incubation at 37 °C and 5% CO₂, isolated monocytes (1 × 10⁵ cells) using the mouse genome (build mm10) using the Cell Ranger toolkit (v3.0.2). Single-cell RNA sequencing parameters were summarized in Supplementary Table 2.

Ex vivo monocyte adhesion assay
C57BL/6j mice were sacrificed in a CO₂ chamber and the aortic valve attached to the aortic sinus was collected in DMEM supplemented with 10% FBS and 50 μg/mL Primocin containing 50 μg/mL of LDL (Kalen Biomedical, #770200) or oxLDL (Kalen Biomedical, #770202) or Dil-VLDL (Kalen Biomedical, #770130) and incubated for 48 hours at 37 °C and 5% CO₂. For imaging, after fixation with 4% paraformaldehyde and co-staining with DAPI, the aortic valve was imaged with a confocal microscope (Nikon). For flow cytometry, the tissue was digested for 30 min with 1000 U/mL collagenase II and 50 μg/mL Primocin containing 50 μg/mL of LDL (Kalen Biomedical, #770200) or oxLDL (Kalen Biomedical, #770202) or Dil-VLDL (Kalen Biomedical, #770130) and incubated for 24 hours at 37 °C, 5% CO₂, and isolated monocytes (5 × 10⁴ cells) using the manufacturer’s protocol [Mouse monocyte isolation kit (BM), Miltenyi Biotec, #130-100-629] were labeled with CFSE cell division tracker kit (BioLegend, #423801) and co-cultured with the aortic valve for an hour of incubation at 37 °C and 5% CO₂. The attached monocytes were imaged using a confocal microscope (Nikon) and counted.

Fluorescence-activated cell sorting (FACS)
Aortic valves were pooled and single cells were isolated as described above. The number of pooled male mice in each experimental group was as follows: C57BL/6j (n = 30), Ldlr⁻/⁻ (n = 24), and Apeo⁻/⁻ group (n = 19). Only live single-cells [propidium iodide (PI) negative single cells] were sorted using BD FACS Aria III and then aligned using the 10x Chromium pipeline to construct the single-cell cDNA library (Supplementary Fig. 6).

scRNA-seq and read processing
The single-cell suspensions were subjected to 3’ single-cell RNA sequencing aiming for target recovery of 3,000 cells (C57BL/6j) or Ldlr⁻/⁻ and 3,000 cells (Apeo⁻/⁻) using Single Cell A Chip Kit, Single Cell 3’ Library, and Gel Bead Kit V2, and 17 Multiplex Kit (10x Genomics). Solutions pertaining to 3’ chemistry and v2 of 10x Genomics were used to barcode individual cells. Libraries were sequenced on an Illumina HiSeq2500 and mapped to the mouse genome (build mm10) using the Cell Ranger toolkit (v3.0.2). Single-cell RNA sequencing parameters were summarized in Supplementary Table 2.

Unsupervised dimensional reduction and clustering
Top-ranked 2000 variably expressed genes were selected using the ‘vsr’ method implemented in FindVariableFeatures function of the Seurat package. These genes were then used to correct the effect of technical batches by applying the FindIntegrationAnchors and IntegrateData functions with a total of 15 or 20 anchors (all single cells, leukocytes, and VICs, 20 anchors, VECs, 15 anchors). Cell clustering and UMAP visualization were then performed using the FindClusters (resolution = 0.3) and RunUMAP functions. Cluster results were visualized using UMAP to verify that the graphically identified clusters were captured. Cell types were assigned based on known marker gene expression, considering the significantly expressed genes in each cell cluster.

Differentially expressed marker gene analysis
We identified significantly expressed genes in each cell cluster using the FindAllMarkers function (default parameters) of the Seurat package. The significance of the difference was determined using the two-sided Wilcoxon rank sum test with Bonferroni correction. We selected marker genes based on the fraction of expressing cells (>25% of cells within either of the two cell groups, marked as pct) and the statistical threshold (log fold change > 0.25, p-value < 0.01) and adjusted p-value (Bonferroni) < 0.01.

Trajectory analysis
The cell state transitions of macrophages were estimated using the Monocle v2 algorithm. The UMI matrix for cells defined as macrophages (LEU_C0, C1, C2, C4, C5, and C7) was submitted to Monocle.

