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Sphingosine 1-phosphate receptor-2 function in myeloid cells regulates vascular inflammation and atherosclerosis

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SUMMARY

Objective—Sphingomyelin deposition and metabolism occurs in the atherosclerotic plaque, leading to the formation of sphingosine 1-phosphate (S1P), which activates G protein-coupled receptors to regulate vascular and immune cells. The role of S1P receptors in atherosclerosis has not been examined.

Methods and Results—We tested the hypothesis that Sphingosine 1-phosphate receptor-2 (S1PR2) regulates atherosclerosis. *Apoe*$^{-/-}$*S1pr2*$^{-/-}$ mice showed greatly attenuated atherosclerosis compared to the *Apoe*$^{-/-}$ mice. Bone marrow transplant experiments indicate that S1PR2 function in the hematopoietic compartment is critical. S1PR2 is expressed in bone marrow-derived macrophages and in macrophage-like foam cells in atherosclerotic plaques. Reduced macrophage-like foam cells were found in the atherosclerotic plaques of *Apoe*$^{-/-}$*S1pr2*$^{-/-}$ mice, suggesting that S1PR2 retains macrophages in atherosclerotic plaques. Lipoprotein profiles, plasma lipids and oxidized LDL uptake by bone marrow-derived macrophages were not altered by the *S1pr2* genotype. In contrast, endotoxin-induced inflammatory cytokine (IL-1β, IL-18) levels in the serum of S1PR2 knockout mice were significantly reduced. Further, treatment of wild-type mice with S1PR2 antagonist JTE-013 suppressed IL-1β and IL-18 levels in plasma.

Conclusion—These data suggest that S1PR2 signaling in the plaque macrophage regulates macrophage retention and inflammatory cytokine secretion, thereby promoting atherosclerosis.

Keywords

atherosclerosis; sphingolipids; sphingosine 1-phosphate; macrophages; inflammation

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INTRODUCTION

Risk factors for atherosclerosis such as hyperlipidemia, smoking, and hypertension injure the vascular endothelium, leading to lipoprotein deposition in the arterial vessel wall [1, 2]. Although cholesterol entry into the atherosclerotic plaque is well accepted to play a major role in atherogenesis, sphingomyelin (SM), which has an affinity for cholesterol in membranes is also deposited. Metabolism of SM by the sphingomyelinase pathway produces sphingolipid metabolites - ceramide, sphingosine and sphingosine 1-phosphate (S1P) [3–5], whose function in vascular disease is not understood. Indeed, inflammatory cytokines stimulate the metabolism of sphingomyelin by inducing the secretion of sphingomyelinase from endothelial cells and macrophage6. However, suppression of SM synthesis with the fungal metabolite myriocin attenuates atherosclerosis in animal models and SM and its metabolites are elevated in plasma of patients with coronary artery disease, suggesting that sphingolipid metabolites are important in atherosclerosis [7–9].

Among the sphingolipid metabolites, extracellular S1P signals via G protein-coupled receptors (S1PR1–5) and regulates vascular permeability, angiogenesis and immune cell trafficking[10–13]. S1P bound to HDL mediates the vascular protective functions of this lipoprotein via the S1PR1[14–16]. On the other hand, clinical studies suggest that serum S1P could be a predictor of obstructive coronary artery disease [17]. Interestingly, FTY720, a structural analog of myriocin and a S1P receptor modulator, inhibits atherosclerosis in mouse models even though the mechanisms involved remain unclear [18]. However, the role of S1P in atherosclerosis is likely complex since S1P receptor subtypes exhibit redundant as well as antagonistic signaling properties [10]. For example, S1PR1 and S1PR2 activate the small GTPases Rac and Rho, respectively, and induce cytoskeletal changes to regulate vascular endothelial adherens junctions and permeability in both positive and negative manner[19–23]. The role of the specific S1P receptors in atherosclerotic vascular disease is virtually unknown. In this report, we describe the pro-atherogenic role of S1PR2. In addition, we provide strong evidence that the atherogenic function of S1PR2 involves macrophage retention in the plaques and pro-inflammatory cytokine production.

