# Retinol-Binding Protein-Dependent Cholesterol Uptake Regulates Macrophage Foam Cell Formation and Promotes Atherosclerosis



Editorial, see p 1355

**BACKGROUND:** Retinol-binding protein 4 (RBP4) is an adipokine that plays decisive roles in glucose metabolism and insulin sensitivity. Elevated circulating RBP4 levels were reported to be associated with increased risk for cardiovascular disease, but the precise role of RBP4 in atherosclerotic diseases and its mechanisms of action remain elusive.

**METHODS:** Serum RBP4 levels of 1683 participants from South China were evaluated and the occurrence of major adverse cardiovascular events was followed up for 5 years. Apolipoprotein E-deficient mice infected with RBP4-overexpressing/silencing adenovirus, J774A.1 macrophages, and primary peritoneal macrophages from RBP4 transgenic mice were used for investigating the function of RBP4 in foam cell formation.

**RESULTS:** Prospective cohort studies revealed that baseline serum RBP4 level was an independent predictor for incidence of adverse cardiovascular events after adjustment for traditional risk factors. Increased RBP4 expression was observed in atherosclerotic lesions of aortic specimens from both humans and apolipoprotein E-deficient mice, and RBP4 was localized to areas rich in macrophage foam cells. RBP4 inhibition attenuated whereas overexpression accelerated atherosclerosis progression in apolipoprotein E-deficient mice. Both treatment with exogenous recombinant RBP4 and overexpression of RBP4 gene promoted macrophage-derived foam cell formation through the activation of scavenger-receptor CD36-mediated cholesterol uptake, and RBP4 transcriptionally upregulated CD36 expression in a manner dependent on jun N-terminal kinase and signal transducer and activator of transcription 1. The tyrosine kinase c-Src was identified as the upstream regulator of jun N-terminal kinase-signal transducer and activator of transcription 1-mediated CD36-dependent cholesterol uptake, and RBP4 challenge was found to alter the membrane distribution of c-Src and cause c-Src to partition into lipid-raft membrane subdomains, where the kinase was activated. Lastly, Toll-like receptor 4, but not retinol or stimulated by retinoic acid 6, mediated the inductive effects of RBP4 in macrophages.

**CONCLUSIONS:** Inclusion of RBP4 levels in traditional models enhances the predictive ability for the incidence of atherosclerotic events. RBP4 promotes atherogenesis by inducing macrophage-derived foam cell formation.

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## Clinical Perspective

### What Is New?

- Despite the link between retinol-binding protein 4 (RBP4) levels and cardiovascular disease risk, few prospective studies have been conducted on the general population, and here we provide evidence that the predictive value of RBP4 for cardiovascular disease is independent and additive to that of traditional risk factors.
- We present experimental evidence that elevated RBP4 expression facilitates macrophage-derived foam cell formation, a hallmark of atherosclerosis, through c-Src-jun N-terminal kinase-signal transducer and activator of transcription 1-CD36-dependent cholesterol uptake and thereby accelerates atherosclerosis progression.

### What Are the Clinical Implications?

- Our findings support the use of RBP4 as a novel and reliable biomarker in the prediction of cardiovascular disease risk.
- The finding that elevated RBP4 promotes atherosclerosis through the induction of foam cell formation increases our understanding of the mechanism of action of RBP4 in the pathophysiology of atherosclerosis and further indicates that lowering RBP4 levels might serve as a new therapeutic approach for targeting cardiovascular disease.

Retinol-binding protein 4 (RBP4), which is mainly secreted from the liver and adipose tissue, is a member of the lipocalin family and the only retinol (vitamin A)-specific transport protein in the circulation.[1,2] As an adipokine, RBP4 was measured in the circulation at levels inversely correlated with insulin sensitivity and were increased in patients with type 2 diabetes mellitus and other metabolic abnormalities.[3,4] Recent animal studies demonstrated that RBP4 upregulation initiated inflammation[5] and impaired endothelial mitochondrial homeostasis,[6] which resulted in endothelial dysfunction, a key feature of atherogenesis. Moreover, epidemiological studies have reported a positive correlation between elevated RBP4 levels and the prevalence of cardiovascular diseases (CVDs), including hypertension,[7] stroke,[8] and coronary heart disease,[9] which indicates a pivotal function of RBP4 in the mediation of cardiovascular health. However, the precise role of RBP4 in the initiation and progression of atherosclerosis, the main contributor to cardiovascular mortality, remains elusive.

A hallmark of atherosclerosis is the formation of macrophage-derived foam cells.[10] Increased uptake of oxidized low-density lipoprotein (oxLDL) and reduced cholesterol efflux leads to the deposition of esterified cholesterol in the cytoplasm of macrophages and promotes the generation of foam cells.[11] In macrophages, oxLDL is taken up by several types of scavenger receptors (SRs), such as class A SRs (SR-AI, SR-AII, SR-AIII) and class B SRs (SR-BI, SR-BII, CD36). Among these receptors, SR-A and CD36 are responsible for >75% of oxLDL internalization.[12] The oxLDL-derived cholesterols imported into macrophages by SRs are further esterified by acyl coenzyme A:cholesterol acyltransferase-1[13] and stored as the lipid droplets that characterize foam cells. Macrophages respond to excessive lipid accumulation mainly through lipid efflux that is mediated by ATP-binding cassette (ABC) transporters ABCA1 and ABCG1 and by SR-BI.[14] Dysregulation of lipid homeostasis in macrophages because of disruptions in any of these aspects leads to foam cell formation and, eventually, the progression of atherosclerosis.[15]

Here, to elucidate the role of RBP4 in atherosclerosis and its underlying mechanism of action, we first evaluated the association between serum RBP4 levels and the incidence of adverse cardiovascular events in a community-based prospective cohort. Next, we examined the effects of RBP4 gain or loss of function on macrophage foam cell formation and atherogenesis in an apolipoprotein E-deficient (apoE$^{-/-}$) mouse model. Concurrently, we used the J774A.1 cell line and primary peritoneal macrophages to investigate the molecular mechanisms of RBP4 involvement in foam cell formation. Our findings reveal a proatherosclerotic role of RBP4 and extend the current knowledge regarding this molecule far beyond that as a marker for diabetes mellitus.

## METHODS

An expanded methods section is available in the online-only Data Supplement.

### Study Population

This human study included 1683 participants from the ongoing cohort of Nutrition and Health of Aging Populations in South China. The study protocol was approved by the Human Research Ethics Committee of Sun Yat-sen University following the principles of the Declaration of Helsinki. Written informed consent was obtained from each participant. Serum full-length RBP4 levels were determined using an enzyme-linked immunosorbent assay kit validated by performing quantitative western blotting analysis in a pilot study (online-only Data Supplement Figure I).

### Animals

All animal experiments were approved by the Animal Care and Utilization Committee of Sun Yat-sen University. We obtained apoE$^{-/-}$ mice on a C57BL/6J background from Jackson Laboratory. Human RBP4-expressing transgenic (RBP4-Tg) mice on a C57BL/6J background were constructed by Cyagen Biosciences and verified as described.[16]



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## Cell Culture and Treatments

The murine macrophage cell line J774A.1 was obtained from American Type Culture Collection (TIB-67). Cells were starved overnight in RPMI 1640 medium containing 0.5% FBS before exposure to various treatments.

## Statistical Analysis

Results are expressed as means±SEM from at least 5 independent experiments. One-way analysis of variance followed by Bonferroni's post hoc test was applied for comparisons among multiple experimental groups. An unpaired Student's *t* test was used to compare 2 groups with a normal distribution. $P<0.05$ was considered significant. SPSS version 22.0 was used for all analyses.

# RESULTS

## Serum RBP4 Level Serves as an Independent Predictor for Adverse Cardiovascular Events

The baseline characteristics and distribution of the 1683 participants (mean age, 61.4 years; 60% male) are shown in online-only Data Supplement Table I. The participants with incidence of major adverse cardiovascular events during the follow-up presented higher risk profiles at baseline than those without, including a comparatively older age, higher prevalence of hypertension and diabetes mellitus, and higher systolic blood pressure, fasting blood-glucose levels, and hsCRP, but lower HDL-c and eGFR. Moreover, participants with incidence of cardiovascular events relatively more frequently used medications, including aspirin, angiotensin converting enzyme inhibitor or angiotensin receptor blocker, and β-blockers.