Ex vivo lipoprotein uptake assay
C57BL/6j mice were sacrificed in a CO₂ chamber and the aortic valve attached to the aortic sinus was collected in DMEM supplemented with 10% FBS and 50 μg/mL Primocin containing 50 μg/mL of LDL (Kalen Biomedical, #770200) or oxLDL (Kalen Biomedical, #770202) or Dil-VLDL (Kalen Biomedical, #770130) and incubated for 48 hours at 37 °C and 5% CO₂. For imaging, after fixation with 4% paraformaldehyde and co-staining with DAPI, the aortic valve was imaged with a confocal microscope (Nikon). For flow cytometry, the tissue was digested for 30 min with 1000 U/mL collagenase II and 90 U/mL DNase I in Ca²⁺/Mg²⁺ DPBS at 37 °C.

Fig. 7 | PPARγ pathway is activated in VECs of hyperlipidemic mice and conserved in human aortic valves. a Average expression map of genes in PPARγ regulon, produced by Single-Cell Regulatory Network Inference and Clustering (SCENIC), for each cluster of leukocytes, VECs, and VICs. b UMAP plot of VICs color-coded by the activity of PPARγ regulon. Gene set activity was calculated by SCENIC. AUC: area under curve. c Immunostaining of PPARγ (red) and endomucin (EMCN, EC marker, green) in aortic valve with sinus from normal (chow diet) and hyperlipidemic mice (ApoE⁻/⁻ and Ldlr⁻/⁻ mice, WD for 16 weeks) (n = 4). DAPI (blue) was used to stain nuclei. The graph represents the relative MI of PPARγ in the VECs. Kruskal-Wallis test with post-hoc Dunn’s test was used. Scale bar: 30 μm. d UMAP plot of 41,326 single cells derived from human aortic valve, colored by the clusters (left) and samples (right). e Average expression map of genes in PPARγ regulon for each cell cluster from human aortic valve. f Pro-inflammatory (top) and cell adhesion molecule (bottom) scores in non-targeting siRNA (NC) and PPARγ targeting siRNA-treated (PPARγ knockdown, KD) human VECs under no (NT) and oxLDL treatment conditions. Each score represents the average expression level of the genes, as shown in Fig. 7g (n = 3). Each box depicts the IQR and median of each score, whiskers indicate 1.5 times the IQR. p, two-sided t-test p-value. g Expression map of pro-inflammatory genes (top) and cell adhesion molecules (bottom). The expression of genes in all samples was scaled by z-transformation. h-j PPARγ IHC in human aortic valves (n = 7 for non-calified, n = 5 for calified). Representative image of PPARγ IHC (top) and H&E stain (bottom) (h), measurement of PPARγ cellular proportion in valvular cells (left) and VECs (right) (i) and the positive correlation between PPARγ VECs of non-calified and the plasma levels of total cholesterol and LDL (j). For (i), two-sided Mann-Whitney test and for (j), the Spearman correlation test were used. Scale bar: 40 μm (left), 20 μm (right). Image data are representative of three independent experiments unless otherwise stated. Data are presented as mean ± SD.

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object was created using the `newCellDataSet` function with the parameter `expressionFamily = negbinomial.size`. Then, the `dispersionTable` function was used to select variably expressed genes based on the variance over the fit curve and an average expression >0.001. The cell trajectory was inferred through dimension reduction and cell ordering using default `Monocle` parameters.

Projection of functional features of macrophage
To demonstrate the functional features of macrophages according to Monocle pseudotime and cell clusters, we used the `ggplot2` (v3.3.3) R package to visualize the expression pattern of pro- and anti-inflammatory scores. Pro-inflammatory represents mean expression of featured genes: `Il1b`, `Tnf`, `Ccl2`, `Cxcl10`, `Cxcl2`, `H2-Afi`, and `Itgax`. Anti-
Fig. 8 | PPARγ activation protects the aortic valve against the inflammation. Adhesion assay of monocytes to ex vivo cultured aortic valve treated with oxLDL and/or inhibition of PPARγ by T0070907 (n = 7 for non-treated, n = 7 for oxLDL, n = 6 for oxLDL+T0070907). Scale bar: 50 μm. a, c, e Pro-inflammatory effect of T0070907 (PPARγ antagonist) on mouse aortic valve in vivo. Ldlr<sup>−/−</sup> mice were intraperitoneally injected daily with vehicle or T0070907 for 10 weeks with a WD feeding. Representative IHC images (b) and flow cytometry analysis presenting percentage of each immune cell subset (n = 6; c). Scale bar: 30 μm. d, f Flow cytometry analysis showing anti-inflammatory effect of PPARγ activation by pioglitazone on mouse aortic valve in vivo. PCKS9-AV injected C57Bl/6j mice were fed with pioglitazone-containing WD or normal WD for 6 weeks. Graphs present percentage of each immune cell subset (n = 10). e Proposed pathogenesis model of the inflammatory score represents mean expression of featured genes: Mrc1, Ly6e, Folr2, Cbr2, and Il10. In the dot plots for inflammatory score and pseudotime, we used geom_smooth function with the parameter method = loess to determine trend line and calculated Pearson’s correlation using cor.test function from the R package stats (v3.6.3). In the boxplots of macrophage functional features in each cluster, we performed one-way ANOVA test using stat_compare_means function with the parameter method = anova from the ggpubr (v0.4.0) R package.