Materials and Methods

A detailed expanded Materials and Methods section is available online at http://atvb.ahajournals.org).

Animals

Mice with targeted disruption of the *S1pr2* gene [24] were maintained on a mixed C57BL/6×129Sv genetic background. Experiments on KO mice were performed with appropriate WT littermate controls. All procedures involving mice were approved by the University of Connecticut Health Center Animal Care Committee. Mice were fed with a high cholesterol diet (TD31857) and atherosclerosis was analyzed after 13 weeks.

Histology and Immunohistochemistry

Serial cross sections of the aortic leaflet were stained with the following antibodies: rat-anti-MOMA-2 (Serotec), polyclonal anti-S1pr2 [25, 26] (1:200).

Bone Marrow Transplantation

Male *Apoe*$^{-/-}$ mice were lethally irradiated with two 550rad doses, 4 hours apart and reconstituted with 5×10^6 bone marrow cells from *Apoe*$^{-/-}$*S1pr2*$^{-/-}$ and *Apoe*$^{-/-}$*S1pr2*$^{+/+}$ donors. Recipients were maintained on normal chow for 4 weeks, then placed on high fat diet for 13 weeks and analyzed for lesion development.

RNA isolation and RT-PCR analysis

RNA was extracted from mouse aortas and quantitative RT-PCR assay was done using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems).

Cytokine measurements

Mouse serum levels of IL-1β, IL-18 (Invitrogen) and TNF-α (e-Bioscience) were measured by Enzyme Linked ImmunoSorbent assay (ELISA).

RESULTS

Requirement for S1PR2 in atherosclerosis

To investigate the role of the S1PR2 in atherosclerosis, we placed *Apoe*$^{-/-}$*S1pr2*$^{-/-}$ mice and *Apoe*$^{-/-}$*S1pr2*$^{+/+}$ littermate controls on a high fat "Western" diet for 13 weeks [24, 27]. Mouse aortae were dissected and stained with Oil Red O to highlight the atherosclerotic plaques of aortic arch, thoracic and abdominal aorta (Figure 1A). *En face* analysis of atheromatous lesions revealed significant and uniform reduction (~70%) in *Apoe*$^{-/-}$*S1pr2*$^{-/-}$ mice compared to *Apoe*$^{-/-}$*S1pr2*$^{+/+}$ littermates (Figure 1B). We also performed Hematoxylin-Phloxin-Saffron (HPS), Trichrome, and Oil Red O staining of serial cross-sections from the aortic sinus to examine the presence of fibrous caps, connective tissue and lipid deposition, respectively (Figure 1C). As shown in Figure 1D, loss of S1PR2 led to a marked reduction (>80%) in atheromatous plaque area (fibrous tissue and collagen content) as well as lipid deposition. Necrotic core from the plaques of the *Apoe*$^{-/-}$*S1pr2*$^{-/-}$ mice was decreased to a similar extent (Figure S1A) [28]. In sharp contrast, nuclear-specific TUNEL staining did not reveal changes in apoptosis (Figure S1B) [29].

Critical role of myeloid S1PR2 in atheroma development

Given that macrophages play major roles in driving atherosclerotic plaque inflammation and progression [2, 30, 31], we examined the requirement for the S1PR2 in the hematopoietic compartment. Immunostaining for the S1PR2 in the aortic sinus showed that S1PR2 is expressed by macrophage-like foam cells in atherosclerotic plaques (Figure 2A). Indeed, bone marrow derived macrophages (BMDM) express high levels of S1PR2 and S1pr1 transcripts. Lack of S1PR2 in the knockout mice did not change the expression levels of *S1pr1, S1pr3* and *S1pr4* receptor transcripts (Figure 2B). Macrophage infiltration into the vessel wall was examined by immunostaining with a macrophage/monocyte specific antibody (MOMA-2). *Apoe*$^{-/-}$*S1pr2*$^{-/-}$ mice showed a significant reduction in macrophage numbers (~80%) (Figure 2C and D). These data suggest that macrophage retention in the atherosclerotic plaques is regulated by the S1PR2.

