Rip2 Deficiency Leads to Increased Atherosclerosis Despite Decreased Inflammation

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Rationale: The innate immune system and in particular the pattern-recognition receptors Toll-like receptors have recently been linked to atherosclerosis. Consequently, inhibition of various signaling molecules downstream of the Toll-like receptors has been tested as a strategy to prevent progression of atherosclerosis. Receptor-interacting protein 2 (Rip2) is a serine/threonine kinase that is involved in multiple nuclear factor-κB (NFκB) activation pathways, including Toll-like receptors, and is therefore an interesting potential target for pharmaceutical intervention.

Objective: We hypothesized that inhibition of Rip2 would protect against development of atherosclerosis.

Methods and Results: Surprisingly, and contrary to our hypothesis, we found that mice transplanted with Rip2−/− bone marrow displayed markedly increased atherosclerotic lesions despite impaired local and systemic inflammation. Moreover, lipid uptake was increased whereas immune signaling was reduced in Rip2−/− macrophages. Further analysis in Rip2−/− macrophages showed that the lipid accumulation was scavenger-receptor independent and mediated by Toll-like receptor 4 (TLR4)−dependent lipid uptake.

Conclusions: Our data show that lipid accumulation and inflammation are dissociated in the vessel wall in mice with Rip2−/− macrophages. These results for the first time identify Rip2 as a key regulator of cellular lipid metabolism and cardiovascular disease. (Circ Res. 2011;109:1210-1218.)

Key Words: atherosclerosis ■ lipids ■ inflammation ■ receptor-interacting protein serine-threonine kinase 2 ■ macrophages

Low-density lipoproteins (LDL) are the major extracellular carriers of cholesterol and, as such, play important physiological roles in cellular function and regulation of metabolic pathways. However, under pathological conditions of hyperlipidemia, cholesterol is diverted from its physiological targets and accumulates in lipid-loaded macrophages (“foam cells”) in the vascular wall.1–4 This pathological deposition of atherogenic lipoproteins activates the inflammatory response that characterizes atherosclerosis. Furthermore, by stimulating the synthesis and secretion of proteoglycans,5,6 this inflammation further accelerates retention of atherogenic lipoproteins. Thus, lipid accumulation and inflammation are closely linked in atherogenesis.7–11

The molecular mechanisms that link retention of atherogenic lipoproteins and activation of the inflammatory response are still unclear. Recent evidence implies a key role for the innate immune system and pathogen pattern-recognition receptors, in particular the membrane-bound Toll-like receptors (TLRs).12 Ligand binding to these receptors results in activation of the proinflammatory transcription factor nuclear factor-κB (NF-κB) and expression of proinflammatory molecules.12,13

The receptor-interacting protein 2 (Rip2) is a serine/threonine kinase that activates NF-κB and is reported to mediate signaling through both TLRs and Nod-like receptors, although its involvement in TLR signaling has been questioned recently.14–18 Furthermore, it was demonstrated recently that the regulation of Rip2 involves a novel feedforward regulatory mechanism: Rip2 not only positively regulates NF-κB activity, but inflammatory cytokines that activate the NF-κB pathway induce increased Rip2 expression.19 These studies thus suggest the therapeutic potential of inhibiting Rip2 to inhibit inflammation and thus protect against the development of atherosclerosis.

Here, we investigated the role of Rip2 in mice prone to developing atherosclerosis. Unexpectedly, we observed that mice transplanted with Rip2−/− bone marrow displayed increased atherosclerosis despite impaired immune signaling.
Further studies in Rip2\(^{-/-}\) macrophages revealed a key role for Rip2 in regulating TLR4-dependent macropinocytosis of LDL in macrophages. On the basis of our results, we suggest that Rip2 has a protective role during atherogenesis.

Methods

Mice

Rip2\(^{-/-}\) mice\(^{15}\) and wild-type littersmates, kindly provided by Dr Paul W. Dempsey (Department of Microbiology, Immunology and Molecular Genetics, UCLA, Los Angeles, CA), and human apolipoprotein B100 (APOB100) transgenic mice on an LDL receptor-deficient (Ldlr\(^{−/-}\)) background (APOB100\(^{×}\)Ldlr\(^{−/-}\))\(^{20,21}\) were housed in a pathogen-free barrier facility and fed rodent chow. All animal studies were approved by the local animal ethics committee.

Bone Marrow Transplantation

Lethally irradiated APOB100\(^{×}\)Ldlr\(^{−/-}\) recipient mice were reconstituted with bone marrow stem cells from Rip2\(^{-/-}\) or wild-type littermate mice as described previously.\(^{22}\)

Blood Analysis

Cholesterol and triglycerides were measured on a Konelab 20 autoanalyzer (Thermo Scientific; Vantaa, Finland). Plasma levels of soluble intercellular adhesion molecule (ICAM) and vascular cellular adhesion molecule (VCAM) were analyzed with ELISA (R&D Systems; Minneapolis, MN). Plasma cytokines were analyzed with a SECTOR Imager 2400 reader (Meso Scale Discovery; Gaithersburg, MD).