Pathway analysis
Cluster-specific gene signatures were categorized according to the gene catalogs in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. For the differentially expressed genes between knockout and wild-type models, gene set analysis was performed using a set of inflammation-related and monocyte-related gene sets selected from Gene Ontology (GO) Biological Process ontology. Gene sets were collected from the MsigDB database using the msigdb (v7.11) package. The gene list was ranked by the fold change. The R package fgesa (v1.12.0)<sup>*</sup> was used to perform gene set enrichment analysis (default parameters) and selected the over-enriched pathways satisfying the adjusted p-value < 0.05.

Transcription factor activity analysis
To investigate transcription factor activity, SCENIC<sup>27</sup> was used with cisTarget databases: mm10_refseq-r80_500bp_up and 100bp_dow n_tss.mce9mr.feather and mm10_refseq-r80_10kb_up and down_tss. mce9mr.feather (SCENIC v1.1.2, which corresponds to RcisTarget v1.6.0, AUCell v1.8.0, and GENIE3 v1.8.0). The input matrix was the log-normalized expression matrix from which genes passed the filtering (default parameters). GENIE3 was used to infer co-expression networks using runGenie3 function. Gene regulatory networks were constructed and scored using the following functions of SCENIC; runSCENIC<sub>1</sub>coexprNetwork2modules, runSCENIC<sub>2</sub>_createRegulons, and run SCENIC<sub>3</sub>_scoreCells.

Selection of cells with high PPARγ regulon expression and their specific genes
The valvular cells were divided into two classes (PPARγ high and low) along the 90th and 10th percentiles of the mean expression of the genes in PPARγ regulon. A total of 543 and 1,621 valvular cells (of these, 181 and 83 cells in VEC_C0, C1, and C2) were classified into PPARγ high and low groups. We calculated the differential expression levels of genes specific to PPARγ high cells compared to low cells using the FindMarkers function (default parameters) of the Seurat package. The significance of the difference was determined using the two-sided Wilcoxon rank sum test with Bonferroni correction. We selected significantly expressed genes based on the fraction of expressing cells (>25% of cells within either of the two cell groups, marked as pct) and the statistical threshold (log fold change > 0.25, p-value < 0.01, and adjusted p-value (Bonferroni) < 0.01).

Sample preparation and bulk tissue RNA sequencing of mouse aortic valves
Male Apoe<sup>−/−</sup> and Ldlr<sup>−/−</sup> mice (6-week-old) fed a WD for 8 weeks (sacrificed at 14-week-old) to induce aortic valve disease with lipid accumulation by hypercholesterolemia. For the control group, chow diet-fed, 14-week-old male C57BL/6j (wild type), Apoe<sup>−/−</sup>, and Ldlr<sup>−/−</sup> mice were used. Mice were euthanized by CO<sub>2</sub> inhalation, and their aortic valves were collected into the cold DPBS. For each sample, isolated aortic valves from 10 mice were pooled into RNAlater Stabilization Solution (Invitrogen, #AM7021). After stabilization, RNA was extracted from aortic valves, using the TRIzol Reagent (Invitrogen, #15596026). cDNA libraries for sequencing were constructed from 0.5 μg of total RNA, using the HiSeq 3000/4000 SBS Kit (Illumina, #FC-410-1003) according to the manufacturer’s protocol. Libraries were sequenced using the Illumina HiSeq 4000 system with 101-bp read length and paired-end reads.

