1. Introduction

Atherosclerosis is a multifactorial disease that is characterized by chronic inflammation of the arterial wall. Both innate and adaptive immune responses play vital roles in the process of atherosclerosis, as reflected by the large number of inflammatory cells within atherosclerotic plaques, including mainly monocyte-derived macrophages and T-lymphocytes. The inflammatory cells that invade lesions produce large amounts of soluble inflammatory cytokines and chemokines, which are critically important in the initiation and development of the disease.

Secreted pro-inflammatory cytokines such as interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α), interleukin (IL)-1, IL-12, IL-18, and many chemokines have pro-atherosclerotic effects, while some anti-inflammatory cytokines such as IL-10 and IL-33 have anti-atherosclerotic effects during all stages of atherosclerosis.

IL-9 was first described as a T cell and mast cell growth factor and has pleiotropic functions in the immune system. Multiple cell types, including T cells, mast cells, and natural killer T cells (NKTs), produce IL-9. IL-9 acts on a variety of cells through the IL-9 receptor (IL-9R), which is composed of IL-9Rα and the common γ chain shared by IL-2, IL-4, and IL-7.
The binding of IL-9 to IL-9R mainly activates JAK1 and JAK3, which leads to the downstream activation of signal transducer and activator of transcription (STAT) 1, 3, and 5.\textsuperscript{9,10}

Accumulated evidence has demonstrated that IL-9 plays an important role in the regulation of inflammatory responses. It has been reported that IL-9 is related to the pathogenesis of allergic inflammation,\textsuperscript{11,12} intestinal parasites,\textsuperscript{13,14} infectious diseases,\textsuperscript{15,16} cancer immunity,\textsuperscript{17,18} and autoimmune inflammatory disorders such as experimental autoimmune encephalomyelitis (EAE).\textsuperscript{19–21} Recently, IL-9 was also found to be increased in plasma and carotid plaques in patients with carotid and coronary atherosclerosis.\textsuperscript{22} However, the role of IL-9 in atherosclerosis remains largely unclear. In this study, we investigated whether IL-9 was involved in the progression of atherosclerosis and further explored the associated mechanisms. We demonstrated that IL-9 aggravated the development of atherosclerosis in ApoE−/− mice partially through induction of VCAM-1 expression and inflammatory cell infiltration.

2. Methods

Expanded Methods descriptions are available in the Supplementary material online.

2.1 Mice and treatment groups

Male C57BL/6 and ApoE−/− mice aged 6–8 weeks weighing 20–25 g were purchased from Beijing University (Beijing, China). The mice were maintained in a specific pathogen-free facility on a 12 h light:12 h dark cycle at 25°C. C57BL/6 and ApoE−/− mice were fed a chow diet or high-fat diet (western type diet, containing 0.15% cholesterol, 21% fat). All animals were cared for in accordance with the NIH guidelines and all animal studies were supported by the Animal Care and Utilization Committee of Huazhong University of Science and Technology. The detailed treatment groups are provided in the Supplementary material online, Methods.

2.2 Patient selection and sample collection

The clinical investigation was performed based on the standards of the Declaration of Helsinki. The experiment was supported by the Ethics Committee of Tongji Medical College of Huazhong University of Science and Technology, and all the selected patients provided written informed consent. The experiment protocols are provided in the Supplementary material online, Methods.

3. Results

3.1 IL-9R is expressed in endothelial cells and aortic tissues

IL-9R is widely expressed in T cells, mast cells, airway smooth muscle cells, epithelial cells, and other cell types.\textsuperscript{23} Recently, IL-9R was also found in human carotid plaques.\textsuperscript{22} However, the expression of IL-9R in endothelial cells and murine aortic tissues has not been previously reported. We investigated the expression of IL-9R in endothelial cells and murine aortic tissues using real-time PCR and western blotting. For detecting the expression of IL-9R in mouse aortic endothelial cells (MAECs), splenocytes were used as a positive control. The results showed that IL-9R was present in MAECs (Supplementary material online, Figure S1A). Meanwhile, IL-9R was expressed in the aortic tissues of C57BL/6 control mice (18 weeks, fed a chow diet or western diet) and ApoE−/− mice (18 weeks, fed a chow diet or western diet), and its expression was similar among the groups independent of atherosclerotic lesions (Supplementary material online, Figure S1B and C). In addition, we determined the expression of IL-9R in the early plaque (western diet for 6 weeks) and advanced plaque (western diet for 24 weeks), and different sites (abdominal aorta and thoracic aorta including the aortic arch) in ApoE−/− mice (western diet for 10 weeks) by RT–PCR and western blotting. The result showed that there was also no significant difference in IL-9R expression among the groups (Supplementary material online, Figure S2).