We generated bone marrow chimeras by transplanting lethally irradiated *Apoe*$^{-/-}$ mice with *Apoe*$^{-/-}$*S1pr2*$^{+/+}$ or *Apoe*$^{-/-}$*S1pr2*$^{-/-}$ bone marrow. Following reconstitution of the hematopoietic system and after 13 weeks on a "western" diet, *en face* analysis demonstrated a significant reduction in atherosclerotic lesion area throughout the aorta (~ 65%) in mice receiving *Apoe*$^{-/-}$*S1pr2*$^{-/-}$ bone marrow cells compared to *Apoe*$^{-/-}$*S1pr2*$^{+/+}$ counterparts (Figure 3A and 3B). Analysis of peripheral blood monocytes (CD11b^{+}, CD115^{+}), polymorphonuclear leukocytes (CD11b^{+}, CD115^{-}, Gr1^{+}), and T- and B-lymphocytes (CD4^{+}, CD8^{+}, or B220^{+}) revealed no differences between *Apoe*$^{-/-}$*S1pr2*$^{+/+}$ and *Apoe*$^{-/-}$*S1pr2*$^{-/-}$ mice (Figure S2). Atherosclerotic lesions at the aortic root showed decreased lipid and macrophage accumulation (Figure 3C and 3D), suggesting that the S1PR2 signaling in macrophages in the *Apoe*$^{-/-}$ mice is sufficient to promote atherosclerosis.

S1PR2 regulates inflammatory changes in macrophages

Body weight, plasma cholesterol, plasma triglyceride and lipoprotein profiles were not different between *Apoe*$^{-/-}$*S1pr2*$^{-/-}$ mice and littermate controls, suggesting that alterations in sterol and triglyceride metabolism are unlikely to account for S1PR2-induced atherosclerosis (Figure S3). In addition, foam cell differentiation *in vitro* was not altered in *S1pr2*$^{-/-}$ cells compared to *S1pr2*$^{+/+}$ control cells (Figure S4). These data suggest that S1PR2 signaling in macrophages rather than the changes in lipid metabolism or uptake is involved.

Although S1PR1 was proposed to regulate anti-inflammatory events, the role of S1PR2 in macrophage-dependent inflammation is not known [32]. In order to test if S1PR2 regulates inflammatory pathways, we measured serum cytokine levels in WT or *S1pr2*$^{-/-}$ mice after treatment with LPS, which activates the TLR4 pathway. JTE-013, the S1PR2 antagonist inhibited LPS-induced IL-1β and IL-18 levels but not TNF-α (Figure 4A). In addition, *S1pr2*$^{-/-}$ mice had significantly reduced serum IL-1β and IL-18 levels compared to control *S1pr2*$^{+/+}$ mice (Figure 4B). However, we did not observe differences in serum TNF-α. These data suggest that S1PR2 function is important in secretion of some inflammatory cytokines (such as IL-1β and IL-18) *in vivo* and thereby promotes atherosclerosis.

DISCUSSION

Despite the transformative nature of HMG CoA reductase inhibitors in the control of hypercholesterolemia and atherosclerosis, efforts continue to identify novel therapeutic agents. Sphingolipid signaling, which appears to be highly significant in atherogenesis [7, 9], is poorly understood at the mechanistic level. In particular, enzymes in the sphingolipid metabolic cascade and the receptors for sphingolipid metabolites may regulate the complex process of atherosclerosis. As such they represent novel opportunities in therapeutic development. Indeed, S1P receptor modulators were shown to be efficacious in clinical trials for the control of autoimmune inflammation in multiple sclerosis [33, 34]. S1P signaling is complex and may have both pro- and anti-atherosclerotic properties [16, 32]. The effect of S1P in atherosclerotic vascular disease might be different depending on the expression of different S1P receptors in the cells of the vessel wall or infiltrating hematopoietic cells. In addition, local production of S1P may also be important.

