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## Disruption of mTORC1 in Macrophages Decreases Chemokine Gene Expression and Atherosclerosis

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### Abstract

**Rationale**—The mammalian target of rapamycin complex 1 (mTORC1) inhibitor, rapamycin, has been shown to decrease atherosclerosis, even while increasing plasma LDL levels. This suggests an anti-atherogenic effect possibly mediated by modulation of inflammatory responses in atherosclerotic plaques.

**Objective**—To assess the role of macrophage mTORC1 in atherogenesis.

**Methods and Results**—We transplanted bone marrow from mice in which a key mTORC1 adaptor, Raptor, was deleted in macrophages by Cre/loxP recombination (*Mac-Rap<sup>KO</sup>* mice) into *Ldlr<sup>-/-</sup>* mice and then fed them the Western-type diet (WTD). Atherosclerotic lesions from *Mac-Rap<sup>KO</sup>* mice showed decreased infiltration of macrophages, lesion size and chemokine gene expression compared with control mice. Treatment of macrophages with minimally modified LDL (mmLDL) resulted in increased levels of chemokine mRNAs and STAT3 phosphorylation; these effects were reduced in *Mac-Rap<sup>KO</sup>* macrophages. While wild-type and *Mac-Rap<sup>KO</sup>* macrophages showed similar STAT3 phosphorylation on Tyr705, *Mac-Rap<sup>KO</sup>* macrophages showed decreased STAT3 Ser727 phosphorylation in response to mmLDL treatment and decreased *Ccl2* promoter binding of STAT3.

**Conclusions**—The results demonstrate cross-talk between nutritionally-induced mTORC1 signaling and mmLDL-mediated inflammatory signaling via combinatorial phosphorylation of STAT3 in macrophages, leading to increased STAT3 activity on the CCL2 (MCP-1) promoter with pro-atherogenic consequences.

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## Keywords

Atherosclerosis; mTORC1; chemokine; macrophage

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## Introduction

The mammalian target of rapamycin (mTOR) is a serine–threonine kinase that as part of the mTORC1 complex regulates anabolic and catabolic processes required for autophagy, RNA translation, protein synthesis, ribosome biogenesis, and cell survival.<sup>1</sup> mTOR acts as a central regulator of cell growth and proliferation by integrating signals from nutrients, energy status, and growth factors, in part by regulating the phosphorylation of p70 S6 kinase (p70S6k) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1).<sup>1</sup> Upstream, the TSC1/2 complex negatively regulates mTORC1 activity.<sup>2</sup> Raptor (regulatory associated protein of mTOR) is an mTOR binding partner that also binds p70S6k and 4E-BP1 and is essential for mTOR signaling.<sup>3, 4</sup> While whole body *Raptor*<sup>KO</sup> mice are embryonic lethal,<sup>5</sup> it has been possible to probe tissue specific functions of mTOR using *Tsc1*<sup>flx/flx</sup> or *Raptor*<sup>flx/flx</sup> (*Rap*<sup>flx/flx</sup>) mice, with *Cre* recombinase expression resulting in increased or decreased mTOR activity respectively.<sup>6</sup>

Rapamycin is an immunosuppressant with potent anti-proliferative and anti-inflammatory effects that is used to prevent cardiac transplantation vasculopathy.<sup>7</sup> However, treatment of patients with rapamycin also leads to increased plasma LDL levels.<sup>8</sup> In mouse models mTORC1 inhibition caused downregulation of LDL receptor levels in liver and decreased the activity of lipoprotein lipase (LPL), leading to higher plasma LDL and triglyceride levels.<sup>9, 10</sup> Using liver specific *Tsc1* knockout (*Li-Tsc1*<sup>KO</sup>) and *Raptor* knockout (*Li-Rap*<sup>KO</sup>) mice, we showed that the mechanism of increased LDL involved mTOR—mediated suppression of *Pcsk9* mRNA resulting in up-regulation of LDLR protein.<sup>9</sup> Paradoxically, the mTORC1 inhibitor, rapamycin, was reported to reduce inflammation and atherosclerosis despite increasing plasma LDL levels.<sup>8</sup> Since LDL levels are usually a dominant factor in atherogenesis, this suggested a potent anti-atherogenic effect of mTORC1 inhibition independent of plasma LDL levels. Inflammation has an important role in atherosclerosis,<sup>11</sup> raising the possibility that rapamycin is working through an anti-inflammatory mechanism.

In view of the central role of macrophages in atherosclerotic inflammation, we tested this hypothesis by crossing *Tsc1*<sup>flx/flx</sup> and *Rap*<sup>flx/flx</sup> mice with *LysM-Cre* mice, resulting in increased and decreased mTORC1 activity in macrophages, respectively; and then transplanted the bone marrow (BM) of these mice into *Ldlr*<sup>-/-</sup> recipients followed by WTD feeding to induce atherogenesis. While studies with the *Mac-Tsc1*<sup>KO</sup> mice were limited by premature death, the *Mac-Rap*<sup>KO</sup> BM transplanted *Ldlr*<sup>-/-</sup> mice displayed reduced macrophage content in atherosclerotic lesions. This appeared to be related to decreased macrophage expression of pro-atherogenic chemokines. Further mechanistic studies revealed that when macrophages were treated with minimally modified LDL (mmLDL), mTORC1 activity amplified the induction of chemokines by increasing IL6 signaling.

## Methods

An expanded Methods section is available in the online data supplement.

### Animal and diet

*Raptor*<sup>flox/flox</sup> (The Jackson Laboratory, stock number 013188) or *Tsc1*<sup>flox/flox</sup> mice were mated with transgenic mice expressing Cre recombinase under the control of the *LysM* promoter (*LysM-Cre*, Jackson Laboratories, stock number 004781) to generate mice with or without *Raptor* (*Mac-Rap*<sup>KO</sup>) or *Tsc1* (*Mac-Tsc1*<sup>KO</sup>) expression in myeloid cells. *Raptor*<sup>flox/flox</sup> (*Rap*<sup>flox/flox</sup>) or *Tsc1*<sup>flox/flox</sup> littermates without the Cre recombinase transgene were used as controls throughout the study. *Tsc1*<sup>flox/flox</sup> mice backcrossed to C57BL/6J for 9 generations were kindly provided by Dr David Kwiatkowski. *Ldlr*<sup>-/-</sup> mice (stock number 002207) were purchased from The Jackson Laboratory. Mice were fed a WTD (21% milk fat, 0.2% cholesterol; Harlan Teklad, TD88137) or chow diet (Purina Mills diet 5053). All protocols were approved by the Institutional Animal Care and Use Committee of Columbia University.

