Maternal High-Fat Diet Exaggerates Atherosclerosis in Adult Offspring by Augmenting Periaortic Adipose Tissue-Specific Proinflammatory Response


Objective—Maternal obesity elicits offspring’s metabolic disorders via developmental modifications of visceral adipose tissue; however, its effect on atherogenesis remains undefined. Perivascular adipose tissue has recently been implicated in vascular remodeling and vasoreactivity. We hypothesize that developmental modifications of perivascular adipose tissue by maternal high-fat diet (HFD) exposure promotes atherosclerosis in adult offspring.

Approach and Results—Eight-week-old female apolipoprotein E-deficient mice were fed an HFD or normal diet (ND) during gestation and lactation. Offspring were fed a high-cholesterol diet from 8 weeks of age. Twenty-week-old male offspring of HFD-fed dams (O-HFD) showed a 2.1-fold increase in atherosclerotic lesion of the entire aorta compared with those of ND-fed dams (O-ND). Although mRNA expressions of interleukin-6, tumor necrosis factor, and monocyte chemotactic protein-1 and accumulation of macrophages in epididymal white adipose tissue were less in O-HFD than in O-ND, thoracic periaortic adipose tissue (tPAT) showed an exaggerated inflammatory response in O-HFD. Intra-abdominal transplantation of tPAT from 8-week-old O-HFD alongside the distal abdominal aorta exaggerated atherosclerosis development of the infrarenal aorta in recipient apolipoprotein E-deficient mice compared with tPAT from O-ND (210%, P<0.01). Although macrophage accumulation was rarely detected in tPAT of 8-week-old offspring, mRNA expression and protein levels of macrophage colony-stimulating factor were markedly elevated in O-HFD (2.3-fold, 3.3-fold, respectively, P<0.05), suggesting that increased macrophage colony-stimulating factor expression contributes to the augmented accumulation of macrophages, followed by the enhanced proinflammatory response.

Conclusions—Our findings demonstrate that maternal HFD exaggerates atherosclerosis development in offspring by augmenting tPAT-specific inflammatory response proceeds by an increased expression of macrophage colony-stimulating factor.

Key Words: adipose tissue • atherosclerosis • developmental biology • inflammation • macrophage

A ccumulating evidence demonstrates that maternal obesity during pregnancy is associated with an increased susceptibility to childhood obesity and metabolic disorders.1–4 Recent epidemiological studies have shown a strong association between maternal obesity and increased morbidity and mortality from cardiovascular events in adult offspring.5 Experimental animal studies also demonstrate that offspring of obese or high-fat diet (HFD)–fed dam are more predisposed to obesity and insulin resistance in their early development, which is associated with greater offspring adiposity.6–11 Visceral adipose tissue is now thought to be an endocrine organ that secretes several factors into circulation, some of which exaggerate systemic inflammation, leading to the development of insulin resistance and cardiovascular diseases.12–16 Nutrient status during fetal development has been shown to modulate genes expression in offspring adipose tissue without alterations in the DNA sequence, which commonly is referred to as epigenetic programming.17–20 However, the underlying mechanism by which developmental modifications of adipose tissue promotes atherosclerosis in offspring remains to be fully elucidated.

Perivascular adipose tissue, one of the ectopic adipose tissues, has been demonstrated to secrete numerous vasoactive molecules involved in vascular remodeling and vasoreactivity.21–25 Takaoka et al found elevated expression of proinflammatory genes and lower adiponectin expression in the perivascular adipose tissue of obese mice and concluded that this contributed to exaggerated neointimal formation after arterial injury.26 Chatterjee et al also found elevated proinflammatory gene expression and diminished adiponectin expression in the perivascular adipose tissue surrounding diseased human coronary arteries.27 Thoracic periaortic adipose tissue (tPAT) surrounding the descending thoracic aorta recently has

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Nonstandard Abbreviations and Acronyms

<table>
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<th>Abbreviation</th>
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<tr>
<td>apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>apolipoprotein E-deficient</td>
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<tr>
<td>CD68</td>
<td>cluster of differentiation 68</td>
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<tr>
<td>HCD</td>
<td>high-cholesterol diet</td>
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<tr>
<td>HFD</td>
<td>high-fat diet</td>
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<tr>
<td>IL-6</td>
<td>interleukin-6</td>
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<tr>
<td>MCP-1</td>
<td>monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>ND</td>
<td>normal diet</td>
</tr>
<tr>
<td>PAT</td>
<td>perivascular adipose tissue</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
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<tr>
<td>IPAT</td>
<td>thoracic peri-aortic adipose tissue</td>
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<td>WAT</td>
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become a focal point of cardiovascular disease risk assessment. Fox et al reported that the volume of tPAT was correlated with the incidence of peripheral arterial disease.28 We have reported previously that tPAT-specific activation of the renin angiotensin system occurred in uninephrectomized apolipoprotein E-deficient (apoE<sup>−/−</sup>) mice and that this was partially responsible for the accelerated atherosclerotic development observed in chronic kidney disease.29 These findings urged us to hypothesize that maternal HFD exerts inflammatory phenotypic alterations of tPAT in adult offspring, thereby contributing to the development of atherosclerosis through their endocrine or paracrine effects on the vasculature.

Here, we examined phenotypic alterations in offspring tPAT by maternal HFD and investigated their roles in atherosclerotic development using a tPAT transplantation model. Maternal HFD exaggerated atherosclerosis development in adult offspring, accompanied by enhanced gene and protein expression levels of proinflammatory cytokines and by augmented accumulation of macrophages in tPAT. Such changes were not observed in epidymal white adipose tissue (WAT). Transplantation of tPAT from offspring of HFD-fed dams significantly exaggerated atherosclerosis development compared with tPAT from offspring of normal diet (ND)–fed dam. Moreover, mRNA expression and protein levels of macrophage colony–stimulating factor (M-CSF) were markedly elevated in 8-week-old offspring of HFD-fed dam, in which macrophage accumulation was observed rarely. Our findings suggest that maternal HFD-induced phenotypic alteration of offspring tPAT contributes to atherosclerosis development by augmenting tPAT-specific proinflammatory response and that therapeutic targeting of the phenotypic changes of tPAT could potentially remediate and prevent cardiovascular diseases in adult offspring.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Atherosclerotic Development Is Exaggerated in Adult Offspring of HFD-Fed Dams