Analysis of Aortas

Whole aortas and aortic roots were analyzed as described previously.\(^{22,23}\)

Macrophage Isolation

Peritoneal macrophages were isolated by flushing the peritoneum of Rip2\(^{-/-}\) or wild-type mice with PBS and were then subjected to cell culture.

Isolation and Modification of LDL Particles

LDL was isolated from human plasma as described previously.\(^{21}\) Minimally oxidized LDL (mmLDL) was prepared by incubating LDL at 4°C for 2 months.\(^{24}\)

In Vitro Analysis of Accumulation and Cytokine Secretion

Peritoneal macrophages were cultured with or without mmLDL (50 \(\mu\)g/mL) for 24 hours. The total Oil Red O-stained surface area was quantified with BioPix software.\(^{25}\) Triglycerides, cholesterol esters, and free cholesterol were analyzed with a straight-phase high-performance liquid chromatography system.\(^{26}\) Cytokine levels in the media were analyzed with a SECTOR Imager 2400 reader (Meso Scale Discovery).

mRNA Expression in Peritoneal Macrophages

Total RNA was extracted with an RNeasy Kit (QIAGEN; Hilden, Germany), and cDNA was synthesized with the high-capacity cDNA Reverse Transcription Kit (Applied Biosystems; Foster City, CA). mRNA expression of genes of interest was analyzed with TaqMan \(\text{M}^\circ\) RNA Expression Assays in an ABI Prism 7900 HT Detection System (Applied Biosystems).

Fluorescence-Activated Cell Sorting Analysis

Peritoneal cells were resuspended in fluorescence-activated cell sorting (FACS) buffer (PBS, 3% FCS, 0.09% NaAz), incubated with Fc Block (2.4G2, BD Bioscience; Erembodegem, Belgium) and then with antibodies directed against surface antigens. After the initial surface-staining step, cells were fixed with paraformaldehyde, permeabilized in FACS buffer (PBS, 3% FCS, 0.09% NaAz) with 0.5% saponin, and then stained for intracellular CD68. A total of 10 000 cells were collected for each staining by use of a FACS Canto II equipped with Diva 6.2 software (BD Biosciences) and were analyzed with FlowJo software (Tree Star; Ashland, OR).

TLR4 and SR-B1 Knockdown

Peritoneal macrophages were transfected with predesigned Silencer Select small interfering RNA (Ambion) with Lipofectamine RNAiMAX (Invitrogen; Carlsbad, CA). The medium was changed after 6 hours, and mmLDL (50 \(\mu\)g/mL) was added to the fresh medium. The cells were analyzed after a further 24 hours. To ensure the specificity of the knockdown, 2 small interfering RNA constructs were used for each gene. Knockdown was confirmed by FACS analysis.

Morphological Analysis of Macropinocytosis

Peritoneal macrophages were permeabilized and then stained with rhodamine phalloidin (Invitrogen Molecular Probes) and DAPI.
To visualize F-actin cytoskeleton and nuclei, respectively. Images were captured with an ApoTome Axioplan 2 imaging system (Carl Zeiss; Göttingen, Germany).

In Vitro Analysis of Macropinocytosis
Peritoneal macrophages were incubated with or without mmLDL and with dextran labeled with Alexa Fluor 488. Fluorescence was assessed with a fluorometer (MDS Analytical Technologies; Sunnyvale, CA).

Statistical Analysis
Data are shown as mean±SEM. Measurements were compared with the 2-tailed t test or Mann-Whitney rank sum test.

Results
Increased Subendothelial Lipid Accumulation Despite Decreased Local and Systemic Inflammation in Mice With Rip2-Deficient Bone Marrow
To investigate the importance of Rip2 in atherogenesis, lethally irradiated APOB100×Ldlr−/− mice were transplanted with Rip2+/− bone marrow.

Figure 3. Increased lipid uptake in macrophages from Rip2+/− mice. A–C, Lipid accumulation was analyzed in wild-type (WT) or Rip2+/− peritoneal macrophages with Oil Red O (ORO) staining after incubation with 50 μg/mL mmLDL (A), native LDL (B), or acetylated LDL (acetyl-LDL; C) for 24 hours (n=6–8 per genotype). Data are from 3 independent experiments. D, Cholesterol ester, triglyceride, and free cholesterol were quantified in WT or Rip2+/− peritoneal macrophages by high-performance liquid chromatography (n=5 per genotype). Data are from 1 experiment. Data are presented as mean±SEM. *P<0.05, **P<0.01, and ***P<0.001 vs WT.
planted with bone marrow cells from Rip2−/− mice or wild-type littermates and fed a chow diet for 6 months. Body weight, plasma cholesterol, and triglycerides did not differ between the 2 groups (data not shown). Surprisingly, however, atherosclerotic lesions both in the whole aorta (measured by en face technique) and in the aortic root were markedly increased in mice that received Rip2−/− bone marrow (Figure 1).