## Results

### Atherosclerosis is decreased in BM transplanted *Mac-Rap*<sup>KO</sup> *Ldlr*<sup>-/-</sup> mice

In a preliminary study, we found that phospho-S6 ribosomal protein (phosphoS6), a downstream target of mTORC1, was increased in peritoneal macrophages from *Ldlr*<sup>-/-</sup> mice fed an atherogenic Western type diet (WTD) compared to chow (Online Figure IA), and also in chow-fed *ob/ob* mice compared to wild type mice (data not shown). To investigate the role of mTORC1 in macrophages, we crossed *Rap*<sup>flox/flox</sup> with *LysM-Cre* mice, which are known to delete targets in macrophages and neutrophils and to a lesser extent in monocytes and myeloid progenitors.<sup>12</sup> For simplicity we refer to these mice as *Mac-Rap*<sup>KO</sup> mice, while recognizing that the knockout is not completely macrophage-specific. In addition, we carried out selected studies in *Mac-Tsc1*<sup>KO</sup> mice; since TSC1 is an upstream inhibitor of mTORC1, these mice have increased mTORC1 activity. However, due to premature mortality at about 4 months of age, we were not able to carry out atherosclerosis studies in these mice. We transplanted *Ldlr*<sup>-/-</sup> mice with *Mac-Rap*<sup>KO</sup> (*Ldlr*<sup>-/-</sup>-*Mac-Rap*<sup>KO</sup>) and *Rap*<sup>flox/flox</sup> (*Ldlr*<sup>-/-</sup>-WT) BM. In peritoneal macrophages, mTORC1 activity reflected by phosphoS6 levels was lower in *Ldlr*<sup>-/-</sup>-*Mac-Rap*<sup>KO</sup> compared with *Ldlr*<sup>-/-</sup>-WT mice (Online Figure IB). Five weeks after BM transplantation, reconstitution of the BM was >90% (Online Figure IC). The body weights, spleen weights, triglyceride levels, and cholesterol levels were similar in the two groups (Figure 1A, Online Figure ID). There was no difference in the lipoprotein cholesterol distribution (Figure 1B). *Ldlr*<sup>-/-</sup> mice transplanted with BM from *Rap*<sup>flox/flox</sup> or *Mac-Rap*<sup>KO</sup> had similar levels of neutrophils, monocytes and Ly6C<sup>hi</sup> monocytes in peripheral blood after 10 weeks of WTD feeding (Online Figure IF). In contrast, the percentages of Ly6C<sup>hi</sup> and F4/80 positive macrophages in spleen were considerably lower in *Ldlr*<sup>-/-</sup>-*Mac-Rap*<sup>KO</sup> than *Ldlr*<sup>-/-</sup>-WT mice at the 10 week timepoint (Online Figure IG).

Following ten weeks of WTD feeding, mice were sacrificed and atherogenesis was assessed in the aortic root. In *Ldlr*<sup>-/-</sup> mice, macrophage deficiency of *Raptor* decreased atherosclerotic

lesion area by 25% compared to the *Ldlr*<sup>-/-</sup>-WT control group (Figure 1C, 1D). To further analyze the lesion phenotype, we examined lesion composition. Morphological analyses of the cross-sectional lesions showed no significant difference in collagen content (Online Figure IIA). In contrast, analysis of macrophage and cholesterol ester content uncovered significant reductions in mac-3<sup>+</sup> immunostaining and oil red O staining in *Mac-Rap*<sup>KO</sup> BM transplanted *Ldlr*<sup>-/-</sup> mice (Figure 1E and Online Figure IIC). The smooth muscle cell and T cell contents remained unchanged between groups as assessed by immunostaining with  $\alpha$ -actin and CD3 antibodies, respectively (Online Figure IIB, IID). These results suggest that decreased macrophage foam cell accumulation in lesions contributes to the reduced development of atherosclerosis in *Ldlr*<sup>-/-</sup>-*Mac-Rap*<sup>KO</sup> mice.

Lesional macrophage accumulation is modulated by many processes such as local proliferation,<sup>13</sup> apoptosis<sup>14</sup> and monocyte recruitment. As shown in Online Figure IIE and IIF, Ki67-positive and TUNEL-positive macrophage staining were similar between the groups, suggesting no difference in local proliferation or apoptosis of macrophages, respectively. In addition, since reduced mTORC1 activity might lead to increased macrophage autophagy, which could contribute to decreased atherosclerosis, we stained lesions for P62, an autophagy marker. There was a 50% decrease in P62 expression, indicative of moderately increased autophagy (Online Figure IIIA, IIIB). However, Razani *et al*<sup>15</sup> showed that a 3.5 fold increase of P62 in *Beclin-Het* autophagy deficient mice was insufficient to change atherosclerosis, while 7-9 fold increases were associated with moderately reduced lesion formation. This suggests that small changes in autophagy such as we observed might not affect atherogenesis.

To further address potential anti-atherogenic mechanisms in *Mac-Rap*<sup>KO</sup> BM transplanted mice, we performed laser capture microscopy (LCM) in lesions and assessed the mRNA expression of pro-atherogenic chemokines and cytokines in macrophage-rich areas. Interestingly, this showed markedly decreased mRNA expression of chemokines *Ccl2/Mcp-1*, *Ccl3/Mip1 $\alpha$* , *Ccl6*, and *Cxcl2* in *Ldlr*<sup>-/-</sup>-*Mac-Rap*<sup>KO</sup> mice compared to control, whereas there were no significant differences in *Il10*, *Tnfa*, *Il12p40* and *Il6* mRNA levels between the two groups (Figure 1F). All of the upregulated chemokines are involved in monocyte recruitment and have been reported to accelerate atherogenesis.<sup>16-18</sup> Consistent with these findings, the level of CCL2 in plasma was decreased to 32% of controls in *Ldlr*<sup>-/-</sup>-*Mac-Rap*<sup>KO</sup> mice (Online Figure IE). These results suggested that the attenuation of the development of atherosclerosis in *Mac-Rap*<sup>KO</sup> BM transplanted mice was at least partly due to decreased macrophage chemokine production, leading to reduced monocyte recruitment into lesions.

To assess monocyte recruitment, we performed adoptive transfer experiments using classical (Ly6C<sup>hi</sup>) monocytes sorted from BM of *Ldlr*<sup>-/-</sup> mice that had been transplanted with *Rap*<sup>fllox/fllox</sup> BM and fed the WTD for 8 weeks. From the donors, 1 $\times$ 10<sup>6</sup> monocytes were labeled with carboxy-fluorescein succinimidyl ester (CFSE) and injected intravenously into *Ldlr*<sup>-/-</sup> mice that had been transplanted with *Rap*<sup>fllox/fllox</sup> or *Mac-Rap*<sup>KO</sup> BM and fed the WTD for 8 weeks. After 2 days, adoptively transferred classical monocytes were quantified in homogenates of the whole aorta recipient mice (Figure 1G). The recruitment of monocytes was significantly decreased in *Ldlr*<sup>-/-</sup> mice transplanted with *Mac-Rap*<sup>KO</sup> BM compared to

*Rap<sup>flox/flox</sup>*-transplanted controls. In addition, to study whether *Raptor* deficiency intrinsically affected monocyte migration in response to chemokines, we sorted classical (Ly6C<sup>hi</sup>) monocytes from BM of *Rap<sup>flox/flox</sup>* and *Mac-Rap<sup>KO</sup>* mice, labeled these monocytes with CFSE and injected them intravenously into *Ldlr<sup>-/-</sup>* mice that had been on WTD for 8 weeks. Monocyte *Raptor* deficiency also significantly decreased the uptake of monocytes in the whole aorta of *Ldlr<sup>-/-</sup>* recipient mice (Figure 1H and Online Figure IIIC, IIID).