3.2 IL-9 is increased in ApoE−/− mice

Next, to examine the involvement of IL-9 in atherosclerosis, we investigated IL-9 levels and mRNA expression in the plasma and aortic arches. We found that IL-9 levels in the plasma of ApoE−/− mice were obviously increased compared with those in C57BL/6 mice (western diet for 10 weeks) (314.41 ± 32.19 pg/mL vs. 150.56 ± 18.11 pg/mL, P < 0.01) (Figure 1A). At the same time, IL-9 mRNA expression in ApoE−/− mice (western diet for 10 weeks) in aortic arches was increased compared with those in C57BL/6 mice (western diet for 10 weeks) (P < 0.05) (Figure 1B).
almost three-fold higher than those in C57 mice (western diet for 10 weeks) (Figure 1B), while RT–qPCR demonstrated a similar pattern of IL-9 expression in early plaque and advanced plaque (Figure 1C).

Multiple cell types, including T cells, and innate immune cells, could produce IL-9. To determine which leukocytes account for the increase of IL-9 in ApoE−/− mice fed a western diet, we performed intracellular cytokine staining combined with staining for some surface markers. Over 60% of the IL-9-secreting leukocytes were CD45+CD3+CD19− innate immune cells, while <40% IL-9+ leukocytes were CD45+CD3+ and CD45−CD19+ lymphocytes (Supplementary material online, Figure S3), indicating that innate immune cells mainly account for the production of IL-9 during atherosclerosis.

3.3 Neutralization of endogenous IL-9 protects against atherosclerosis development

Then, to determine the impact of IL-9 in atherosclerosis, the anti-IL-9 neutralizing mAb was used to block endogenous IL-9 in ApoE−/− mice (6–8 weeks, fed a western diet containing 0.15% cholesterol and 21% fat). The results showed that the aortic plaque burden was significantly smaller in mice treated with IL-9 mAb (3.68 ± 0.51%) compared with the isotype control group (6.46 ± 0.94%) or PBS group (6.31 ± 0.88%) (P < 0.05) (Figure 2A and B). Meanwhile, the plaque size in the aortic root was obviously decreased in mice treated with IL-9 mAb (240.18 ± 12.46 × 103 μm²) compared with the isotype control group (371.40 ± 28.84 × 103 μm²) or PBS group (384.88 ± 20.48 × 103 μm²) (P < 0.01) (Figure 2C and D). At the same time, IL-9 mAb treatment significantly decreased the areas of Mac-3+ macrophage (Figure 2G and H) and CD3+ T cell (Figure 2E and F) infiltration in plaques compared with the isotype control group or PBS group. In addition, treatment with IL-9 mAb did not affect body weight, plasma total cholesterol, triglycerides, or high-density lipoprotein cholesterol (HDLC) (Table 1).

3.4 Treatment with rIL-9 exacerbates atherosclerosis development

Then, to further demonstrate the role of IL-9 in the development of atherosclerosis, we treated the ApoE−/− mice (6–8 weeks, fed a western diet containing 0.15% cholesterol and 21% fat) with recombinant IL-9 for 10 weeks. Administration of rIL-9 led to a significant elevation of circulating IL9 levels in plasma compared with PBS group (629.40 ± 105.53 pg/mL vs. 310.30 ± 34.93 pg/mL, P < 0.01) (Supplementary material online, Figure S4), while we found no change in the number of mast cells in the adventitia in mice treated with rIL9, suggesting that at this dosage and time, IL-9 did not promote mast cell proliferation and activation (Supplementary material online, Figure S5). In addition, treatment with rIL-9 did not affect body weight, plasma total cholesterol, triglycerides, or high-density lipoprotein cholesterol (HDLC) (Table 2).