Immunohistochemical analyses of the aortic root showed similar amounts of macrophages and smooth muscle cells in the lesions in both groups (Figures 2A and 2B). In contrast, the number of T cells was significantly reduced in the lesion areas in mice that received Rip2−/− bone marrow (Figure 2C). However, the number of circulating monocytes, B cells, and T cells did not differ between groups (Figure 2D). Analysis of CD4+; CD8+, and regulatory T-cell populations showed that there were no significant differences between the groups (Online Figure I, A). ICAM-1, a known NF-κB target gene, was decreased significantly in lesions in mice that received Rip2−/− bone marrow (Figures 2E and 2F). In contrast, VCAM-1 was not altered significantly (Figure 2F).

We also investigated the effect of Rip2 deficiency on systemic inflammation and observed lower levels of soluble ICAM, VCAM, and the proinflammatory cytokines CXCL1 (Gro1/CK) and interleukin-6 in plasma from mice that received Rip2−/− bone marrow (Figures 2G–J). Even though systemic inflammation was decreased in mice that received Rip2−/− bone marrow, mice from both groups were still capable of responding with a strong inflammatory reaction when challenged with lipopolysaccharide (Online Figure I, B).

Altogether, these data show that mice transplanted with Rip2−/− bone marrow display increased atherosclerotic lesions despite decreased inflammation both locally in the aorta and systemically.

Increased Lipid Uptake Despite Suppressed Immune Signaling in Rip2−/− Macrophages
To determine whether the subendothelial lipid accumulation in mice transplanted with Rip2−/− bone marrow could be explained by increased lipid uptake in macrophages, we investigated lipid uptake in peritoneal macrophages isolated from Rip2−/− or wild-type mice. Quantification of the total Oil Red O–stained surface area after incubation with 50 μg/mL mmLDL for 24 hours (left) or from mice fed a high-fat (“Western”) diet (HF diet) and cultured without mmLDL for 24 hours (right). The cells were then analyzed for the endoplasmic reticulum stress marker phosphorylated PERK and the internal standard GAPDH normalized to GAPDH expression. Data are from 1 experiment. E, Apoptosis in WT and Rip2−/− macrophages with and without incubation of 50 μg/mL mmLDL for 48 hours were analyzed with terminal dUTP nick end-labeling (TUNEL) staining (n=3 per group). Data are presented as mean±SEM. *P<0.05, **P<0.01, and ***P<0.001 vs WT.
**Rip2**/**−/−** macrophages than in wild-type macrophages under basal conditions (Figure 3D).

The NF-κB activation status was also reduced in **Rip2**/**−/−** macrophages after stimulation with lipopolysaccharide (Figure 4A), in agreement with previous studies.14,15,27 To determine the effect of **Rip2** deficiency on the inflammatory response of macrophages, we analyzed levels of proinflammatory cytokines in the culture media of peritoneal **Rip2**/**−/−** or wild-type macrophages after incubation with and without 50 μg/mL mmLDL for 24 hours. No significant differences in cytokines were seen in the media of cells incubated in the absence of mmLDL, except for a slightly reduced concentration of tumor necrosis factor-α in the medium of **Rip2**/**−/−** macrophages (Figure 4B). As expected, we observed significantly reduced concentrations of tumor necrosis factor-α, interferon-γ, monocyte chemoattractant protein-1, and interleukin-6 in media from **Rip2**/**−/−** macrophages incubated with mmLDL (Figure 4B). Thus, **Rip2** is involved in the inflammatory response induced by the cellular uptake of mmLDL in macrophages.

To investigate whether the impaired immune signaling in **Rip2**/**−/−** macrophages induced a switch to the more anti-inflammatory M2 macrophage phenotype, we analyzed peritoneal macrophages with a FACS assay for the M1 and M2 markers interleukin-10, respectively. However, we did not detect a difference in phenotype between **Rip2**/**−/−** and wild-type macrophages (Figures 4C and 4D).

Lipid accumulation in atherosclerotic lesions has been reported to induce endoplasmic reticulum stress and apoptosis in macrophages, which can lead to exacerbation of the lesions.28 We tested whether the increased lipid levels in **Rip2**/**−/−** macrophages induced endoplasmic reticulum stress by analyzing the marker phospho-PERK in peritoneal macrophages. There was no difference in endoplasmic reticulum stress between **Rip2**/**−/−** and wild-type macrophages from mice that received a chow diet (Figure 4E); however, **Rip2**/**−/−** macrophages from mice fed a high-fat diet actually had lower levels of endoplasmic reticulum stress than wild-type macrophages under basal conditions (Figure 4D).

**Increased TLR4 and SR-B1 in**

**Rip2**/**−/−** **Macrophages**

To elucidate the mechanism for the increased lipid uptake in **Rip2**/**−/−** macrophages, we analyzed the expression of several...
key regulators of lipid metabolism in peritoneal macrophages isolated from Rip2−/− or wild-type mice. We observed significantly increased mRNA levels of scavenger receptor (SR) class B type 1 (SR-B1) and TLR4 and decreased mRNA levels of fatty acid synthase, an important regulator of de novo lipogenesis, in Rip2−/− peritoneal macrophages. No differences in mRNA expression were seen for SR-A, CD36, ABCA1 (ATP-binding cassette, subfamily A, member 1), ABCG1 (ATP-binding cassette, subfamily G, member 1), or very low-density lipoprotein receptor; TLR2; or stearoyl-coenzyme A desaturase and carnitine palmitoyltransferase I (Figure 5A). FACS analysis showed significantly higher surface expression of TLR4 and TLR4-MD2, a trend toward higher levels of SR-B1 (P=0.11), and no differences in protein levels for SR-A, CD36, ABCG1, very low-density lipoprotein receptor, or TLR2 in Rip2−/− peritoneal macrophages (Figure 5B). Protein expression of scavenger receptors SR-A, SR-B1, and CD36 was also ascertained with Western blotting, which confirmed the results from FACS analysis (Figures 5C and 5D).