Serum full-length RBP4 levels were significantly higher in participants with than without major adverse cardiovascular events at baseline (median, 53.5 μg/mL [interquartile range, 30.4–81.7 μg/mL] versus 32.8 μg/mL [interquartile range, 20.8–53.6 μg/mL]; $P<0.001$) (online-only Data Supplement Table I). Compared with patients in the lowest quartile of RBP4 level, those in the highest quartile presented a significantly increased risk for an adverse event (hazard ratio, 1.87; 95% confidence interval [CI], 1.64–2.25; $P<0.001$). After adjustment for the well-established risk factors for CVD, increased full-length RBP4 level remained a significant predictor for the risk of major adverse cardiovascular events (hazard ratio, 1.47; 95% CI, 1.19–1.68; $P=0.008$) (Table). Cross-validated estimates of incremental predictive value showed that adding RBP4 to the traditionally used risk factors improved the C-statistic from 0.745 (95% CI, 0.731–0.761) to 0.763 (95% CI, 0.750–0.783; $P=0.003$). Furthermore, Kaplan–Meier analysis revealed a graded increase in the risk of cardiovascular events according to the quartile of RBP4 level (online-only Data Supplement Figure II).

## RBP4 Exhibits Increased Expression in Atherosclerotic Tissues and Colocalizes With Macrophages

Immunohistochemical staining of aortic samples from patients with CVD (Figure 1A, left) and the atherosclerotic mouse model (Figure 1A, right) revealed an enhanced ac-

**Table. Hazard Ratios for Incidence of Major Cardiovascular Events According to Quartiles of Serum RBP4 Levels**

| | Serum RBP4 Level | | | | | | |
|---|---|---|---|---|---|---|---|
| | Quartile 1 | Quartile 2 | | Quartile 3 | | Quartile 4 | |
| Risk of Event | Reference | Hazard Ratio (95% CI) | *P* Value | Hazard Ratio (95% CI) | *P* Value | Hazard Ratio (95% CI) | *P* Value |
| Unadjusted hazard ratio | 1.00 | 1.27 (1.03–1.62) | 0.042 | 1.51 (1.25–1.89) | 0.001 | 1.87 (1.64–2.25) | <0.001 |
| Adjusted hazard ratio | | | | | | | |
| Model 1 | 1.00 | 1.17 (0.94–1.48) | 0.173 | 1.38 (1.12–1.74) | 0.023 | 1.73 (1.48–2.03) | <0.001 |
| Model 2 | 1.00 | 1.08 (0.85–1.39) | 0.382 | 1.26 (0.97–1.55) | 0.191 | 1.58 (1.32–1.80) | <0.001 |
| Model 3 | 1.00 | 1.03 (0.78–1.31) | 0.565 | 1.18 (0.86–1.34) | 0.357 | 1.47 (1.19–1.68) | 0.008 |

A major adverse cardiovascular event was defined as death, myocardial infarction, or stroke. The quartiles of serum RBP4 levels were as follows: Quartile 1, <22.7 μg/mL; Quartile 2, 22.7–36.3 μg/mL; Quartile 3, 36.4–57.8 μg/mL; and Quartile 4, >57.8 μg/mL. Hazard ratios and *P*-values shown are for the comparison with Quartile 1. In Model 1, hazard ratios were adjusted for established, traditional risk factors: age, sex, BMI, smoking status, systolic blood pressure, fasting blood glucose, prevalence of hypertension and diabetes mellitus, LDL-c, HDL-c, and log-transformed hsCRP levels. In Model 2, hazard ratios were adjusted for all factors in Model 1, plus log-transformed eGFR levels and usage of medications, including aspirin, statins, ACE inhibitor, ARB, or β-blockers. In Model 3, hazard ratios were adjusted for all factors in Model 2, plus disease extent assessed using angiography. ACE indicates angiotensin-converting enzyme; ARB, angiotensin receptor blocker; BMI, body mass index; CI, confidence interval; eGFR, estimated glomerular filtration rate; HDL-c, high-density lipoprotein cholesterol; hs-CRP, high-sensitivity C-reactive protein; LDL-c, low-density lipoprotein cholesterol; and RBP4, retinol-binding protein 4.

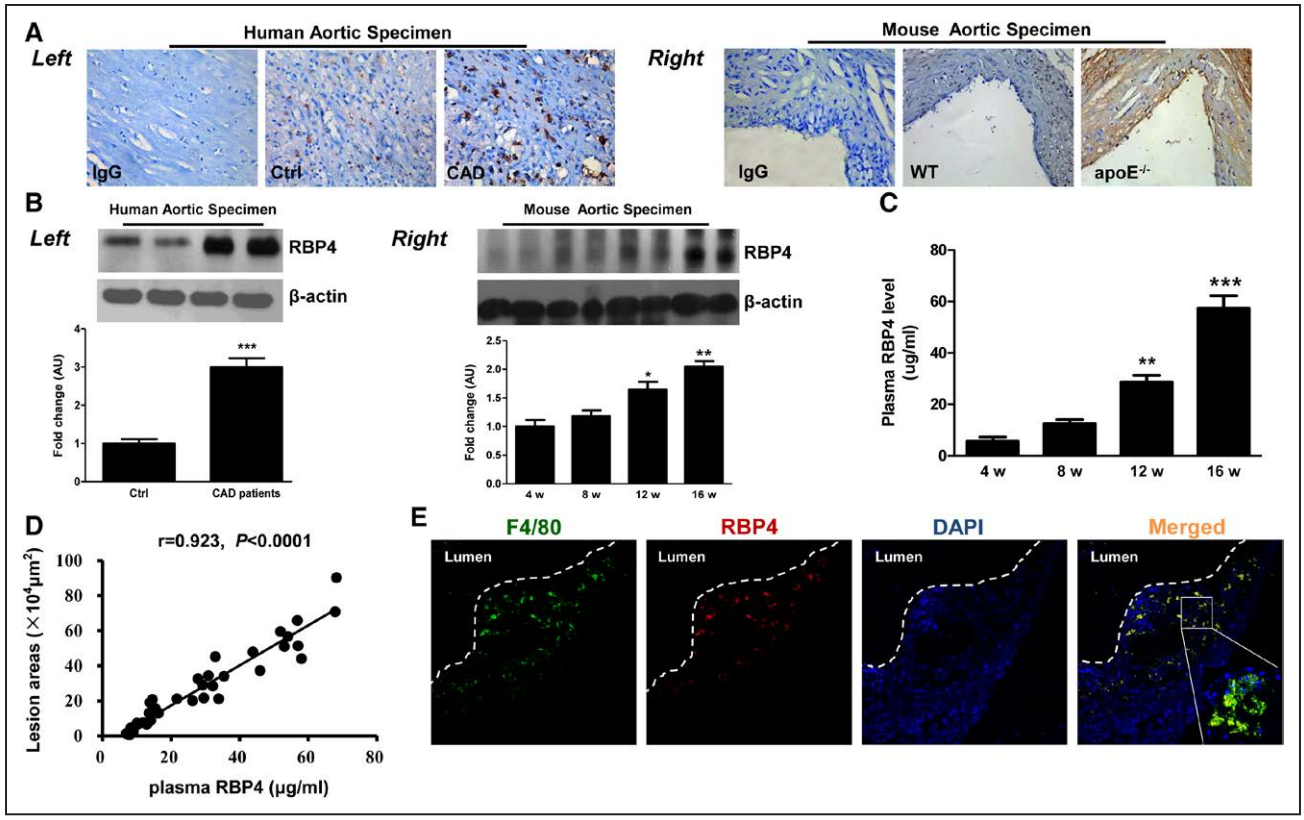


**Figure 1. RBP4 exhibits increased expression in atherosclerotic lesions and colocalizes with macrophages.** **A,** Immunohistological analysis of RBP4 expression in human (**left**) and mouse (**right**) aortic samples (magnification, ×400). **B,** Representative western blots showing RBP4 levels in human (**left**) and mouse (**right**) aortic plaques. *N*=8 for each group. ***$P$<0.001 non-CVD versus CVD patients (**left**). *$P$<0.05, **$P$<0.01 versus apoE$^{-/-}$ mice fed on high-cholesterol diet for 4 weeks (**right**). **C,** Serum RBP4 levels determined using ELISA in apoE$^{-/-}$ mice fed on high-cholesterol diet for 4, 8, 12, and 16 weeks. *N*=8 to 10 for each time point. **$P$<0.01, ***$P$<0.001 versus apoE$^{-/-}$ mice fed on high-cholesterol diet for 4 weeks. **D,** Pearson's correlation between serum RBP4 levels and atherosclerotic lesion areas. **E,** Colocalization of RBP4 and macrophages in atherosclerotic lesions. Green fluorescence: F4/80, a macrophage marker; red fluorescence: RBP4 protein. *N*=8 (magnification, ×400). apoE indicates apolipoprotein E; CVD, cardiovascular disease; ELISA, enzyme-linked immunosorbent assay; and RBP4, retinol-binding protein 4.

cumulation of RBP4 in atherosclerotic lesions. RBP4 protein levels were ≈3.5-fold higher in patients with CVD than in participants without CVD (Figure 1B**,** left) and ≈2.3-fold higher in apoE$^{-/-}$ mice than in control mice (Figure 1B**,** right). Moreover, serum RBP4 levels increased gradually with atherosclerosis progression (Figure 1C) and were significantly correlated with areas of aortic lesions (Figure 1D) in apoE$^{-/-}$ mice. The RBP4 gene was exclusively expressed in macrophages (online-only Data Supplement Figure III), and, more importantly, immunofluorescence analysis revealed that RBP4 colocalized with macrophages (labeled by the marker F4/80) in aortic lesions (Figure 1E), which indicated that RBP4 was specifically upregulated in atherosclerotic lesions that were rich in macrophages.