The lesion area was first determined in aortas pinned out en face. The aortic plaque burden was significantly greater in mice treated with rIL-9 compared with the PBS-treated group (10.62 ± 1.29% vs. 5.76 ± 0.74%, P < 0.01) (Figure 3A and B). Meanwhile, the plaque size in the aortic root was analysed. The atherosclerotic lesion area was obviously increased in mice treated with rIL-9 compared with the PBS-treated group (520.94 ± 41.54 × 103 μm² vs. 363.96 ± 30.97 × 103 μm², P < 0.01) (Figure 3C and D). Simultaneously, rIL-9 treatment significantly increased the areas of Mac-3+ macrophage and CD3+ T cell infiltration in plaques (Figure 3E and F). In addition, on better characterization of the lesions, we performed SM-22α, Masson, Sirius red, Haematoxylin–Eosin (H&E), and TUNEL stainings of lesions, and found no significant difference in the content of smooth muscle cells (SMC), collagen, necrotic cores, and apoptotic cells between the two groups (Figure 2E and F and Supplementary material online, Figure S6), indicating that IL-9 increased plaque size independent of them.

3.5 Treatment with rIL-9 does not alter T cell or monocyte subset profiles

To explore the immunological profile of mice treated with rIL-9, splenic T cell and periphery monocyte subsets were investigated by flow cytometry. Flow cytometric analysis of splenic cells showed that treatment with rIL-9 did not affect T cell subsets, including Th1, Th2, Th17, and T regulatory cells (Tregs) (Supplementary material online, Figure S7). At the same time, the concentration of IFN-γ, IL-4, IL-17A, and IL-10 in the culture supernatants and plasma were also not altered in mice treated with rIL-9 (Supplementary material online, Figure S8). In addition, treatment with rIL-9 did not affect the percentage of CD11b+Ly6G−Ly6C+ monocytes or Ly6Cint and Ly6Chigh monocyte subsets in the peripheral blood of mice (Supplementary material online, Figure S9). It has been reported that IL-9 can enhance the suppressive functions of Tregs and consequently alleviate inflammatory responses. However, in our study, we found no significant difference in the suppressive functions of Tregs after rIL-9 treatment (Supplementary material online, Figure S10).

3.6 IL-9 increases VCAM-1 expression in vivo

It is known that VCAM-1 plays an important role in initiation and progression of atherosclerosis. Interestingly, in our study, we found that the VCAM-1+ positive area in plaques and the proportion of VCAM-1+ endothelial cells in the total endothelial cell populations were obviously increased in mice treated with rIL-9 relative to the PBS-treated group, and decreased in mice treated with IL-9 mAb compared with the isotype control group or PBS group (Figure 4A–C). Meanwhile, VCAM-1 mRNA expression in the aortic arches of mice treated with rIL-9 was markedly higher, while VCAM-1 mRNA expression in the aortic arches of mice treated with IL-9 mAb was significantly lower (Figure 4D). In addition, the concentration of sVCAM-1 in the plasma of mice treated with rIL-9 was obviously increased compared with that in the PBS-treated group, whereas the concentration of sVCAM-1 in the plasma of mice treated with IL-9 mAb was obviously decreased compared with the isotype group or PBS group (Figure 4E).