The mean maternal body weight of HFD-fed dam on day 1 after delivery was greater than that of ND-fed dam (25.4±0.5 versus 21.6±0.7 g, P<0.01; Figure IA in the online-only Data Supplement). There was no significant difference in the mean litter size between the 2 groups. The mean weight of offspring did not differ between the 2 groups before and after a high-cholesterol diet (HCD) (Figure IB in the online-only Data Supplement). The hemodynamic parameters and lipid profiles did not differ between the 2 groups in both sex (Figures IC and ID in the online-only Data Supplement). The atherosclerotic lesion area in 20-week-old male offspring of HFD-fed dams (O-HFD) was markedly greater than that of ND-fed dams (O-ND) (210%, P<0.01; Figures 1A and 1B). Likewise, the atherosclerotic lesion area in female O-HFD was increased modestly, but significantly, compared with that of female O-ND (P<0.05; Figures 1A and 1B). We further examined the sex difference by using 2-way analysis of variance. The proatherogenic effect of maternal obesity was significantly greater in male offspring rather than in female offspring (P<0.01; Figure 1B). We next examined the atherosclerosis development in aortic root of male offspring, which was not surrounded by tPAT. The percentages of plaque area, oil-red O-staining area, and MOMA-2–positive area were equivalent between the 2 groups (Figures 1C and 1D). These results suggest that developmental modifications by maternal HFD promote atherosclerosis development in adult offspring in a region-specific manner.

Maternal HFD Does Not Promote the Inflammatory Response in Male Offspring Epididymal WAT

First, we examined the effect of maternal HFD on the phenotypic alterations in visceral WAT of male offspring. Although the weight of the epididymal WAT pads before starting an HCD was significantly higher in O-HFD than in O-ND without a difference in adipocyte size (Figures IIA–IIC in the online-only Data Supplement), after feeding an HCD, the difference in pad weights disappeared, with smaller adipocyte size in O-HFD than in O-ND. Expression levels of adipocyte differentiation-related genes after an HCD were markedly elevated in both groups; however, no difference could be observed between the 2 groups (Figure IID in the online-only Data Supplement). Along with the decreased adipocyte size in O-HFD, tumor necrosis factor-α (TNF-α) mRNA expression and protein levels of monocyte chemotactic protein-1 (MCP-1) in O-HFD were significantly lower than those in O-ND (Figures 2A and 2B). The mRNA expression levels of cluster of differentiation 68 (CD68) and F4/80 tended to be lower in O-HFD (Figure 2C). Further, macrophage accumulations assessed by the numbers of Mac-2–positive and CD68–positive cells were significantly reduced in O-HFD (Figure 2D). These findings indicated that maternal HFD does not elicit an inflammatory response in offspring visceral WAT.

Maternal High-Fat Diet Exaggerates the Inflammatory Response in Male Offspring tPAT

Next, we examined the effect of maternal HFD on the phenotypic alterations in tPAT of male offspring. tPAT pad weight and histomorphological appearance did not show a discernible difference between the 2 groups (Figures IIIA...
Expression levels of adipocyte differentiation-related genes did not show any difference between the 2 groups before and after an HCD (Figure IIC in the online-only Data Supplement). In contrast, MCP-1 gene expression after an HCD was increased to a greater extent in O-HFD than that in O-ND (Figure 3A). Similarly, the protein levels of TNF-α and MCP-1 were significantly higher in O-HFD than those in O-ND (Figure 3B). The mRNA expressions of anti-inflammatory genes (interleukin [IL]-4, IL-10, and TGF-β) were significantly increased in O-HFD compared with those in O-ND (Figure 3C); however, percent changes after HCD was much smaller than those in proinflammatory genes. Consistent with these findings, the gene expression levels of the monocye/macrophage markers and the accumulation of Mac-2–positive and CD68-positive cells were markedly increased in O-HFD (Figures 3D and 3E). The MCP-1 mRNA expression was significantly correlated with the plaque area ($r=0.83$, $P<0.05$), whereas TNF-α mRNA expression showed a positive, but not significant, correlation with plaque area ($r=0.36$, $P=0.35$; Figure 3F). We further analyzed the MCP-1 mRNA expression levels and correlation with the plaque area in female offspring. Although MCP-1 mRNA expression was markedly augmented in male O-HFD compared with male O-ND, there was no significant difference between the 2 groups in female offspring (Figure 3G). Consistently, MCP-1 mRNA expression was not significantly correlated with percent plaque area in female offspring (Figure 3H). These findings suggest that maternal HFD elicits a tPAT-specific inflammatory response in adult offspring in a sex-specific manner and that inflammatory expression patterns are closely implicated in the decrease in overall plaque area in female offspring.