### Chemokine expression induced by mmLDL is regulated by mTORC1

Oxidation or other modifications of LDL are thought to have an important role in atherogenesis.<sup>19</sup> Minimally modified LDL (mmLDL) prepared by incubating LDL with cells expressing 15-lipoxygenase, displays many pro-atherogenic properties.<sup>20</sup> To investigate the mechanism of the regulation of chemokines by mTORC1 in macrophages, we treated BM derived macrophages (BMDM) from *LysmCre*, *Mac-Rap<sup>KO</sup>* and *Rap<sup>flox/flox</sup>* mice with mmLDL. In *LysmCre* and *Rap<sup>flox/flox</sup>* macrophages, mmLDL treatment resulted in induction of *Tnfa*, *Il6*, *Il10*, *Il12p40*, and chemokine gene expression. In *Mac-Rap<sup>KO</sup>* macrophages, there was a similar response of *Il-6*, *Il10*, *Il12p40*, and *Tnfa* genes (Figure 2A). However the expression of *Ccl2*, *Ccl3*, *Ccl6*, *Ccl7*, and *Cxcl2* was reduced by 48%, 41%, 61%, 63%, and 63%, respectively, compared with floxed controls (Figure 2B). An additional control group consisting of macrophages from *LysM-Cre* mice showed no difference in responses compared to the *Rap<sup>flox/flox</sup>* controls. Consistent with these findings, CCL2 levels in the cell culture media were decreased by 50% (Figure 2C).

The findings in *Mac-Rap<sup>KO</sup>* mice suggested that the anti-inflammatory effects of mTORC1 inhibition may be mediated via repression of chemokine production. To further test this hypothesis, we isolated BMDM from *Mac-Tsc1<sup>KO</sup>* and *Tsc1<sup>flox/flox</sup>* mice and measured gene expression. mmLDL increased the levels of chemokine expression in macrophages of both genotypes but the effects were significantly more pronounced for *Mac-Tsc1<sup>KO</sup>* than control macrophages (Online Figure IVA). While there were trends toward higher levels of induced *Tnfa* and *Il-6* mRNAs in the *Mac-Tsc1<sup>KO</sup>* mice (Online Figure IVB), these effects were less pronounced than for the chemokine genes. As expected, rapamycin treatment reversed these effects, but not for *Tnfa* and *Il-6* (Online Figure IVA and IVB).

### Mechanism of the regulation of chemokine expression by mTORC1

To further examine the mechanisms mediating the modification of chemokine expression by mTORC1, we explored whether the transcriptional repressor BCL6, which has a key role in regulating macrophage chemokine expression, was involved.<sup>21-24</sup> Barish *et al*<sup>22</sup> employed macrophages from *Bcl6* knockout mice and showed that BCL6 containing promoter complexes constrained inflammatory cytokine and chemokine responses to mmLDL. However, the BCL6 protein and mRNA expression were not changed by macrophage *Raptor* deficiency (Figure 3A and 3C). Interestingly, the sequence of the BCL-6 consensus binding site overlaps that of STAT transcription factor-binding sites,<sup>25, 26</sup> and competition between BCL-6 and STATs likely modulates innate immunological functions.<sup>26</sup> STAT3 is phosphorylated by JAK tyrosine kinases at Tyr705 which transduces the signals of IL6. However, STAT3 also requires phosphorylation on Ser727 to achieve maximal transcriptional activity via formation of stable STAT3-STAT3-DNA complexes.<sup>27, 28</sup>

Several reports suggested that mTORC1 is responsible for STAT3 serine phosphorylation.<sup>29, 30</sup> Here, we found that STAT3 Ser727 phosphorylation in response to mmLDL was reduced in *Mac-Rap*<sup>KO</sup> macrophages (3.2-fold increase in controls, 2.0-fold increase in *Mac-Rap*<sup>KO</sup> macrophages,  $p < 0.05$ ); as expected, S6 phosphorylation was impaired in *Mac-Rap*<sup>KO</sup> macrophages (Figure 3A, 3B). In contrast, the phosphorylation of STAT3 at Tyr705 was similar in the two groups (Figure 3A, 3B). As shown by immunofluorescence confocal microscopy, nuclear phosphoSTAT3 Ser727 staining was increased by mmLDL and this was reduced by *Raptor* deficiency (Online Figure V), which is consistent with that of lesional macrophages (Figure 3C and 3D).

Opposite to *Mac-Rap*<sup>KO</sup> macrophages, the phosphorylation of S6 induced by mmLDL was increased by 2.3-fold in *Mac-Tsc1*<sup>KO</sup> BMDM compared to controls (Online Figure VIA, VIB). Moreover, the phosphorylation of STAT3 at Ser727 was induced 2.2-fold in *Tsc1* knockout BMDM compared to the *Tsc1*<sup>fllox/fllox</sup> group, while Tyr705 was not influenced (Online Figure VIA, VIB). Pre-incubation with rapamycin reversed the induction of both phosphoS6 and phosphoSTAT3 Ser727 (Online Figure VIA, VIB). However, the phosphorylation of STAT3 at Tyr705 was not influenced by TSC1 deficiency or rapamycin treatment (Online Figure VIA, VIB).

### **mTOR activation enhances the competition between STAT3 and BCL-6 on the Ccl2 promoter**

To further investigate the underlying mechanism of the regulation of chemokines by mTORC1, we co-transfected a *Bcl6* expression plasmid and *Ccl2* promoter-reporter plasmid into HEK-293T cells. Compared with the empty vehicle transfection control, the luciferase activity was reduced by *Bcl6* transfection. Co-transfection of wild-type STAT3 (CA-STAT3) reversed the reduction of *Bcl6*, while neither dominant negative (DN-STAT3) nor Ser727 mutated versions of STAT3 (STAT3A) had any effect on the decreased luciferase activity caused by *Bcl6* (Figure 4A). This suggests that phosphorylation of Ser727, as mediated by mTORC1, is essential for the ability of STAT3 to reverse the inhibitory effect of BCL-6 on the chemokine promoter. We next co-transfected RAW macrophages with the *Ccl2* promoter plasmid and either control or *Bcl6* plasmid. Luciferase activity induced by IL6 was partly reversed by *Bcl6* expression (Online Figure VIIA). Notably, quantitative chromatin-immunoprecipitation (ChIP) assays showed impaired enrichment of STAT3 protein at the *Ccl2* promoter in *Mac-Rap*<sup>KO</sup> BMDM compared with control BMDM in response to mmLDL treatment (Figure 4B). *Cyclin D2* (CCND2) and *Gapdh* were performed as positive and negative control, respectively (Online Figure VIIB). Together, these experiments suggest that the phosphorylation of Ser 727 of STAT3 enhances its ability to counteract the effect of BCL-6 at the *Ccl2* promoter.