3.7 IL-9 induces VCAM-1 expression in murine aortic endothelial cells mainly via a STAT3-dependent pathway

Having demonstrated that IL-9 induces VCAM-1 expression in vivo, we next investigated whether IL-9 could induce VCAM-1 expression in MAECs in vitro. To determine the effect of IL-9 on VCAM-1 expression, MAECs were first treated with different concentration of rIL-9 for 12 h, and we found that the mRNA expression of VCAM-1 was in a concentration-dependent manner and the optimum concentration of IL-9-induced MAECs VCAM-1 expression was 100 ng/mL (Figure 5A). Then, we treated MAECs with rIL-9 (100 ng/mL) for different periods of time, and found that VCAM-1 mRNA expression increased as early as 3 h after rIL-9 treatment, rose to a peak at 12 h, and subsequently
Figure 2 Neutralization of endogenous IL-9 decreases atherosclerotic plaque size and macrophage and T-cell accumulation. ApoE−/− mice fed a western diet were treated with PBS \((n = 6)\), isotype control antibody \((6)\), or anti-IL-9 antibody \((n = 6)\) for 10 weeks. (A) Representative images of oil red O staining in aorta. (B) Quantification of aortic lesion area as a percentage of total aortic area in the different groups. (C) Representative images of oil red O staining in sections of the aortic root; scale bar = 200 μm. (D) Absolute quantification of the lesion area in the aortic roots of different groups. (E) Representative images of sections stained with specific antibodies for detecting T cells \((\text{CD3}, n = 6)\), black arrows show CD3-positive T cells; scale bar = 50 μm. (F) Quantitative analysis of the numbers of CD3-positive T cells in plaques in three groups. (G) Representative images of sections stained with specific antibodies for detecting macrophages \((\text{Mac-3}, n = 6)\), scale bar = 50 μm. (H) Quantitative analysis of percentages of Mac-3 staining-positive areas in plaques in three groups. *\(P < 0.05\), **\(P < 0.01\) vs. the isotype control antibody group or PBS group.
began to decrease (Figure 5B), and the protein expression of VCAM-1 obviously increased at 6 h and reached a peak at 24 h (Figure 5C). To confirm the increased VCAM-1 expression in MAECs after IL-9 stimulation, the adhesion of monocytes to endothelial cells was evaluated. Monocytes labelled with CFSE were added to the MAECs after treatment with rIL-9 for 24 h. Treatment with rIL-9 significantly increased monocyte–endothelial adhesion compared with the control, while neutralization of VCAM-1 using anti-VCAM-1 antibody increased monocyte–endothelial adhesion (Figure 5D). Moreover, inflammatory cell infiltration including CD3⁺ T cell and macrophages in plaques compared with isotype antibody-treatied mice, while mice treated with rIL-9 and anti-VCAM-1 neutralizing antibody showed a significant decrease in the area of Mac-3⁺ macrophages and CD3⁺ T cell infiltration in plaques compared with isotype antibody-treated mice, while mice treated with rIL-9 and anti-VCAM-1 neutralizing antibody showed a significant decrease in the area of Mac-3⁺ macrophages and CD3⁺ T cell infiltration in plaques compared with isotype antibody (Supplementary material online, Figure S12). In addition, the treatment did not alter body weight, plasma total cholesterol, triglycerides, or high-density lipoprotein cholesterol (HDL-C) (Table 3).

### Table 1  Body weight and plasma cholesterol in ApoE−/− mice (1)

<table>
<thead>
<tr>
<th>Group</th>
<th>PBS (n = 6)</th>
<th>Isotype (n = 6)</th>
<th>αIL-9 mAb (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, g</td>
<td>29.68 ± 0.78</td>
<td>31.01 ± 1.41</td>
<td>31.2 ± 0.85</td>
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<td>Total cholesterol, mmol/L</td>
<td>35.3 ± 2.50</td>
<td>34.49 ± 2.35</td>
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<td>Triglycerides, mmol/L</td>
<td>1.85 ± 0.49</td>
<td>1.79 ± 0.28</td>
<td>1.89 ± 0.30</td>
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<td>HDL-cholesterol, mmol/L</td>
<td>0.53 ± 0.20</td>
<td>0.61 ± 0.12</td>
<td>0.59 ± 0.15</td>
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Values are expressed as mean ± SEM.

### Table 2  Body weight and plasma cholesterol in ApoE−/− mice (2)

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<th>Group</th>
<th>PBS (n = 12)</th>
<th>rIL-9 (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, g</td>
<td>28.52 ± 1.23</td>
<td>28.33 ± 0.92</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>35.15 ± 1.60</td>
<td>36.89 ± 1.25</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>2.04 ± 0.23</td>
<td>2.28 ± 0.25</td>
</tr>
<tr>
<td>HDL-cholesterol, mmol/L</td>
<td>0.48 ± 0.03</td>
<td>0.42 ± 0.04</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM.