## RBP4 Inhibition Attenuates but Overexpression Accelerates Atherosclerosis Progression

To investigate the role of RBP4 in the pathogenesis of atherosclerosis, adenovirus constructs were generated to deliver RBP4 gene (Ad-RBP4) or shRNA (Ad-shRBP4) into apoE$^{-/-}$ mice. Compared with the serum RBP4 level in null-infected mice (19.39±6.03 μg/mL), RBP4 levels were markedly decreased and increased in mice infected with, respectively, Ad-shRBP4 (6.39±2.55 μg/mL) and Ad-RBP4 (67.44±7.99 μg/mL) (online-only Data Supplement Figure IVA). Furthermore, RBP4 protein expression was decreased in peritoneal macrophages isolated from Ad-shRBP4-treated mice but increased in macrophages from Ad-RBP4-infected mice (online-only Data Supplement Figure IVB). *En face* oil red O staining of the entire aorta showed a 45% reduction in the atherosclerotic lesion area in Ad-shRBP4-treated mice but a ≈2.2-fold increase in the lesion area in Ad-RBP4-treated mice (Figure 2A). Histological evaluation verified that atherosclerotic fatty streak lesions were markedly mitigated and aggravated in, respectively, Ad-shRBP4- and Ad-RBP4-treated mice (Figure 2B). Accordingly, cholesterol ester accumulation was decreased and prominently increased, respectively, in the aorta of Ad-shRBP4- and Ad-RBP4-treated mice (Figure 2C). Similarly, both macro-


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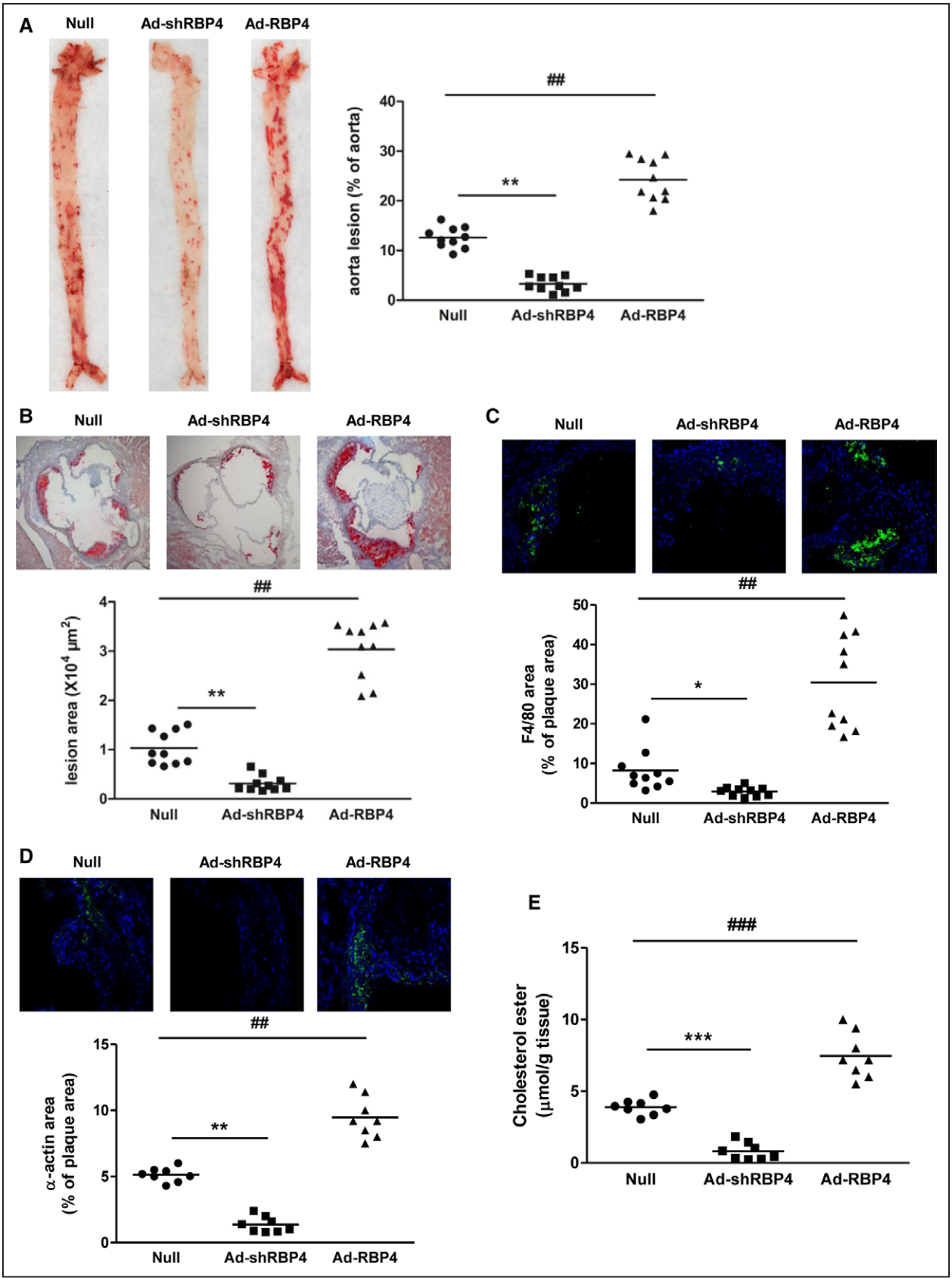


**Figure 2. RBP4 inhibition attenuates but overexpression promotes atherosclerotic plaque formation in apoE$^{-/-}$ mice.** Adult apoE$^{-/-}$ mice treated with adenovirus to silence RBP4 expression or overexpress RBP4 were maintained on a high-cholesterol diet for 8 weeks before analysis (*N*=10 for each group). **A**, *En face* staining of the entire aorta. **B**, Cross-sections (*Continued*)

phage infiltration (Figure 2D) and smooth muscle proliferation (Figure 2E) in the atherosclerotic lesion area of the aortic sinus were alleviated in the Ad-shRBP4 group but exacerbated in the Ad-RBP4 group compared with the null-treatment group. Serum lipid profiling revealed that triglyceride levels were increased in Ad-RBP4-treated mice, but cholesterol levels, including total cholesterol, high-density lipoprotein cholesterol, and LDL cholesterol, were not significantly altered (online-only Data Supplement Table II). Collectively, these data suggest that RBP4 plays a critical role in the susceptibility to atherosclerosis progression.

## RBP4 Stimulates Macrophage Cholesterol Uptake and Foam Cell Formation Through CD36 Induction

Next, we examined the potential role of RBP4 in the formation of macrophage-derived foam cells. In RBP4-Tg mice that were generated, the circulating RBP4 concentration was ≈4-fold higher than that in their wild-type (WT) littermates (online-only Data Supplement Figure V). Ex vivo studies revealed that lipid accumulation and cholesterol uptake in peritoneal macrophages cultured in RBP4-Tg serum were increased by ≈3-fold and ≈2-fold, respectively, whereas these effects were abolished when RBP4-depleted WT serum was used (online-only Data Supplement Figure VIA and VIB). However, cholesterol efflux showed no notable change (online-only Data Supplement Figure VIC). In accordance with these findings, recombinant RBP4 treatment caused a dose-dependent induction of lipid accumulation (Figure 3A) and oxLDL uptake (Figure 3B) but produced no effect on efflux (Figure 3C) in J774A.1 macrophages. To delineate the mechanism by which RBP4 promoted foam cell formation, we examined the alterations of SRs and transporters, which are regarded as key mediators in cholesterol homeostasis during foam cell formation. Treatment of J774A.1 macrophages with exogenous RBP4 enhanced CD36 expression at both mRNA and protein levels in a dose-dependent manner but exerted no marked effect on SR-AI expression (Figure 3D and 3E), which was further confirmed by immunofluorescence measurement (Figure 3F). Furthermore, CD36 transcriptional activity was significantly enhanced on RBP4 stimulation (Figure 3G). Conversely, RBP4 did not affect the expression of either transporters responsible for cholesterol efflux, including ABCA1, ABCG1, and SR-BI (online-only Data Supplement Figure VIIA and VIIB), or the enzyme required for cholesterol esterification (online-only Data Supplement Figure VIIC and VIID). Moreover, similar findings were obtained using peritoneal macrophages derived from RBP4-Tg mice and their WT littermates (online-only Data Supplement Figure VID–VII). Collectively, these results showed that RBP4 promoted macrophage foam cell formation through the induction of modified LDL uptake.

