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Vascular smooth muscle- and myeloid cell-derived integrin α9β1 does not directly mediate the development of atherosclerosis in mice

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Abstract

Background and aims: Sushi, von Willebrand factor type A, EGF pentraxin domain-containing protein 1 (SVEP1), an extracellular matrix protein, is a human coronary artery disease locus that promotes atherosclerosis. We previously demonstrated that SVEP1 induces vascular smooth muscle cell (VSMC) proliferation and an inflammatory phenotype in the arterial wall to enhance the development of atherosclerotic plaque. The only receptor known to interact with SVEP1 is integrin α9β1, a cell surface receptor that is expressed by VSMCs and myeloid lineage-derived monocytes and macrophages. Our previous *in vitro* studies suggested that integrin

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Author contributions

I.-H.J. and N.O.S. conceived of the study. I.-H.J. performed animal and *in vitro* experiments. I.- H.J., J.S.E., K.H.B., J.M.A., Z.X., A.A., and N.O.S. designed and interpreted the experiments. J.S.E., Z.X., and A.A. generated critical reagents. I.-H.J. and N.O.S. wrote the manuscript. All authors reviewed and provided critical editing of the manuscript.

Conflict of interest

N.O.S. has received investigator-initiated research funds from Regeneron Pharmaceuticals unrelated to the content of this study. I.-H.J., J.S.E., A.A., and N.O.S. are listed as co-inventors on patent applications related to SVEP1. The other authors have no conflicts.

Declaration of interests

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: In-Hyuk Jung, Jared S. Elenbaas, Arturo Alisio, and Nathan O. Stitziel are listed as co-inventors on patent applications related to SVEP1. The other authors have no conflicts.

CRediT author statement

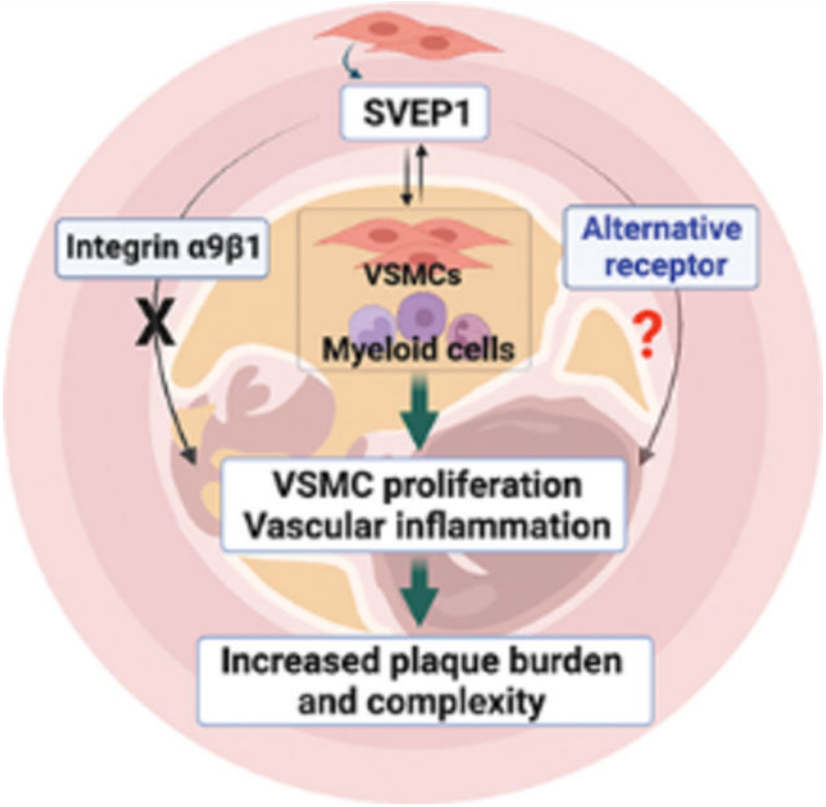
In-Hyuk Jung: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Visualization, Writing – Original Draft, Writing – Review & Editing. **Jared S. Elenbaas**: Methodology, Investigation, Resources, Writing – Review & Editing. **Kendall H. Burks**: Methodology, Investigation, Writing – Review & Editing. **Junedh M. Amrute**: Methodology, Investigation, Writing – Review & Editing. **Zhang Xiangyu**: Methodology, Investigation, Resources, Writing – Review & Editing. **Arturo Alisio**: Methodology, Resources, Supervision, Writing – Review & Editing. **Nathan O. Stitziel**: Conceptualization, Supervision, Funding acquisition, Writing – Original Draft, Writing – Review & Editing.