3.8 Neutralization of VCAM-1 prevents the IL-9-induced increase in plaque size

The above results demonstrated that rIL-9 can induce VCAM-1 expression in vivo and in vitro. Furthermore, VCAM-1 plays an important role in atherosclerosis. To determine whether VCAM-1 is directly related to the increase in plaque formation after rIL-9 treatment, we conducted a VCAM-1 neutralization study. Mice that were treated with rIL-9 and an isotype control antibody showed a significant increase in plaque area in both the aorta and aortic root relative to the isotype group (10.52 ± 1.13 vs. 6.08 ± 0.64%, P < 0.01; 530.18 ± 33.83 vs. 377.56 ± 21.88 × 10³ μm², P < 0.01, respectively) (Figure 6A–D). Furthermore, mice treated with rIL-9 that received anti-VCAM-1 neutralizing antibody revealed an obvious decrease in plaque area in both the aorta and aortic root relative to rIL-9 and isotype-matched antibody group (6.78 ± 0.63 vs. 10.52 ± 1.13%, P < 0.01; 388.63 ± 27.24 vs. 530.18 ± 33.83 × 10³ μm², P < 0.01, respectively), but significant increase in plaque area compared with mice treated with only anti-VCAM-1 mAb group (6.78 ± 0.63 vs. 4.06 ± 0.43%, P < 0.05; 388.63 ± 27.24 vs. 300.51 ± 18.37 × 10³ μm², P < 0.05, respectively), indicating that VCAM-1 is directly involved in the pro-atherogenic effects of IL-9, but neutralization of VCAM-1 did not completely reverse the increase in plaque size caused by IL-9 (Figure 6A–D). Meanwhile, mice that were treated with rIL-9 and isotype control antibody showed a marked increase in the area of Mac-3⁺ macrophages and CD3⁺ T cell infiltration in plaques compared with isotype antibody-treated mice, while mice treated with rIL-9 and anti-VCAM-1 neutralizing antibody showed a significant decrease in the area of Mac-3⁺ macrophages and CD3⁺ T cell infiltration in plaques compared with mice treated with rIL-9 and isotype antibody (Supplementary material online, Figure S12). In addition, the treatment did not alter body weight, plasma total cholesterol, triglycerides, or high-density lipoprotein cholesterol (HDL-C) (Table 3).

3.9 CD4⁺IL-9⁺ T cells and IL-9 are increased in patients with acute coronary syndrome

Having revealed the role of IL-9 in atherosclerosis in ApoE−/− mice, we next determined whether IL-9 is involved in atherosclerosis in humans. We detected the percentages of CD4⁺IL-9⁺ T cells and the concentration of IL-9 in culture supernatants in 66 acute coronary syndrome (ACS) patients, 37 chronic stable angina (CSA) patients, and 55 chest pain syndrome (CPS) patients by flow cytometry and ELISA. The basic clinical characteristics of the enrolled patients are shown in Table 4. The percentage of CD4⁺IL-9⁺ T cells within the CD4⁺ T cell populations was significantly higher in the patients with ACS (0.70 ± 0.037%) than in those with CSA (0.41 ± 0.029%) or CPS (0.36 ± 0.023%) (P < 0.001), whereas there was no obvious difference between the CSA and CPS groups (P > 0.05) (Figure 7A and B). Moreover, the absolute number of peripheral CD4⁺IL-9⁺ T cells was also significantly higher in patients with ACS than in those with CSA or CPS (Supplementary
The levels of IL-9 in the culture supernatants was also obviously higher in patients with ACS (77.01 ± 10.72 pg/mL) (P < 0.001) than in those with CSA (37.61 ± 5.65 pg/mL) and CPS (32.05 ± 3.84 pg/mL) (Figure 7C).

As we have shown in Table 4, the study population in the three groups were different in their use of aspirin, clopidogrel, β-blockers, statins, ACE-I or ARBs, or nitrates, incidence of current smoking. To adjust the data on IL-9 or CD4^+IL-9^+T cells for above these variables, we performed a multiple linear regression analysis, and found that the use of medication and current smoking status did not influence IL-9 or CD4^+IL-9^+T cells (Supplementary material online, Table S2).