### **The role of mTORC1 and IL6 in the induction of chemokine gene expression by mmLDL**

STAT3 plays a critical role in the IL6 signaling pathway. To further confirm the role of mTORC1/STAT3 and investigate the effect of IL6 in mmLDL-induced chemokine production, we employed IL6 and its neutralizing antibody. When macrophages were incubated with mmLDL in the presence of the IL6 neutralizing antibody, there was no induction of chemokine gene expression, indicating a key role of mmLDL induced IL-6



expression in chemokine production (Figure 5A, Online Figure VIIIA). Next, we asked if the effects of IL6 on chemokine gene expression could be influenced by mTORC1 deficiency. As expected, phosphorylation of STAT3 at both Ser727 and Tyr705 was induced by IL6 treatment. Different from intact phospho-STAT3Tyr705, STAT3Ser727 was reduced by Raptor deficiency (Online Figure VIIIB and VIIC). Furthermore, unlike in control macrophages, IL-6 did not induce chemokine gene expression in *Mac-Rap<sup>KO</sup>* cells (Figure 5B, Online Figure VIID).

Since chemokines produced by macrophages in lesions are thought to promote monocyte-macrophage recruitment to lesions, we assessed the function of our findings on chemokine expression in a macrophage migration assay. In response to mmLDL treatment, 1.6-fold more macrophages were attracted by *Rap<sup>flox/flox</sup>* BMDMs in a transwell macrophage migration assay compared to *Mac-Rap<sup>KO</sup>* BMDMs (Figure 5C and Online Figure VIIIE). Consistent with our *in vivo* study (Figure 1H), *Raptor* deficiency intrinsically reduced macrophage migration to CCL2/MCP-1 (Online Figure VIIF). Pre-incubation with the IL6 antibody substantially reduced macrophage migration in control BMDMs, and to a lesser extent in *Mac-Rap<sup>KO</sup>* macrophages (Figure 5C, 5D), consistent with the idea that mmLDL induced IL6, which in turn led to increased chemokine secretion in an mTORC1 dependent fashion.

## Discussion

The mTOR inhibitor, rapamycin, has potent immunosuppressive effects and has been widely used to inhibit transplant rejection. Rapamycin blocks cell cycle progression and migration of T lymphocytes and thus inhibits acquired immune responses.<sup>31</sup> Rapamycin has also been found to influence innate immune responses with suppression of chemokine production and variable effects on inflammatory gene expression.<sup>32, 33</sup> In humans and experimental animals, rapamycin suppresses transplant vasculopathy, a specialized form of concentric atherosclerosis with prominent T-cell involvement.<sup>7, 8</sup> There is also evidence that rapamycin decreases atherosclerosis in *ApoE<sup>-/-</sup>* mice,<sup>8</sup> despite having adverse effects on VLDL/LDL cholesterol levels. We found prominent induction of mTORC1 activity in macrophages isolated from WTD-fed mice, suggesting an effect of over-nutrition to increase mTORC1 activity similar to what occurs in the liver.<sup>34</sup> We then used a cell-specific knockout approach to demonstrate a regulatory role for macrophage mTORC1 in chemokine gene expression and atherogenesis. mTORC1 was found to amplify the effect of IL6/STAT3 on *Ccl2* gene expression, uncovering a novel cross-talk between inflammatory signaling pathways induced by mmLDL and mTORC1 activation produced by over-nutrition, in macrophages of atherosclerotic lesions (Online Figure IX).

While many cellular factors could potentially contribute to the decreased macrophage content in atherosclerotic lesions of *MacRap<sup>KO</sup>* mice, including macrophage autophagy, proliferation or apoptosis, our studies suggested a predominant role of reduced lesional macrophage chemokine expression and monocyte recruitment into lesions. Studies using *Ccr2<sup>-/-</sup>* BM have suggested a predominant role of CCL2 in promoting monocyte emergence from BM;<sup>35</sup> however, other studies in which lesional *Ccl2/Mcp-1* expression is altered indicate that the gradient of CCL2 between lesion and blood may also play a role in



monocyte recruitment into lesions,<sup>36</sup> consistent with our observations. In addition to *Ccl2*, *Ccl3/Mip1a* was reduced in *MacRap<sup>KO</sup>* macrophages; CCL3 receptors CCR1 and CCR5 may also contribute to the recruitment of classical monocytes into atherosclerotic lesions.<sup>16</sup> Moreover, studies have shown that not only the expression of chemokines by an inflammation site but also by monocytes themselves has a role in the recruitment and migration of monocytes suggesting autocrine or paracrine effects of chemokines.<sup>37-40</sup> This may explain the reduced recruitment of CFSE labeled monocytes obtained from *MacRap<sup>KO</sup>* donors versus *Rap<sup>fllox/fllox</sup>* controls into the aortas of WTD fed *Ldlr<sup>-/-</sup>* mice. Our studies suggest a dual defect involving both decreased chemokine production by plaque macrophages, as well as a cell intrinsic effect of raptor deficiency in migrating monocyte/macrophages, contributing to decreased monocyte/macrophage migration into plaques.

As reported<sup>41, 42</sup> treatment of macrophages with mmLDL induced expression of several different chemokine genes, *Ccl2*, *Ccl3* and *Ccl7*, which have all been implicated in atherogenesis.<sup>43,44, 45</sup> In this study, we used a mildly oxidized form of LDL to study the mechanisms underlying the suppression of chemokine expression in *Mac-Rap<sup>KO</sup>* macrophages. As shown in this study, mmLDL was found to induce *Il6* gene expression and blocking antibodies revealed that the IL6 signaling pathway was required for induction of chemokines by mmLDL. As expected, IL6 treatment led to phosphorylation of STAT3 on Tyr705. This response was not affected by *Raptor* deficiency. Rather a second site, Ser727, was dependent on *Raptor* deficiency. Previous studies have shown that phosphorylation of Serine 727 on STAT3 is required for full transcriptional responses.<sup>27, 28, 46</sup> Although IL6 production induced by mmLDL was intact in *Raptor*-deficient macrophages, IL6 signaling was decreased due to the reduced phosphorylation of its downstream target STAT3 at Ser727 which resulted in decreased mmLDL induced chemokine expression. Thus, the impact of macrophage mTORC1 activity was to amplify the effect of mmLDL/IL-6 on chemokine gene expression. As shown in FigS10, the binding of STAT3 to the chemokine promoter, exemplified by *Ccl2*, led to displacement of the transcriptional co-repressor BCL-6, leading to induction of chemokine gene expression. These findings are consistent with previous studies demonstrating a key role of BCL-6 in macrophage chemokine expression and atherosclerosis<sup>23, 47</sup> and in mediating the effects of mmLDL on chemokine gene expression.<sup>22</sup> Our studies show an important interface between mTORC1 signaling and the mmLDL/IL-6/STAT3 signaling axis, mediated by combinatorial STAT3 signaling and leading to an amplification of pro-atherogenic macrophage chemokine gene expression.