To further evaluate the regulatory role of RBP4 in CD36 induction, endogenous RBP4 was inhibited using a specific siRNA. RBP4 silencing caused a notable reduction of CD36 protein expression (Figure 3H) in J774A.1 macrophages, which resulted in ≈80% decrease in cholesterol uptake (Figure 3I). Furthermore, cholesterol uptake in peritoneal macrophages from WT mice was markedly induced when the cells were cultured in RBP4-supplemented media, but this phenomenon was not observed in CD36$^{-/-}$ macrophages (Figure 3J), which implied that the ability of RBP4 to stimulate cholesterol uptake depended on CD36.

## RBP4 Promotes CD36-Mediated Cholesterol Uptake Through Jun N-Terminal Kinase (JNK)-Signal Transducer and Activator of Transcription 1 (STAT1) Signaling Pathway

To identify the transcription factors involved in RBP4-induced CD36 upregulation, we tested the role of STAT1, a potential transcription activator in the mediation of foam cell formation.[17] RBP4 treatment dose-dependently augmented STAT1 phosphorylation at Tyr701 (Figure 4A), which in turn promoted STAT1 nuclear translocation (Figure 4B) and binding to CD36 promoter (Figure 4C). Furthermore, CD36 promoter activity was increased by 3.9-fold on RBP4 stimulation, but the response was absent when STAT1 phosphorylation was inhibited pharmacologically, and this outcome led to a sharp decrease in CD36 transcriptional activation (Figure 4D) and expression (Figure 4E), as well as a reduction in cholesterol uptake (Figure 4F). To directly test the role of the STAT-binding site of CD36 promoter in mediating the RBP4 effect, we performed site-directed mutagenesis: mutation of the putative STAT-binding site at nucleotide −107 relative to the transcription start site attenuated RBP4-induced CD36 promoter activity (Figure 4G), which suggested that STAT1 activation is necessary for RBP4-stimulated CD36 transcription.

STAT1 was reported to represent a potential downstream target of JNK,[18] a kinase closely involved with human atherosclerotic plaque formation.[19] Thus, we evaluated the role of JNK in RBP4-induced cholesterol uptake. Both recombinant RBP4 treatment (online-only Data Supple-

**Figure 2 Continued.** of aortic sinuses stained with oil red O. **C,** Cholesterol ester levels in arteries. **D,** Macrophage infiltration and (**E**) smooth muscle proliferation in aortic sinus determined by immunostaining for F4/80 and α-actin, respectively (magnification, ×400). **P*<0.05, ***P*<0.01, ****P*<0.001 null versus Ad-shRBP4. ##*P*<0.01, ###*P*<0.001 null versus Ad-RBP4. apoE indicates apolipoprotein E; and RBP4, retinol-binding protein 4.

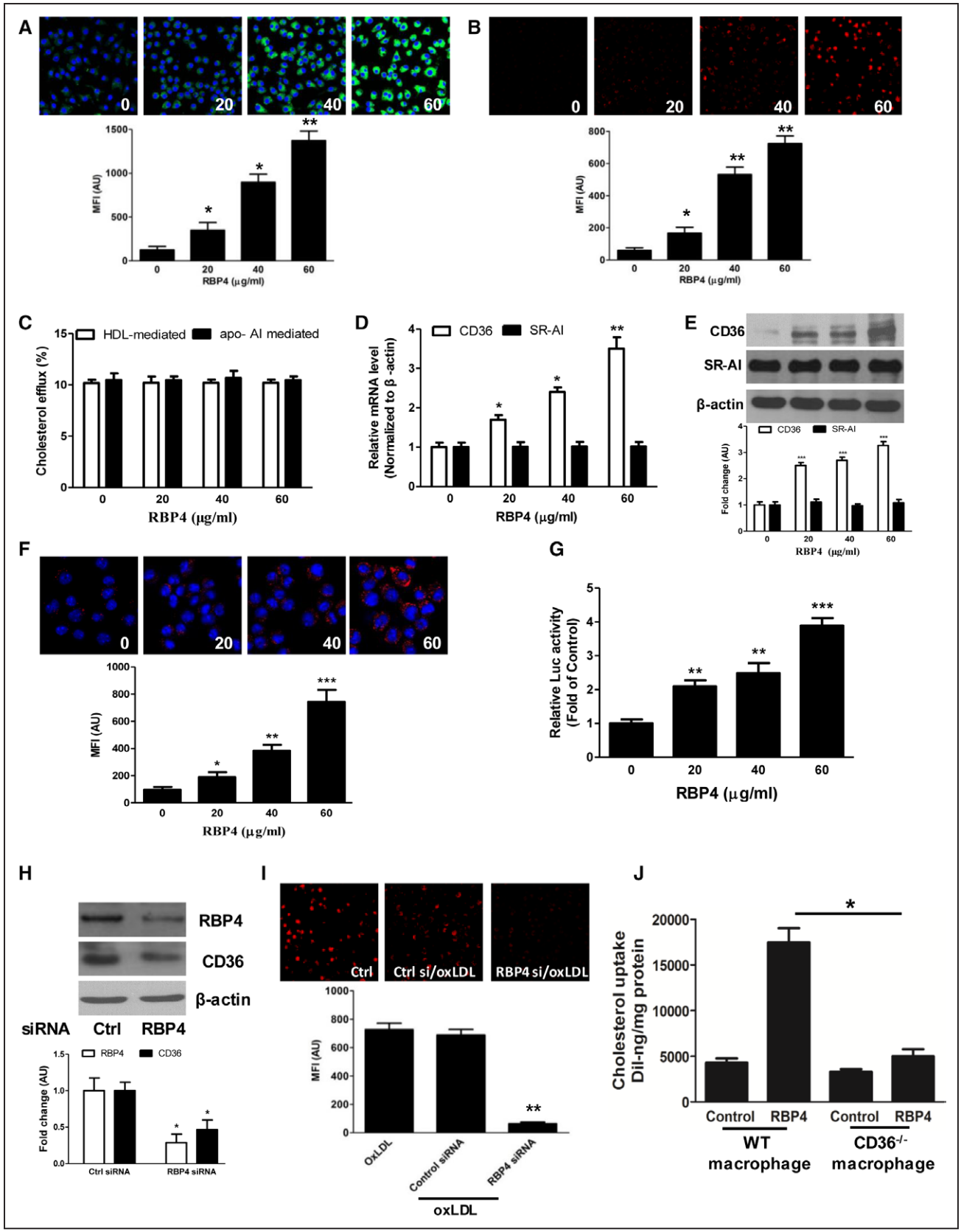


**Figure 3. RBP4 promotes cholesterol uptake through upregulation of CD36.**
**A** through **E**, J774A.1 cells were treated with RBP4 (20, 40, 60 μg/mL) for 24 h before analysis. **A**, Lipid accumulation and (**B**) cholesterol uptake were determined using Bodipy 493/503 and Dil-oxLDL, respectively (magnification, ×400). **C**, HDL and apo-AI-mediated cholesterol efflux were assayed. **D** and **E**, Cell lysates were subjected to real-time PCR and western blotting (*Continued*)

ment Figure VIIIA) and RBP4 gene overexpression (online-only Data Supplement Figure VIIIB) robustly enhanced JNK activation, as indicated by its increased phosphorylation at Thr183/Tyr185. Conversely, pretreatment of cells with SP600125, a JNK-specific inhibitor, markedly reversed the RBP4-dependent induction of STAT1 phosphorylation (Figure 4H), CD36 transcriptional activation (Figure 4I) and expression (Figure 4J), and cholesterol uptake (Figure 4K). By contrast, pharmacological inhibition of p38 and ERK did not affect either CD36 expression or cholesterol uptake in response to RBP4 treatment (data not shown). These results indicate that JNK-STAT1-CD36 signaling is required for RBP4-induced foam cell formation.

## c-Src Is Required for JNK-STAT1 Activation by RBP4

c-Src is a nonreceptor tyrosine kinase that phosphorylates tyrosine residues in numerous proteins.[20] Thus, we examined the role of c-Src in RBP4-induced JNK and STAT1 activation and CD36 expression in macrophages. RBP4 treatment of J774A.1 cells dose-dependently increased the level of Tyr416-phosphorylated c-Src (the activated form of c-Src) (Figure 5A) and c-Src kinase activity (Figure 5B), and this result was further validated in peritoneal macrophages isolated from RBP4-Tg mice and WT littermates (online-only Data Supplement Figure IXA and IXB). Notably, RBP4-mediated JNK and STAT1 activation (Figure 5C and 5D), CD36 induction (Figure 5E), and enhancement of cholesterol uptake (Figure 5F) were all almost completely reversed after pretreatment of macrophages with PP2, a c-Src-family kinase inhibitor, but not its structurally similar control compound PP3. These findings were validated using c-Src gene knockdown (online-only Data Supplement Figure IXC–IXF). Furthermore, PP2 treatment also suppressed CD36 induction and cholesterol uptake in peritoneal macrophages from RBP4-Tg mice (Figure 5G and 5H). Together, these results demonstrated a central role of c-Src in RBP4-induced macrophage foam cell formation.