In this report, we demonstrate that the S1PR2 promotes atherosclerosis in the *Apoe*$^{-/-}$ mouse model. It is known that this receptor subtype, which potently activates the Rho GTPase, induces vascular permeability in endothelial cells [21]. It is also associated with pathologic angiogenesis and is required for abnormal vascular tuft formation in the retinopathy of prematurity in the mouse [22]. Bone marrow transplant experiments in this report clearly indicate that the function of S1PR2 in the macrophage compartment is critical in the promotion of atherosclerosis. Although the function of S1PR2 in endothelial cells may also contribute to atherosclerosis, it appears that macrophage may be the important cell type in which S1PR2 signaling is critical.

The function of S1PR2 in hematopoietic cells is less understood. Recent studies showed that S1PR2 inhibits chemoattractant-induced motility in murine primary macrophages *in vitro* and may limit macrophage infiltration into the inflamed peritoneum[35]. In these studies, we showed that S1PR2 induced the cAMP/ protein kinase A pathway to inhibit macrophage motility *in vitro*. Given that macrophage accumulation in the atherosclerotic plaque is significantly reduced in *S1pr2*$^{-/-}$ mice, it is likely that S1PR2 in the macrophage regulate trafficking in and out of the plaque. Myeloid adhesion proteins (VLA-4 and CD18) which are important in monocyte-endothelial cell adhesion, were not altered in bone marrow-derived macrophages isolated from *S1pr2*$^{-/-}$ mice (Figure S5). *In vitro* studies do not

support the hypothesis that S1P is a direct chemoattractant for myeloid cells. In fact, it is known that chemokine signals, i.e., those induced by CCL2 (MCP-1) and CXCL11 (SDF-1) regulate the recruitment of monocytes into atherosclerotic plaques [36]. In contrast, S1P action on S1PR2 inhibit motility of macrophages, suggesting that S1PR2 may retain these macrophage / foam cells in the atherosclerotic plaques. Interestingly, the related S1PR1 receptor is known to promote egress of immune cells (especially T and B cells) from lymphoid organs into lymph or blood 37. Thus, S1PR2 may be a retention promoting receptor in myeloid cells.

In addition, we show that the S1PR2 is needed for TLR4-induced expression and secretion of proinflammatory cytokines IL-1β and IL-18. It is known that these two cytokines are important for atherosclerosis [38]. Thus, our findings suggest that specific inhibition of macrophage S1PR2 by pharmacological antagonists may be useful as adjuncts in the control of atherosclerotic vascular disease. Due to the pharmacokinetic and solubility properties of JTE-013, a pharmacologic antagonist for S1PR2 that is not optimal for chronic administration, its effect on atherosclerosis cannot be assessed at present. However, acute administration of JTE-013 was able to inhibit TLR4-stimulated IL-1β and IL-18 release *in vivo*. Development of a specific S1PR2 antagonist with better *in vivo* properties will address pharmacologic utility of this novel pro-atherogenic signaling pathway.

Interestingly, S1PR2 did not regulate TNF-α secretion. In contrast to IL-1β and IL-18, which are secreted by the caspase-dependent inflammasome pathway [39], TNF-α is produced by the classical ER/ Golgi secretory pathway. Thus, S1PR2 signaling may selectively regulate the non-classical cytokine secretion pathway.