**tPAT-Specific Inflammatory Response**

**Dose not Effect on the Circulating Inflammatory Adipocytokines**

To elucidate the mechanisms by which the proinflammatory property of tPAT in O-HFD mice promotes the development of atherosclerosis, we measured the circulating concentration
Figure 2. Maternal high-fat diet dose not promote the inflammatory response in epididymal white adipose tissue of male offspring. A, Quantitative PCR analysis of the mRNA expression levels of proinflammatory cytokines in the epididymal white adipose tissue of male offspring. Values are the means±SE relative to those in 8-week-old O-ND. Each group had ≥6 mice. *P<0.05 vs 8-week-old O-ND. **P<0.01 vs 8-week-old O-ND. #P<0.05 vs 8-week-old O-HFD. ##P<0.01 vs 8-week-old O-HFD. ¶P<0.05 vs 20-week-old O-ND. O-ND: Offspring of normal diet-fed dam. B, Tissue concentrations of IL-6, TNF-α, and MCP-1 in the epididymal white adipose tissue of male offspring. Values are the means±SE for ≥5 mice in each group. *P<0.05 vs O-ND. C, Quantitative PCR analysis of the mRNA expression levels of CD68 and F4/80. Values are the means±SE relative to those in 8-week-old O-ND. Each group had ≥6 mice. *P<0.05 vs 8-week-old O-ND. **P<0.01 vs 8-week-old O-ND. #P<0.05 vs 8-week-old O-HFD. ##P<0.01 vs 8-week-old O-HFD. D, Immunohistochemical staining and quantitative analysis of Mac-2–positive (a) and CD-68–positive (b) cells. Arrow indicates Mac-2–positive cells. Arrow-head indicates CD68–positive cells. The scale bar shows 10-μm intervals. Values are the means±SE for ≥6 mice in each group. *P<0.05 vs 8-week-old O-ND. #P<0.01 vs 8-week-old O-HFD. ¶P<0.05 vs 20-week-old O-ND. ¶¶P<0.01 vs 20-week-old O-ND. IL-6 indicates interleukin-6; MCP-1, monocyte chemotactic protein-1; O-FD, offspring of high-fat diet-fed dam; and TNF-α, tumor necrosis factor-α.
Figure 3. Maternal high-fat diet promotes the inflammatory response in the thoracic periaortic adipose tissue (tPAT) of male offspring. A, Quantitative PCR analysis of the mRNA expression levels of proinflammatory cytokines in the tPAT of male offspring. Values are the mean±SE relative to those in 8-week-old O-ND. Each group had ≥6 mice. *P<0.05 vs 8-week-old O-ND. #P<0.05 vs 8-week-old O-HFD. ##P<0.01 vs 8-week-old O-HFD. ¶P<0.05 vs 20-week-old O-ND. B, Tissue concentrations of IL-6, TNF-α, and MCP-1 in the tPAT of male offspring. Values are the mean±SE for ≥5 mice in each group. *P<0.05 vs O-ND. C, Quantitative PCR analysis of the mRNA expression levels of anti-inflammatory cytokines in the tPAT of male offspring. Values are the mean±SE relative to those in 8-week-old O-ND. Each group had ≥6 mice. #P<0.01 vs 8-week-old O-HFD. ¶P<0.05 vs 20-week-old O-ND. D, Quantitative PCR analysis of the mRNA expression levels of CD68 and F4/80. Values are the mean±SE relative to those of 8-week-old O-ND. Each group had ≥6 mice. *P<0.05 vs 8-week-old O-ND. **P<0.01 vs 8-week-old O-ND. ¶P<0.05 vs 20-week-old O-HFD. ¶¶P<0.01 vs 20-week-old O-ND. E, Immunohistochemical staining and quantitative analysis of Mac-2–positive (a) and CD68–positive (b) cells. Arrow indicates Mac-2–positive cells. Arrowhead indicates CD68–positive cells. The scale bar shows 10-μm intervals. Values are the mean±SE for ≥6 mice in each group. *P<0.01 vs 8-week-old O-ND. **P<0.01 vs 8-week-old O-HFD. ¶P<0.05 vs 20-week-old O-ND. F, Correlation of percent plaque area with the mRNA expression of inflammatory cytokines in male offspring. G, Quantitative PCR analysis of the mRNA expression levels of MCP-1 in male and female offspring. Values are the mean±SE relative to those of male 8-week-old O-ND. Each group had ≥6 mice. *P<0.05 vs 8-week-old male O-ND. H, Correlation of percent plaque area with MCP-1 mRNA expression in female offspring. IL-4 indicates interleukin-4; IL-6, interleukin-6; IL-10, interleukin-10; MCP-1, monocyte chemotactic protein-1; O-HFD, offspring of high-fat diet-fed dam; O-ND, offspring of normal diet-fed dam; TGF-β, transforming growth factor-β; and TNF-α, tumor necrosis factor-α.
Figure 3 (Continued)

E  a  b

O-ND (8wk)  O-HFD (8wk)

O-ND (20wk)  O-HFD (20wk)

Isotype

C

Number of MAC-2-positive cells (cells/mm²)

D

Number of CD68-positive cells (cells/mm²)

(\(r=0.83\))

\(p<0.05\)

n=8

\(r=0.36\)

\(p=0.35\)

n=8

Relative mRNA levels of TNF-α

Percent plaque area in entire aorta

Relative mRNA levels of MCP-1

Percent plaque area in entire aorta

(\(r=0.25\))

\(p=0.46\)

n=10

MCP-1

Male  Female

(\(r=0.83\))

\(p<0.05\)

n=8
of adipocytokines related to atherogenesis. The serum concentrations of IL-6, TNF-α, and MCP-1 were comparable between O-ND and O-HFD mice (Figure IV in the online-only Data Supplement), suggesting that proinflammatory tPAT in O-HFD is not likely to exhibit an atherogenic action through its endocrine effect.

**Intra-Abdominal Transplantation of tPAT From O-HFD Exaggerates Atherosclerosis Development in Infrarenal Aorta**

To investigate whether tPAT of O-HFD exerts proatherogenic action in a paracrine manner, tPAT was harvested from 8-week-old O-ND or O-HFD, in which inflammatory response rarely was detected, and was transplanted into 20-week-old apoE−/− mice fed an HCD from 8 weeks of age (tPAT-O-ND and tPAT-O-HFD, respectively). Representative picture of harvested tPAT and the transplantation site were shown in Figures VA and VB in the online-only Data Supplement. Immunohistological images showed the engraftment of transplanted tPAT and its relative position with abdominal aorta (Figure VC in the online-only Data Supplement). There was no significant difference in the atherosclerotic lesion of the entire aorta among the 3 groups (Figures 4A and 4B). However, atherosclerosis in the infrarenal aorta was exaggerated significantly in tPAT-O-HFD compared with sham and tPAT-O-ND, whereas the atherosclerosis lesion in the suprarenal aorta did not differ among the 3 groups (Figures 4C and 4D). Hemodynamic parameters and lipid profiles did not differ among the 3 groups (data not shown). These findings suggest that tPAT-specific inflammatory response in O-HFD exhibits proatherogenic action through direct paracrine effect on the vasculature.