In this study, we have used mmLDL and focused on the modulation of IL6 signaling pathway induced chemokines secretion by mTORC1 signaling. There is increasing evidence for the involvement of IL6 in human atherosclerosis. Inflammatory markers such as C-reactive protein (CRP) have an independent predictive value for CHD incidence, and IL6 is a key inflammatory cytokine promoting CRP production.<sup>48, 49</sup> Recent human genome wide association studies (GWAS) have shown that single nucleotide polymorphisms (SNPs) in the *Il6 receptor* gene are associated with coronary artery disease (CAD),<sup>50</sup> indicating the relevance of the IL6 signaling pathway in human disease. These data suggest that our studies conducted in a murine atherosclerosis model, and with human mmLDL in cell culture, could potentially have human relevance and suggest an anti-atherogenic role of mTORC1

inhibition in macrophages. mTOR inhibition has potential widespread applications in the treatment of human disease. In addition to transplant rejection, there may be beneficial effects on aging and cancer.<sup>51, 52</sup> However, whole body mTORC1 inhibition may have adverse effects such as increased LDL levels.<sup>9</sup> Our study suggests a potential role of targeted mTORC1 inhibition in macrophages in the treatment of atherosclerosis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Nonstandard Abbreviations and Acronyms

<b>mTORC1</b>	Mammalian target of rapamycin complex 1
<b>TSC1</b>	tuberous sclerosis complex 1
<b>IL6</b>	Interleukin 6
<b>CCL2</b>	Chemokine (C-C motif) ligand 2
<b>TNF<math>\alpha</math></b>	Tumor necrosis factor $\alpha$
<b>p70S6k</b>	p70 S6 kinase
<b>STAT3</b>	Signal transducer and activator of transcription
<b>Bcl6</b>	B cell lymphoma 6

<b>WTD</b>	Western type diet
<b>LDLR</b>	Low density lipoprotein receptor
<b>mmLDL</b>	Minimally modified LDL
<b>ox-LDL</b>	Oxidized LDL
<b>BMDM</b>	Bone marrow derived macrophage
<b>CAD</b>	coronary artery disease
<b>CFSE</b>	carboxy-fluorescein succinimidyl ester
<b>4E-BP1</b>	eukaryotic initiation factor 4E-binding protein 1
<b>Raptor</b>	regulatory associated protein of mTOR
<b>BM</b>	Bone marrow
<b>phosphoS6</b>	phospho-S6 ribosomal protein
<b>CRP</b>	C-reactive protein
<b>GWAS</b>	genome wide association studies
<b>SNPs</b>	single nucleotide polymorphisms



## Novelty and Significance

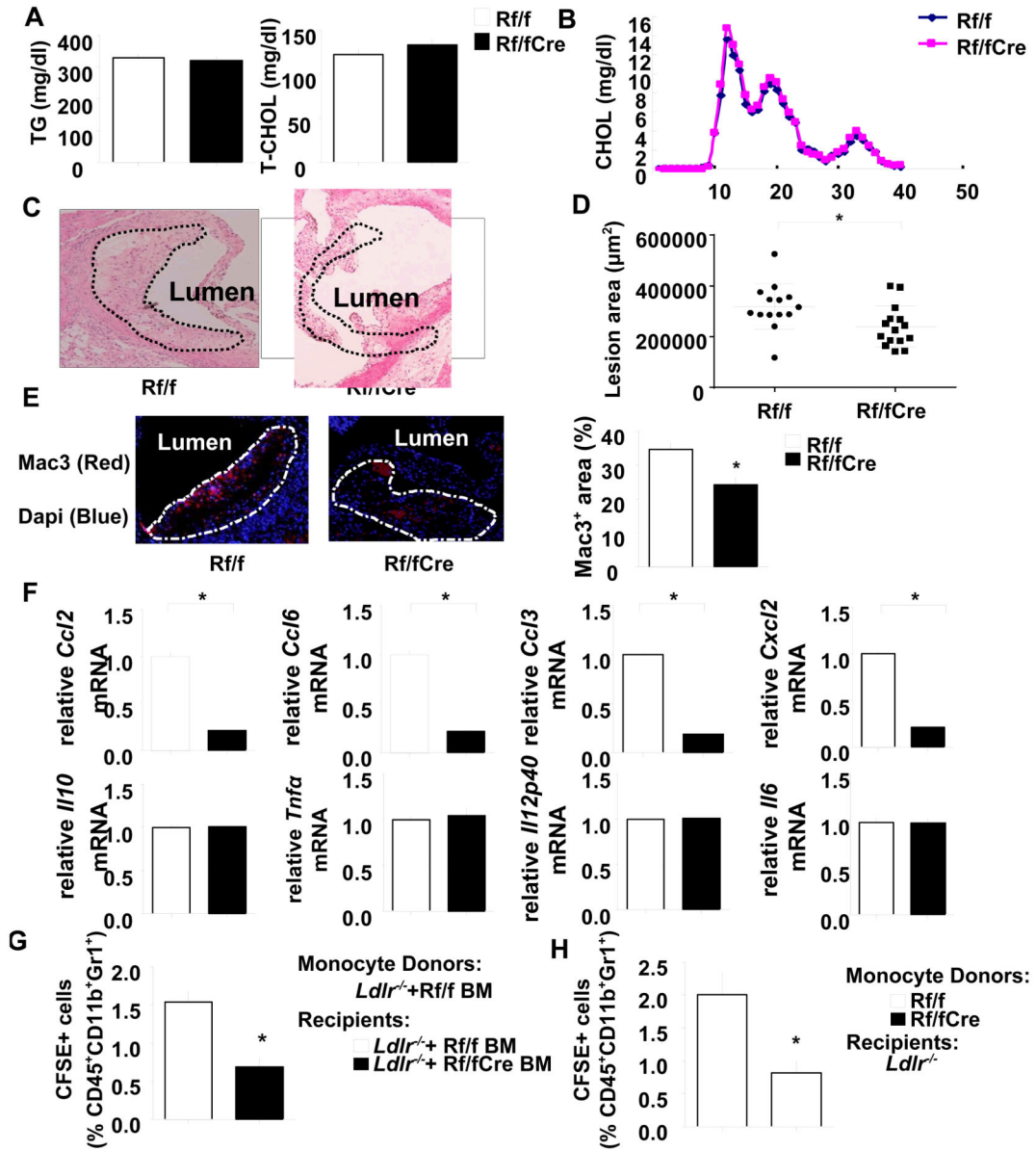
### What is Known?

- Global administration of the mTORC1 inhibitor, rapamycin, decreases atherosclerosis in mice.
- The anti-inflammatory properties of rapamycin could be involved in its athero-protective effects, but direct evidence is lacking.