**TLR4 Mediates Lipid Uptake in Rip2−/− Macrophages**

To investigate whether TLR4 or SR-B1 mediates the increase in lipid accumulation in Rip2−/− macrophages, we knocked down these proteins in Rip2−/− or wild-type peritoneal macrophages with small interfering RNA and incubated the cells with 50 μg/mL mmLDL for 24 hours. Transfection with small interfering RNA against TLR4 or SR-B1 reduced the TLR4 and SR-B1 protein levels by 85% and 78%, respectively. TLR4 knockdown totally abolished the increase in lipid accumulation in Rip2−/− macrophages (Figures 6A and 6B). In contrast, SR-B1 knockdown did not affect lipid uptake in Rip2−/− macrophages (Figure 6C). Thus, TLR4 but not SR-B1 is essential for the increased lipid uptake of mmLDL seen in Rip2−/− macrophages.

**Constitutively Activated TLR4-Dependent Macropinocytosis in Rip2−/− Macrophages**

TLR4 per se cannot promote lipid uptake, but mmLDL was recently shown to promote lipid accumulation in macrophages by TLR4-dependent macropinocytosis (which is characterized by actin polymerization and cytoskeletal rearrangements). We therefore investigated whether Rip2 deficiency induces TRL4-dependent macropinocytosis in macrophages. By staining peritoneal macrophages for F-actin, we showed that Rip2 deficiency induced cytoskeletal rearrangements and spreading even under basal conditions (Figure 7A). We also analyzed uptake of fluorescent dextran as a measure of macropinocytosis and showed that dextran accumulation was increased significantly in Rip2−/− peritoneal macrophages compared with wild-type macrophages both in the absence and presence of 50 μg/mL mmLDL (Figure 7B). Knockdown of TLR4 with small interfering RNA reduced the increased uptake of dextran in Rip2−/− peritoneal macrophages (Figure 7C). Our results thus show that Rip2 deficiency induces a constitutively active TLR4-dependent macropinocytosis in macrophages.

A previous study by Kanters et al showed that deficiency in IκB kinase-β (IKK-β) in macrophages led to increased atherosclerosis despite decreased inflammation. To examine whether deficiency in classic NF-κB signaling would result in
a phenotype similar to that of Rip2−/− macrophages, we knocked down IKK-β and NEMO (NF-κB essential modulator) in wild-type peritoneal macrophages. Knockdown of either IKK-β or NEMO did not affect lipid accumulation in the presence of mmLDL (Figure 7D), TLR4 mRNA levels (Figure 7E), or macrophage spreading (data not shown), which suggests that the regulatory effects of Rip2 are mediated through an NF-κB–independent mechanism.

mmLDL induces TRL4-dependent macropinocytosis in wild-type macrophages by inducing recruitment of spleen tyrosine kinase (Syk) to TLR4.29 We therefore tested whether the TRL4-dependent macropinocytosis in Rip2−/− peritoneal macrophages was also Syk dependent. We showed that activation of Syk (as measured by Western blotting of phosphorylated Syk) was increased in Rip2−/− macrophages (Online Figure III). In addition, we showed that incubation with the Syk inhibitor piceatannol (30 μmol/L) abolished lipid accumulation in Rip2−/− macrophages cultured under basal conditions (Figure 7F), which indicates that the constitutively active TRL4-dependent macropinocytosis in Rip2−/− macrophages is Syk dependent.

Our findings suggest that Rip2 or a Rip2-dependent signaling pathway may negatively regulate TRL4/Syk-dependent macropinocytosis. Consistent with this hypothesis, incubation of wild-type peritoneal macrophages with 50 μg/mL mmLDL for 24 hours resulted in a reduction of Rip2 expression by 31.0±0.13% (n=5; P<0.01). Collectively, these results show that induction of TLR4-dependent macropinocytosis in macrophages by mmLDL is paralleled by a reduction in Rip2 expression and reveal a key role for Rip2 in the regulation of TLR4-dependent macropinocytosis of LDL in macrophages.

**Discussion**

In the present report, we studied the consequence of Rip2 deficiency in an atherosclerotic mouse model. Surprisingly, we found that subendothelial lipid accumulation was increased in aortas from mice that received Rip2−/− bone marrow despite impaired immune signaling. We also showed that lipid uptake was increased, whereas immune signaling was reduced, in Rip2−/− macrophages. Further analysis in Rip2−/− macrophages showed that lipid accumulation was dependent on TLR4 and that Rip2 deficiency induced TLR4-dependent macropinocytosis. The present study interestingly illustrates a mouse model with dissociated lipid and inflammation levels in the atherosclerotic lesions. In addition, these results identify Rip2 for the first time as a key regulator of cellular lipid metabolism.

