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## Supplemental Information

# Macrophage miR-34a Is a Key Regulator of Cholesterol Efflux and Atherosclerosis

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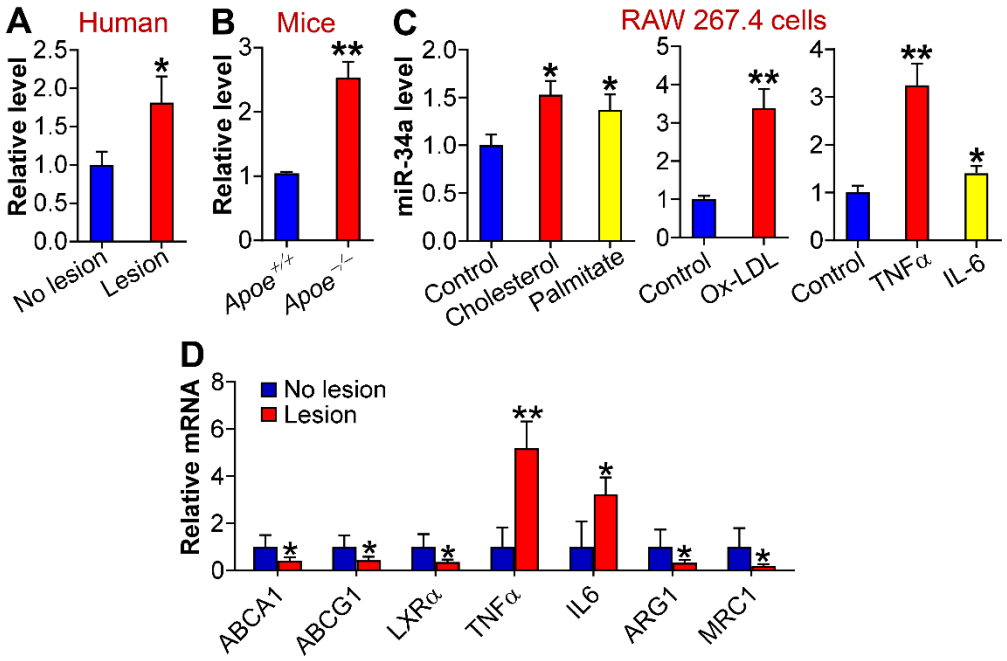


**Figure S1. MiR-34a is increased in human or mouse atherosclerotic lesions and is up-regulated by inflammatory lipids and cytokines**

(A) MiR-34a expression was measured in the atherosclerotic lesions and surrounding lesion-free aortas of human carotids (n=8 or 9). (B) 10 weeks-old wild-type (*Apoe*$^{+/+}$) mice or *Apoe*$^{-/-}$ mice were fed a chow diet or a Western diet for 12 weeks, respectively. MiR-34a expression was determined in atherosclerotic lesions of Western diet-fed *Apoe*$^{-/-}$ mice (n=6) or the aortas of chow-fed *Apoe*$^{+/+}$ mice (n=4). (C) RAW267.4 cells were treated for 24 h with vehicle, cholesterol (10 μg/ml), palmitate (300 μg/ml) (left panel, n=4), oxidized LDL (ox-LDL; 50 μg/ml) (middle panel, n=4) or TNFα (25 ng/ml) or IL-6 (25 ng/ml) (right panel, n=4). MiR-34a levels were quantified by qRT-PCR. (D) mRNA levels of genes in the atherosclerotic lesions and surrounding lesion-free aortas of human carotids (n=8-9). A two-tailed Student's *t*-test was used for statistical analysis. **P*<0.05, ***P*<0.01