α9β1 was necessary for SVEP1-induced VSMC proliferation and inflammation; however, the underlying mechanisms mediated by integrin α9β1 in these cell types during the development of atherosclerosis remain poorly understood.

Methods and Results: Here, using cell-specific gene targeting, we investigated the effects of the integrin α9β1 receptor on VSMCs and myeloid cells in mouse models of atherosclerosis. Interestingly, we found that depleting integrin α9β1 in either VSMCs or myeloid cells did not affect the formation or complexity of atherosclerotic plaque in vessels after either 8 or 16 weeks of high fat diet feeding.

Conclusions: Our results indicate that integrin α9β1 in these two cell types does not mediate the *in vivo* effect of SVEP1 in the development of atherosclerosis. Instead, our results suggest either the presence of other potential receptor(s) or alternative integrin α9β1-expressing cell types responsible for SVEP1 induced signaling in the development of atherosclerosis.

Graphical Abstract



1. Introduction

Despite widespread awareness of cardiovascular disease, rupture of unstable atherosclerotic plaques leading to myocardial infarction (MI) remains a leading cause of morbidity and mortality worldwide [1–6]. Understanding the pathophysiology of atherosclerosis and identifying novel potential therapeutic targets for the prevention and treatment of coronary artery disease (CAD) has long been emphasized. To identify potential novel therapeutic

targets, we previously performed a large-scale association study of protein altering variation across the genome to identify specific CAD-associated genes. This led to the identification of a highly conserved missense polymorphism in *SVEP1* (p.D2702G) that associated with an increased risk of CAD in humans without a change in plasma lipid concentrations [7]. SVEP1 is a secreted extracellular matrix (ECM) protein, and the only protein currently known to directly interact with SVEP1 is integrin α9β1 [8].

Integrins are transmembrane heterodimeric receptors consisting of α and β subunits that can mediate the adhesive interactions of cells with ECM proteins and neighboring cells [9, 10]. Emerging evidence suggests that the local microenvironment modulates the expression profile of cell-surface integrins and consequently plays a major role in regulating local susceptibility to atherosclerosis [11]. For example, integrin α1β1 regulates VSMC specific phenotypic modulation [12], and whole body deletion or antibody blockade of integrin α1β1 attenuates atherosclerosis by reducing the migration of leukocytes to plaque lesions and inducing a stable plaque phenotype [13]. Similarly, integrin α5β1 mediates oxidized low density lipoprotein-induced inflammation, and inhibiting integrin α5β1 results in reduced atherosclerosis at early time points [14]. Although deletion of integrin α2β1 has no effect on macrophage infiltration or subsequent development of atherosclerosis [15], integrin α8β1 is required for maintenance of the VSMC contractile phenotype [16, 17] and integrin α8β1 deletion aggravates plaque formation and induces neointimal thickening [18]. In the context of functional study of integrin α9β1, knocking down integrin α9β1 in VSMCs resulted in switching from a contractile to a synthetic phenotype [19]. Despite these previous studies, the mechanisms mediated by integrin α9β1 during the pathogenesis of atherosclerosis remain undefined.

We previously found that SVEP1, whose only known direct protein interaction is with integrin α9β1, promotes the development of atherosclerosis and increases plaque complexity in mice [20]. In the developing plaque, *SVEP1* is expressed by VSMCs and we found that VSMC-derived SVEP1 promoted VSMC proliferation and dysregulation of key differentiation pathways. Beyond its influence on VSMCs, we also found that whole body and VSMC-specific depletion of SVEP1 within the arterial wall reduced the recruitment of monocytes into the developing plaque, ultimately resulting in lower plaque macrophage content. Due to the close association of SVEP1 with integrin α9β1, we further explored if integrin α9β1 – which is expressed by VSMCs as well as myeloid lineage cells including monocytes and macrophages – might mediate the SVEP1-induced effects on cell behavior. Indeed, our previous *in vitro* results suggested that integrin α9β1 might be necessary for the SVEP1-mediated effects on VSMC proliferation and leukocyte recruitment.