In summary, the results of this study provide the first molecular genetic and pharmacologic evidence to support the hypothesis that S1PR2 pathway is a crucial step in the development of atherosclerotic disease. We speculate that similar mechanisms may be involved in human cardiovascular disease. Thus, the specific inhibition of S1PR2 function could lessen lesion formation and facilitates plaque stability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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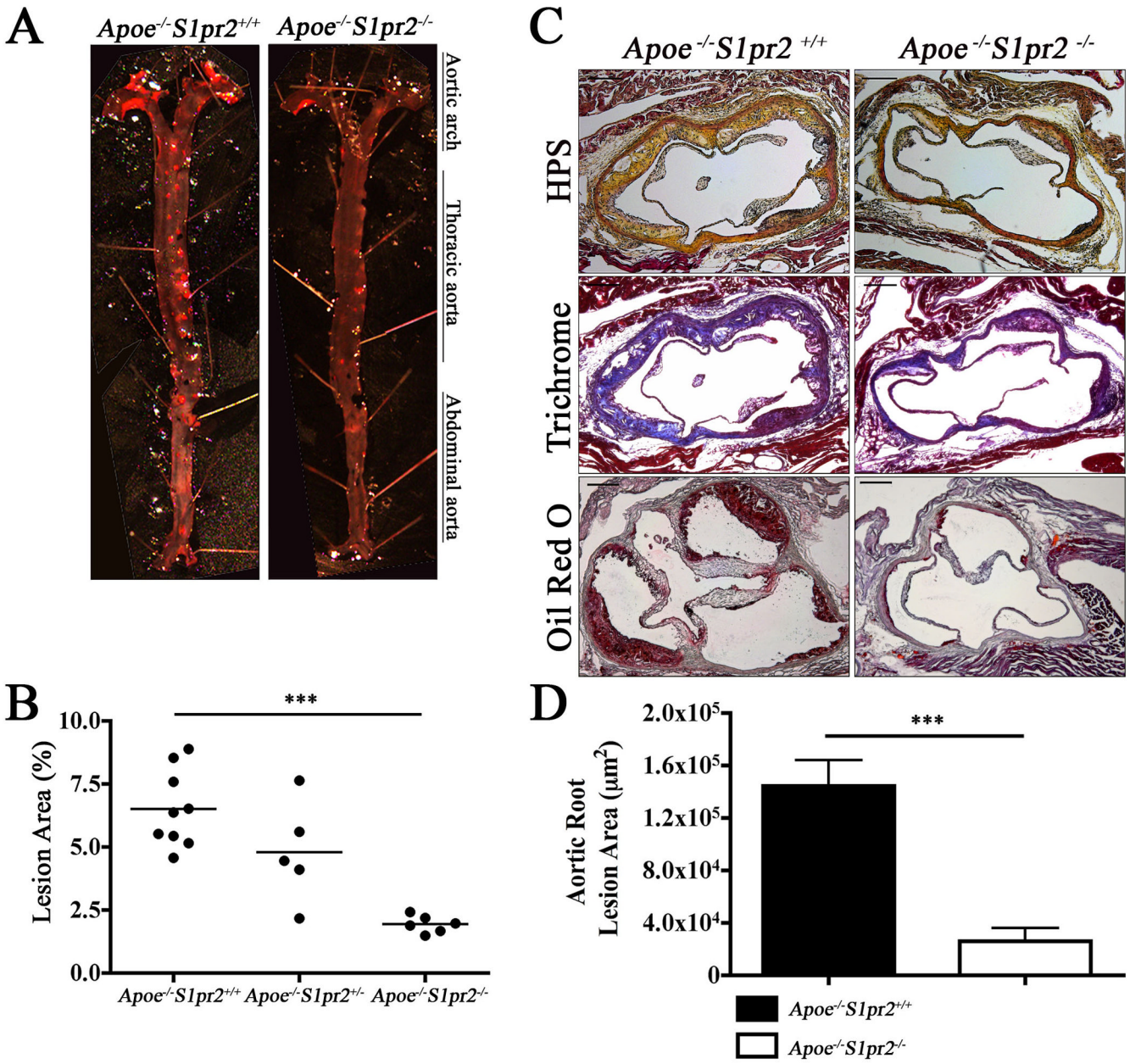

Figure 1. Requirement for S1PR2 in murine atherosclerosis

(A) Aortae from *Apoe*$^{-/-}$*S1pr2*$^{+/+}$ *(n=9)*, *Apoe*$^{-/-}$*S1pr2*$^{+/-}$ *(n=5)* and *Apoe*$^{-/-}$*S1pr2*$^{-/-}$ *(n=6)* mice were stained with Oil Red O to visualize plaques. *En face* analysis is shown from a representative aorta. **(B)** Quantitative analysis of aortae indicates eduction of atherosclerotic plaques in the entire aortic tree of *Apoe*$^{-/-}$*S1pr2*$^{-/-}$ *(n=6)* compared to *Apoe*$^{-/-}$*S1pr2*$^{+/+}$ *(n=9, P* < 0.0001). **(C)** Aortic root cross-sections of *Apoe*$^{-/-}$*S1pr2*$^{+/+}$ *(n=5)* and *Apoe*$^{-/-}$*S1pr2*$^{-/-}$ *(n=5)* stained with Hematoxylin Phloxin and Saffron (HPS) to characterize fibrotic areas, Trichrome to visualize collagen and Oil Red O to stain for lipid deposition. **(D)** Lack of the receptor markedly reduced the plaque area (*P < 0.001*). Scale bar, 100μm.