Inflammation was reduced in mice that received Rip2−/− bone marrow, as expected. Several circulating proinflammatory mediators were reduced, eg, interleukin-6 and CXCL1; however, mice that received Rip2−/− bone marrow still had
normal frequencies of circulating monocytes, B cells, and T cells. Local inflammation in the vessel wall was also decreased, as shown by a marked reduction in ICAM-1 expression in atherosclerotic lesions; however, there was no difference in VCAM-1 expression in these lesions. This discrepancy is surprising but is supported by recent data showing that VCAM-1, unlike ICAM-1, can be regulated separately from NF-κB, for example, by shear stress.\(^{31,32}\)

The present in vitro studies showed that lipid accumulation was increased in Rip2\(^{−/−}\) macrophages; however, we did not detect increased expression of any of the major receptors that mediate macrophage lipid uptake (eg, SR-A and CD36), in agreement with results showing that foam cell formation in vivo may proceed even in the absence of SR-A and CD36.\(^{33}\) In contrast, we showed that the increased lipid accumulation seen in Rip2\(^{−/−}\) macrophages was instead linked to increased expression of TLR4. Knockdown of TLR4 in peritoneal macrophages completely abolished the increased lipid accumulation seen in Rip2\(^{−/−}\) macrophages. Thus, the present results suggest that Rip2 normally functions as a negative regulator of TLR4 in macrophages.

Further investigation showed that TLR4 promoted Syk-dependent macrophage spreading and uptake of lipids via pinocytosis. TLR4/Syk-dependent macropinocytosis has been observed previously in mmLDL-stimulated macrophages.\(^{29,34}\) However, a different phenotype was observed in Rip2\(^{−/−}\) macrophages, because TLR4/Syk-dependent macropinocytosis in these cells did not require induction by mmLDL but was constitutively activated. Our results thus show that impaired Rip2 activation in macrophages results in constitutive signaling through TLR4/Syk, which induces macrophage spreading and uptake of lipids via pinocytosis. Rip2 may thus be considered a novel key regulator of lipid metabolism. Interestingly, previous studies identified overlapping roles for several host proteins (eg, MyD88 and other signaling molecules downstream of TLRs) in lipid trafficking and innate immunity and suggested that certain proteins may have the ability to mediate coupling between lipid metabolism and immune signaling under permissive conditions.\(^{35}\)

The role of inflammation in atherosclerosis has been a widely emphasized feature over the past decade. Although improved treatment of hyperlipidemia has dramatically reduced mortality due to complications of atherosclerosis, further efforts to develop pharmaceuticals to treat atherosclerosis are needed. Immunopharmacological intervention against atherosclerosis attracts major interest. Rip2 recently has been suggested to be a key target for immune intervention to atherosclerosis,\(^{19}\) as well as several other pathological disorders.\(^{36,37}\) However, the results of the present study clearly demonstrate that immunosuppression targeting Rip2 may have unexpected negative consequences, eg, subendothelial lipid accumulation.

In conclusion, our work identifies Rip2 as a novel player in the modulation of macrophage lipid metabolism and cardiovascular disease. Furthermore, we demonstrate that mice with Rip2\(^{−/−}\) macrophages have increased atherosclerotic lesions in the aorta, despite a suppressed innate immune system. Our results yield important insights into the underlying association between lipid accumulation and inflammation in atherogenesis.

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**Disclosures**

None.

**References**


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**Novelty and Significance**

**What Is Known?**

- Lipid accumulation and inflammation are both involved in the development of atherosclerosis.
- The molecular mechanisms linking lipid accumulation and inflammation to atherosclerosis are poorly understood, but Toll-like receptors (TLRs) and nuclear factor-xB (NF-xB) are thought to play a key role.
- Receptor-interacting protein 2 (Rip2) is a serine/threonine kinase that activates NF-xB and could therefore be expected to protect against the development of atherosclerosis.

**What New Information Does This Article Contribute?**

- Mice transplanted with Rip2-deficient bone marrow–derived cells show a surprising dissociation between lipid accumulation and inflammation, with increased aortic lipid accumulation despite decreased local and systemic inflammation.
- Rip2-deficient macrophages have increased lipid uptake despite suppressed immune signaling.
- TLR4-dependent macrophocytosis mediates lipid uptake in Rip2-deficient macrophages.