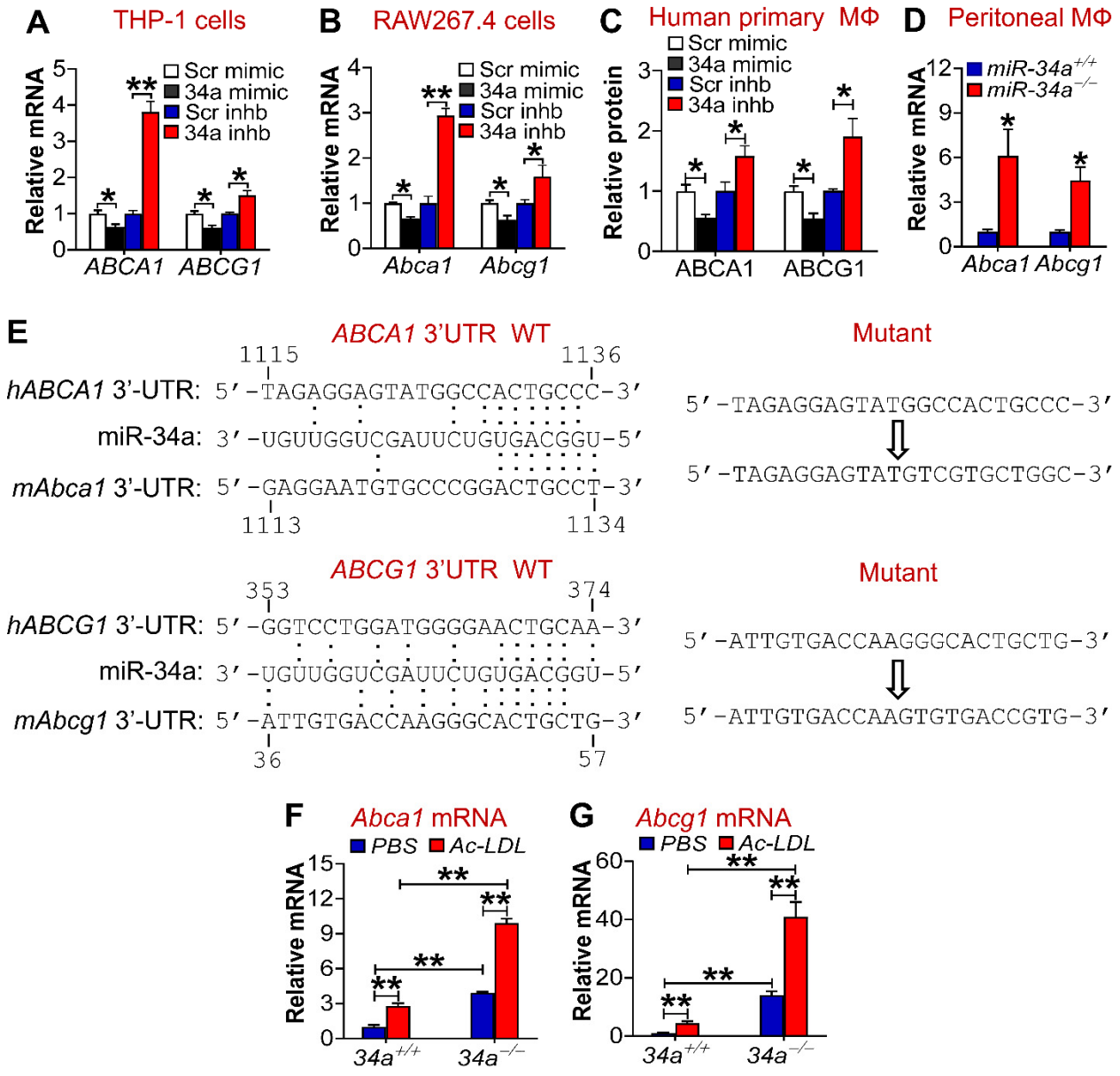


**Figure S2. MiR-34a regulates *ABCA1* and *ABCG1* expression in macrophages**
(A-C) THP-1 cells (A), RAW267.4 cells (B) or human primary macrophages (C) were transfected with 75 nM of miR-34a mimics, miR-34a inhibitors (34a inhb), or scramble controls (n=4-6). mRNA levels were determined. (D) Peritoneal macrophages were isolated from chow-fed *miR-34a*$^{+/+}$ or *miR-34a*$^{-/-}$ mice. *Abca1* and *Abcg1* mRNA levels were determined (n=4). (E) The binding sites for miR-34a in the 3'UTRs of human or mouse *ABCA1*/*Abca1* (top panel; left) or *ABCG1/Abcg1* (bottom panel; left) are presented. The mutant sequences of 3'UTRs are shown on the right. (F and G) Peritoneal macrophages were isolated from chow-fed *miR-34a*$^{+/+}$ mice or *miR-34a*$^{-/-}$ mice, and then treated with either PBS or acetylated-LDL (Ac-LDL; 50 μg/ml) for 24 h (n=4). *Abca1* (F) or *Abcg1* (G) mRNA levels were determined. All the data are expressed as mean±SEM. A two-tailed Students' *t*-test was used for statistical analysis. **P*<0.05, ***P*<0.01

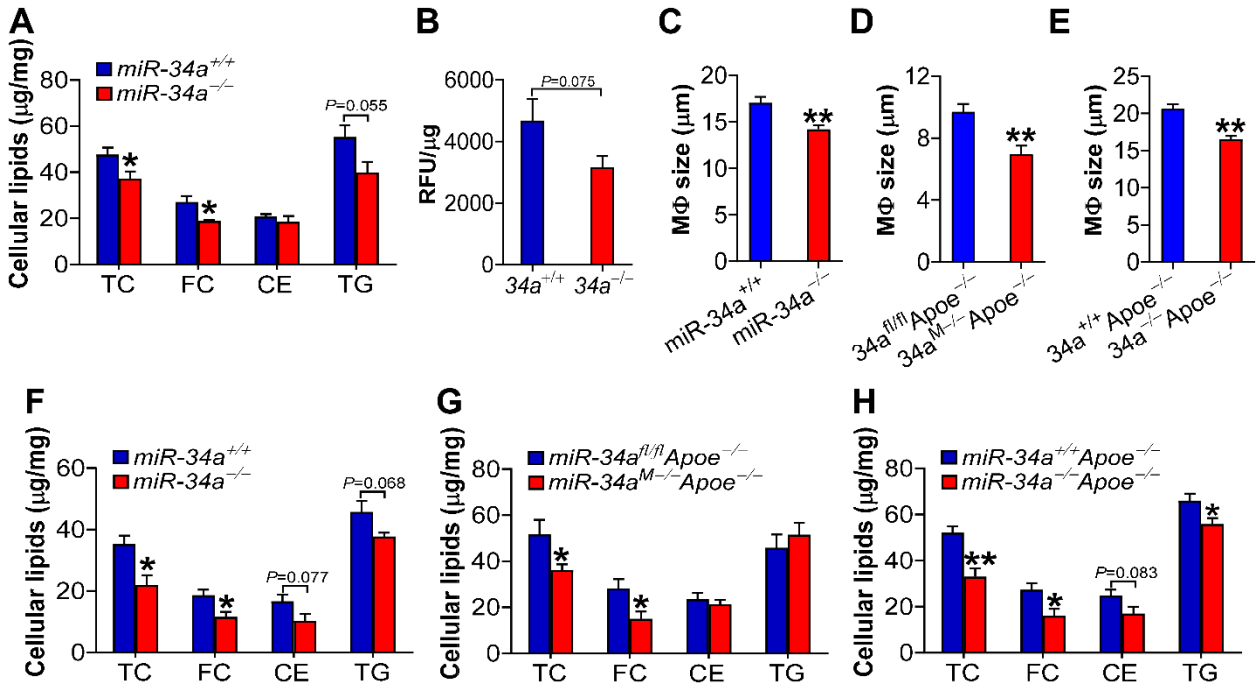


**Figure S3. MiR-34a ablation reduces free cholesterol levels in macrophages**
(A and B) Mouse peritoneal macrophages were isolated from chow-fed *miR-34a*$^{+/+}$ or *miR-34a*$^{-/-}$ mice, and then treated with Ac-LDL (50 μg/ml) for 24 h (A) or 48 h (B). In (A), cellular lipid levels were quantified (n=5). In (B), macrophages were also treated with DiI-LDL (10 μg/ml) for the last 4 h to investigate cholesterol uptake (n=8). RFU, relative fluorescence units. (C-H) Peritoneal macrophages were isolated from the following mice that had been fed a Western diet for one month: *miR-34a*$^{+/+}$ or *miR-34a*$^{-/-}$ mice (C, F), *34a*$^{fl/fl}$*Apoe*$^{-/-}$ or *miR-34a*$^{M-/-}$*Apoe*$^{-/-}$ mice (D, G) or *miR-34a*$^{+/+}$*Apoe*$^{-/-}$ or *miR-34a*$^{-/-}$*Apoe*$^{-/-}$ mice (E, H). Macrophage size (C-E) (n=7) and cellular lipid levels (F-G) (n=5) were determined. TC, total cholesterol. FC, free cholesterol. CE, cholesterol esters. TG, triglycerides. All the data are expressed as mean±SEM. A two-tailed Students' *t*-test was used for statistical analysis. **P*<0.05, ***P*<0.01