## RBP4 Promotes c-Src Membrane Translocation and Activation

To further explore the mechanism by which RBP4 promotes c-Src activation, we examined the distribution of phospho-Tyr416 c-Src before and after RBP4 treatment relative to that of flotillin-2, a protein that associates with sphingolipid- and cholesterol-enriched membrane microdomains and serves as a lipid-raft marker. Whereas untreated cells exhibited extremely weak colocalization of phospho-Tyr416 c-Src and flotillin-2, this colocalization was strongly enhanced after RBP4 treatment (Figure 6A). Immunoblotting analysis further confirmed that phosphorylated c-Src was dose-dependently accumulated in lipid rafts on RBP4 exposure (Figure 6B). To demonstrate the potential involvement of lipid rafts in RBP4-mediated signaling, we applied methyl-β-cyclodextrin (β-MCD),[21] which effectively disrupts lipid rafts. Whereas incubation of cells with β-MCD strongly disrupted lipid rafts, incubation with α-MCD, an inactive β-MCD analog that cannot deplete cholesterol, exerted no effect on rafts (Figure 6C). Pretreatment of cells with β-MCD drastically inhibited c-Src translocation to lipid rafts and activation in response to RBP4 stimulation, but α-MCD failed to produce this effect (Figure 6D). Furthermore, RBP4-induced c-Src activation was markedly suppressed after β-MCD treatment (Figure 6E), which led to an abrogation of the activation of JNK (Figure 6F) and STAT1 (Figure 6G) and the induction of CD36 (Figure 6H). These effects were not detected in response to α-MCD treatment. Collectively, our results suggest that interference with lipid-raft organization abolished c-Src recruitment to this membrane microdomain and altered RBP4-mediated cholesterol-uptake signaling.

## Toll-Like Receptor 4 (TLR4) Partially Mediates the Effect of RBP4 on CD36-Dependent Cholesterol Uptake

Because retinol produces multiple physiological effects, we examined whether the effect of RBP4 on macrophage cholesterol accumulation depended on retinol. Treatment of J774A.1 macrophages with retinol-free RBP4 (or apo-RBP4, whose preparation was confirmed by the results shown in online-only Data Supplement Figure X) promoted CD36 expression (Figure 7A) and cholesterol uptake (Figure 7B) to a similar level as did treatment with retinol-bound RBP4 ("holo-RBP4"), which excluded the potential participation of retinol in this process. The only identified high-affinity cell-surface receptor of RBP4

**Figure 3 Continued.** analysis to determine (**D**) mRNA and (**E**) protein levels of SRs (CD36, SR-AI). **F**, RBP4-induced CD36 activation, as determined by immunofluorescence detection (magnification, ×1600). **G**, CD36 transcriptional activity was determined using the luciferase assay. Data are presented as means±SEM from at least 5 independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001 versus untreated cells. **H** and **I**, J774A.1 macrophages were transfected with RBP4-specific siRNA to silence endogenous RBP4 expression, and (**H**) CD36 protein expression and (**I**) cholesterol uptake were determined. **P*<0.05, ***P*<0.01 Ctrl siRNA versus RBP4 siRNA treatment. **J**, Quantification of cholesterol uptake in peritoneal macrophages from WT and CD36$^{-/-}$ mice; cells were cultured in RBP4-deficient or RBP4-supplemented media. *N*=8 for each group. **P*<0.05 RBP4-treated WT macrophages versus RBP4-treated CD36$^{-/-}$ macrophages. apo indicates apolipoprotein; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; HDL, high-density lipoprotein; oxLDL, oxidized low-density lipoprotein; PCR, polymerase chain reaction; RBP4, retinol-binding protein 4; SR, scavenger receptor; and WT, wild-type.


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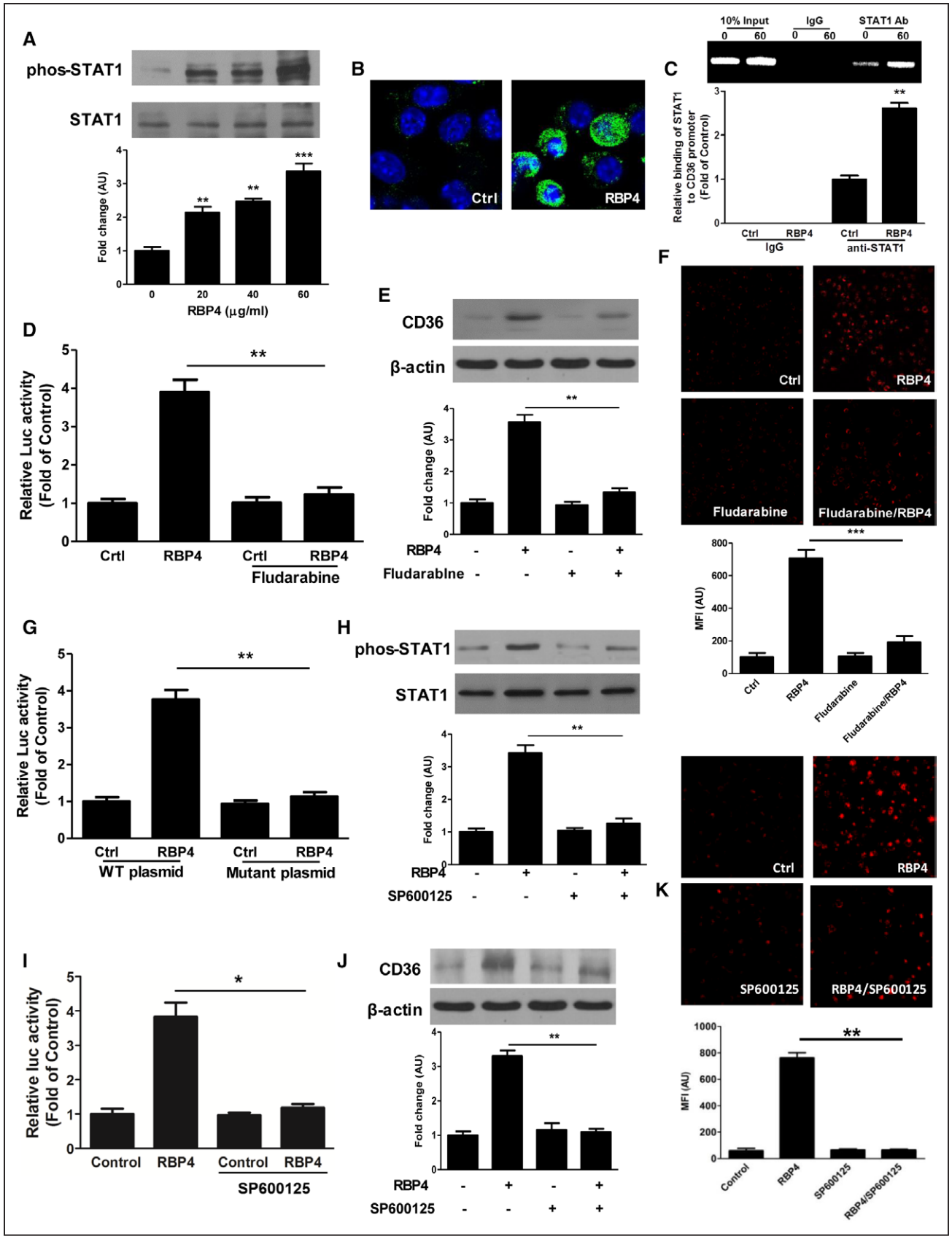


**Figure 4. RBP4-induced CD36 upregulation depends on JNK-STAT1 signaling.**
**A**, J774A.1 macrophages were treated with RBP4 (20, 40, 60 μg/mL) for 24 h and the phosphorylation level of STAT1 was determined. ***P*<0.01, ****P*<0.001 versus untreated cells. **B**, Activation and nuclear translocation of phospho-STAT1 (*Continued*)

is stimulated by retinoic acid 6 (STRA6), which functions in the uptake of retinol from holo-RBP4.[22] Inhibition of STRA6 expression by using a specific siRNA (online-only Data Supplement Figure XIA) exerted no marked effect on either c-Src activation (online-only Data Supplement Figure XIB) or CD36 expression and cholesterol uptake (online-only Data Supplement Figure XIC and XID), which indicated that STRA6 might not be involved in RBP4-mediated cholesterol uptake in macrophages.