Atherosclerosis is both a lipid disorder and a chronic inflammatory disease. Macrophages play a central role in the atherogenic process modulating both lipid metabolism and immune responses. Recent research suggests that there is cross talk between signaling pathways that regulate inflammation and lipid metabolism in macrophages; however, molecular mechanisms linking these pathways are poorly understood. Rip2 is an established mediator of inflammatory signaling, involved in multiple NF-xB signaling pathways (e.g., TLRs). Inhibition of inflammation has been suggested as a strategy to prevent progression of atherosclerosis, and Rip2 has been suggested as a key target for immune intervention. Therefore, we investigated the role of Rip2 in lipid metabolism and development of atherosclerosis in mice. Surprisingly, we found that mice transplanted with Rip2−/− bone marrow–derived cells displayed increased aortic atherosclerosis despite impaired inflammation. Moreover, lipid uptake was increased in Rip2−/− macrophages, whereas immune signaling was reduced. We also showed that lipid accumulation in Rip2−/− macrophages was scavenger-receptor independent but mediated by TLR4-dependent lipid uptake. Our study demonstrates for the first time that Rip2 is a key regulator of cellular lipid metabolism and is important for cardiovascular disease.
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Supplemental Material

Methods

Mice. Rip2^{−/−} mice\(^1\) and wild-type littersmates (backcrossed 7 times on C57BL/6J BomTac background), kindly provided by Dr Paul W. Dempsey (Dept. of Microbiology, Immunology and Molecular Genetics, UCLA, Los Angeles, CA), and human apoB100 transgenic mice on Ldlr^{−/−} background (APOB100×Ldlr^{−/−} mice; backcrossed 7 times on C57BL/6J BomTac background)\(^2,4\) were housed in a pathogen-free barrier facility (12-h light/12-h dark cycle) and fed rodent chow. All animal studies were approved by the local animal ethics committee.

Bone Marrow Transplantation. One week before and two weeks after transplantation, 8-week-old APOB100×Ldlr^{−/−} recipient mice were given acidified water supplemented with neomycin (100 mg/L) and polymyxin B sulfate (10 mg/L). The mice were irradiated with 9 Gy and reconstituted with bone marrow stem cells from Rip2^{−/−} or wild-type mice as previously described.\(^5\) The transplantation efficiency was 95.3±1.7% (n=12) for mice reconstituted with Rip2-deficient bone marrow and 99.1±0.9% (n=17) for mice reconstituted with wild-type bone marrow. To determine the chimerism in transplanted mice, we took advantage of the fact that the donor bone marrow was Ldlr^{+/+}, whereas recipient bone marrow was Ldlr^{−/−} as described by Kanters et al.\(^6\) Mice were sacrificed 6 months after transplantation for atherosclerosis studies. Mice were sacrificed 9 weeks after transplantation for analyses of circulating immune cells, T-cell compartment and immune response.

Blood Analysis. Blood was obtained after a 4 h fast the day before the mice were sacrificed. Cholesterol and triglycerides were measured on a Konelab 20 autoanalyzer (Thermo, Vantaa, Finland). Plasma levels of soluble intercellular adhesion molecule (ICAM) and vascular cellular adhesion molecule (VCAM) were analyzed with ELISA (R&D systems, Minneapolis, MN). Plasma cytokines were analyzed with a SECTOR Imager 2400 reader (Meso Scale Discovery, Gaithersburg, MD).

Analysis of Aortae. Whole aortae were pinned out by en face technique and fixed in 70% ethanol for 5 min, stained with 0.5% Sudan IV for 6 min and differentiated for 3 min in 80% ethanol. The aortic roots were embedded in OCT Tissue-Tec medium, frozen in dry ice and isopentane, cut into 10-µm-thick cross sections, and stained with 0.5% Oil Red O.\(^7\) Lesion areas were quantified using KS-400 software (Zeiss). Immunohistochemistry was done with antibodies against α-actin, Mac-2, CD4/CD8, ICAM and VCAM\(^5\) on cross sections of aortic sinuses.

LPS Stimulation of Transplanted Mice. 9 weeks after transplantation, mice were given an intraperitoneal injection of LPS (5mg/kg). 6h after the injection, blood and tissues were collected. RNA was isolated using the RNeasy Protect Animal Blood System and RNeasy Tissue Kit (QIAGEN, Hilden, Germany) and cDNA and Taqman performed as described below.

Macrophage Isolation. Peritoneal macrophages were isolated by flushing the peritoneum of Rip2^{−/−} or wild-type mice with PBS. Cells were collected and cultured for 2 h at 37°C in serum-free RPMI 1640 media supplemented with sodium pyruvate (2 mmol/L), non-essential amino acids, penicillin (100 U/mL) and streptomycin (100 mg/L). The cells were washed three times with PBS and cultured at 37°C in RPMI 1640 media containing 10% FCS supplemented with sodium pyruvate (2 mmol/L), non-essential amino acids, penicillin (100 U/mL) and streptomycin (100 mg/L).

Isolation and Modification of LDL Particles. LDL was isolated from human plasma as described previously.\(^3\) Minimally oxidized LDL (mmLDL) was prepared by incubating LDL at 4°C for 2 months.\(^8\)

In Vitro Analysis of Lipid Accumulation and Cytokine Secretion. Peritoneal macrophages were cultured with or without mmLDL (50 µg/ml) for 24 h. The total Oil Red O surface area was quantified using the BioPix software.\(^9\) Triglycerides, cholesterol esters and free cholesterol were analyzed using a straight phase HPLC system with light-scattering detection according to Homan et al.\(^10\) The lipid classes were quantified using external standards. Cytokine levels in the media were analyzed with a SECTOR Imager 2400 reader (Meso Scale Discovery).