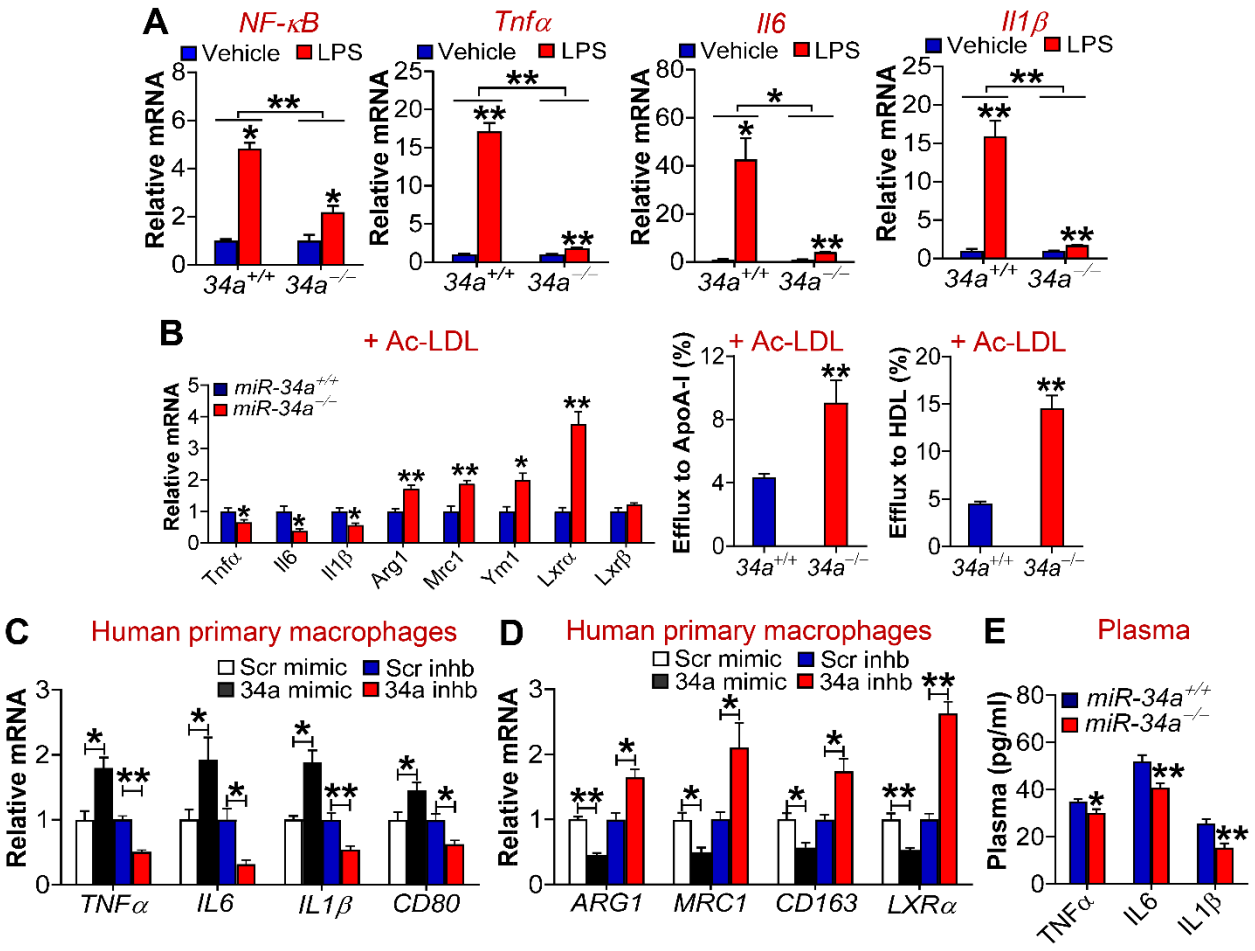


**Figure S4. MiR-34a inhibition regulates macrophage polarization in both human and mouse primary macrophages**

(A and B) Mouse peritoneal macrophages were isolated from *miR-34a*$^{+/+}$ or *miR-34a*$^{-/-}$ mice, and then treated with either vehicle or LPS (100 ng/ml) for 6 h (A), or Ac-LDL (50 μg/ml) for 24 h (B) (n=4 per group). mRNA levels were determined (A, B (left panel)) and cholesterol efflux to ApoA-I or HDL was analyzed (B; middle and right panels). (C and D) Human primary macrophages were transfected with 75 nM miR-34a mimics, miR-34a inhibitors (34a inhb), or scramble controls (n=4 per group). After 24 h, mRNA levels were determined. (E) Plasma cytokine levels were determined in chow-fed *miR-34a*$^{+/+}$ or *miR-34a*$^{-/-}$ mice (n=8). All the data are expressed as mean±SEM. A two-tailed Students' *t*-test was used for statistical analysis. **P*<0.05, ***P*<0.01