In the context of these results, we sought to determine whether integrin α9β1 depletion *in vivo* would alter neointima formation and phenocopy SVEP1 depletion in an animal model of atherosclerosis, thereby clarifying if integrin α9β1 mediated either the cell autonomous or cell non-autonomous effects of SVEP1 in the pathogenesis of atherosclerosis. To address these hypotheses, we investigated the role of integrin α9β1 in atherosclerotic plaque development by generating VSMC and myeloid cell lineage-specific targeted mouse models separately.

2. Materials and methods

2.1 Mice

Itga9$^{flox/flox}$ (*Itga9*$^{fl/fl}$) mice [21] were a gift from D. Sheppard (University of California San Francisco, United States) and L. Van De Water (Albany Medical College, United States). Briefly, we crossed *Itga9*$^{fl/fl}$ mice with *Myh11-CreER*^{T2} (#019079, the Jackson laboratory, United States) to generate *Itga9*$^{fl/+}$*Myh11-CreER*^{T2} mice. *Itga9*$^{fl/+}$*Myh11-CreER*^{T2} male mice were then crossed with *Apoe*$^{-/-}$ females. We maintained *Itga9*$^{fl/+}$*Myh11-CreER*^{T2}*Apoe*$^{-/-}$ males and *Itga9*$^{fl/+}$*Apoe*$^{-/-}$ females as breeders to generate experimental *Itga9*$^{fl/fl}$ *Myh11-CreER*^{T2}*Apoe*$^{-/-}$ (*Itga9* $^{SMC-/-}$) and control *Itga9*$^{+/+}$*Myh11-CreER*^{T2}*Apoe*$^{-/-}$ (*Itga9*$^{SMC+/+}$) littermates. To activate Cre-recombinase, mice were injected intraperitoneally with 1 mg of tamoxifen (Sigma-Aldrich, United States) in 0.1 ml of peanut oil (Sigma-Aldrich, United States) for 10 consecutive days starting at 6 weeks of age. Tamoxifen treatment was performed with all experimental and control mice. *LysM-Cre* (kindly provided from B. Razani from Washington University School of Medicine, United States) mice were crossed with *Itga9*$^{fl/fl}$ mice to generate *Itga9*$^{fl/+}$*LysM-Cre* mice. *Itga9*$^{fl/+}$*LysM-Cre* mice were then crossed with *Apoe*$^{-/-}$. *Itga9*$^{fl/+}$*LysM-CreApoe*$^{-/-}$ mice were maintained as breeders to generate experimental *Itga9*$^{fl/fl}$ *LysM-CreApoe*$^{-/-}$ (*Itga9*$^{MYE-/-}$) and control *Itga9*$^{+/+}$*LysM-CreApoe*$^{-/-}$ (*Itga9*$^{MYE+/+}$) littermates. All mice were housed in a pathogen-free environment at the Washington University School of Medicine animal facility and maintained on a 12-hour light/ 12-hour dark cycle with a room temperature of 22° ± 1°C.

2.2 Diet and tissue preparation for assessment of atherosclerosis

All experimental mice were fed a diet containing 21% fat by weight and 0.2% cholesterol (#TD88137, Envigo Teklad, United States) for 8 and 16 weeks starting at 8 weeks of age. After HFD feeding, blood was collected from the retro-orbital plexus after 12 hr of fasting. Detailed tissue processing including plasma, aortic roots, and aortas including whole arteries from the aortic arch to the iliac for *en face* analysis, brachiocephalic arteries (BCA) as well as thoracic arteries was described previously [20]. Total cholesterol, triglycerides, and glucose in plasma were measured using the appropriate kit (all purchased from Cell Biolabs, Inc, United States). Briefly, isolated aortas were opened longitudinally, following fixation with 4% paraformaldehyde for *en face* analysis. Aortas were then stained with 0.5% Oil Red O in propylene glycol (Sigma-Aldrich, United States), followed by de-staining with 85% propylene glycol to reduce background staining. For analysis of plaque in the aortic root, isolated hearts were fixed with 4% paraformaldehyde, and embedded into optimal cutting temperature (OCT) compound (Sakura Finetek, Japan). Tissues were sectioned at 5 μm, and further processed for Oil Red O staining. Measurement of plaque was performed using 6–8 sections per artery to get the average value of size. The atherosclerotic plaque area was digitized and calculated using AxioVison (Carl Zeiss, Germany).

For details regarding preparation and culture of BMDMs and pMACs, quantitative real time PCR, immunohistochemistry and immunofluorescent staining, flow cytometry, RNAscope *in situ* hybridization, and statistical analysis, please see the supplementary data.