### What New Information Does This Article Contribute?

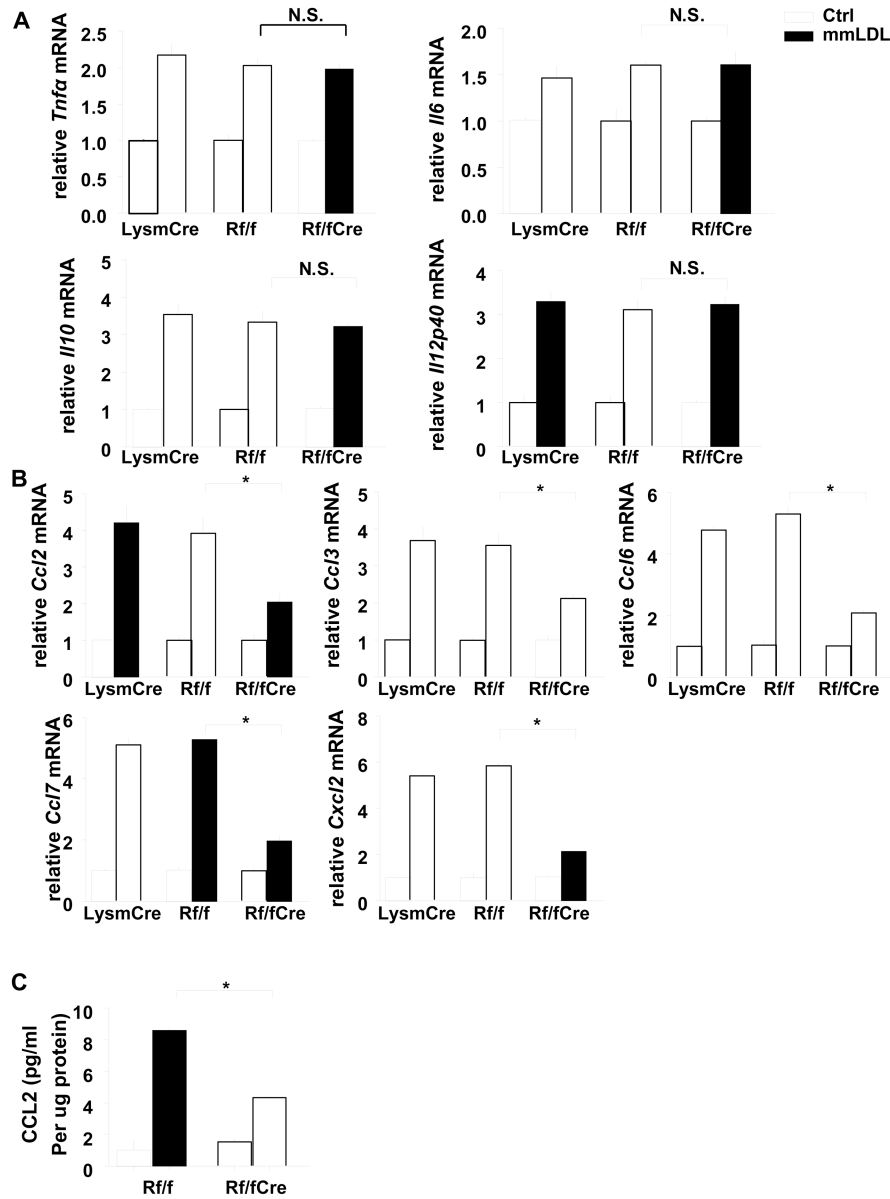
- Deletion of the *Raptor* gene in macrophages decreases mTOR activity, chemokine gene expression in plaques, and attenuates atherosclerosis.
- The induction of CCL2 expression by minimally modified LDL was decreased in low mTORC1 activity macrophages due to lower STAT3 phosphorylation at Serine727, as well as increased binding with the transcription repressor Bcl6.
- These findings suggest the presence of pro-atherogenic cross talk between nutritional and inflammatory signaling pathways in macrophages.

The mammalian target of rapamycin complex 1 (mTORC1) inhibitor rapamycin has been shown to decrease atherosclerosis, even while increasing plasma LDL levels. This finding suggests that inhibition of mTORC1 exerts an anti-atherogenic effect mediated by modulation of the inflammatory response in atherosclerotic plaques independent of plasma LDL cholesterol levels. We evaluated this hypothesis by investigating the contribution of macrophage mTORC1 to the development of atherosclerosis in mice. We found that macrophage *Raptor* deficiency decreased atherosclerosis, macrophage accumulation, and chemokine gene expression in atherosclerotic lesions independent of plasma lipid profile. In macrophages lacking both *Raptor* and *Tsc1*, the expression of chemokine genes induced by mmLDL and IL6 was modulated by mTORC1 activity. Activation of mTORC1 activity led to an increase in STAT3 phosphorylation at Ser727 site and repressed binding of BCL6 on *Ccl2* promoter. These results provide direct evidence showing that the activation of macrophage mTORC1 by Western diet is pro-atherogenic in mice. Thus, targeted inhibition of mTORC1 in macrophages may be useful in the treatment of atherosclerosis.