**Transplanted tPAT Graft of O-HFD Exerts Proinflammatory Properties in Recipient Mice**

We further investigated the relation of the transplanted tPAT graft with the exaggerated atherosclerosis development in recipient mice. The mRNA expression levels of TNF-α were markedly higher in the tPAT graft from tPAT-O-HFD compared with that from sham and tPAT-O-ND (Figure 5A). Likewise, protein levels of TNF-α were significantly higher in the tPAT graft from tPAT-O-HFD (Figure 5B). The expressions of anti-inflammatory genes (IL-4, IL-10, and TGF-β) were not different among the 3 groups (Figure 5C). The mRNA expressions of F4/80 and CD68 were significantly elevated in the tPAT graft from tPAT-O-HFD (Figure 5D), accompanied by an augmented accumulation of Mac-2-positive cells and CD68-positive cells (Figure 5E). Percent oil-red-staining area in the infrarenal aorta was significantly correlated with the tissue concentration of TNF-α in tPAT graft (r=0.65, P<0.05; Figure 5F). These findings indicate that isolated tPAT from O-HFD could exhibit a similar inflammatory response in recipient apoE−/− mice as endogenous tPAT in O-HFD.

**M-CSF Expression in Offspring tPAT Is Exaggerated by Maternal High-Fat Diet**

To investigate the underlying mechanism of the tPAT-specific inflammatory response in O-HFD, we focused on the augmented accumulation of macrophages. At 8 weeks of age, accumulation of Mac-2-positive cells as well as CD68-positive cells rarely could be observed in both O-ND and O-HFD (Figure 3E). Considering that the number of circulating monocytes did not differ between the 2 groups (Figure VLA and VIB in the online-only Data Supplement), it is likely that adipocytokines produced by tPAT facilitate the migration of circulating monocytes into tPAT and promote differentiation/proliferation of monocytes in tPAT. Gene expression of MCP-1 did not differ between the 2 groups of 8-week-old mice (Figure 3A). In contrast, the mRNA and protein levels of M-CSF were markedly increased in O-HFD compared with O-ND at both 8 and 20 weeks of age (Figures 6A and 6B). On the other hand, M-CSF mRNA expression in epididymal WAT and serum concentration of M-CSF did not differ between the 2 groups at both 8 and 20 weeks of age (Figures 6C and 6D). These findings suggest that the tPAT-specific increased

![Figure 4](http://atvb.ahajournals.org/)

**Figure 4.** Intra-abdominal transplantation of thoracic periaortic adipose tissue (tPAT) exaggerates atherosclerotic lesion development. **A,** Representative images of oil-red-stained entire aortas and infrarenal aortas (insets, at higher magnification) from sham control (a, c), tPAT-O-ND (b, d), and tPAT-O-HFD (c, e). Arrow indicates the right renal artery. The scale bar shows 3-mm intervals. Quantitative analysis of the atherosclerotic lesion area in the entire aorta (B), in the suprarenal aorta (C), and in the infrarenal aorta (D). Values are the mean±SE for 6 mice in each group. *P<0.01 vs sham control. #P<0.01 vs tPAT-O-ND. tPAT-O-HFD indicates apoE−/− mice transplanted with tPAT from offspring of high-fat diet-fed dam; and tPAT-O-ND, apoE−/− mice transplanted with tPAT from offspring of normal diet-fed dam.
Figure 5. Transplanted graft exhibits proinflammatory properties in recipient mice. A, Quantitative PCR analysis of the mRNA expression levels of proinflammatory cytokines in transplanted tPAT graft. Values are the mean±SE relative to those in sham control. Each group had ≥6 mice. *P<0.01 vs sham control. #P<0.01 vs tPAT-O-ND. B, Tissue concentrations of IL-6 and TNF-α in transplanted tPAT graft. Values are the mean±SE for ≥5 mice in each group. *P<0.01 vs sham. #P<0.05 vs tPAT-O-ND. C, Quantitative PCR analysis of the mRNA expression levels of anti-inflammatory cytokines in transplanted tPAT graft. Values are the mean±SE relative to those in sham control. Each group had ≥6 mice. *P<0.05 vs sham control. D, Quantitative PCR analysis of the mRNA expression levels of CD68 and F4/80. Values are the mean±SE relative to those of sham control. Each group had ≥6 mice. *P<0.05 vs sham control. E, Immunohistochemical staining and quantitative analysis by guest on July 8, 2017 http://atvb.ahajournals.org/ Downloaded from
expression of M-CSF is closely implicated with the enhanced macrophage accumulation in tPAT.

**Discussion**

In this study, we demonstrated for the first time that maternal HFD accelerated atherosclerosis development in adult offspring, in which tPAT-specific exaggerated accumulation of macrophages and subsequent increase in proinflammatory cytokines expression were involved substantially. Intra-abdominal transplantation of the tPAT from offspring of HFD-fed dam significantly exaggerated atherosclerotic development in apoE−/− mice, suggesting the causal effect of tPAT-specific inflammatory response on atherosclerosis development in adult offspring. Further, tPAT-specific increased expression of M-CSF during early development is likely to initiate an inflammatory response by promoting the migration and differentiation/proliferation of monocytes in tPAT. Our findings demonstrate that the tPAT-specific proinflammatory response induced by maternal HFD substantially contributes to the atherosclerosis development in adult offspring and provide a new insight into the mechanism underlying the offspring morbidity and mortality from cardiovascular diseases by maternal nutrient status.

Maternal obesity increases the susceptibility to offspring obesity and type 2 diabetes mellitus by modulating the properties of visceral adipose tissue.6–8 Murine models demonstrated that visceral adipose tissue of offspring of obese dam showed an increase in the expression of genes related with adipocyte differentiation and lipogenesis.9 We also examined the phenotypic alterations of epididymal WAT and tPAT; however, expressions of adipocyte differentiation-related genes were not different between O-ND and O-HFD before and after an HCD (Figures IID and IIE in the online-only Data Supplement). These findings suggest that maternal HFD is unlikely to modulate offspring adipocyte properties when offspring are fed an HCD and raise the possibility that enhanced accumulation of macrophages primarily contributes to the tPAT-specific inflammatory response in offspring.