3. Results

3.1 VSMC-specific Itga9 depletion does not alter atherosclerotic plaque development

We previously found that *Itga9* was expressed by VSMCs in the mouse aortic root as well as human arterial tissue [20]. To confirm its expression in our current mouse models, we performed *in situ* hybridization on arterial tissues. Consistent with our previous results, we found that *Itga9* was widely expressed in the aortic root, brachiocephalic artery (BCA), thoracic artery from 8-week-old wild type animals (Supplementary Fig. 1A). We also observed *Itga9* expression in atherosclerotic plaque (Supplementary Fig. 1B), mostly colocalizing with VSMCs. Given the hypothesis that VSMC-derived cells are the predominant source of *Itga9* in arterial tissue, we generated $Apoe^{-/-}$ mice with VSMC-specific *Itga9* knockout ($Itga9^{flox/flox}Myh11cre^{ER/T2}Apoe^{-/-}$; hereafter referred to as $Itga9^{SMC-/-}$) along with wild type controls ($Itga9^{+/+}Myh11cre^{ER/T2}Apoe^{-/-}$; hereafter referred to as $Itga9^{SMC+/+}$). Due to its broad expression in multiple cell types in vascular tissue including endothelial cells [22–24], VSMCs [25], and myeloid cells [26, 27], ITGA9 expression was assessed by immunofluorescence staining first in BCA sections from 8-week-old mice to obviate concerns surrounding cellular complexity. Although we detected ITGA9 expression in endothelium from both genotype groups, $Itga9^{SMC-/-}$ mice showed negligible ITGA9 protein expression in VSMCs compared to control mice (Supplementary Fig. 2A).

We then sought to determine whether VSMC-specific depletion of integrin α9β1 would phenocopy VSMC-specific depletion of SVEP1 by feeding $Itga9^{SMC+/+}$ and $Itga9^{SMC-/-}$ mice a high fat diet (HFD) for either 8 or 16 weeks. VSMC-specific deficiency of *Itga9* did not alter body weight (Fig. 1A), plasma total cholesterol, triglycerides, or glucose (Fig. 1B) at either time point. We confirmed that ITGA9 was depleted in VSMCs from atherosclerotic plaques of the aortic root from $Itga9^{SMC-/-}$ mice as compared to $Itga9^{SMC+/+}$ animals, while endothelial ITGA9 expression was unchanged between genotype groups (Supplementary Fig. 2B). We were unable to detect any differences in the size of atherosclerotic plaque in either the aortic arch or the whole aorta by *en face* preparation (Fig. 1C), and aortic root regions (Fig. 1D) between experimental groups. Beyond size, atherosclerotic plaques from $Itga9^{SMC-/-}$ mice did not show any changes in area staining positive for VSMCs (Fig. 1E), macrophages (Supplementary Fig. 3A), necrotic core (Supplementary Fig. 3B), or collagen (Supplementary Fig. 3C), all indicators of plaque characteristics. Together, these data suggest that integrin α9β1 expressed on VSMC is likely not mediating the effects of VSMC-specific depletion of SVEP1 that results in smaller and less complex plaques at these time points.

3.2 Myeloid cell lineage specific Itga9 depletion does not alter atherosclerotic plaque development

It has been recently reported that integrin α9β1 is expressed on neutrophils and that myeloid lineage-specific deficiency inhibits arterial thrombosis [26] along with improving the short- and long-term effects of stroke [27] in mice. Therefore, to determine if myeloid cell-specific *Itga9* deficiency affects atherosclerotic plaque development, we generated $Itga9^{flox/flox}LysMcre$ mice in the hyperlipidemic $Apoe^{-/-}$ background

(*Itga9*$^{flox/flox}$*LysMcreApoe*$^{-/-}$; hereafter referred to as *Itga9*$^{MYE-/-}$) along with wild type control animals (*Itga9*$^{+/+}$*LysMcreApoe*$^{-/-}$; hereafter referred to as *Itga9*$^{MYE+/+}$). We first confirmed the lack of *Itga9* expression in macrophages differentiated in the presence of macrophage-colony stimulating factor from the bone marrow (BMDM) as well as on thioglycolate induced-peritoneal macrophages (pMAC) isolated from *Itga9*$^{MYE-/-}$ mice (Supplementary Fig. 4A). Immunofluorescence staining also demonstrated a lack of ITGA9 protein on BMDMs from *Itga9*$^{MYE-/-}$ mice (Supplementary Fig. 4B).