**Analysis of Apoptosis in Peritoneal Macrophages and in RAW 264.7 Cells Overexpression Rip2.**

Peritoneal macrophages were seeded on glass slips, cultured with or without mmLDL (50 µg/ml) for 24 h and then fixed with formaldehyde. RAW 264.7 cells were seeded on glass slips and cells were transfected with Rip2 cDNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The medium was changed after 6 h and the cells were fixed with formaldehyde after a further 24 h. Apoptosis was analyzed using ApoTag Fluorescein In Situ Apoptosis Detection Kit (Millipore, Temecula, CA). Images were captured using an ApoTome Axioplan 2 imaging system (Carl Zeiss, Göttingen, Germany) and apoptosis was quantified using the BioPix software.

**mRNA Expression in Peritoneal Macrophages.** Peritoneal macrophages were cultured with or without mmLDL (50 µg/ml) for 24 h, washed with PBS and total RNA was extracted using RNeasy Kit (QIAGEN, Hilden, Germany). cDNA was synthesized using the high capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) with random primers. mRNA expression of genes of interest was analyzed with TaqMan real-time PCR in an ABI Prism 7900 HT Detection System (Applied Biosystems). The following TaqMan Gene Expression assays were used: interleukin (IL)-6 _mm00446190, IL-10 _mm00439614, scavenger receptor class A (SR-A) _mm00446214, SR-B1 _mm00450236, CD36 _mm00432401, ATP-binding cassette sub-family G member 1 (ABCG1) _mm0043739, very-low-density lipoprotein receptor (VLDLr) _mm00443281, TLR4 _mm00445273, TLR2 _mm00442346, fatty acid synthase (FAS) _mm00662319, stearoyl-CoA desaturase-1 (SCD1) _mm00772290, carnitine palmitoyltransferase I (CPT1) _mm00550438, Rip2 _mm00446816, TNFα _mm00443258, mRNAs. The following TaqMan Gene Expression assays were used: interleukin (IL)-6 _mm00446190, IL-10 _mm00439614, scavenger receptor class A (SR-A) _mm00446214, SR-B1 _mm00450236, CD36 _mm00432401, ATP-binding cassette sub-family G member 1 (ABCG1) _mm0043739, very-low-density lipoprotein receptor (VLDLr) _mm00443281, TLR4 _mm00445273, TLR2 _mm00442346, fatty acid synthase (FAS) _mm00662319, stearoyl-CoA desaturase-1 (SCD1) _mm00772290, carnitine palmitoyltransferase I (CPT1) _mm00550438, Rip2 _mm00446816, TNFα _mm00443258, mRNAs.

**FACS Analysis.** Peritoneal cells were resuspended in FACS buffer (PBS, 3% FCS, 0.09% NaAz) and then incubated with Fc block (2.4G2, BD Bioscience, Erembrougdem, Belgium) for 20 min at room temperature to avoid nonspecific binding via Fc-receptor interactions. For FACS analysis in blood, blood was lysed in Red Blood Cell Lysis Solution (MACS, Fisher Scientific) and then centrifuged at 300 x g for 10 min and resuspended in FACS buffer and Fc block. For surface-expressed antigens, the following antibodies were used: HMC class II (FITC-conjugated, BD Pharmingen), CD206 (Alexa Fluor 647-conjugated, BioLegend), CD36 [allophycocyanin (APC)-conjugated, BD Bioscience], SR-A [fluorescein (FITC)-conjugated, Lifespan Biosciences, Seattle, WA], ABCG1 (non-conjugated, Abcam, Cambridge, UK), SR-B1 (non-conjugated, Novus Biologicals), VLDLr (non-conjugated, Clone 6A6, Santa Cruz Biotechnology, Santa Cruz, CA), TLR4 (Clone UT41, Alexa fluor 488-conjugated, eBioscience, San Diego, CA), TLR4-MD2 (Clone MTS510, APC-conjugated, eBioscience) and TLR2 (Clone 6C2, non-conjugated, Santa Cruz Biotechnology). For analysis of immune cells in blood, the following antibodies were used without the following intracellular staining: CD11b (PerCP-Cy5.5-conjugated), CD5 (PE-conjugated), CD19 (APC-conjugated), CD25 (PE-conjugated), CD4 (FITC-conjugated), CD8 (APC-conjugated), CD3 (PerCP-Cy5.5-conjugated), all from BD Pharmingen.

All incubation times were 45 min at 4°C, followed by washing steps in FACS buffer. VLDLr and TLR2 were detected using a goat anti-mouse IgG F(ab\')2 conjugated to APC (Santa Cruz Biotechnology) and ABCG1 and SR-B1 were detected using a biotinylated rat anti-rabbit antibody (Zymed Invitrogen, Carlsbad, CA) followed by addition of Streptavidin-APC (BD Bioscience).