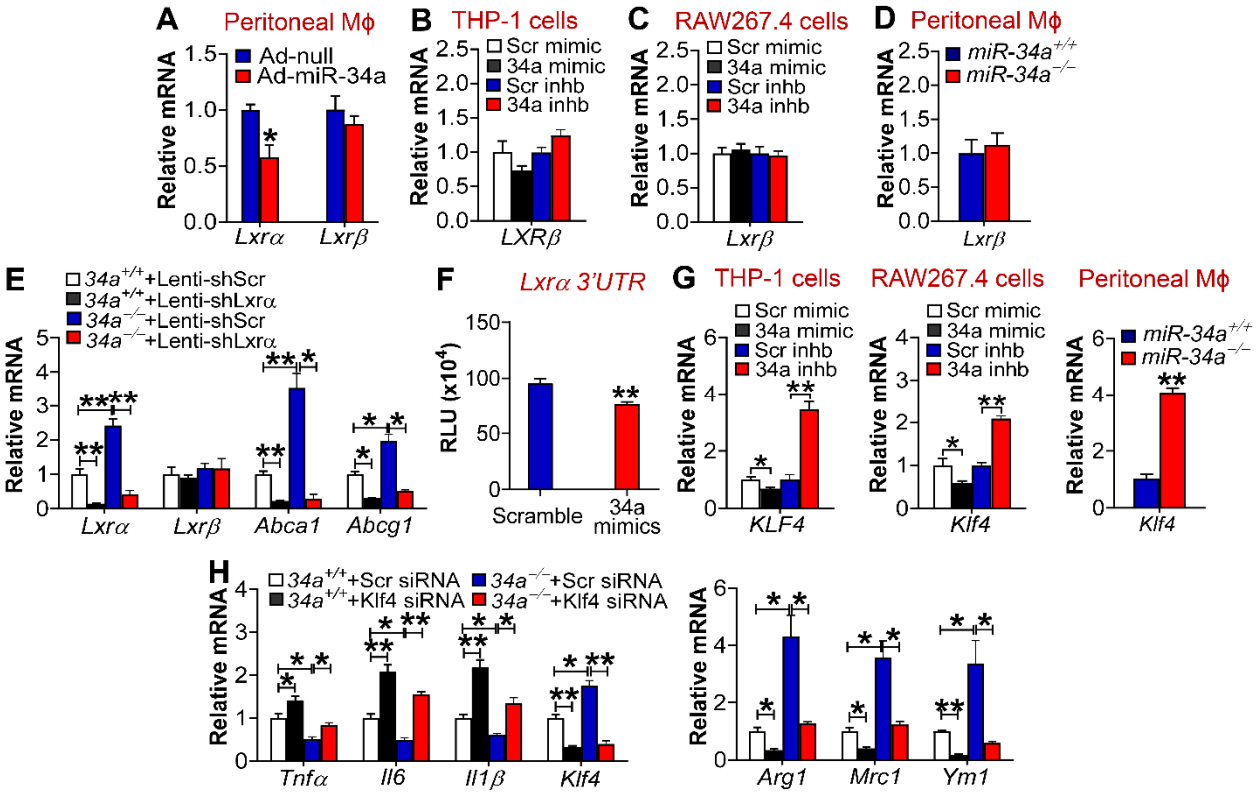


**Figure S5. MiR-34a regulates LXRα and KLF4, but not LXRβ in macrophages**
(A) Peritoneal macrophages were isolated from C57BL/6 mice and then infected with Ad-null or Ad-miR-34a for 24 h. mRNA levels were quantified (n=4). (B, C) THP-1 cells (B) or RAW267.4 cells (C) were treated with 75 nM miR-34a mimics, inhibitors or their scramble controls for 24 h (n=4-6). mRNA levels were determined. (D) Peritoneal macrophages were isolated from chow-fed *miR-34a*$^{+/+}$ or *miR-34a*$^{-/-}$ mice, and mRNA levels were determined (n=4). (E) Peritoneal macrophages were isolated from *miR-34a*$^{+/+}$ or *miR-34a*$^{-/-}$ mice, and then infected with lentiviruses expressing shRNA against scramble sequences (Lenti-shScr) or *Lxrα* (Lenti-shLxrα) for 48 h. mRNA levels were determined (n=3). (F) A plasmid expressing *Lxrα* 3'UTR was transfected into HepG2 cells together with miR-34a mimics or scramble controls. After 36 h, relative luciferase units (RLU) were determined (n=8). (G) THP-1 cells (left panel) or RAW267.4 cells (middle panel) were treated for 24 h with 75 nM miR-34a mimics, inhibitors or scramble controls (n=4-6). Peritoneal macrophages were isolated from chow-fed *miR-34a*$^{+/+}$ or *miR-34a*$^{-/-}$ mice (right panel) (n=4). *KLF4/Klf4* mRNA levels were determined. (H) Peritoneal macrophages were isolated from chow-fed *miR-34a*$^{+/+}$ or *miR-34a*$^{-/-}$ mice, and then transfected with 75 nM of siRNAs against scramble sequences (Scr) or *Klf4* (n=3). After 24 h, mRNA levels were quantified. All the data are expressed as mean±SEM. A two-tailed Students' *t*-test was used for statistical analysis. **P*<0.05, ***P*<0.01

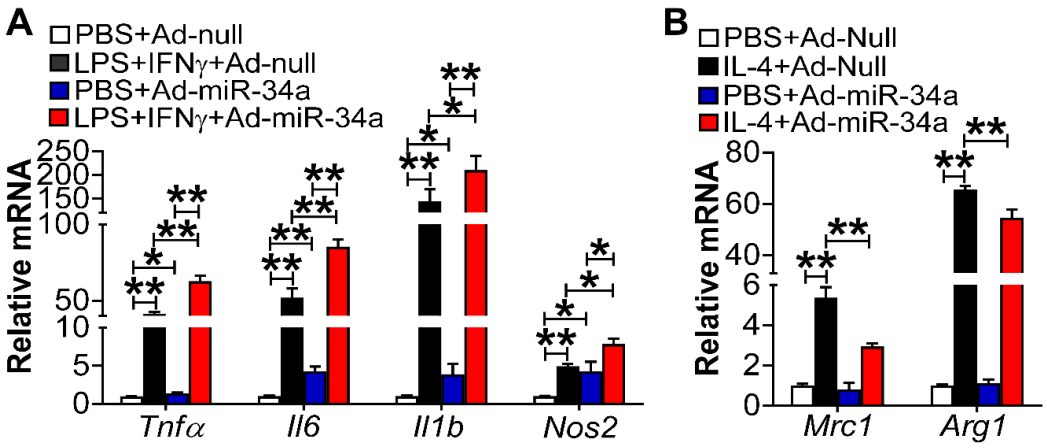


**Figure S6. MiR-34a over-expression regulates M1/M2 macrophage polarization**
(A and B) Peritoneal macrophages were isolated from C57BL/6 mice and then infected with Ad-null or Ad-miR-34a for 24 h, followed by treatment for 24 h with vehicle, LPS (1 μg/ml) plus IFNγ (50 ng/ml) (A) or IL-4 (20 ng/ml) (B). mRNA levels were quantified (n=4). All the data are expressed as mean±SEM. A two-way ANOVA test was used for statistical analysis. **P*<0.05, ***P*<0.01