Human aortic VEC isolation and culture conditions
Human aortic valves from the patients were collected in a cold, sterile saline solution. After incubation with collagenase II solution [600 U/mL in EBM-2 Endothelial Cell Growth Basal Medium-2 (Lonza, #CC-3156) with 10% FBS and GA-1000 (11000, Lonza, #CC-4083)] for 10 min at 37 °C, human aortic VECs were isolated from the aortic valve tissue, by a gentle rolling of pre-soaked sterile cotton swaps, according to a previous report by Gould & Butcher<sup>70</sup>. Isolated VECs were cultured in EGM-2 Endothelial Cell Growth Medium-2 BulletKit (Lonza, #CC-3162), supplemented with 10% FBS in a humidified CO<sub>2</sub> incubator (5% CO<sub>2</sub>, 37 °C). When 90% confluency was reached, cells were trypsinized and then subcultured, at a 1:3 ratio. Gelatin-coated cell culture flasks (T25 for passage 0 and T75 for passage 1 or higher) were used for cell culture. VECs at passage 1-2 were used for further experiments.

PPARG gene silencing in human aortic VECs by siRNA transfection
Human aortic VECs (5 × 10<sup>5</sup> cells per sample) at passage 1–2, were transfected with siRNA targeting PPARγ (AccuTarget Predesigned siRNA, Bioneer, pooling of 3 siRNA, #SDO-1001: siRNA #5468-1, -2, and -3) or negative control siRNA (Bioneer, #SN-1003) by electrophoresis (750 nm of final siRNA concentration, 1 pulse, voltage: 1300 V, width: 30 ms) using Neon Transfection System (Invitrogen). The human aortic VECs were then transferred into gelatin-coated six-well plates with EGM-2 medium supplemented with 10% FBS. 9 h after the transfer, media changes were performed. And 48 h after transfection (including serum starvation for 1h), cells were treated with or without oxLDL (150 μg/mL, Kalen Biomedical, #770252), for 24 h and then subjected to RNA extraction for RNA sequencing.

Library construction and RNA sequencing of human aortic VECs
Total RNA was extracted from human aortic VECs at passage 2–3, using the TRIzol Reagent (Invitrogen, #15596026). cDNA libraries for sequencing were constructed from 1 μg of total RNA, using a TruSeq
Stranded mRNA LT Sample Prep Kit (Illumina, #RS-122-2101 & RS-122-2102) according to the manufacturer’s protocol. Libraries were sequenced using the Illumina NovaSeq 6000 system with paired-end (2 × 100 bp) reads.

**Bulk RNA sequencing data processing**

The RNA reads were aligned to the reference sequences (mouse, GRCm38; human, GRCh38) and quantified as transcripts per million (TPM) using STAR (v2.7.5a) and RSEM (v1.3.1) using the function rsem-calculate-expression with the following parameters:--paired-end--star-estimate-rspd. The TPM count for the genes in each sample was log-normalized and used in the log2 scale TPM plus 1, for subsequent analyses.

**PPARG knockdown-specific gene analysis**

Differentially expressed genes between the two sample groups (NT-PPARG KD versus NT-NC, and OxLDL-PPARG KD versus OxLDL-NC) were identified using the DESeq function (default parameters for Wald test) of the DESeq2 (v.1.26.0) package. We selected genes based on the statistical threshold (>1.5 fold change, p-value < 0.05, and adjusted p-value < 0.05).

**scRNA-seq analysis of human aortic valve**

We obtained raw 3’ scRNA-seq data on human aortic valve leaflets from two healthy and four stenosis donors. Sequencing libraries were mapped to the human genome (build GRCh38) using the Cell Ranger toolkit (v3.0.2). Then, we selected single cells satisfying the following two quality measures: mitochondrial genes (<10%) and gene count (range from 200 to 5,000) from the R package Seurat v3.1.1 (https://satijalab.org/seurat/). The UMI matrix on the selected cell barcodes was log-normalized using the NormalizeData function and used in downstream analysis after z-transformation using the ScaleData function. The top-ranked 2000 variably expressed genes were selected using the ‘svt’ methods implemented in FindVariableFeatures function of the Seurat package. These genes were then used to correct the effect of technical batches by applying the FindIntegrationAnchors and IntegrateData functions with a total of 20 anchors. Cell clustering and UMAP visualization were then performed using the FindClusters (resolution = 0.5) and RunUMAP functions. Cluster results were visualized using UMAP to verify that the graphically identified clusters were captured. Cell types were assigned based on known marker gene expression, considering the significantly expressed genes in each cell cluster.