We have demonstrated that IL-9 can increase VCAM-1 expression in ApoE−/− mice. However, the relationship between IL-9 and VCAM-1 in humans was not previously determined. In this study, the concentration of sVCAM-1 was determined in the plasma of patients in whom IL-9 was also determined. The concentration of sVCAM-1 was significantly higher in ACS patients (654.49 ± 32.74 ng/mL) (P < 0.001) than in those with CSA (560.62 ± 31.33 ng/mL) or CPS (456.86 ± 17.43 ng/mL), while there was also significant difference between the CSA and CPS groups (P < 0.05) (Figure 7D). Spearman’s correlation test demonstrated a moderate positive correlation between IL-9 and sVCAM-1 in the enrolled patients (r = 0.401, P < 0.001) (Figure 7E).
4. Discussion

The current study demonstrates a key role for IL-9 in mediating atherosclerotic plaque formation in ApoE−/− mice. The data revealed that IL-9R was expressed in MAECs and aortic tissues, and IL-9 was increased in plasma and aortic arches in ApoE−/− mice. Treatment with rIL-9 markedly increased the plaque area, which was related to an increase in VCAM-1 expression and infiltration of macrophages and T cells. Treatment with anti-IL-9 mAb induced the opposite effect. In vitro studies revealed that treatment with rIL-9 could induce VCAM-1 expression mainly via a STAT3-dependent pathway in MAECs, subsequently increasing monocytes adhesion of endothelial cells. Furthermore, the VCAM-1 neutralization study confirmed that IL-9 aggravated atherosclerotic plaque formation partially via increased VCAM-1 expression. In addition, we also found that IL-9 and CD4+IL-9+ T cells were obviously increased in patients with acute coronary syndrome, and the concentrations of IL-9 in culture supernatants and sVCAM-1 in plasma showed a moderate positive correlation in the enrolled patients.

Recently, emerging evidence has indicated that IL-9 has a role in regulating immune responses and is involved in the pathogenesis of various...
inflammatory diseases. However, little is known about the role of IL-9 in the development of atherosclerosis. Our data, for the first time, showed that IL-9R was expressed in MAECs and aortic tissues. Moreover, we found that the levels of IL-9 in the plasma and aortic arches were significantly higher in atherosclerotic mice, especially those fed a western type diet. These data suggested that IL-9 might affect the development of atherosclerosis by interacting with IL-9R. We investigated the cellular source of IL-9 in ApoE/ mice. As suggested by previous study, the main cellular sources of IL-9 are T cell subsets, especially Th2 cells, Th17 cells, Treg cells, and a recently identified Th9 cells. However, we found that innate immune cells other than T or B cells were the main source of IL-9 in ApoE/ mice. There have also been reported that innate immune cells including mast cells and NKTs, could produce IL-9. Recently, using IL-9 fate reporter mice, it is reported that IL-9 production in vivo was mainly stemmed from innate lymphoid cells (ILCs), and rapidly lost in papain-induced lung inflammation.

To study the effect of IL-9 directly, we treated ApoE/ mice with exogenous rIL-9 and anti-IL-9 mAb for 10 weeks. We discovered that administration of rIL-9 markedly increased plaque size, as accompanied by increased macrophage and T cell infiltration, whereas administration of anti-IL-9 mAb obviously reduced plaque size, indicating a key role for IL-9 in atherosclerotic plaque formation. The mechanisms of IL-9 effects in atherosclerosis are further studied. The downstream effect of IL-9 has been primarily associated with mast cells that acts as a growth factor of mast cells and increase the activity. However, we found that both the number and activity of mast cells in the atherosclerotic plaque were not altered by IL-9, indicating that IL-9 aggravated the progression of atherosclerosis independent of mast cell activation and expansion.

There are also some indications that IL-9 exerts its effects by targeting the T cell subsets. In the EAE model, IL-9 could induce Th17 differentiation and enhance the suppressive functions of Treg cells. In vitro experiments demonstrated that IL-9 together with TGF-β1 can cause naïve CD4+ T cells to differentiate into Th17 cells. However, in ApoE/ mice, we found that treatment with rIL-9 did not affect the number of T-cell subsets including Th1, Th2, Th17, and Treg cells and their capacity to secret cytokines or suppress proliferation. This discrepancy may be a result of the pleiotropic functions of IL-9, the differences in the disease models or the dependence of T-cell subsets differentiation on the cytokines milieu of the microenvironment rather than a single cytokine.