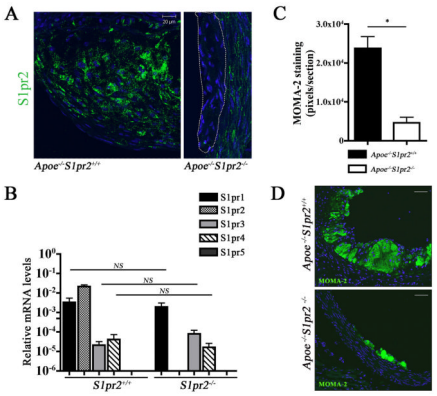

Figure 2. S1PR2 is expressed and regulates macrophage/ foam cell retention in the plaque

(A) Aortic root cross-sections were immunostained for S1PR2 with the affinity-purified antibody. Note positive signals in the atherosclerotic lesion of *Apoe*$^{-/-}$*S1pr2*$^{+/+}$ mice. Small plaques (encircled by dashed line) found in *Apoe*$^{-/-}$*S1pr2*$^{-/-}$ animals are negative for S1PR2 expression. Scale bar, 20μm. **(B)** Expression of S1P receptor transcripts in bone marrow-derived macrophages was determined by Q RT-PCR *(n=3)*. **(C),(D)** Aortic root sections from *Apoe*$^{-/-}$*S1pr2*$^{+/+}$ *(n=3)* and *Apoe*$^{-/-}$*S1pr2*$^{-/-}$ *(n=3)* were immunostained with MOMA-2 (*P* < *0.05*) to visualize macrophage/ foam cells. Scale bar, 50μm.