Adipose tissue macrophages play a major role in adipose tissue inflammation.30–34 The number of Mac-2–positive and CD68-positive cells in both epididymal WAT and tPAT was increased remarkably after an HCD; however, the extent of Mac-2–positive and CD68-positive cells in epididymal WAT was less in O-HFD than in O-ND, whereas O-HFD showed greater accumulation of macrophages to a greater extent than O-ND. Considering that the number of circulating monocytes did not differ between O-ND and O-HFD (Figures VIA and VIB in the online-only Data Supplement), migration of monocytes into adipose tissue seems to be more abundant in tPAT than in WAT. Recruitment of circulating monocytes into adipose tissue largely depends on the tissue concentration of chemotactic cytokines.35–38 In 20-week-old offspring, the expression level of the chemotactic cytokine, MCP-1, was significantly higher in tPAT of O-HFD than of O-ND; however, macrophages produce and secrete various kinds of inflammatory cytokines, such as TNF-α, which also promote adipocytes to secrete chemotactic cytokines.39 Therefore, we examined the expression level of MCP-1 in 8-week-old offspring, in which Mac-2–positive and CD68–positive cells rarely could be detected. Unexpectedly, MCP-1 mRNA expression in tPAT could not be detected in either O-HFD or O-ND, suggesting that MCP-1 is unlikely to be associated with macrophage recruitment in an early developmental period. The expression level of M-CSF is developmentally regulated during fetal and neonatal periods.40 M-CSF has been shown to be involved in macrophage differentiation/proliferation as well as monocyte migration and subsequently promotes atherosclerosis.41 M-CSF mRNA and protein expressions at 8 weeks of age were significantly higher in

![Figure 6](http://atvb.ahajournals.org/)

**Figure 6.** Maternal high-fat diet exaggerates M-CSF expression in thoracic periaortic adipose tissue (tPAT) of male offspring. **A**, Quantitative real-time PCR analysis of the mRNA expression levels of M-CSF in the tPAT of male offspring. Values are the means±SE relative to that in 8-week-old O-ND. Each group had ≥6 mice. **P<0.05 vs 8-week-old O-ND.** ¶¶**P<0.05 vs 8-week-old O-HFD. **B**, Tissue concentrations of M-CSF in the tPAT of male offspring. Values are the means±SE for ≥5 mice in each group. **P<0.05 vs 8-week-old O-ND.** ¶P<0.05 vs 20-week-old O-ND. **C**, Quantitative real-time PCR analysis of the mRNA expression levels of M-CSF in the epididymal white adipose tissue of male offspring. Values are the means±SE relative to those in 8-week-old O-ND. Each group had ≥6 mice. **P<0.05 vs 8-week-old O-ND.** ¶P<0.05 vs 20-week-old O-ND. **D**, Circulating concentration levels of M-CSF in male offspring. Values are the means±SE for ≥6 mice in each group. **P<0.05 vs 8-week-old O-ND.** #**P<0.05 vs 8-week-old O-HFD. M-CSF indicates macrophage colony-stimulating factor; O-ND, offspring of normal diet-fed dam; and O-HFD, offspring of high-fat diet-fed dam.
tPAT of O-HFD than of O-ND, whereas epididymal WAT did not show any difference between the 2 groups. This finding suggests that tPAT-specific augmented expression of M-CSF seems to be primarily implicated in enhanced accumulation of macrophages. The tPAT concentrations of M-CSF in 20-week-old offspring were not significantly higher than those in 8-week-old offspring. In contrast, the mRNA and protein expression levels of TNF-α and MCP-1 were markedly increased in 20-week-old offspring along with the increased accumulation of lesion macrophages (Figures 3A, 3D, and 3E). These findings suggest that tPAT M-CSF play a crucial role in the early stage of atherosclerosis by promoting the accumulation of monocytes/macrophages in the vessel wall. On the other hand, proinflammatory cytokines, such as TNF-α and MCP-1, which are predominantly released from accumulated macrophages, are likely to contribute to plaque progression. Consistent with this notion, the M-CSF mRNA expression was not significantly correlated with the plaque area (r=0.19, P=0.61; Figure VII in the online-only Data Supplement), whereas a significant positive correlation between MCP-1 mRNA expression and percent plaque area was observed (r=0.83, P<0.05; Figure 3F).

Recently, Chang et al developed a smooth muscle cell–specific PPARγ-deficient mouse model, in which absence of tPAT markedly reduced the thermogenic capacity and energy expenditure of excessive nutrition.42 The authors demonstrated that atherosclerosis development was significantly exaggerated when the animals were housed at 16°C, but not at 22°C, because of the intravascular temperature–associated endothelial dysfunction and impaired lipid clearance. They also showed that mRNA expression levels of UCP-1 and PGC-1α were significantly higher in tPAT from control mice housed at 16°C than at 22°C, suggesting that cold-mediated activation of the tPAT exerted antiatherogenic effects. In our experimental model, mice were housed at 22°C, and UCP-1 mRNA expression level in endogenous tPAT did not show any difference between the 2 groups before and after HCD feeding. Therefore, it is not likely that antiatherogenic action of tPAT is impaired in O-HFD; however, analysis of proatherogenic action of tPAT using a genetically tPAT-specific deficient mice model needs to be performed in the future study.

In this study, the effect of tPAT on atherosclerosis was investigated focusing on the descending thoracic aorta surrounding by tPAT, in which the degree of atherosclerosis was not so high compared with that in proximal aorta when fed an ND. Therefore, we applied a modified Western-type diet (13.6% fat, 1.25% cholesterol), but not a Western-type diet (21% milk fat, 0.2% total cholesterol), to make it easy to investigate the tPAT-associated atherosclerosis development. When apoE−/− mice were fed a HCD, hypercholesterolemia-associated bone marrow monocytesis was significantly augmented compared with those in ND-fed apoE−/− mice.43 However, the number of circulating monocytes was equivalent between the 2 groups (Figure VI in the online-only Data Supplement). Notably, the augmented expression of M-CSF in tPAT of O-HFD could be observed before starting an HCD. These findings suggest that augmented expression of M-CSF and its effect on atherosclerosis is not likely to be attributable to the artificial effect of high level of cholesterol diet.