TLR4 activation has been reported to enhance JNK phosphorylation and cytokine secretion in macrophages.[23] Therefore, we tested whether TLR4 is necessary for RBP4-induced cholesterol uptake. TLR4 inhibition by using a specific siRNA (Figure 7C) potently suppressed the phosphorylation of c-Src (Figure 7D), JNK (Figure 7E), and STAT1 (Figure 7F), which led to diminished CD36 induction (Figure 7G) and oxLDL uptake (Figure 7H). These results suggest that TLR4 is at least partially responsible for RBP4-induced c-Src-JNK-STAT1-CD36 signal transduction in macrophages.

## DISCUSSION

This study provides novel evidence indicating that RBP4 upregulation markedly exacerbates atherosclerosis through the induction of foam cell formation. We first demonstrated that in a cohort of middle-age and elderly Chinese people, the predictive value of serum RBP4 level for CVD risk was independent and additive to those of established, traditional risk factors. This finding supports the application of serum RBP4 as a novel and reliable biomarker for CVD risk evaluation. The results of in vivo and in vitro studies further demonstrated that elevated RBP4 facilitated macrophage-derived foam cell formation through the activation of c-Src-JNK-STAT1-CD36-mediated cholesterol uptake in a TLR4-dependent manner and thus accelerated atherosclerosis progression (summarized in Figure 7I). Thus, lowering RBP4 levels might serve as a promising therapeutic approach for CVD prevention and treatment.

Increasing numbers of human studies have indicated that circulating RBP4 level is associated with established cardiovascular risk factors such as dyslipidemia[24] and hypertension[7] and is also correlated with the thickness of carotid intimamedia[25] and prevalence of CVD.[26] However, given the nature of cross-sectional studies, previous work has confirmed neither the causal effect nor the predictive value of RBP4 levels. A few prospective studies conducted on the Chinese population were restricted to patients with diabetes mellitus[27] or featured a limited sample size and short follow-up period,[28] which considerably restricted the generalization of their conclusions. Nevertheless, in accord with a recent prospective cohort study conducted on only white females,[9] we observed that in the general Chinese population, inclusion of full-length RBP4 levels markedly enhanced the predictive ability for CVD risk compared with traditional models, which further confirmed the predictive value of RBP4 for CVD. Although the absolute level of circulating RBP4 remains broadly defined because of heterogeneity in race, sex, and study design, 30 to 60 μg/mL has been the typical range reported in people predisposed to CVDs,[25,26] which agrees with the results from our cohort (online-only Data Supplement Table I). Therefore, the 20 to 60 μg/mL RBP4 dose applied in our in vitro models encompasses the clinically relevant range of serum RBP4 reported in patients with CVDs. Moreover, this dosage is comparable to those used in previous studies, in which a different macrophage cell line was used.[23] This approach allowed us to determine how incremental changes in RBP4 levels might cause marked changes in the induction of foam cell formation.

Dysfunctional adipose tissue in obesity is now widely accepted to lead to an unbalanced production of adipokines that exert diverse adverse effects on the cardiovascular system mainly by modulating lipid metabolism or systemic inflammation.[29] Thus, identifying the association between adipokines and CVD risk and clarifying the plausible underlying mechanisms of their action will be crucial for preventing and treating CVDs. To our knowledge, our study provides the first experimental evidence that RBP4 upregulation notably exacerbated and RBP4 downregulation markedly delayed the progression of atherosclerosis, which were accompanied with increased

**Figure 4 Continued.** on RBP4 (60 μg/mL) treatment (magnification, ×3200). **C,** Untreated cells and cells incubated with RBP4 (60 μg/mL) were analyzed for STAT1 binding to CD36 promoter by using the chromosome-immunoprecipitation assay. **D** through **F**, After transfection with empty vector or pGL3-CD36 promoter plasmid, J774A.1 macrophages were pretreated with or without fludarabine (50 μmol/L) for 12 h and then stimulated with RBP4 (60 μg/mL) for another 24 h before measuring (**D**) CD36-promoter luciferase activity, (**E**) CD36 protein level, and (**F**) cholesterol uptake (magnification, ×400). ***P*<0.01, ****P*<0.001 RBP4-treated versus fludarabine/RBP4-treated cells. **G**, Cells were transfected with empty vector or the pGL3-CD36 promoter plasmid with or without a mutated STAT-binding site and then treated with RBP4 (60 μg/mL) for 24 h before measuring luciferase activity. ***P*<0.01 WT plasmid/RBP4-treated versus mutant plasmid/RBP4-treated cells. **H** through **K**, Cells were treated with RBP4 (60 μg/mL) or SP600125 (10 μmol/L) for 24 h before measuring (**H**) STAT1 phosphorylation, (**I**) CD36-promoter luciferase activity, (**J**) CD36 protein level, and (**K**) cholesterol uptake (magnification, ×400). Data are presented as means±SEM from at least 5 independent experiments. **P*<0.05, ***P*<0.01 RBP4-treated versus RBP4/SP600125-treated macrophages. JNK indicates jun N-terminal kinase; RBP4, retinol-binding protein 4; STAT1, signal transducer and activator of transcription 1; and WT, wild-type.


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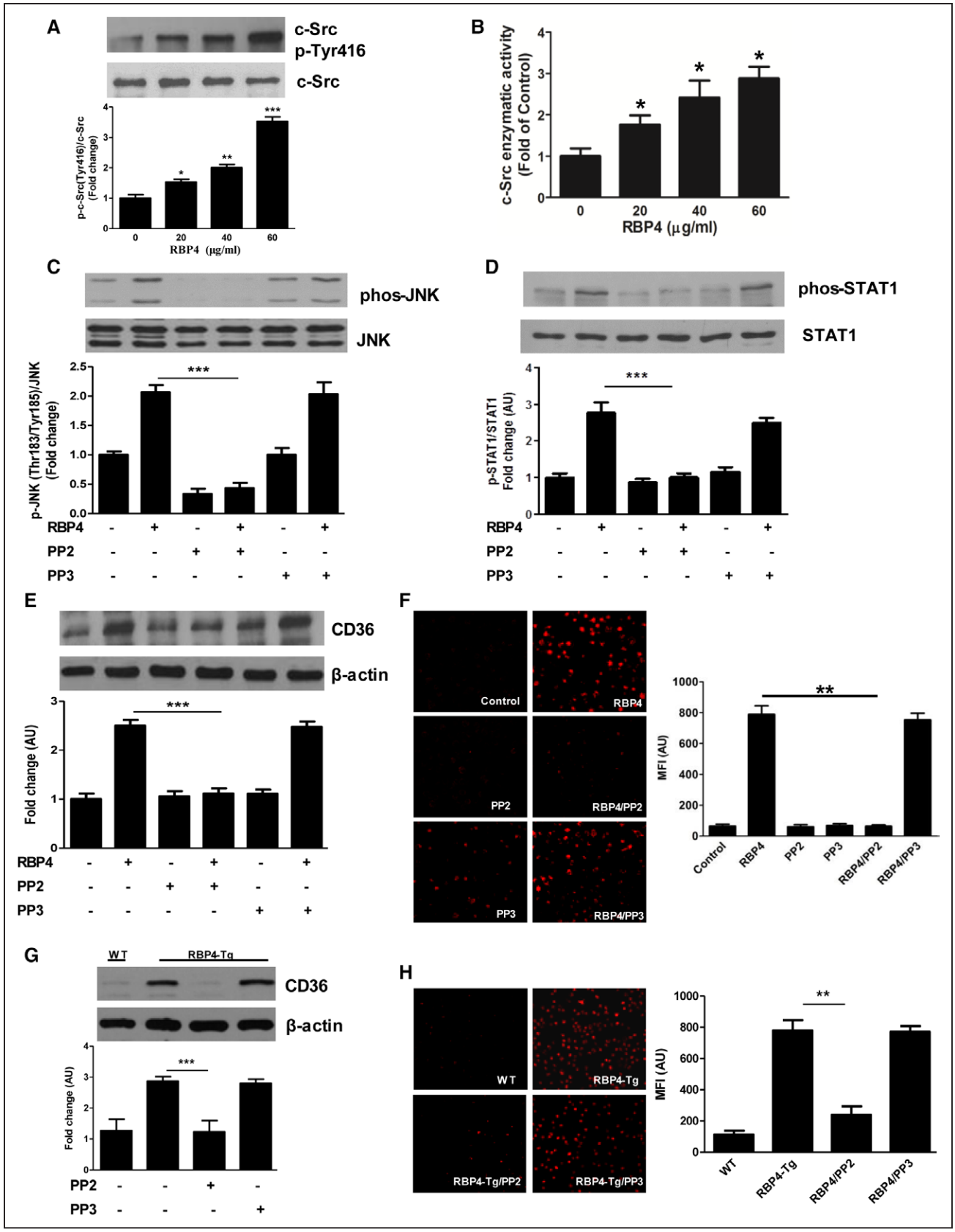