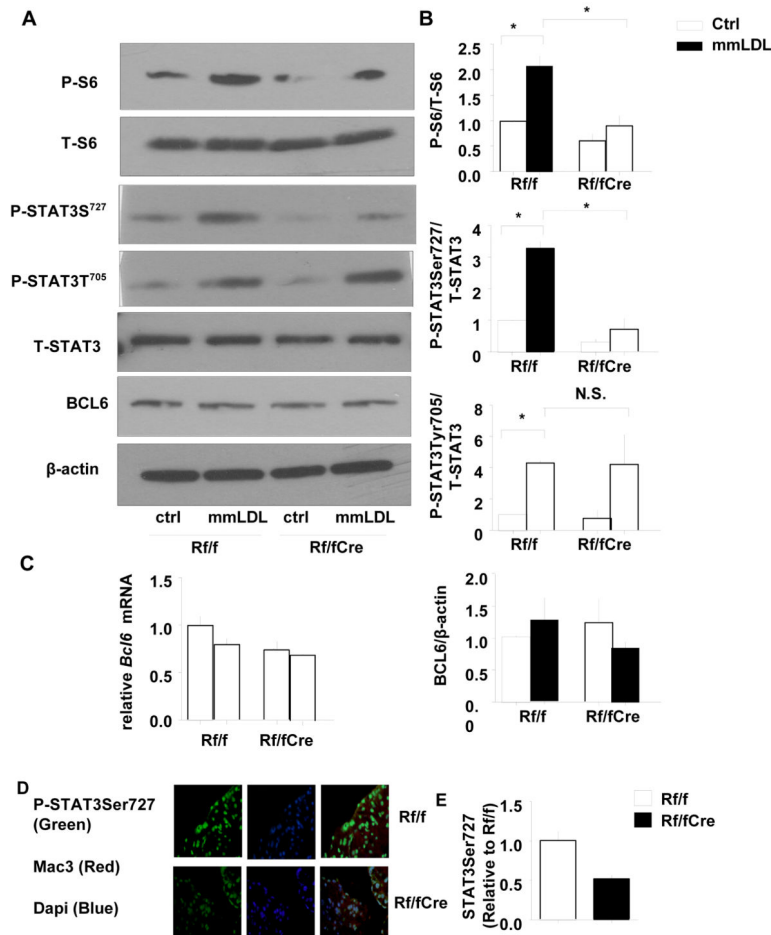


**Figure 1. Plasma lipid levels and atherosclerotic lesions in the aortic root of *Ldlr*<sup>-/-</sup> mice transplanted with *Raptor*<sup>flox/flox</sup> or *Mac-Rap*<sup>KO</sup> BM**  
Mice were fed WTD for 10 weeks. **A.** Plasma triglycerides and total cholesterol levels were measured in *Ldlr*<sup>-/-</sup> mice with *Raptor*<sup>flox/flox</sup> (*Rf/f*) or *Mac-Rap*<sup>KO</sup> (*Rf/fCre*) BM transplantation. N=5-8. **B.** Cholesterol lipoprotein distribution as determined by fast performance liquid chromatography on pooled plasma samples. N=5-8 per pool. **C.** H&E staining of representative aortic root sections. Atherosclerotic lesions are demarcated by the dashed lines. N=14-15. **D.** Quantification of lesions by morphometric analysis. **E.** Sections were stained with a Mac3 antibody for the presence of macrophages (left), and values represent the percentages of Mac3<sup>+</sup> area/total lesion area (right). N=5. **F.** mRNA levels of the indicated targets were assayed in LCM-captured RNA obtained from atherosclerotic lesions. The data were normalized to the expression level of  $\beta$ -actin mRNA. N=5-7. \*P<0.05. **(G)** Classical monocytes sorted from *Ldlr*<sup>-/-</sup> mice transplanted with *Rap*<sup>flox/flox</sup> BM

were labeled with CFSE, and injected intravenously into *Ldlr*<sup>-/-</sup> mice that had been transplanted with BM from either *Rap*<sup>flox/flox</sup> or *Mac-Rap*<sup>KO</sup> mice ; (H) monocytes from *Rap*<sup>flox/flox</sup> or *Mac-Rap*<sup>KO</sup> mice were transplanted into *Ldlr*<sup>-/-</sup> mice. CFSE labeled cells (as a percentage of CD45<sup>+</sup>CD11b<sup>+</sup>Gr1<sup>+</sup> cells) in the aorta were quantified by flow cytometry. N=5. \*P < 0.05.

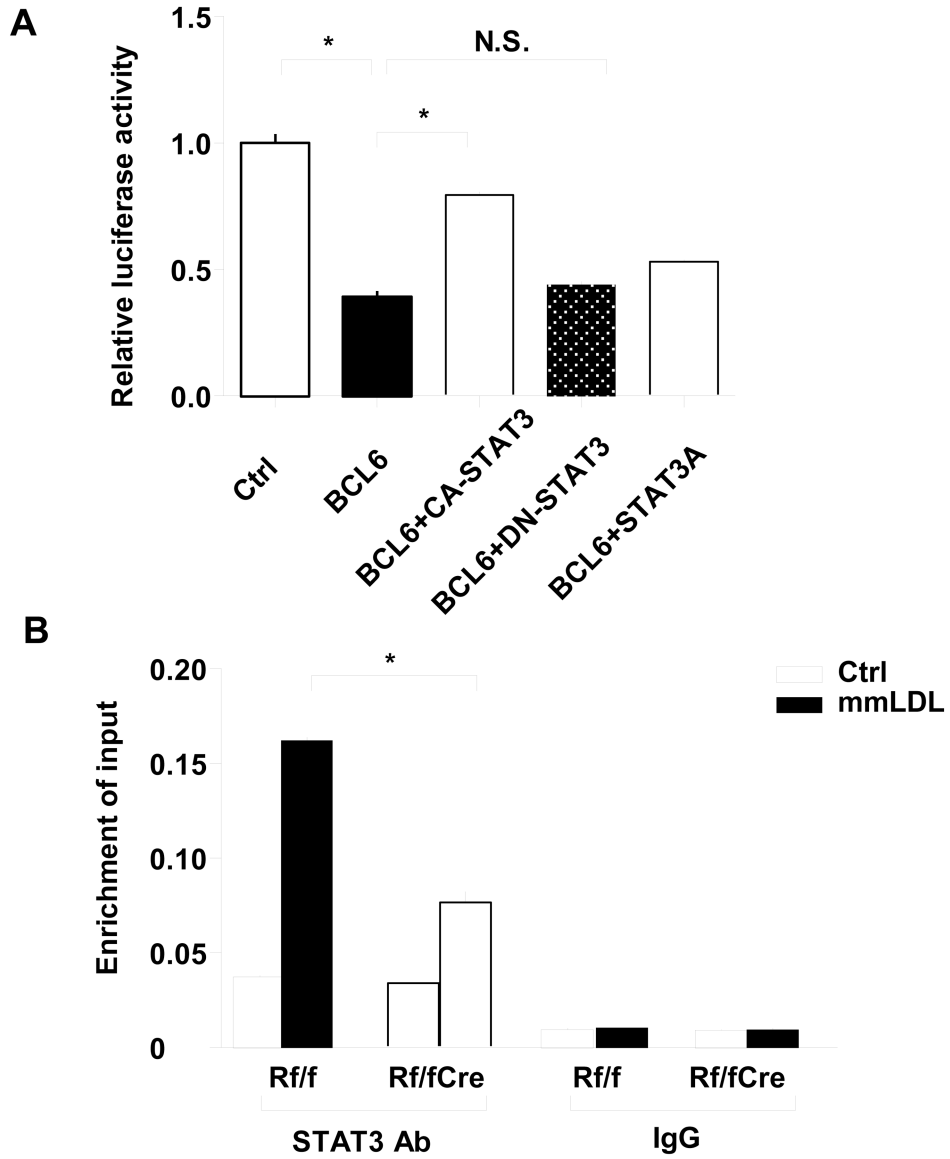


**Figure 2. mmLDL mediated inflammatory gene expression in bone marrow derived macrophages (BMDMs) from *LysmCre*, *Raptor<sup>fllox/fllox</sup>* and *Mac-Rap<sup>KO</sup>* mice**  
**A.** BMDMs from *LysmCre*, *Raptor<sup>fllox/fllox</sup>* (*Rff/f*) and *Mac-Rap<sup>KO</sup>* mice (*Rff/fCre*) were treated with mmLDL (50ug/ml) for 2 hours. Expression levels of *Tnfa*, *Il6*, *Il10* and *Il12p40* were measured by quantitative -PCR and normalized to expression levels observed without treatment (Ctrl). **B.** *Ccl2*, *Ccl3*, *Ccl6*, *Ccl7* and *Cxcl2* gene expression in BMDMs of *LysmCre*, *Rff/f* and *Rff/fCre* mice after 2 hours of mmLDL (50 µg/ml) treatment. N=3, \*P<0.05 **C.** BMDMs were treated with mmLDL for 2 hours, washed with PBS and then incubated with fresh media for another 18 hours. CCL2 in the media was measured by ELISA and values were normalized to total cellular protein. N=3, \*P<0.05, N.S., not significant