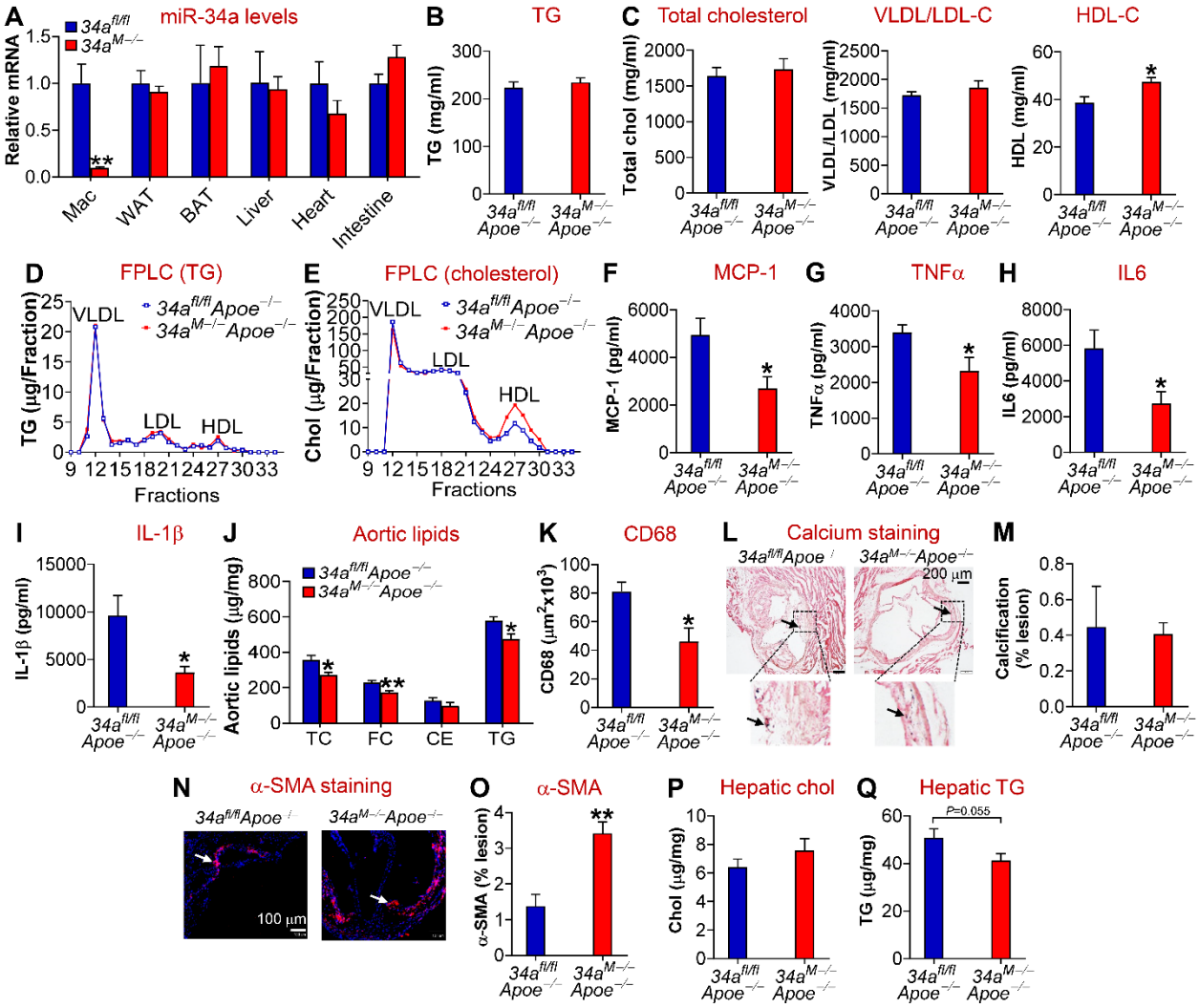


**Figure S7. Effect of macrophage-selective miR-34a deletion in *Apoe*$^{-/-}$ mice on lipid metabolism, inflammation, plaque calcification or plaque smooth muscle content**

(A) MiR-34a levels in peritoneal macrophages or different tissues of *miR-34a*$^{fl/fl}$ mice or *miR-34a*$^{fl/fl}$*LyzM-Cre* (*34a*$^{M-/-}$) mice (n=3). (B-Q) *34a*$^{fl/fl}$*Apoe*$^{-/-}$ mice and *34a*$^{M-/-}$*Apoe*$^{-/-}$ mice were fed a Western diet for 18 weeks (n=8). Plasma levels of TG (B), total cholesterol (C, left panel), VLDL/LDL-C (C, middle panel) and HDL-C (C, right panel) were quantified. Plasma TG (D) or cholesterol (E) lipoprotein profiles were determined by FPLC. Plasma levels of MCP-1 (F), TNFα (G), IL6 (H) and IL-1β (I) as well as aortic lipid levels (J) were analyzed (n=7). CD68 protein levels in the lesions of aortic roots were determined (K). Aortic roots were stained with a Von Kossa kit (L) and the calcification areas in the plaques were quantified (M) (n=3). Aortic roots were also immunostained using an antibody against α-SMA (N) and the α-SMA-positive areas in the plaques were quantified (O) (n=3-6). In addition, hepatic cholesterol (P) or TG (Q) levels were quantified (n=8). See also Figure S8. In (L), arrows point to the calcification sites and the bottom panels show enlarged images. In (N), arrows point to α-SMA-positive areas. All the data are expressed as mean±SEM. A two-tailed Students' *t*-test was used for statistical analysis. **P*<0.05, ***P*<0.01

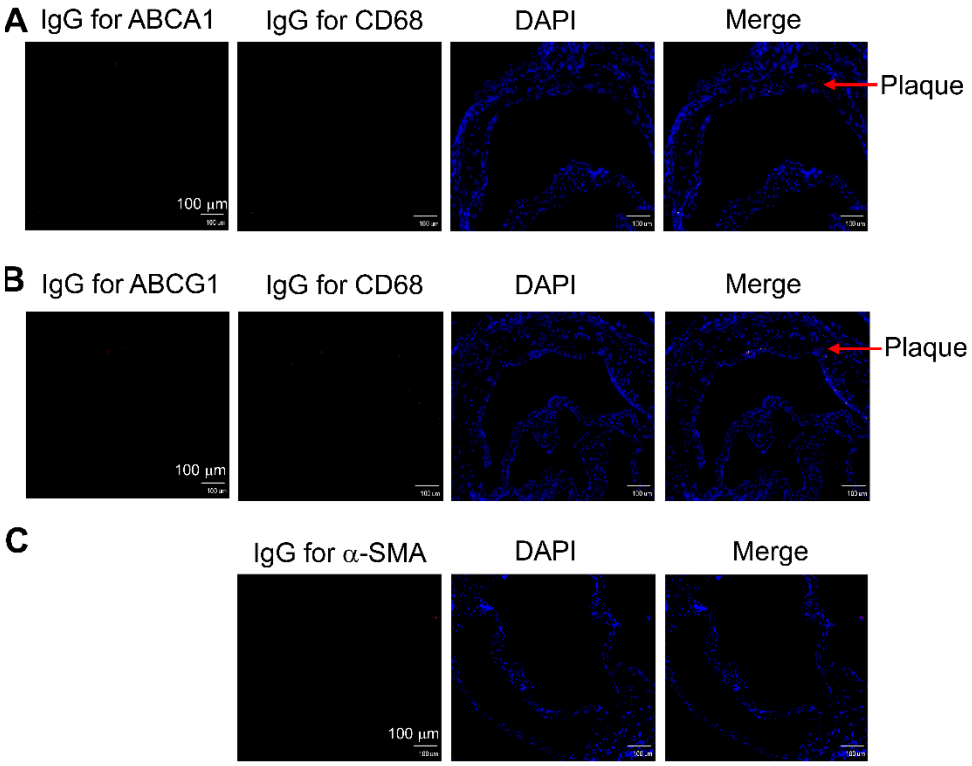


**Figure S8. Negative controls for ABCA1, ABCG1, CD68 or α-SMA antibodies**
*34a*$^{fl/fl}$*Apoe*$^{-/-}$ mice and *34a*$^{M-/-}$*Apoe*$^{-/-}$ mice were fed a Western diet for 18 weeks (n=8). Immunostaining of aortic roots was performed using IgG for ABCA1 (A), ABCG1 (B), α-SMA (C) or CD68 (A, B). The same sections were also stained with DAPI. Arrows point to the plaques.