Epigenetic programing in early life exerts a profound effect on the susceptibility to cardiovascular diseases in late adulthood by affecting the expression of genes involved in atherogenesis.17-20 Epigenetic changes detected in the atherosclerotic lesions have been characteristic of smooth muscle cells and of endothelial cells as well as immune cells.44,45 We, therefore, examined the mRNA expression levels of VCAM-1 and ICAM-1 in the aorta of 8-week-old offspring and found that VCAM-1 mRNA expression was significantly reduced in O-HFD (Figure VIII in the online-only Data Supplement), suggesting that endothelial dysfunction is not likely to be involved in offspring atherosclerosis in this model. Bekkering et al reported that brief exposure of isolated human monocytes to oxidized low-density LDL induced a long-term proinflammatory response via epigenetic histone modification.46 A recent study by Singer et al demonstrated that hematopoietic stem cells from BM of obese mice have the sustained capacity to preferentially generate inflammatory adipose tissue macrophages.47 Furthermore, Kampen et al reported that bone marrow cells of Western-type diet–fed mice exhibited hypomethylation of genes encoding Pu.1 and IRF8, key regulators of monocyte proliferation and macrophage differentiation, and increased susceptibility to atherosclerosis in bone marrow–transplanted mice accompanied by the increased number of circulating F4/80+ monocytes.48 These findings suggest the possibility that gene expression of M-CSF, a key mediator for the differentiation/proliferation of myeloid lineage cells,49 is likely to be regulated via epigenetic programing; however, precise mechanism needs to be investigated in the future study.

Sex-specific difference in offspring outcomes has been less studied in humans than in animal models.50 Human study reported by Mingrone G et al indicated that male offspring of obese mothers showed higher values of insulin sensitivity, but both significant, than female offspring; however, population size examined was extremely small, and underlying mechanism remains undefined.51 Consistent with our results, Dahlhoff M et al reported that male offspring of HFD-fed dam was more susceptible to adverse offspring outcomes than female of HFD-fed dam.52 Adverse offspring outcomes induced by in utero environmental insult are often more prominent in male than female offspring.50 Females may be more sensitive and adaptable to the intrauterine environment; however, precise underlying mechanisms remain to be elucidated.

In conclusion, our results showed that a tPAT-specific inflammatory response elicited by maternal HFD is involved substantially in atherosclerosis development in adult offspring and that tPAT-specific enhanced expression of M-CSF may initiate the exaggerated macrophage accumulation in tPAT. Our findings shed a new light on the emerging role of developmental modifications of tPAT and suggest that reverse programming of tPAT may be a useful new therapeutic strategy for the prevention of cardiovascular diseases.

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Disclosures

None.

References


Maternal High-Fat Diet Exaggerates Atherosclerosis in Adult Offspring by Augmenting Periaortic Adipose Tissue-Specific Proinflammatory Response

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Materials and Methods

Experimental Animals

All experiments were performed by strictly adhering to the “Directive 2010/63/EU” of the European Parliament and to the Guidelines for Animal Experiments of the Kyoto Prefectural University of Medicine, following approval by a local university ethics review board.

*ApoE*−/− mice (C57BL/6) were obtained from Taconic Co., Ltd. (Germantown, NY, USA). C57BL/6-Tg(CAG-EGFP)C14-Y01-FM131Osb mice were kindly provided by Dr. M. Okabe (Osaka University). Eight-week-old female *apoE*−/− mice were mated with lean male *apoE*−/− mice and were switched to a high-fat diet (energy content: 62% fat, 18.2% protein, and 19.6% carbohydrate; Oriental Yeast Co., Tokyo, Japan) (HFD) or a normal diet (12.0% fat, 28.9% protein, 59.1% carbohydrate; Oriental Yeast Co., Tokyo, Japan) (ND) throughout pregnancy and lactation. All offspring were weaned at 4 weeks of age and were fed an ND until the age of 8 weeks, then were switched to a high-cholesterol diet (13.6% fat, 1.25% cholesterol; Oriental Yeast Co., Tokyo, Japan) (HCD) until the age of 20 weeks. The animals were housed in a room that was maintained at 22°C under a 12-hr light/dark cycle and were provided with drinking water *ad libitum*. At 20 weeks of age, the area of the atherosclerotic lesion in the aorta was evaluated. Before harvesting the tissues (aortas), mice were euthanized by trans-cardiac perfusion under anesthesia induced by isoflurane (2%, 0.2 mL/min). In transplantation experiments, mice were anaesthetized using isoflurane throughout the surgery. The depth of anesthesia was confirmed by lack of tail pinch response. A laparotomy was performed under sterile conditions with the assistance of an operating stereomicroscope. A periaortic fat pad surrounding the descending thoracic aorta (tPAT) was harvested from 8-week-old male offspring of ND-fed dam (O-ND) or of HFD-fed dam (O-HFD), and subsequently was transplanted over the endogenous infrarenal abdominal periaortic adipose tissue of 20-week-old recipient *apoE*−/− mice fed a high-cholesterol diet for 12 weeks. Finally, the musculofascial and skin incisions were sutured. Sham surgeries of control animals were performed in the same manner, but without transplantation of tPAT. Eight weeks after transplantation, the atherosclerotic lesion area was evaluated.

Hemodynamic analysis
Mean blood pressure and heart rates were measured under conscious and unrestrained conditions using a programmable sphygmomanometer (BP-98A; Softron, Tokyo, Japan).

**Plasma lipid analysis**
Measurements of total cholesterol, triglyceride, LDL-cholesterol, and HDL-cholesterol were outsourced to SRL, Tokyo, Japan.

**Quantitative measurement of atherosclerotic lesions**
Mice were euthanized, and atherosclerotic lesions were analyzed. The entire aortic lesion area in each animal was expressed as the percentage of lesion area per total aortic area. Atherosclerotic lesions in the aortic root were examined at several locations, each separated by 100 µm. Imaging and analysis of oil-red O-stained aortas was accomplished using Image J Software (http:rsbweb.nih.govij).