**Figure 5. RBP4 induces c-Src activation in macrophages.**

**A**, **B**, J774A.1 macrophages were treated with RBP4 (20, 40, 60 μg/mL) for 24 h and then c-Src phosphorylation at Tyr416 (**A**) and kinase activity (**B**) were determined. **P*<0.05, ***P*<0.01, ****P*<0.001 versus untreated cells. **C** through **F**, J774A.1 (*Continued*)

**Figure 5 Continued.** macrophages were pretreated with the c-Src inhibitor PP2 (10 μmol/L) or the control compound PP3 (10 μmol/L) for 2 h before incubation with RBP4 (60 μg/mL), after which (**C**) JNK phosphorylation, (**D**) STAT1 phosphorylation, (**E**) CD36 expression, and (**F**) cholesterol uptake were assayed. $^{**}P<0.01$, $^{***}P<0.001$ RBP4-treated versus RBP4/PP2-treated macrophages. **G**, **H**, Peritoneal macrophages from WT and RBP4-Tg mice were isolated and treated with PP2 or PP3 for 2 h before measuring (**G**) CD36 protein levels and (**H**) cholesterol uptake. Data are presented as means±SEM from at least 5 independent experiments. $^{**}P<0.01$, $^{***}P<0.001$ RBP4-Tg cells versus PP2-treated RBP4-Tg cells. JNK indicates jun N-terminal kinase; RBP4, retinol binding protein 4; STAT1, signal transducer and activator of transcription 1; and WT, wild-type.

and reduced macrophage-rich fatty streaks, respectively. This finding indicates that RBP4 might function as a previously unrecognized participant in atherosclerosis development. However, although we showed that RBP4 was exclusively expressed in macrophages to better delineate its specific role through macrophages, bone marrow from RBP4-Tg or RBP4-deficient mice should be transplanted into apoE$^{-/-}$ mice. To avoid potentially confounding the effects of apoE-expressing bone marrow,[30] in future studies, apoE$^{-/-}$ bone marrow cells may need to

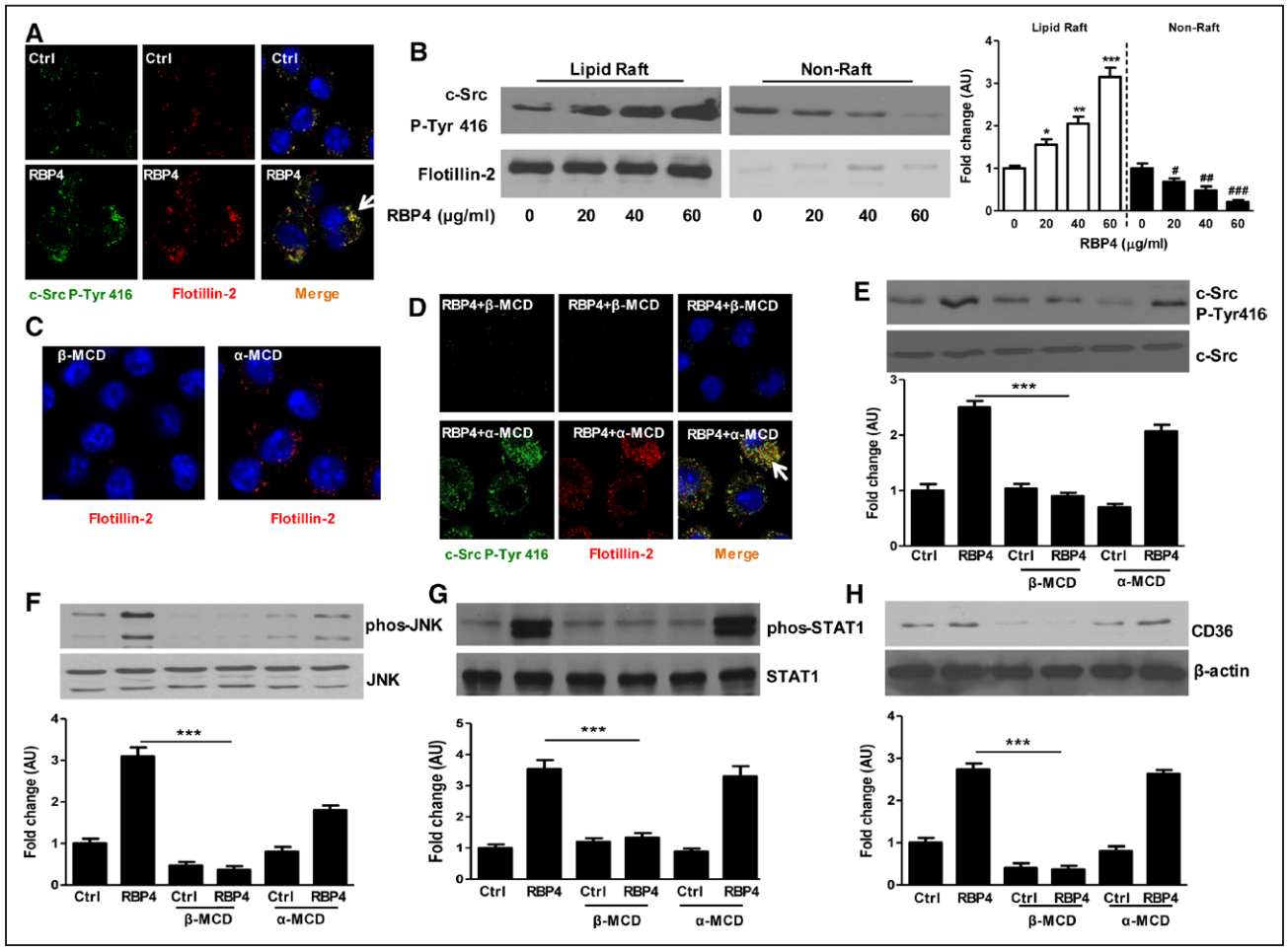


**Figure 6. Lipid rafts are required for RBP4-induced c-Src activation.**
**A,** J774A.1 macrophages were treated with RBP4 for 24 h, fixed, and then stained with antibodies against phospho-Tyr416 c-Src (activated c-Src; green) or flotillin-2 (lipid rafts; red) (magnification, ×3200). **B,** Phosphorylation of c-Src in both lipid rafts and nonraft fractions was examined through western blotting. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus untreated cells in lipid rafts; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ versus untreated cells in nonraft fractions. **C,**) J774A.1 cells were treated with β-MCD or α-MCD (20 mmol/L) for 30 minutes, and then lipid rafts were identified by immunostaining with anti-flotillin-2 antibody; red fluorescence: lipid rafts (magnification, ×3200). **D through H,** J774A.1 macrophages were incubated with β-MCD or α-MCD (20 mmol/L) for 30 minutes and then treated with RBP4 (60 μg/mL) for 24 h. **D,** Activated c-Src and flotillin-2 were detected through immunofluorescence; green fluorescence: phospho-Tyr416 c-Src; red fluorescence: lipid rafts (magnification, ×3200). Western blotting analysis of the phosphorylation levels of (**E**) c-Src, (**F**) JNK, and (**G**) STAT1 and (**H**) the expression of CD36. Data are presented as means±SEM from at least 5 independent experiments. $^{***}P<0.001$ RBP4-treated versus RBP4/β-MCD treated cells. β-MCD indicates methyl-β-cyclodextrin; JNK, jun N-terminal kinase; RBP4, retinol-binding protein 4; and STAT1, signal transducer and activator of transcription 1.