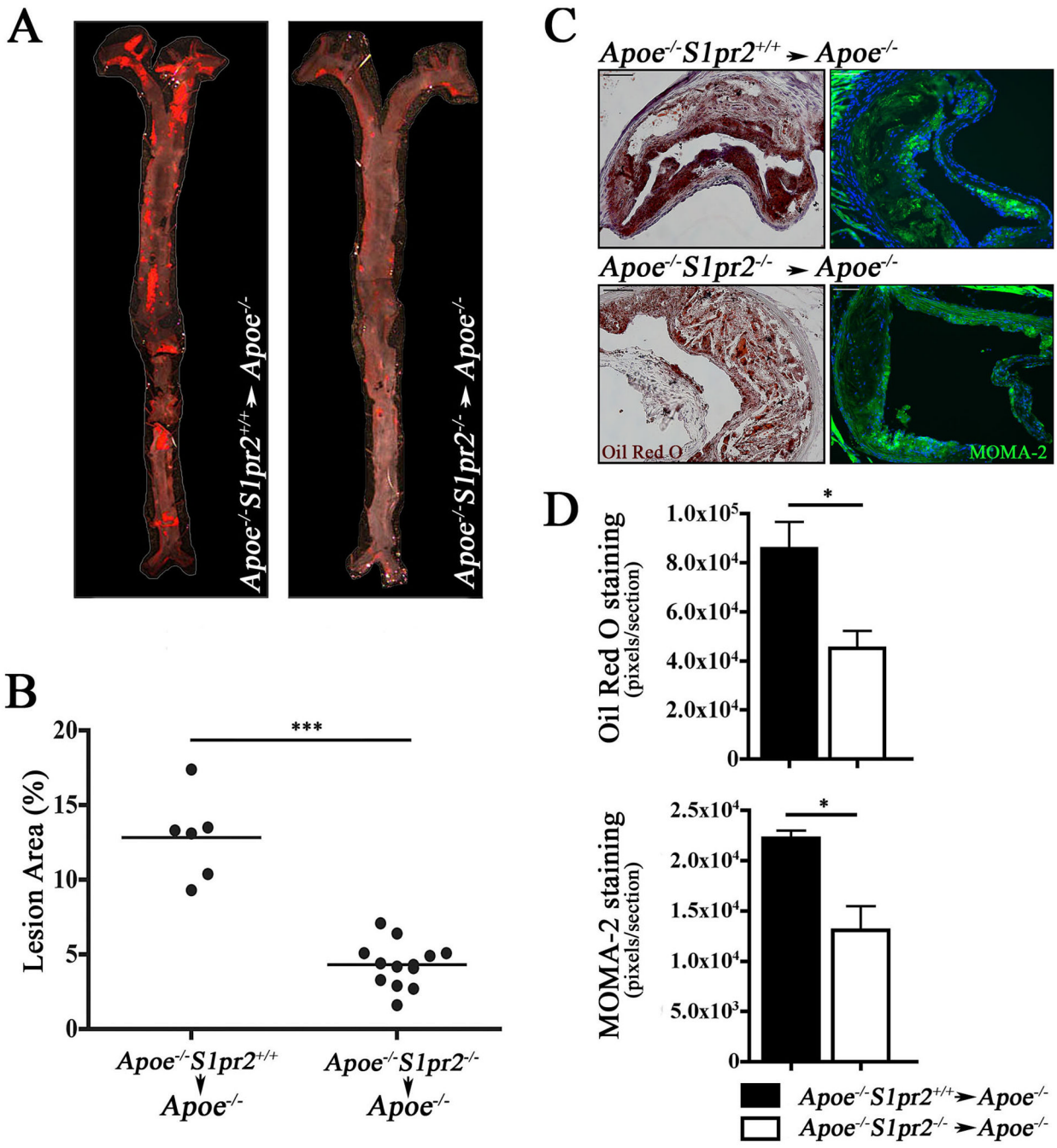

Figure 3. Myeloid S1PR2 mediates pro-atherosclerotic effects

(A) *En face* Oil Red O staining analysis of aortae upon transplantation of *Apoe*$^{-/-}$*S1pr2*$^{+/+}$ *(n=6)* or *Apoe*$^{-/-}$*S1pr2*$^{-/-}$*(n=13)* bone marrow cells to *Apoe*$^{-/-}$*S1pr2*$^{+/+}$ recipients. Mice were placed on high fat diet for 13 weeks and analyzed as described. **(B)** Quantification of *en face* analysis of atherosclerotic plaques. (*P < 0.0001*). **(C)** MOMA-2 (monocytes and macrophages) and Oil Red O (lipid accumulation) staining in aortic root lesions from *Apoe*$^{-/-}$*S1pr2*$^{+/+}$ *(n=3)* or *Apoe*$^{-/-}$*S1pr2*$^{-/-}$ *(n=3)* bone marrow transplanted to *Apoe*$^{-/-}$ *S1pr2*$^{+/+}$ recipients and placed on high fat diet for 13 weeks. Scale bar, 100μm. **(D)** Quantitative analysis of aortic root lesions. Lipid accumulation (*P < 0.04*) and inflammatory

component ($P < 0.03$) of the plaque were significantly reduced in $Apoe^{-/-}S1pr2^{+/+}$ animals transplanted with $Apoe^{-/-}S1pr2^{-/-}$ bone marrow cells.