**Immunohistochemistry**
Epididymal white adipose tissue (WAT), tPAT surrounding the descending thoracic aorta 1 mm proximal to the diaphragm, and transplanted tPAT graft were quickly removed after PBS perfusion, embedded in paraffin, and immunofluorescently labeled. For the staining Mac-2 or CD68, a combination of anti-mouse Mac2 antibody (CEDARLANE, Burlington, Ontario, Canada) and Alexa Flour 488-conjugated secondary antibodies (Invitrogen, Carlsbad, California, USA), or a combination of anti-CD68 (abcam, Cambridge, United Kingdom) and Alexa Flour 555-conjugated secondary antibodies (Invitrogen, Carlsbad, California, USA) was used, respectively. For the staining of GFP, a combination of anti-GFP antibody (B-2) (Santa Cruze Biotechnology, Dallas, Texas, USA) and Alexa Flour 488-conjugated secondary antibodies (Invitrogen, Carlsbad, California, USA) was used. For the staining of MOMA-2, tissue samples of aortic root were embedded in optimal cutting temperature compound, and were quick-frozen in liquid nitrogen. Anti-mouse MOMA-2-FITC (MOMA-2) antibodies (AbD serotec, Kidlington, United Kingdom) were used. Nuclei were labeled using DAPI solution (DOJINDO, Kumamoto, Japan), and the sections were examined using a LSM 510 META confocal microscope (Carl Zeiss, Jena, Germany). For a negative control, non-immune immunoglobulin and Alexa Flour 488-conjugated secondary antibodies (Invitrogen, Carlsbad, California, USA).
were used. The number of Mac2-positive cells was assessed using data collected from 3 sections of each animal (6 animals in each group).

**Real-time PCR**

Total RNA was extracted from adipose tissue and reverse transcribed to prepare cDNA. Real-time PCR was performed on a Thermal Cycler Dice (Takara Bio, Shiga, Japan), using SYBR Premix Ex Taq 2 (Takara Bio, Shiga, Japan). Dissociation curves were examined for the aberrant formation of primer dimers. PCR-amplified products were electrophoresed on 2% agarose gels to confirm the presence of a single amplicon. Data were expressed as gene expression levels relative to those of control.

**Enzyme-linked immunosorbent (ELISA) assay**

Blood from the right ventricular was collected in tubes containing citrate at a final concentration of 0.01 M. Serum was separated by centrifugation at 1,000 x g for 20 min and was stored -80°C. Adipose tissue was homogenized in 600 µL of buffer containing PBS and 0.04% Tween 80 using a rotary homogenizer. Homogenized tissue samples were centrifuged at 10,000 x g for 10 min, and the supernatants were collected. The amounts of IL-6, TNF-α, MCP-1, and M-CSF present in the serum were estimated using commercial ELISA kits (M6000B, MTA00B, and MJE00, R&D Systems, Minneapolis, MN. GWB-ZZD125, GenWay, San Diego, CA). The levels of IL-6, TNF-α, MCP-1, and M-CSF in the adipose tissue were estimated using ELISA kits (#88-7064-22 and #88-7324-22, eBioscience, San Diego, CA. ab100733, abcam, Cambridge, MA. GWB-ZZD125, GenWay, San Diego, CA). The assays were carried out according to the manufacturer’s instructions.

**Flow cytometry analysis**

To determine the number of circulating monocytes, peripheral blood cells were incubated with a cocktail of monoclonal antibodies (BD, San Jose, CA, USA) against T cells (CD90, 53-2.1), B cells (B220, RA3-6B2), NK cells (CD49b, HMa2 and NK1.1, PK136), granulocytes (Ly-6G, 1A8), myeloid cells (CD11b, M1/70), and monocyte subsets (Ly-6C, AL-21), as previously described (1). Monocyte numbers were calculated as total leukocytes multiplied by percent cells within the monocyte gate of the mononuclear cell fraction.
Statistical analysis

We performed the Kolmogorov-Smirnov Test for the normality of all continuous variables, and if the p-value were more than 0.05, which indicated all data was normal distribution and could be expressed as mean ± SE. Mean values were compared using ANOVA. If a statistically significant effect was found, Tukey-Kramer test was performed to analyze the differences between the groups. Significant differences among groups for dependent variables were detected by using two-way (maternal diet [normal diet versus high-fat diet] gender [male offspring versus female offspring]) analysis of variance. \( P < 0.05 \) was considered statistically significant.

Reference

Supplemental Material

Maternal high-fat diet exaggerates atherosclerosis in adult offspring by augmenting periaortic adipose tissue-specific proinflammatory response

Wakana: Maternal high-fat diet exaggerates atherosclerosis

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Supplemental Figure

Supplemental Figure I

Body weight, hemodynamics, and lipid profiles. (A) Body weights of dams on day 1 after delivery show a modest, but significant, increase in HFD-fed dam compared with ND fed-dam. Values are the mean ± SE for at least 6 mice in each group. \( *P < 0.01 \) vs. ND fed-dam. ND: normal chow. HFD: high-fat diet. (B) Offspring body weights show a significant increase after a high-cholesterol diet, but without discernible difference between O-ND and O-HFD. Values are the mean ± SE for at least 6 mice in each group. \( *P < 0.01 \) vs. 8-week-old O-ND. \( \#P < 0.01 \) vs. 8-week-old O-HFD. O-ND: Offspring of normal diet-fed dam. O-HFD: Offspring of high-fat diet-fed dam. (C) Mean blood pressure and heart rate before and after a high-cholesterol diet show no significant difference between O-ND and O-HFD. Values are the mean ± SE for at least 6 mice in each group. O-ND: Offspring of normal diet-fed dam. O-HFD: Offspring of high-fat diet-fed dam. (D) Lipid profiles before and after a high-cholesterol diet show no significant difference between O-ND and O-HFD. Values are the mean ± SE for at least 6 mice in each group. \( *P < 0.01 \) vs. 8-week-old O-ND. \( \#P < 0.01 \) vs. 8-week-old O-HFD. O-ND: Offspring of normal diet-fed dam. O-HFD: Offspring of high-fat diet-fed dam. T-chol: total cholesterol. LDL-chol: low-density lipoprotein cholesterol. HDL-chol: high-density lipoprotein cholesterol. TG: triglyceride.