**Meta-analysis of endothelial cell diversity**

We obtained 3’ scRNA-seq data on normal aorta, and heart, from a C57BL/6 model mouse. UMI matrices were collected from published studies using the 10x Genomics platform. ECs were selected by in silico sorting based on the expression of Pecam1 and Cd68 genes after cell quality control. We performed integrated dimensional reduction and clustering for aortic, heart, and valvar endothelial cells, including the data generated by us.

**Histology and plasma lipid measurement**

Mouse hearts were perfused with 4% paraformaldehyde phosphate buffer solution, followed by tissue fixation for 2 h (O.C.T. compound embedded) or 16 h (paraffin-embedded) with the same solution as that used for perfusion. For histological analysis, Hematoxylin and Eosin (H&E) staining (BBC Biomedical, #MA0101035), PPARγ immunostaining, and RNA in situ hybridization were performed using paraffin sections. Standard tissue processing was performed to prepare the paraffin-embedded tissue. Processed heart tissue was embedded in paraffin and then sectioned into 4 μm sections. For the measurement of human PPARγ valvar cells, the average percentage of PPARγ valvar cells at least five random fields (22,500 μm² per field) was used. For the measurement of human PPARγ VECs, the percentage of total PPARγ VECs in total VEC count was used.

Frozen sections were used for Oil Red O staining and immunofluorescence. Fixed mouse hearts were embedded in O.C.T compound (Sakura Finetek, #4583) and frozen at –80 °C for 4 h. Frozen tissue blocks were serially sectioned into 7 μm sections from the beginning to the end of the aortic valve. To measure the aortic valve lesion area, frozen sections at 77 μm intervals were Oil Red O stained using a Fat stain kit (BBC Biomedical, #SSK5019), according to the manufacturer’s protocol. The lesion area was quantified as a percentage of the Oil Red O-positive area in the total aortic valve area using NIS Elements (v.4.30, Nikon) and Photoshop (v22.3.0, Adobe).

For whole-mount aortic valve Oil Red O staining, Fat stain kit (BBC Biomedical, #SSK5019) was used with a modified procedure. The aortic valve was rinsed with 100% propylene glycol and the tissue was stained with Oil Red O working solution for an hour at 37 °C. After brief washing with 85% propylene glycol, the stained tissue was mounted on a glass slide and imaged using brightfield microscopy (Nikon, Eclipse 50i with DS-R2).

To analyze the lipid profile, blood was obtained from the retro-orbital sinus. Plasma was collected from blood and centrifugated at 2,500 × g for 20 min at RT. The levels of total cholesterol, triglycerides, HDL, and LDL were analyzed using an automated blood chemical analyzer (Hitachi). For an assessment of comparative analysis between Ldlr<sup>−/−</sup> and Apeor<sup>−/−</sup> mice fed WD feeding for 4-, 8-, or 12-weeks, the data and tissue-sections from Kim et al., were used.

**Immunostaining**

Mouse hearts were fixed for 2 h in 4% paraformaldehyde phosphate buffer solution. Aortic valves were harvested from the fixed hearts and placed in a 96-well plate. For whole-mount immunostaining, aortic valves were permeabilized with 0.5% Triton X-100 for 30 min and then non-specific binding was blocked using 1% normal donkey serum for 1 hour. Subsequently, the aortic valves were incubated in primary antibodies (1:200, see Supplementary Data 5) in 1% normal donkey serum for 16 h at 4 °C with gentle shaking. The next day, valves were washed using DPBS with gentle shaking, and then incubated with secondary antibodies (1:400, see Supplementary Data 5) or Alexa Fluor 594- and 594- (1:400, Invitrogen, #S12227), following the same procedure as that in primary antibody incubation. The valves were then washed with DPBS and mounted on slides using coverslips and mounting medium with DAPI (Vector Laboratories, #H-1200-10). Images were acquired as Z-stacks using a confocal microscope (Nikon; or Zeiss, LSM 780). The acquired images were analyzed using NIS Elements (v.4.30, Nikon), ZEN (v8.1, Zeiss), ImageJ (v1.53c, National Institutes of Health, NIH), and Imaris (v9.0.2, Bitplane).