**Figure 3. Regulation of STAT3 phosphorylation by *Raptor* deficiency in BMDMs**

**A.** Western blot analysis of S6 and STAT3 phosphorylation and BCL6 expression.  $\beta$ -Actin was used as internal control. P-, phosphorylated; T-, total **B.** Quantification of protein levels  $N=3$ ,  $*P < 0.05$ . Rf/f vs. Rf/f+mmLDL, Rf/f+mmLDL vs. Rf/fCre+mmLDL, one-way ANOVA, Bonferroni post-test. N.S., not significant **C.** Measurement of macrophage *Bcl6* mRNA by qPCR.  $N=3$  **D.** Sections of the aortic root were double-stained with Mac3 and phospho-STAT3Ser727 antibodies. **E.** Relative fluorescence intensity for phospho-STAT3Ser727 expression (green) in the macrophage-dense areas (red) of the lesions is shown. Quantification of phospho-STAT3Ser727 in lesion macrophage area normalized to the value of Rf/f.  $N=5$ .  $*P < 0.05$ .

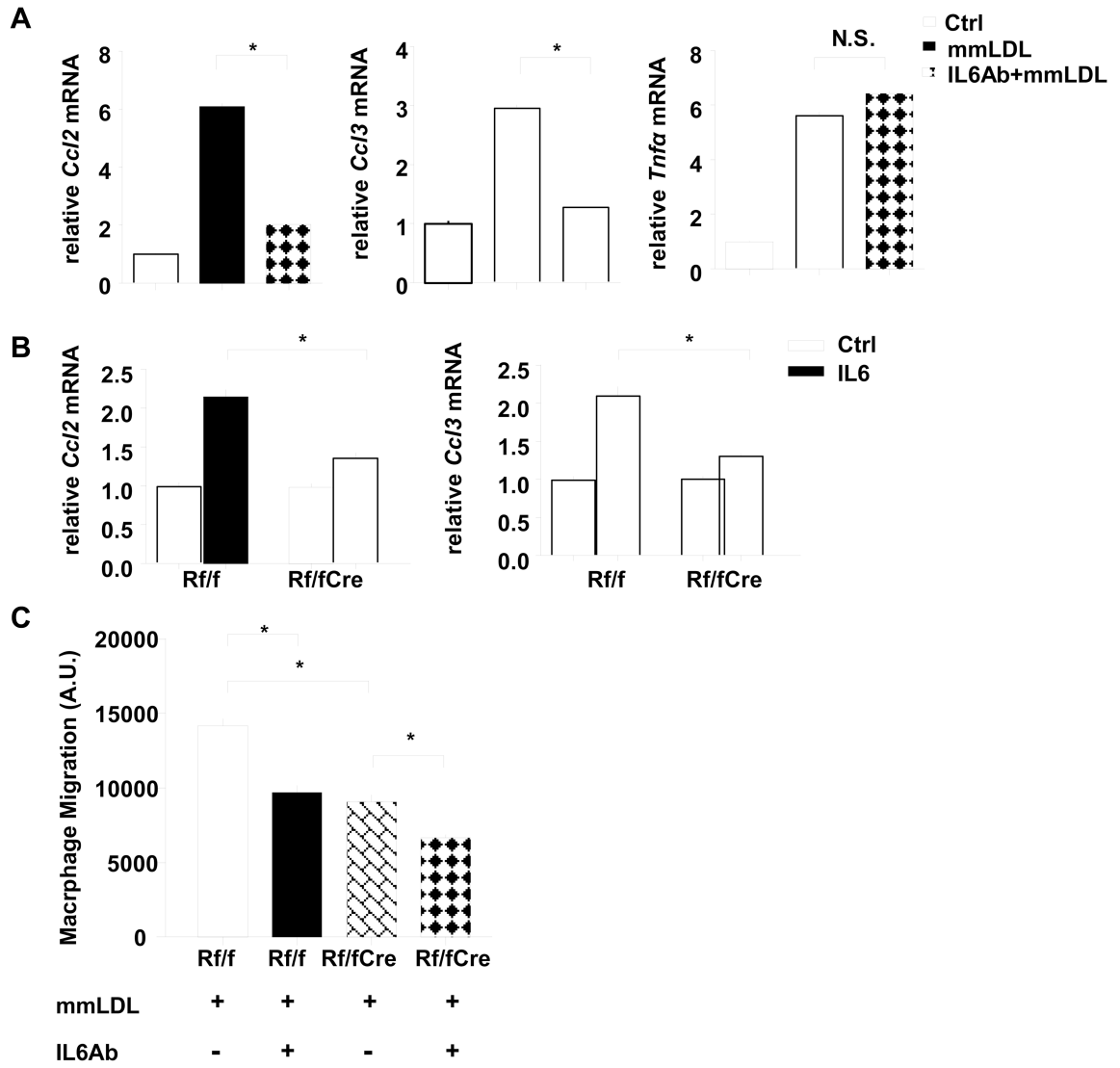


**Figure 4. Mechanism of CCL2 regulation by Raptor**

**A.** *Ccl2* promoter inserted into a reporter system was cotransfected with CA-STAT3, DN-STAT3 or STAT3A plasmids in the presence or absence of *Bcl6* plasmid in 293 cell lines. An empty vectors were used as normalization control. N=3, \*P < 0.05. NS, not significant

**B.** ChIP-qPCR analysis of STAT3 binding on the *Ccl2* genomic locus in BMDMs from *Rf/f* or *Rf/fCre* mice with or without mmLDL treatment using STAT3 antibody or rabbit IgG. The fold-enrichment of the *Ccl2* locus was determined by qPCR and calculated as percentage of input. Data are from three independent experiments. N=3, \*P < 0.05.





**Figure 5. Chemokine expression and migratory response induced by mmLDL was blocked by IL6 neutralizing antibody in BMDMs from wild type mice**

**A.** Expression levels of chemokines and *Tnfa* were measured by quantitative RT-PCR in wild type BMDMs 2 hours after mmLDL (50 µg/ml) treatment with or without a 1 hour rat IgG against IL-6 or isotype control (0.5 µg/ml) pretreatment and normalized to Ctrl. N=3 **B.** BMDMs from *Rf/f* and *Rf/fCre* mice were treated with IL6 (20 ng/ml) for 2 hours. Expression levels of chemokines were measured by quantitative PCR and normalized to expression levels observed without treatment. N=3 **C.** Macrophage migration assay was performed in which BMDMs from *Rf/f* and *Rf/fCre* mice were added to bottom wells and pre-treated with mmLDL (50 µg/ml) and rat IgG against IL-6 or isotype control for 24 hours. The migrated macrophages were quantified by fluorescence spectroscopy. N=3, \*P < 0.05. one-way ANOVA, Bonferroni post-test. N.S., not significant.