Many studies have demonstrated that monocytes have an active role in atherosclerosis. Monocytes circulating in the blood can infiltrate into the plaque, where they could become macrophages, release pro-inflammatory cytokines, and accelerate atherosclerotic plaque formation. Mouse monocytes are a heterogeneous population and can be divided into at least two different subsets: Ly6Chigh monocytes and Ly6C low monocytes. It has been reported that Ly6Chigh monocytes dominate hypercholesterolaemia-associated monocytecytosis and give rise...
Neutralization of VCAM-1 prevents the IL-9-induced increase in plaque size. ApoE−/− mice fed a western diet were treated with PBS (n = 6), isotype control antibody (n = 6), anti-VCAM-1 antibody (n = 6), rIL-9 and isotype control antibody (n = 8), or rIL-9 and anti-VCAM-1 antibody (n = 8) for 10 weeks. (A) Representative images of oil red O staining in aorta. (B) Quantification of aortic lesions as a percentage of the total aortic area in the five groups. (C) Representative images of oil red O staining in sections of the aortic root, scale bar = 200 μm. (D) Absolute quantification of the lesion area in aortic roots in five groups.

Table 3  Body weight and plasma cholesterol in ApoE−/− mice (3)

<table>
<thead>
<tr>
<th>Group</th>
<th>PBS (n = 6)</th>
<th>Isotype (n = 6)</th>
<th>αVCAM-1 mAb (n = 6)</th>
<th>rIL-9 + isotype (n = 8)</th>
<th>rIL-9 + αVCAM-1 mAb (n = 8)</th>
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<tr>
<td>Weight, g</td>
<td>28.46 ± 1.02</td>
<td>27.85 ± 0.88</td>
<td>28.12 ± 0.78</td>
<td>29.14 ± 0.96</td>
<td>28.86 ± 0.93</td>
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<td>Total cholesterol, mmol/L</td>
<td>37.68 ± 2.08</td>
<td>36.54 ± 1.94</td>
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<td>37.46 ± 1.79</td>
<td>36.91 ± 1.82</td>
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<td>Triglycerides, mmol/L</td>
<td>2.28 ± 0.40</td>
<td>2.19 ± 0.28</td>
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<td>HDL-cholesterol, mmol/L</td>
<td>0.39 ± 0.09</td>
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</table>

Values are expressed as mean ± SEM.
to macrophages in atheromata in ApoE−/− mice. Therefore, monocytes and their subsets were investigated in our study. The results demonstrated that the percentages of monocytes and their subsets were not altered after rIL-9 treatment.

VCAM-1 is an immunoglobulin superfamily member that mediates leucocyte adhesion. The high level of VCAM-1 expression on the membrane of endothelial cells is an important marker of endothelial activation. Moreover, endothelial activation is considered the most important step in the initiation of atherosclerosis. Our data revealed that IL-9 could promote VCAM-1 expression in ApoE−/− mice, which might cause macrophage and T-cell infiltration and aggravate the development of atherosclerosis.

Our in vitro experiments further confirmed that IL-9 could induce VCAM-1 expression in MAECs, consequently leading to a significant increase in monocyte–endothelial adhesion. The combination of IL-9 with VCAM-1 expression in MAECs, consequently leading to a significant increase in plaque area compared with mice treated with only anti-VCAM-1 mAb group, indicating that in addition to increased VCAM-1 expression and consequent inflammatory cell infiltration, there are other mechanisms underlying the pro-atherogenic effects of IL-9 in ApoE−/− mice that must be further investigated. As a result, in the present study, we hypothesized that IL-9 significantly increased the plaque area in ApoE−/− mice at least partially through increased VCAM-1 expression, which caused macrophage and T-cell infiltration into the plaques.

Thus far, a number of studies in humans have demonstrated that IL-9 and Th9 cells are associated with allergy diseases and anti-tumour immunity. Our study demonstrated that the concentration of IL-9 in culture supernatants and the percentage of CD4+CD97 T cells were increased in patients with acute coronary syndrome. Simultaneously, the concentration of sVCAM-1 in plasma was also obviously increased in patients with coronary heart diseases (CAD). The concentrations of sVCAM-1 have been consistently shown to be a strong independent predictor of future fatal cardiovascular event in patients with established CAD. In consistent with animal experiments, we found that IL-9 and sVCAM-1 had a moderate positive correlation in the enrolled patients. However, additional details regarding the roles of IL-9 and CD4+IL-9+ T cells in inflammatory atherosclerotic diseases in humans need to be further investigated.