For immunostaining of CD68, vimentin or PROX1, frozen sections of aortic valves were rehydrated with DPBS, and endogenous peroxidase was quenched with 3% hydrogen peroxide and blocked with 1% normal donkey serum for 1 h. After blocking, the sections were incubated with a mixture of anti-CD68 (Bio-Rad, #MCA1957) and anti-vimentin (Abcam, #ab92547) antibodies or anti-PROX1 (R&D Systems, #AF2727) antibodies (all 1:200) in 1% normal donkey serum for 16 h at 4 °C. After washing, slides were incubated in a mixture of HRP anti-rat IgG (Jackson ImmunoResearch, #712-035-153) and Alexa Fluor 647 anti-rabbit IgG (Invitrogen, #A-31573) or Alexa Fluor 488 anti-goat IgG (Invitrogen, #A-11055) antibodies (all 1:400) in 1% normal donkey serum for 12 h at 4 °C. To detect CD68, the slides were treated with an Alexa Fluor 594-tyramide signal amplification kit (Invitrogen, #T20935) according to the manufacturer’s protocol. In immunostaining of CD68 and vimentin, the slides were incubated for 1 h with 1 μM BODIPY 493/503 (Invitrogen, #D3922) solution to stain lipid droplets. Lastly, the slides were washed with DPBS and coveredslipped using a mounting medium with DAPI (Vector Laboratories, #H-1200-10).
To immunostain PPARγ, the paraffin sections of aortic valves were deparaffinized, rehydrated, and double-boiled for 10 minutes in the Diva Decloaker (Biocare Medical, #DV2004) solution for antigen retrieval. After endogenous peroxidase quenching with 3% hydrogen peroxide and blocking with 1% normal donkey serum, the slides of mouse samples were incubated with anti-PPARγ (1:50, Cell Signaling Technology, #2435) and anti-EMCN antibodies (1:200, Abcam, #ab106100), whereas the slides of human samples were incubated only with the anti-PPARγ antibody (1:50, Cell Signaling Technology, #2435), in 1% normal donkey serum for 20 h at 4 °C. For the mouse samples, after primary antibody incubation, the slides were washed and then incubated with Cy3 anti-rabbit IgG (1:200, Jackson ImmunoResearch, #711-165-152) and Alexa Fluor 488 anti-mouse IgG (1:200, Jackson ImmunoResearch, #715-545-151) in 1% normal donkey serum in PBST (DPBS with 0.1% Tween 20), the slides were incubated with Alexa Fluor 488 anti-rabbit IgG (1:200, Invitrogen, #A-21208) in 1% normal donkey serum for 4 h at room temperature. The slides were coveredslipped using a mounting medium with DAPI (Vector Laboratories, #H-1200-10), after brief washing with DPBS. For human samples, VECTASTAIN Elite ABC-HPK Kit (Vector Laboratories, #PK-6101) and DAB Substrate (Vector Laboratories, #SK-4105) were used, according to the manufacturer’s protocol.

For immunocytochemistry of CD31, human aortic VECs at passage 2–3 were cultured in gelatin-coated, four-well chamber slides (Thermo Scientific, #1542562PK). The cells in slides were briefly washed with DPBS, fixed with 4% paraformaldehyde, and then permeabilized with 0.5% Tween 20 in DPBS. After blocking with 1% normal donkey serum in PBST (DPBS with 0.1% Tween 20), the slides were incubated with anti-CD31 antibody (1:200, BioLegend, #303101) in 1% normal donkey serum in PBST for 16 h at 4 °C. After that, the slides were washed and then incubated with Alexa Fluor 488 anti-mouse IgG (1:200, Jackson ImmunoResearch, #715-545-151) in 1% normal donkey serum in PBST for 2 h at room temperature. After brief washing with DPBS, the slides were coveredslipped using mounting medium with DAPI (Vector Laboratories, #H-1200-10).

Fluorescence images were captured using a confocal microscope (Nikon) or fluorescence microscope (Nikon, Eclipse 50i with DS-Ri2). Brightfield microscopy (Nikon) was used to obtain bright field images. Captured images were analyzed using NIS Elements (Nikon), ImageJ (NIH), and Imaris (Bitplane). For the quantification of PPARγ, ImageJ (NIH) was used.