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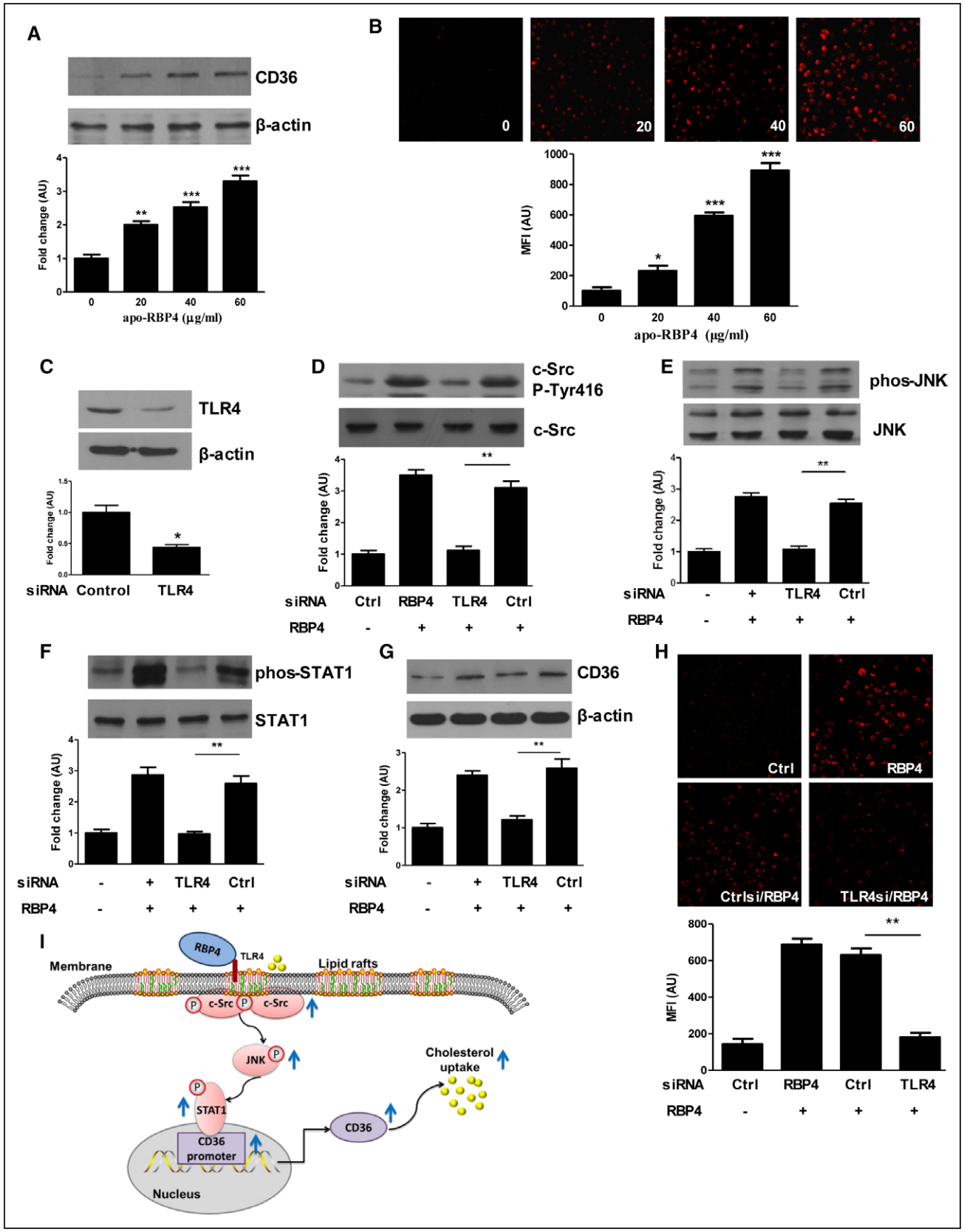


**Figure 7. RBP4 effects are retinol-independent but TLR4-dependent.**

**A**, **B**, J774A.1 macrophages were treated with apo-RBP4 (20, 40, 60 μg/mL) for 24 h and then (**A**) CD36 induction and (**B**) cholesterol uptake were determined (magnification, ×400). **P*<0.05, ***P*<0.01, ****P*<0.001 versus untreated cells. **C** through **H**, J774A.1 macrophages were transfected with scrambled or TLR4-targeting siRNA (100 nmol/L) and then treated (*Continued*)

be transduced with an RBP4 overexpression or deficient lentivirus before transplantation.

The formation of macrophage foam cells, which is characterized by massive accumulation of cholesterol esters, is a feature of both early and late atherosclerotic lesions, and the disruption of lipid homoeostasis is regarded as the main contributor to foam cell formation.[31,32] Our results further confirmed that RBP4 upregulation (by means of recombinant RBP4 treatment, RBP4 gene transfection, or application of serum derived from RBP4-Tg mice) substantially accelerated the formation of macrophage-derived foam cells through the activation of CD36, a key SR involved in mediating cholesterol uptake. Collectively, these results suggest that RBP4 regulates a unifying cell-signaling pathway that promotes both SR expression and modified LDL cholesterol uptake. Thus, our findings extend the main role of RBP4 far beyond that as an insulin-resistant adipokine.

As potent cytoplasmic transcription factors, STAT-family proteins mediate the transmission of the signals of numerous cytokines from the cell membrane to nucleus by binding to the promoter region of target genes.[33] In the atherosclerotic milieu, STAT1 was reported to become activated and modulate CD36 expression and foam cell formation.[17] Accordingly, our results showed that STAT1 binding to CD36 promoter was markedly augmented on RBP4 exposure. Furthermore, both pharmacological inhibition of STAT1 activation and mutagenesis of its binding site abrogated the activation of CD36 transcription, which confirmed that STAT1 functions as a key transcriptional modulator of RBP4-induced CD36 upregulation. The phosphorylation of STAT proteins, mainly at Tyr701 in STAT1, is considered to be a prerequisite for STAT nuclear import and transcriptional function.[34] JNK, a multifunctional kinase that belongs to a subfamily of mitogen-activated protein kinases, has been reported to be involved in regulating STAT-family proteins.[18] Furthermore, JNK inhibition in apoE$^{-/-}$ mice was shown to hamper atherosclerosis progression partly though the suppression of CD36- and SR-AI-mediated foam cell formation.[35] Here, we found that RBP4 acted as a positive regulator of JNK activation, and that targeted inhibition of JNK abolished STAT1 phosphorylation, which led to impaired transcriptional activation of CD36 and thus reduced cholesterol uptake. These data collectively indicate that the JNK-STAT1 signaling pathway was responsible for the observed inductive effects of RBP4 on CD36 upregulation.

The nonreceptor tyrosine kinase c-Src plays a vital role in myriad facets of cell physiology, including angiogenesis.[36] We demonstrated here that RBP4 treatment promoted c-Src activation by triggering Tyr416 phosphorylation. More importantly, pharmacological and genetic inhibition of c-Src activation almost eliminated RBP4-mediated JNK and STAT1 phosphorylation, and thus hindered cholesterol uptake through CD36. As a myristoylated protein, c-Src localizes both within and outside lipid rafts before translocating to the plasma membrane.[37] We also showed here that RBP4 treatment increased the accumulation of Tyr416-phosphorylated c-Src within lipid rafts, where the kinase was more active than in the general pool of cellular c-Src.[38] Furthermore, β-MCD-mediated cholesterol depletion blocked c-Src translocation to lipid rafts and hampered the activation of JNK-STAT1 signaling, which resulted in an inhibition of RBP4-induced CD36 upregulation. These findings indicate that lipid-raft integrity is crucial for RBP4-mediated downstream signaling in macrophages.

Retinol presents diverse biological properties,[39] and a well-established function of RBP4 is to deliver retinol from the liver to peripheral tissues by binding to the cell-membrane receptor STRA6.[40] Therefore, it is critical to differentiate whether the effects of RBP4 depend on retinol or STRA6. As in recent work showing that RBP4 induced inflammation through a retinol-independent mechanism,[8] we have provided evidence here that retinol-free RBP4 increased CD36 expression and cholesterol uptake in macrophages to a similar extent as retinol-bound RBP4, which excludes the possibility of retinol participation in macrophage foam cell formation. Moreover, in contrast to a previous report of RBP4 binding to STRA6 in adipocytes,[41] our results have shown that STRA6 silencing affected neither CD36-mediated cholesterol uptake nor c-Src activation in response to RBP4. Thus, RBP4 can activate the c-Src-JNK-STAT1-CD36 signaling pathway in a retinol- and an STRA6-independent manner. The participation of TLR signaling in promoting atherosclerosis has long been supported by animal studies,[42] particularly the participation of TLR4 signaling, which was reported to be involved in macrophage foam cell formation.[43] Consistent with previous work indicating that RBP4 stimulates proinflammatory cytokine release partly by activating TLR4 in macrophages,[23] we have shown here that TLR4 activation also functions in the CD36 signaling pathway elicited by RBP4. However, additional in-depth investigation is required to elucidate the precise mechanism

**Figure 7 Continued.** with RBP4 (60 μg/mL); after we measured (**C**) TLR4 siRNA efficacy, (**D**) c-Src phosphorylation, (**E**) JNK phosphorylation, (**F**) STAT1 phosphorylation, (**G**) CD36 protein levels, and (**H**) cholesterol uptake. Data are presented as means±SEM from at least 5 independent experiments. **P*<0.05, ***P*<0.01 Ctrl siRNA/RBP4 versus TLR4 siRNA/RBP4 treatment. **I,** Schematic summary depicting the molecular mechanism of RBP4 function in macrophage cholesterol uptake and foam cell formation. JNK indicates jun N-terminal kinase; RBP4, retinol-binding protein 4; STAT1, signal transducer and activator of transcription 1; and TLR4, Toll-like receptor 4.

governing the interaction between RBP4 and TLR4 and to identify other receptors potentially responsible for the action of RBP4.

In conclusion, this study has shown that RBP4 plays a previously unrecognized pathophysiological role in macrophage foam cell formation and atherogenesis by upregulating CD36 expression and cholesterol uptake though the c-Src-JNK-STAT1 signaling pathway. Our findings shed new light on the role of RBP4 and identify it as a promising therapeutic target for preventing the progression of atherosclerotic vascular disease.

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## DISCLOSURES

None.

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## FOOTNOTES

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