We next sought to determine whether myeloid cell lineage-specific integrin α9β1 depletion would phenocopy VSMC-specific depletion of SVEP1 by feeding *Itga9*$^{MYE+/+}$ and *Itga9*$^{MYE-/-}$ mice a HFD for 8 and 16 weeks. Similar to VSMC-specific depletion, there were no differences in body weight (Fig. 2A), plasma total cholesterol, triglycerides, or glucose (Fig. 2B) at either time point between genotype groups. In atherosclerotic plaque from aortic root tissues, we confirmed there was negligible colocalization of ITGA9 with the macrophage marker Mac3 in *Itga9*$^{MYE-/-}$ mice when compared with control *Itga9*$^{MYE+/+}$ animals (Supplementary Fig. 4C) while ITGA9 expression on endothelial layers from both genotype groups was unchanged. As with the VSMC-specific model, we also did not observe any significant differences in the size of atherosclerotic plaques from the aortic arch or whole aorta by *en face* preparation (Fig. 2C) as well as in the aortic root (Fig. 2D). We found no differences between genotype groups in advanced plaque phenotypes including macrophage staining (Fig. 2E), VSMC staining (Supplementary Fig. 5A), necrotic core (Supplementary Fig. 5B), and collagen content (Supplementary Fig. 5C).

To determine if our results might be confounded by altered proportions of circulating monocytes, we investigated whether myeloid lineage-specific *Itga9* deletion affected the composition of monocyte subsets as well as other leukocytes. Using flow cytometry (gating strategy provided in Supplementary Fig. 6A), we found Ly6C^{hi} and Ly6C^{low} monocytes showed high expression of ITGA9 compared to regulatory T (Treg) cells, neutrophils, and platelets in peripheral blood (Supplementary Fig. 6B). After feeding *Itga9*$^{MYE+/+}$ and *Itga9*$^{MYE-/-}$ mice a HFD for 8 and 16 weeks, we did not appreciate any difference between genotype groups in the percentage of blood leukocyte subsets including Tregs, neutrophils, total monocytes (including both Ly6C^{hi} and Ly6C^{low}), and platelets at either the 8 (Supplementary Fig. 6C) or 16 (Supplementary Fig. 6D) week time point. Together, these data suggest that myeloid lineage-specific Itga9 knockout does not affect the development of atherosclerosis, plaque complexity, or the composition of circulating leukocyte subsets in the peripheral blood at these time points.

4. Discussion

As primary transmembrane receptors responsible for transducing signals between the ECM and cytoskeleton, integrins have broad biological functions. The effect of integrin α9β1 on VSMC phenotype switching [19] and vascular remodeling [25] has recently been described. Among myeloid cells, integrin α9β1 is expressed on neutrophils, and interactions with its ligands VCAM-1 and tenascin-C are critical for neutrophil migration [28]. In the context of cardiovascular disease, previous studies have used murine models of arterial thrombosis [26]

and ischemic stroke [27] to investigate the function of integrin α9β1 in neutrophils. Beyond this, it is not known if integrin α9β1 impacts the pathogenesis of atherosclerosis.

Our previous investigation of SVEP1 raised the possibility that it might interact with integrin α9β1 on VSMCs by an autocrine mechanism and on circulating monocytes and neointimal macrophages by a paracrine mechanism to promote atherosclerosis [20]. To determine if integrin α9β1 was indeed responsible for mediating the pro-atherogenic effect of SVEP1, we generated atheroprone animals with tissue specific *Itga9* depletion in these two cell types and studied the development of atherosclerosis at 8 and 16 weeks of HFD after confirming that integrin α9 mRNA and protein levels were depleted in both models (although a caveat in any Cre-expression model is that Cre-mediated recombination may not be fully effective in all Cre-expressing cells). Unexpectedly, we did not observe any differences between genotype groups in the size or complexity of atherosclerotic plaques in either model at either time point, suggesting that integrin α9β1 in these cell types does not have a significant impact on the pathogenesis of atherosclerosis.