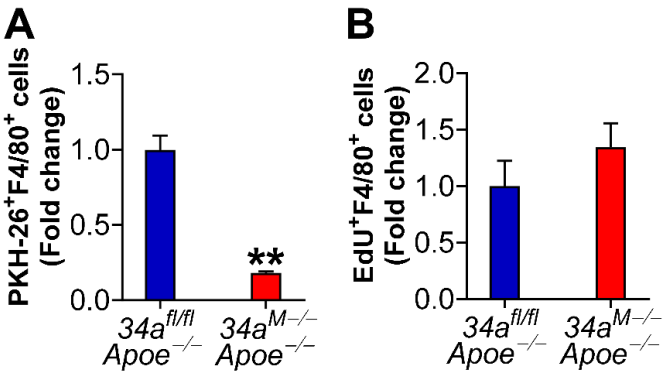


**Figure S9. Macrophage-selective miR-34a deletion reduces monocyte infiltration in *Apoe*$^{-/-}$ mice**

(A) *34a*$^{fl/fl}$*Apoe*$^{-/-}$ mice and *34a*$^{M-/-}$*Apoe*$^{-/-}$ mice were fed a Western diet for 12 weeks and then injected with 4x10$^6$ PKH-26$^+$ monocytes (see Materials and Methods). PKH-26$^+$F4/80$^+$ macrophages in the aortas were analyzed by flow cytometry (n=8). (B) *34a*$^{fl/fl}$*Apoe*$^{-/-}$ mice and *34a*$^{M-/-}$*Apoe*$^{-/-}$ mice were fed a Western diet for 12 weeks and then injected with EdU (2 mg/kg/d) for 3 days. EdU$^+$F4/80$^+$ macrophages in the aortas were analyzed by flow cytometry (n=6). All the data are expressed as mean±SEM. A two-tailed Students' *t*-test was used for statistical analysis. ***P*<0.01

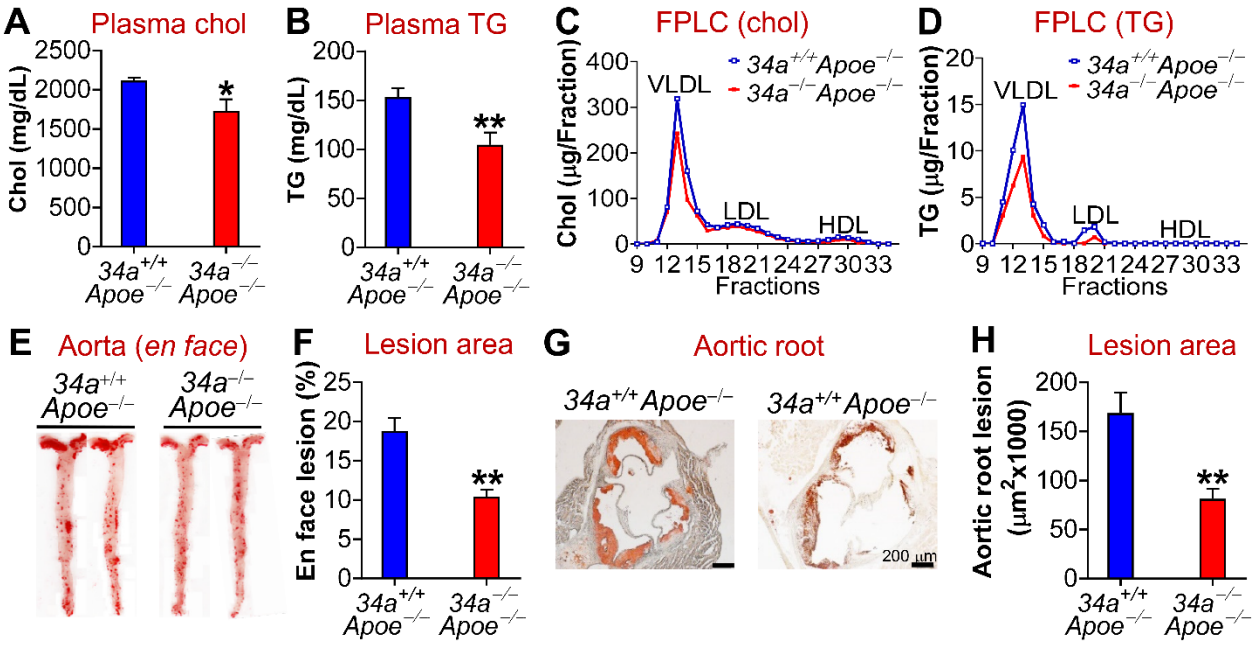


**Figure S10. Loss of miR-34a in *Apoe*$^{-/-}$ mice improves dyslipidemia and attenuates the development of atherosclerosis**

(A-H) *miR-34a*$^{+/+}$*Apoe*$^{-/-}$ mice and *miR-34a*$^{-/-}$*Apoe*$^{-/-}$ mice were fed a Western diet for 18 weeks. Plasma cholesterol (A) or TG (B) levels were quantified (n=7). Plasma cholesterol (C) or TG (D) lipoprotein profiles were analyzed by FPLC. *En face* aortas were stained by Oil Red O (ORO) (E) and the plaque size was analyzed (F) (n=7). Aortic roots were also stained by ORO (G) and the plaque size was determined (H) (n=7)All the data are expressed as mean±SEM. A two-tailed Students' *t*-test was used for statistical analysis. **P*<0.05, ***P*<0.01