After the initial surface staining step, cells were fixed using 2% paraformaldehyde for 30 min at room temperature and stored at 4°C. For intracellular staining, paraformaldehyde-fixed cells were permeabilized in FACS buffer (PBS, 3% FCS, 0.09% NaAz) with 0.5% saponin for 10 min on ice, followed by incubation with Fc block for 20 min at room temperature. Phycoerythrin (PE) conjugated rat anti-mouse CD68 (FA-11, AbD Serotec, Oxford, UK) (as a marker of macrophages) or the matched PE conjugate rat anti-mouse isotype control (YTH71.3, AbD Serotec) was added. After incubation for 45 min at 4°C, cells were washed twice in permeabilization buffer followed by an additional wash in FACS buffer. 10,000 cells were collected for each staining using a FACSCanto II equipped with the Diva 6:2 software (BD Bioscience) and were analyzed using the FlowJo software (Tree Star, Ashland, OR).

**Immunoblot.** Peritoneal macrophages were cultured with or without mmLDL (50 µg/ml) for 24 h, and washed 3 times in PBS and treated with cell lysis buffer (Cell Signaling Technology, Danvers, MA) according to the manufacturer’s protocol. For NFκB-activation analysis, peritoneal macrophages were cultured with or without LPS for 20 minutes, treated with cell lysis buffer and flash-frozen in liquid...
nitrogen. Equal amounts of total protein were loaded onto a NuPAGE 4-12% Bis-Tris Gel (Novex, Invitrogen, Carlsbad, CA). The proteins were transferred to nitrocellulose membranes and incubated with antibodies that recognized phosphorylated PERK (phospho-PERK) (Cell Signaling Technology), Macrophage Scavenger Receptor 1 (MSR1, SR-A) (Abcam, Cambridge, UK), Scavenging Receptor SR-B1 (Abcam), CD36 (Abcam), phosphorylated-IkBα (Cell Signaling Technology), phosphorylated spleen tyrosine kinase (phospho-Syk (Tyr525/526)) (Cell Signaling Technology) and GAPDH (Ambion, Applied Biosystems, Foster City, CA). Proteins were visualized with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) and ECL Western Blotting System (Amersham Biosciences). Bands were quantified with Multi Gauge V3.0 (Fujifilm Life Sciences) and normalized to GAPDH.

**TLR4 and SR-B1 Knockdown.** Peritoneal macrophages were transfected with pre-designed Silencer Select siRNA (Ambion) using Lipofectamine RNA Max (Invitrogen, Carlsbad, CA). The medium was changed after 6 h and mmLDL (50 µg/mL) was added to the fresh medium. The cells were analyzed after a further 24 h. To ensure the specificity of the knockdown, two siRNA constructs were used for each gene. The knockdown was confirmed by FACS analysis.

**Morphological Analysis of Macropinocytosis.** Peritoneal macrophages were seeded on glass slips, cultured for 4 days and then fixed with formaldehyde. Cells were permeabilized in PBS containing 10% saponin and 0.7% gelatin solution from fish skin (Sigma-Aldrich, St Louis, MO) and then stained with Rhodamin phalloidin (Invitrogen Molecular Probes) and DAPI (Sigma-Aldrich) to visualize F-actin cytoskeleton and nuclei, respectively. Images were captured using an ApoTome Axioplan 2 imaging system (Carl Zeiss, Göttingen, Germany).

**In Vitro Analysis of Macropinocytosis.** Peritoneal macrophages were incubated with or without mmLDL (50 µg/mL) and with dextran labeled with Alexa Fluor 488 (200 µg/mL) in the media for 16 h. The cells were washed with PBS and lysed with sodium hydroxide solution (0.1 mol/L sodium hydroxide, 0.1% SDS). The fluorescence was assessed with a fluorometer (MDS Analytical Technologies), and normalized to protein content.

**Statistical Analysis.** Data are shown as means ± SEM. Measurements were compared with the two-tailed t-test or Mann-Whitney rank sum test.
Online Figure I. Circulating T-cells and response to inflammatory stimulus in *APOB×Ldlr<sup>−/−</sup>* mice transplanted with WT or *Rip2<sup>−/−</sup>* bone marrow. A, FACS analysis of T-cells in blood. Regulatory T-cells were gated based on CD4<sup>+</sup> and CD25<sup>+</sup> (n=3 per group). B, Inflammatory response in mice with and without injection of LPS (5mg/kg). Data are presented as mean ± SEM; **P < 0.01 vs. basal WT, ***P < 0.001 vs. basal WT, #P < 0.05 vs. LPS-stimulated WT.
Online Figure II

A

Online Figure II. Increased apoptosis in RAW264.7 macrophages with Rip2 overexpression. Apoptosis in 264.7 macrophages with Rip2 overexpression for 24h was analyzed with TUNEL staining (n=3 per group). Data are presented as mean ± SEM; ***P < 0.001 vs. control.

Online Figure III

A

B

Online Figure III. Increased activation of Syk in macrophages from Rip2⁻/⁻ mice. 
A. Representative Western blot of phosphorylated Syk in WT or Rip2⁻/⁻ peritoneal macrophages. 
B. Quantification of phosphorylated Syk from Western blots (n=4 per genotype). Data are presented as mean ± SEM; *P < 0.05 vs. WT.
References