As an ECM protein, SVEP1 is causally associated with numerous human diseases including CAD, hypertension, and type 2 diabetes [29], and the only protein known to interact with SVEP1 is integrin α9β1. Interestingly, a recent genome-wide association study found that a protein altering variant in *SVEP1* strongly associates with adenosine diphosphate (ADP)-induced platelet aggregation [30]; integrin α9β1 is not expressed on platelets (Supplementary Fig. 6B), suggesting that at least one or more additional SVEP1 receptors likely exist. Further studies are needed to identify the SVEP1 binding partners that might mediate its effect on platelets.

Lethal radiation followed by bone marrow transplantation causes a complete loss of VSMC investment in blood vessels of neural crest origin including BCA, carotid artery, and aortic arch [31]. As a result, we did not pursue studies on the combined effect of depleting integrin α9β1 on VSMCs and myeloid cells simultaneously in an *in vivo* model. Additional studies in other animal models and cell types will be needed to further investigate the roles of integrin α9β1 in vascular biology. For example, lymphatic endothelial cells (LECs) express integrin α9β1 and blocking integrin α9β1 signaling in LECs reduced lymphocyte egress in a model of autoimmune encephalomyelitis [24] while other studies have found that the interaction between integrin α9β1 and vascular endothelial growth factors (VEGF-C and -D) mediates adhesion and migration of endothelial cells in lymphangiogenesis [32]. In addition, the interaction of integrin α9β1 with fibronectin-EIIIA [33], and EMILIN1, an ECM multidomain glycoprotein [34], are crucial for lymphatic valve formation. Paralleling these observations of integrin α9β1 expression and mechanistic activity in LECs, we found that intimal endothelial cells of BCA and aortic root tissues express ITGA9. As a result, we cannot exclude the possibility that arterial endothelial cell-derived ITGA9 could play a role in regulating endothelial dysfunction or pro-inflammatory endothelial activation during atherosclerotic lesion initiation and progression.

Similarly, because we evaluated the effect of integrin α9β1 depletion on atherosclerosis at 8 and 16 weeks of HFD (time points we previously found reduced atherosclerotic plaque burden and complexity in models of SVEP1 deficiency), we cannot exclude a role for

integrin α9β1 in the earliest stages of disease. For example, the ECM protein fibronectin containing extra domain A (Fn-EDA) is an endogenous ligand for integrin α9β1 [35] and it appears that endothelial cell-derived Fn-EDA contributes to early atherosclerosis after only 4 weeks of HFD [36]. The popular apolipoprotein E-deficient mouse model of atherosclerosis develops atherosclerotic plaque quickly [37] which could theoretically also contribute to the masking of a subtle role for integrin α9β1 in the earliest stages of disease. Regardless, although we cannot rule out an effect of integrin α9β1 in terms of interaction with Fn-EDA in the earliest stages of atherosclerotic plaque development, our results provide strong evidence that depleting integrin α9β1 on the surface of VSCMs and myeloid cells does not phenocopy the effect of depleting SVEP1. Along with the recent discovery that SVEP1 may play a role in platelet reactivity, our results support further studies focused on discovering novel receptors for SVEP1 that might mediate its effect on the development of CAD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- Integrin α9β1 is the only known receptor for the atherogenic ECM protein SVEP1
- Depleting Integrin α9β1 in key cell types does not alter atherosclerosis
- Other receptors mediating the effect of SVEP1 on atherosclerosis likely exist