Based on our study that IL-9 could exacerbate atherosclerosis development in mice and increased in patients with ACS, targeting IL-9 pathway may provide a new therapeutic strategy for coronary artery disease. Recently, MEDI-528, a monoclonal antibody targeting IL-9, has been shown to alleviate mild-to-moderate asthma without major safety concerns. Therefore, further studies are needed to determine the therapeutic potential of MEDI-528 for the treatment of coronary artery disease.

Our study has some limitations. First, we conclude the results by administering exogenous rIL-9 or anti-IL-9 neutralizing mAb, the results should be confirmed by using IL-9 transgenic or IL-9/IL-9R knock-out mice. Secondly, the role of IL-9 in advanced plaques and plaque instability has not been addressed in this study. At last, the mechanisms underlying the pro-atherosclerotic effects of IL-9 have not completely illuminated, and other mechanisms in addition to increased VCAM-1 expression should be further investigated.

In summary, our study is the first to demonstrate that IL-9 aggravates the progression of atherosclerosis at least partially by inducing VCAM-1 expression, which mediates the infiltration of lesion-associated macrophages and T cells into atherosclerotic lesions. These results indicate

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**Table 4 Clinical characteristics of study population**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>ACS (n = 66)</th>
<th>CSA (n = 37)</th>
<th>CPS (n = 55)</th>
<th>P-value</th>
</tr>
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<tr>
<td>Age (years)</td>
<td>56.8 ± 1.33</td>
<td>54.5 ± 1.64</td>
<td>55.4 ± 1.31</td>
<td>0.549</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>44/22</td>
<td>26/11</td>
<td>34/21</td>
<td>0.691</td>
</tr>
<tr>
<td>Risk factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>38 (57.6)</td>
<td>23 (62.2)</td>
<td>21 (45.5)</td>
<td>0.230</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>25 (37.9)</td>
<td>19 (51.4)</td>
<td>16 (29.1)</td>
<td>0.098</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>46 (69.7)</td>
<td>24 (64.9)</td>
<td>15 (27.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hyperlipidemia, n (%)</td>
<td>28 (42.4)</td>
<td>13 (35.1)</td>
<td>30 (54.5)</td>
<td>0.161</td>
</tr>
<tr>
<td>Medications</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin, n (%)</td>
<td>57 (86.4)</td>
<td>34 (91.9)</td>
<td>19 (34.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Clopidogrel, n (%)</td>
<td>56 (84.8)</td>
<td>16 (43.2)</td>
<td>13 (23.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Beta-blockers, n (%)</td>
<td>33 (50.0)</td>
<td>31 (83.8)</td>
<td>6 (10.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ACE-I/ARB, n (%)</td>
<td>26 (39.4)</td>
<td>27 (73.0)</td>
<td>15 (27.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Statins, n (%)</td>
<td>60 (90.9)</td>
<td>30 (81.1)</td>
<td>23 (41.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Nitrates, n (%)</td>
<td>44 (66.7)</td>
<td>8 (21.6)</td>
<td>7 (12.7)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM or number or percentage of enrolled patients.

ACS, acute coronary syndrome; CSA, chronic stable angina; CPS, chest pain syndrome; ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker.
Figure 7  CD4+IL-9+ T cells and IL-9 are increased in patients with acute coronary syndrome. A total of 158 patients including 66 ACS patients, 37 CSA patients, and 55 CPS patients were enrolled in this study, and the percentage of CD4+IL-9+ T cells as well as the IL-9 and sVCAM-1 levels were determined by flow cytometry and ELISA, respectively. (A) Representative dot plots illustrating the proportion of CD4+IL-9+ T cells in the total CD4+ T cell populations in each group. (B) Quantitative analysis of the percentage of CD4+IL-9+ T cells in the total CD4+ T cell populations in the different groups. (C) The concentration of IL-9 in culture supernatants of the different groups. **P < 0.01 vs. the CSA or CPS group. (D) The level of sVCAM-1 in plasma was detected by ELISA. *P < 0.05 vs. the CPS group and **P < 0.01 vs. the CSA or CPS group. (E) The correlation between IL-9 in culture supernatants and sVCAM-1 in plasma in the enrolled patients (n = 158) (r = 0.401, P < 0.001).
that control of IL-9 might be beneficial for controlling the inflammation associated with atherosclerosis.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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Conflict of interest: none declared.

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References

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