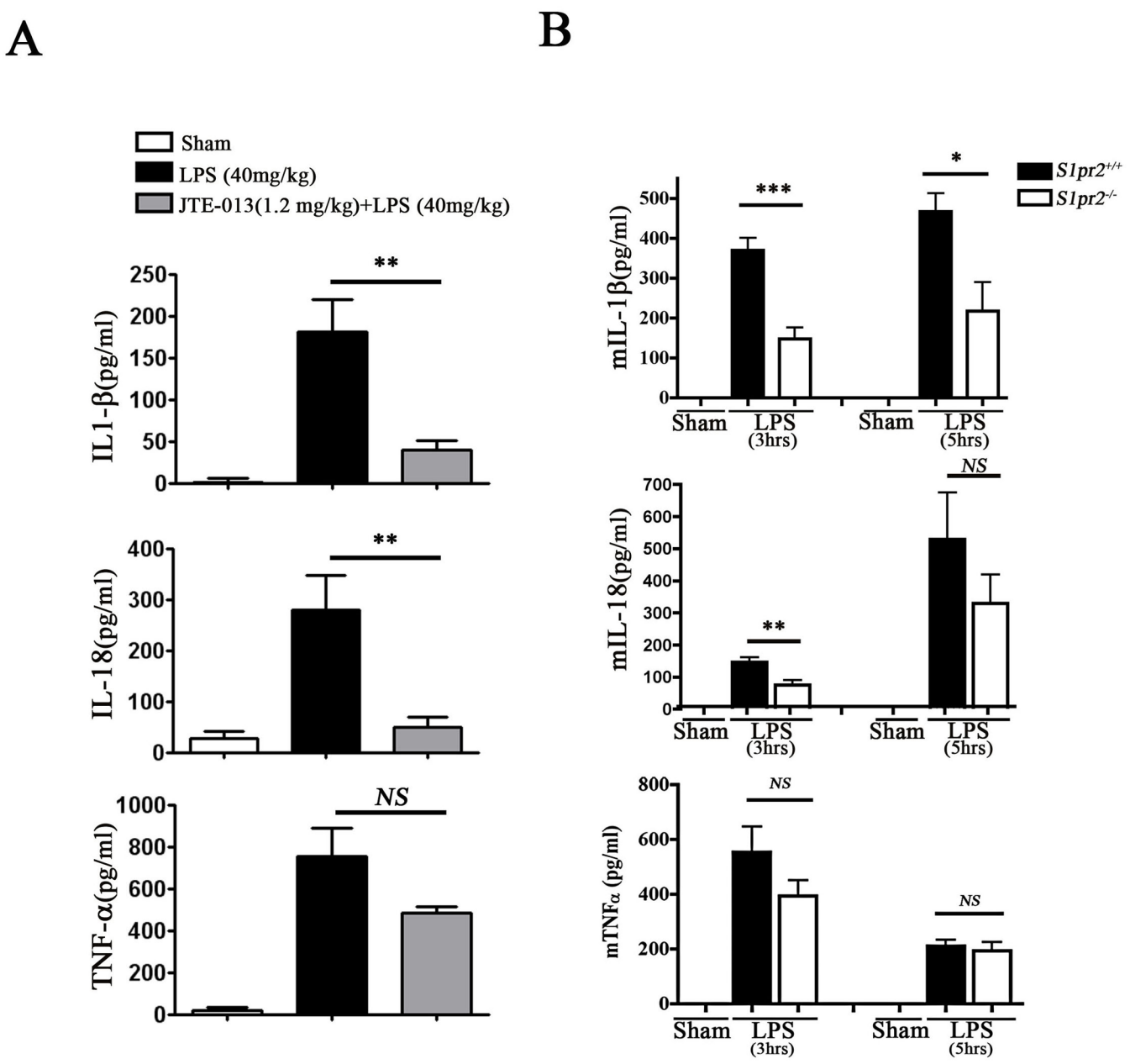

Figure 4. S1PR2 regulates pro-atherosclerotic cytokine release *in vivo*

(A) Mice were treated with LPS (40mg/kg) for 3hrs and plasma cytokine levels were quantified (*n=6–7*). Mice pretreated with the S1PR2 antagonist (JTE-013, 1.2mg/kg, 30min pretreatment, *n=7–9*) had reduced serum IL-1β (*P < 0.003*) and IL-18 (*P < 0.007*) levels. TNF-α levels were not changed significantly. **(B)** Mice were treated with LPS (40mg/kg) for 3hrs and plasma cytokine levels were quantified. *S1pr2*$^{-/-}$ mice had reduced serum IL-1β and IL-18 compared to control group *S1pr2*$^{+/+}$ mice (*n=6*, *P < 0.001* and *P < 0.01*, respectively). At 5hrs of LPS treatment, the difference in serum IL-1β levels between WT and KO animals is still significant (*n=6*, *P < 0.02*).