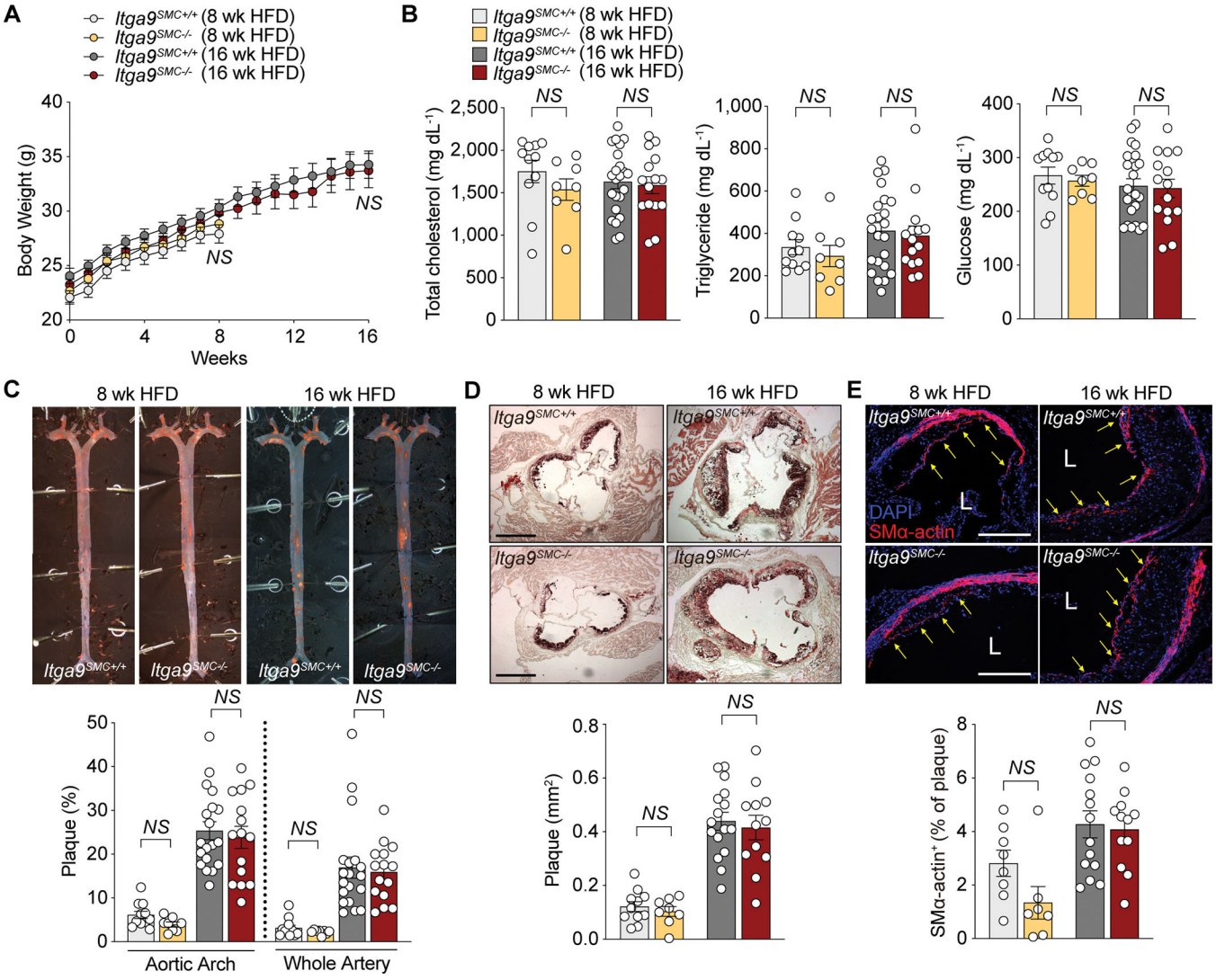

Fig. 1.

VSMC-derived *Itga9* deficiency does not regulate atherosclerosis in mice. (A) Body weight of $Itga9^{SMC+/+}$ and $Itga9^{SMC-/-}$ mice during HFD feeding for 8 and 16 weeks. (B) Lipid profiling in plasma including total cholesterol, triglycerides, and glucose in mice. (C) Oil red O-stained *en face* aortas. Quantification of stained area in each aortic arch and whole aorta. (D) Oil red O-stained aortic root sections. Quantification of stained area. Scale bar, 500 μm. (E) SMα-actin immunofluorescence staining of aortic roots. Quantification of SMα-actin as a percentage of a plaque. Scale bar, 200 μm. L, lumen. $n = 8$ to 21 per group in (A), $n = 8$ to 23 per group in (B), $n = 8$ to 19 per group in (C), $n = 8$ to 16 per group in (D), and $n = 8$ to 14 per group in (E). Data were shown as means ± SEM and analyzed with one-way ANOVA (A) or unpaired nonparametric Mann-Whitney test (B to E). *NS*, not significant.