Western blotting analysis
Human aortic VECs, at passage 2–3, were lysed in RIPA buffer (GenDEPOT, #R4100-010) containing a protease inhibitor cocktail (GenDEPOT, #P3200-001). Protein samples were separated by SDS-PAGE and transferred into the polyvinylidene fluoride (PVDF) membrane (Millipore, #IPVH00010). After blocking with 5% skim milk in tris-buffered saline (TBST), the membrane was incubated with an anti-PPARγ antibody (1:500, Santa Cruz Biotechnology, #sc-7273) in 5% skim milk for 1 h at 4 °C, and then incubated with HRP anti-mouse IgG antibody (1:5000, BioLegend, #405306) in 5% skim milk, for 2 h at room temperature. After obtaining the PPARγ signal, antibodies were stripped from the membrane using BlotFresh Western Blot Stripping Reagent (SigmaGen, #SL100324) according to the manufacturer’s protocol. The membrane was blocked again with 5% skim milk and incubated with anti-GAPDH antibody (1:1000, Cell Signaling Technology, #2118S, 4 h at 4 °C) as the primary antibody, and then incubated with HRP anti-rabbit IgG antibody (1:10,000, Jackson ImmunoResearch, #711-035-152, 1 h at room temperature). Immobilon Western HRP substrate (Millipore, #WBSTUF0100) was used for signal development, and a chemiluminescence imaging system (Fusion SL, Vilber Lourmat, France) with software (Evolution-Capt, v17.04a, Vilber Luormat) was used for signal capture and image processing. ImageJ (NIH) was used for image analysis.

RNA in situ hybridization (RNAscope®)
To detect in situ expression of marker genes, RNA in situ hybridization (RNAscope, ACDbio) was used, according to the manufacturer’s instructions (RNAscope® 2.5 HD Duplex Reagent Kit, ACDbio, #322430 and ACDbio user manual document #322452 & 322500). Formalin-fixed paraffin sections of 4 µm thickness were used for the RNAscope assay. Amplified probes were detected by brightfield (C1, C2) or fluorescence (C2) microscopy (Nikon) and analyzed using the NIS Elements (Nikon) and ImageJ (NIH). For the quantification of Cd31, ImageJ (NIH) was used. The following probes were used in this study: Meox1 (ACDbio, #530641-C2), Id4 (ACDbio, #447861-C2), Rspo3 (ACDbio, #402011), Chad (ACDbio, #458851), Sppl1 (ACDbio, #435191-C2), Fgfr3 (ACDbio, #440771-C2), Cd36 (ACDbio, #464431-C2), Cx3cl1 (ACDbio, #426211-C2), Csf1 (ACDbio, #315621), Cple3b (ACDbio, #539561-C2), Dpep1 (ACDbio, #480831), Polr2ra (ACDbio, #312471-C2), Pibd (ACDbio, #313911), and DapB (ACDbio, #310043 & 310043-C2).

Statistical analysis
Statistical methods used in the scRNA-seq analysis are addressed in each section of Materials and Methods. GraphPad Prism (v9.4.1, GraphPad) was used to perform statistical analysis and graph representation of all in vivo and in vitro data. Unless otherwise stated, data are shown as mean ± standard deviation (SD), and the nonparametric analysis was used because of the small sample size (n < 30). Two-sided Mann–Whitney test was used for the comparison between two groups, and Kruskal-Wallis test with post-hoc Dunn’s test was used for comparing three or more groups. For correlation analysis, the Spearman correlation test was used. The p value of two-sided Mann–Whitney test, post-hoc Dunn’s test, and Spearman correlation test with correlation coefficient (r) is presented on the respective graph. Statistical significance was set at p < 0.05.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
All data associated with this study are present in the main text or the supplementary materials. The RNA-seq data generated in this study have been deposited in the GEO database under accession code “GSE180278”, “GSE205587” and “GSE206927”. The publicly available RNA-seq data used in this study are available in the BioProject database under accession code “PRJNA626459”, and in the ArrayExpress database under accession code “E-MTAB-7149”, “E-MTAB-8077”, “GRC383” and “GRCm38” were used for the reference genome. All other relevant data supporting the key findings of this study are available within the article and its Supplementary Information files or from the corresponding author upon reasonable request. Source data are provided with this paper.

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Competing interests

The authors declare no competing interests.

Additional information

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