Supplemental Figure II

The effect of maternal high-fat diet on the epididymal white adipose tissue in offspring. (A) Epididymal adipose tissue weight in offspring before and after a high-cholesterol diet. Values are the mean ± SE for at least 6 mice in each group. \( *P < 0.01 \) vs. 8-week-old O-ND. \( \#P < 0.01 \) vs. 8-week-old O-HFD. O-ND: Offspring of normal diet-fed dam. O-HFD: Offspring of high-fat diet-fed dam. (B) Representative images of hematoxylin-eosin stained sections of the epididymal adipose tissue used for quantification of adipocyte sizes. The scale bar shows 50-µm intervals. O-ND: Offspring of normal diet-fed dam. O-HFD: Offspring of high-fat diet-fed dam. (C) Quantification of mean adipocyte area. Values are the mean ± SE for at least 6 mice in each group. \( \#P < 0.05 \) vs. 20-week-old O-HFD. (D) Quantitative PCR analysis of the mRNA expression levels of adipocyte differentiation-related genes in the epididymal adipose tissue. Values are the
mean ± SE relative to those in 8-week-old O-ND. Each group had at least 6 mice. *P < 0.05 vs. 8-week-old O-ND. **P < 0.01 vs. 8-week-old O-ND. †P < 0.01 vs. 8-week-old O-HFD.

Supplemental Figure III
The effect of maternal high-fat diet on the thoracic periaortic adipose tissue in offspring. (A) Thoracic periaortic adipose tissue weight in offspring before and after a high-cholesterol diet. Values are the mean ± SE for at least 6 mice in each group. *P < 0.05 vs. 8-week-old O-ND. O-ND: Offspring of normal diet-fed dam. O-HFD: Offspring of high-fat diet-fed dam. (B) Representative images of hematoxylin-eosin stained sections of thoracic periaortic adipose tissue. The scale bar shows 50-µm intervals. O-ND: Offspring of normal diet-fed dam. O-HFD: Offspring of high-fat diet-fed dam. (C) Quantitative PCR analysis of the mRNA expression levels of adipocyte differentiation-related genes in the thoracic periaortic adipose tissue. Values are the mean ± SE relative to those in 8-week-old O-ND. Each group had at least 6 mice. *P < 0.05 vs. 8-week-old O-ND. †P < 0.01 vs. 8-week-old O-HFD.

Supplemental Figure IV
Maternal high-fat diet does not affect the circulating levels of inflammatory adipocytokines in offspring. Circulating concentration levels of IL-6, TNF-α, and MCP-1 show high concentrations in 20-week-old offspring compared with 8-week-old offspring in each group, but without a significant difference between the two groups. Values are the mean ± SE for at least 6 mice in each group. *P < 0.05 vs. 8-week-old O-ND. **P < 0.01 vs. 8-week-old O-ND. †P < 0.05 vs. 8-week-old O-HFD. ‡P < 0.01 vs. 8-week-old O-HFD. IL-6: interleukin-6. TNF-α: tumor necrosis factor-α. MCP-1: monocyte chemotactic protein-1.

Supplemental Figure V
Intra-abdominal transplantation site of tPAT and the relative position of abdominal aorta and tPAT graft. (A) Representative picture of the harvested tPAT from 8-week-old mice. The scale bar shows 3-mm intervals. (B) Illustration showing the transplantation site in recipient mice. (C) Representative images of endogenous tPAT (a) and transplanted tPAT graft (b-g). The tPAT of GFP-transgenic mice was transplanted into wild-type mice. The scale bar shows 50-µm (a,c,d-f), 100-µm (b), and 20-µm (g) intervals, respectively. HE:

Supplemental Figure VI
The effect of maternal high-fat diet on the number of circulating monocytes in offspring. (A) The number of Lin^−CD11b^+ monocytes was examined by flow cytometry. Values are the mean ± SE for at least 6 mice in each group. ^P < 0.05 vs. 8-week-old O-HFD. O-ND: Offspring of normal diet-fed dam. O-HFD: Offspring of high-fat diet-fed dam. Lin^-: CD90^−B220^− CD49b^− NK1.1^− Ly-6G^−. (B) The number of Lin^−CD11b^+Ly6C^hi^ monocytes was examined by flow cytometry. Values are the mean ± SE for at least 6 mice in each group. *P < 0.05 vs. 8-week-old O-ND. #P < 0.01 vs. 8-week-old O-HFD. Lin^-: CD90^−B220^− CD49b^− NK1.1^− Ly-6G^−.

Supplemental Figure VII
M-CSF mRNA expression level does not correlate with the plaque development. M-CSF mRNA expression in tPAT of 20-week-old male offspring does not correlate with the percent plaque area in entire aorta.

Supplemental Figure VIII
The effect of maternal high-fat diet on mRNA expression of VCAM-1 and ICAM-1 in the aorta of offspring. (A) and (B) Quantitative PCR analysis of the mRNA expression levels of VCAM-1 and ICAM-1 in the descending thoracic aorta of 8-week-old offspring. Values are the mean ± SE relative to those in 8-week-old O-ND. Each group has at least 6 mice. *P < 0.01 vs. 8-week-old O-ND. O-ND: Offspring of normal diet-fed dam. O-HFD: Offspring of high-fat diet-fed dam.
Supplemental Figure II

A

(mg/g BW)

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B

C

Mean adipocyte size

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D

Relative mRNA levels

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Supplemental Figure IV

**Notes:**
- **IL-6:**
  - O-ND 8wk: 0 pg/ml
  - O-HFD 8wk: 5 pg/ml
  - O-ND 20wk: 10 pg/ml
  - O-HFD 20wk: 15 pg/ml

- **TNF-α:**
  - O-ND 8wk: 0 pg/ml
  - O-HFD 8wk: 5 pg/ml
  - O-ND 20wk: 10 pg/ml
  - O-HFD 20wk: 15 pg/ml

- **MCP-1:**
  - O-ND 8wk: 0 pg/ml
  - O-HFD 8wk: 0 pg/ml
  - O-ND 20wk: 0 pg/ml
  - O-HFD 20wk: 0 pg/ml

**Significance Levels:**
- **O-ND vs O-HFD:**
  - *: p < 0.05
  - **: p < 0.01
  - ##: p < 0.001

**Groups:**
- O-ND
- O-HFD
Supplemental Figure VI

**A**

Monocytes

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**B**

Ly6C$^{hi}$ monocytes

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* O-ND vs O-HFD
# O-ND vs O-HFD
Supplemental Figure VII

Percent plaque area in entire aorta vs. Relative mRNA levels of M-CSF.

r=0.19
p=0.61
n=8
Supplemental Figure VIII

(A) Relative mRNA levels of VCAM1 in O-ND and O-HFD conditions.

(B) Relative mRNA levels of ICAM1 in O-ND and O-HFD conditions.