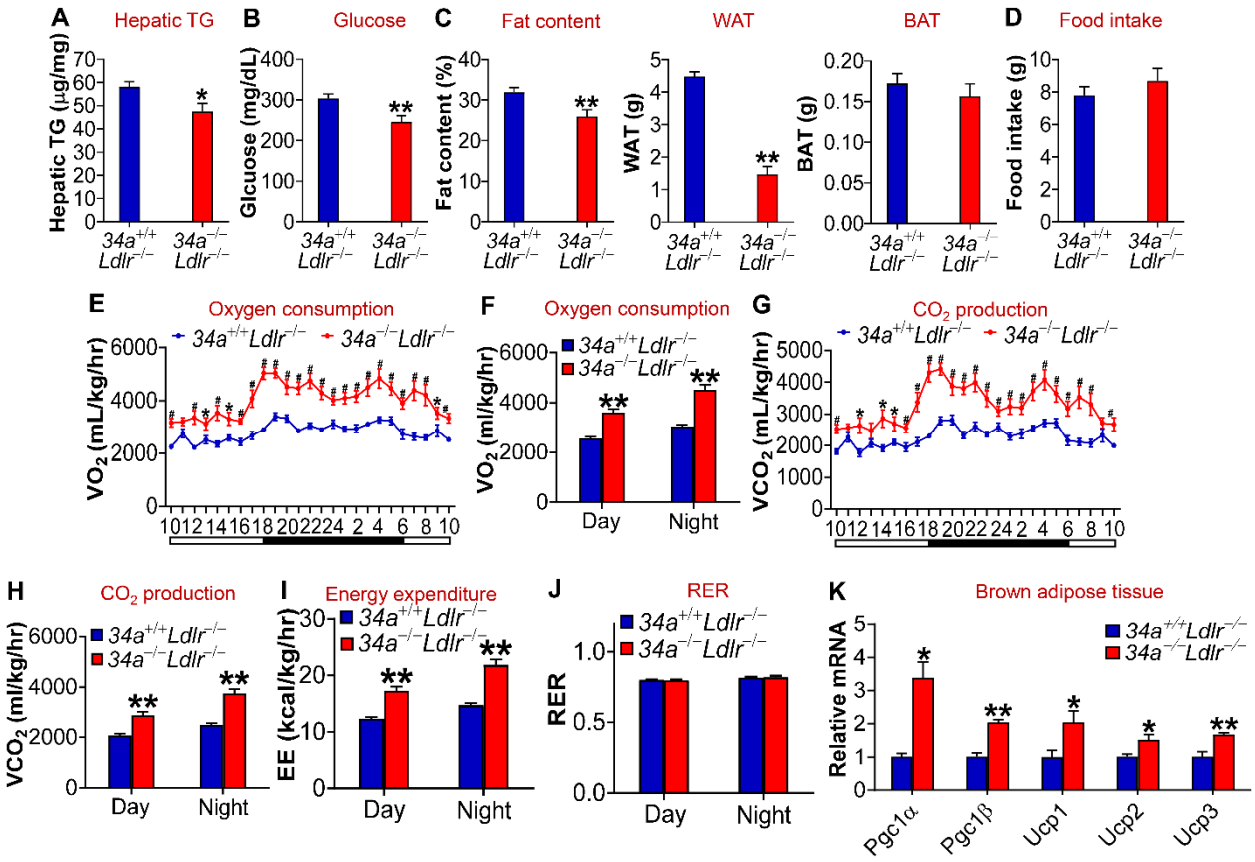


**Figure S11. Loss of miR-34a in *Ldlr*$^{-/-}$ mice improves metabolic homeostasis and enhances energy expenditure**

(A-K) *miR-34a*$^{+/+}$*Ldlr*$^{-/-}$ mice and *miR-34a*$^{-/-}$*Ldlr*$^{-/-}$ mice were fed a Western diet for 18 weeks. Hepatic TG (A, n=11-15) or plasma glucose (B, n=14-15) levels were determined. Body fat content (C, left panel), white adipose tissue (WAT) weight (C, middle panel), and brown adipose tissue (BAT) weight (C, right panel) were analyzed (n=10-12). Food intake (g/day) (D), oxygen consumption (E), average oxygen consumption (F), $CO_2$ production (G), average $CO_2$ production (H), energy expenditure (I), and respiration exchange rates (RER) (J) during day or night time were determined (n=7-8). mRNA levels in BAT were quantified (K) (n=4). All the data are expressed as mean±SEM. A two-tailed Students' *t*-test was used for statistical analysis. **P*<0.05, ***P*<0.01