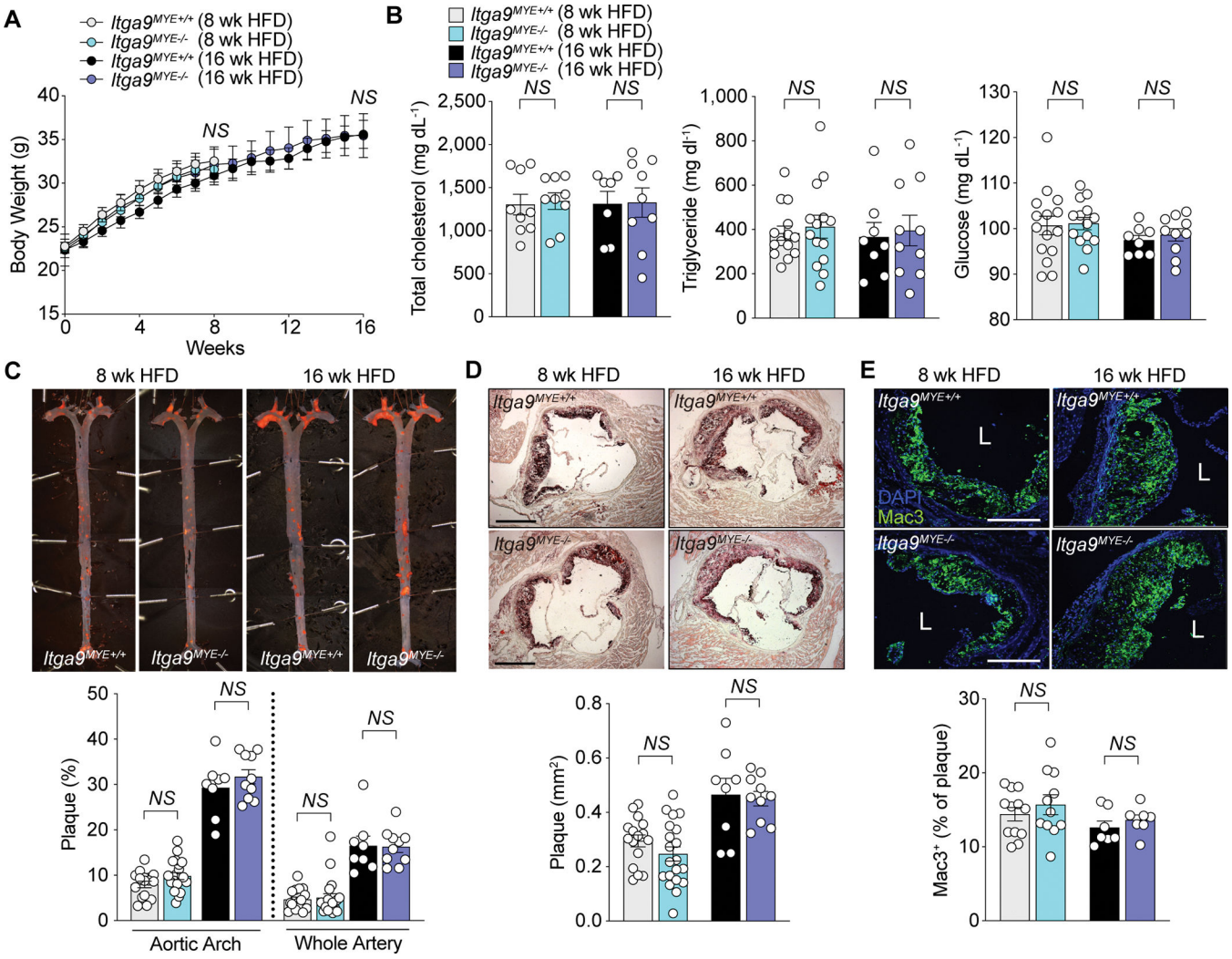

Fig. 2.

Myeloid cell lineage-specific *Itga9* deficiency does not regulate atherosclerosis in mice. (A) Body weight of *Itga9*$^{MYE+/+}$ and *Itga9*$^{MYE-/-}$ mice during HFD feeding for 8 and 16 weeks. (B) Lipid profiling in plasma including total cholesterol, triglycerides, and glucose in mice. (C) Oil red O-stained *en face* aortas. Quantification of stained area in each aortic arch and whole aorta. (D) Oil red O-stained aortic root sections. Quantification of stained area. Scale bar, 500 μm. (E) Mac3 immunofluorescence staining of aortic roots. Quantification of Mac3 as a percentage of a plaque. Scale bar, 200 μm. L, lumen. n = 6 to 22 per group in (A), n = 7 to 15 per group in (B), n = 8 to 20 per group in (C), n = 8 to 20 per group in (D), and n = 7 to 12 per group in (E). Data were shown as means ± SEM and analyzed with one-way ANOVA (A) or unpaired nonparametric Mann-Whitney test (B to E). *NS*, not significant.