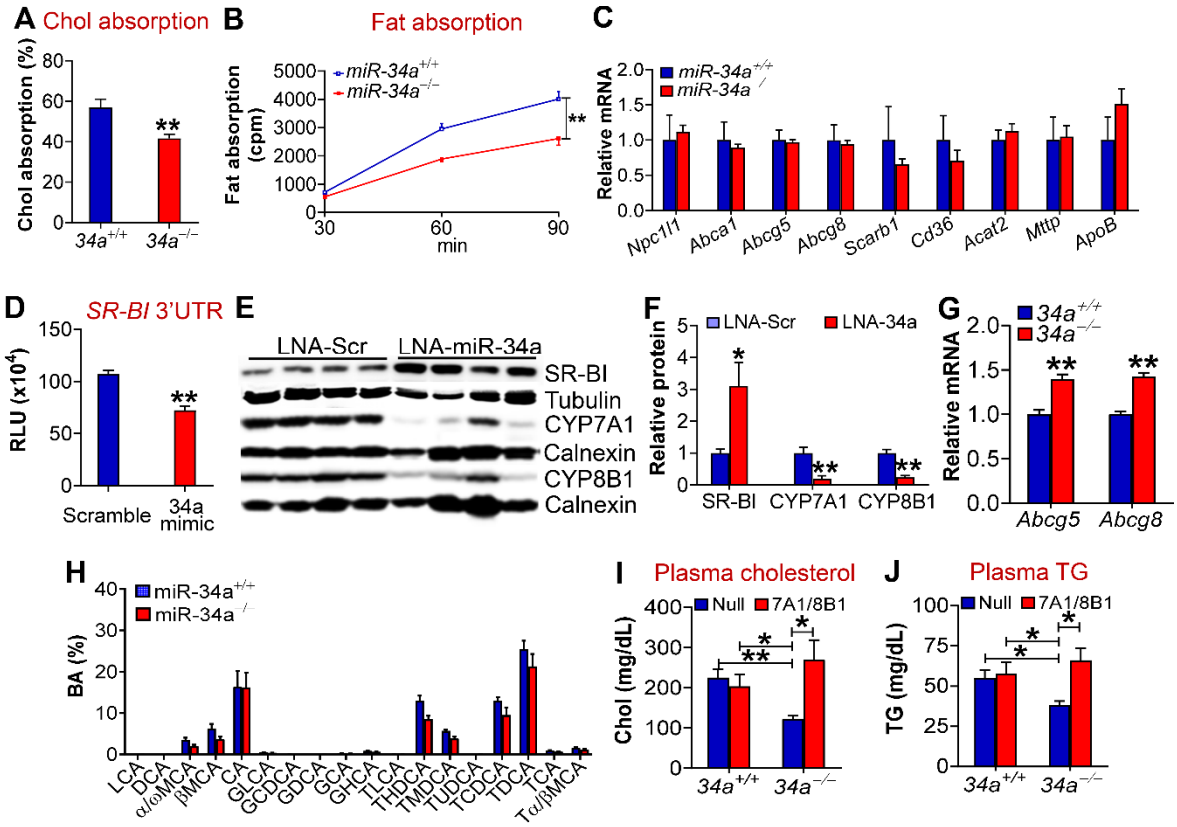


**Figure S12. Loss of miR-34a inhibits cholesterol or fat absorption, and hepatic CYP7A1 and CYP8B1 expression**

(A-C) *miR-34a*$^{+/+}$ mice or *miR-34a*$^{-/-}$ mice were fed a Western diet for 18 weeks. Cholesterol absorption was performed using dual-isotope ratio method (n=7-10) (A). Fat absorption was carried out after i.v. injection of Tyloxapol and gavage with [$^3$H]triolein (n=5-6) (B). mRNA levels in the intestine were quantified by qRT-PCR (n=8) (C). (D) The plasmid expressing *SR-BI* 3'UTR was transfected into HepG2 cells together with 75 nM scramble oligos or miR-34a mimics. After 36 h, relative luciferase units (RLU) were determined (n=8). (E and F) C57BL/6 mice were i.p. injected with LNA-Scr or LNA-miR-34a (10 mg/kg) once a week for 5 weeks. Western blotting was performed (E) and protein levels were quantified (F) (n=4-5). (G) Hepatic mRNA levels in *miR-34a*$^{+/+}$ or *miR-34a*$^{-/-}$ mice were quantified (n=8). (H) Bile acid composition in the bile collected from *miR-34a*$^{+/+}$ mice or *miR-34a*$^{-/-}$ mice was analyzed (n=8). The overall abundance of measured bile acids was set as 100% in each bile sample. (I, J) *miR-34a*$^{+/+}$ or *miR-34a*$^{-/-}$ mice were fed a Western diet for 18 weeks, and then injected i.v. with Ad-Empty (Null) or Ad-CYP7A1 plus Ad-CYP8B1 (7A1/8B1). After 7 days, plasma cholesterol (I) or triglyceride (J) levels were quantified (n=6). In (A, C-J), a two-tailed Students' *t*-test was used for statistical analysis. In (B), a two-way ANOVA was used for statistical analysis. **P*<0.05, ***P*<0.01

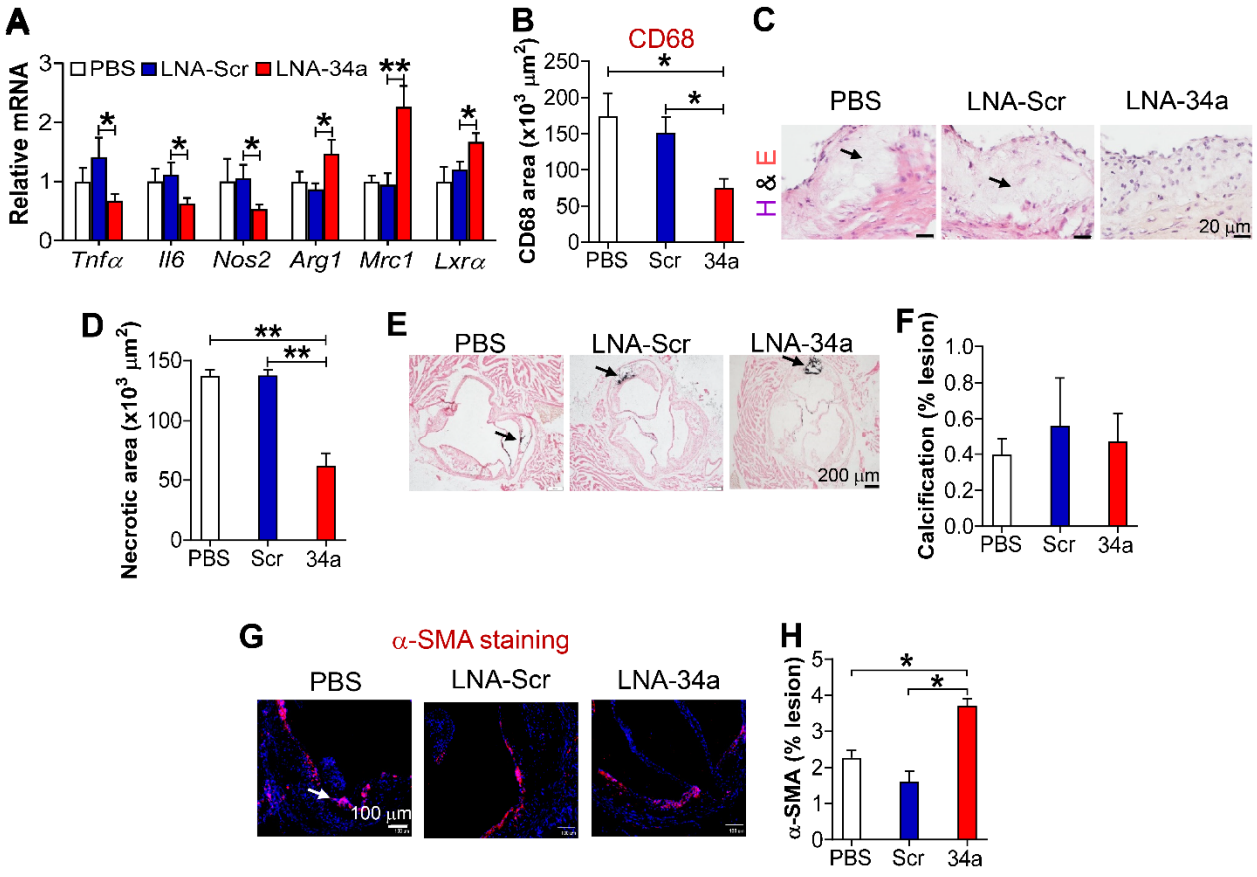


**Figure S13. MiR-34a inhibition regulates M1/M2 macrophage polarization, plaque stability and smooth muscle content in the plaques of *Ldlr*$^{-/-}$ mice**

(A-H) *Ldlr*$^{-/-}$ mice were i.p. injected with PBS, LNA-Scr or LNA-miR-34a as described in the legend of Figure 6. mRNA levels in the plaques of aortic roots were determined by qRT-PCR (n=6-7) (A). CD68 protein expression in aortic roots was analyzed (n=5) (B). Aortic roots were stained by H & E (C) and the necrotic areas were quantified (n=3) (D). Calcium in the aortic roots was stained using a Von Kossa Stain kit (E) and calcification levels in the plaques were determined (F). In addition, aortic roots were immunostained using an α-SMA antibody (G) and α-SMA-positive areas in the plaques were quantified (H). In (C), the arrows point to necrotic areas. In (E), the arrows point to calcification areas. In (G), the arrow points to α-SMA-positive area. The lesion size in the LNA-34a group was reduced. All the data are expressed as mean±SEM. A two-tailed Students' *t*-test was used for statistical analysis. **P*<0.05, ***P*<0.01