SUPPLEMENTAL MATERIAL

Detailed Methods

Reagents

We purchased mouse IL6 from Sigma-Aldrich, CCL2 ELISA kit and IL6 neutralizing antibody from R&D system. mmLDL was produced by incubating human native LDL with fibroblasts overexpressing human 15-lipoxygenase, as described[1]. Possible contamination with endotoxin was tested with a LAL chromogenic kit (Lonza), and only mmLDL preparations with endotoxin levels < 0.25 EU/mg protein were used in the study.

Bone Marrow Transplantation

At 8 weeks of age, *Ldlr*$^{-/-}$ mice were transplanted with *Mac-Rap*^{KO} or *Raptor*$^{flox/flox}$ BM. BM transplantation was performed as previously described [2]. Mice were allowed to recover for 5 weeks after BM transplantation and then fed Western-type diet for 10 weeks. Genomic DNA was isolated from blood by Ultraclean Blood spin DNA isolation kit (Mo-Bio laboratories, CA, USA) 5 weeks after transplantation and used for verification of the efficacy of bone marrow reconstitution. Reconstitution efficacy was determined by qPCR. Primer used for *Ldlr* alleles were forward 5'-GCG ATG GAT ACA CTC ACT GC and reverse 5'-CCA TAT GCA TCC CCA GTC TT. The relative amounts of *Ldlr* were normalized to the copy number of housekeeping gene *β-actin*.

Adoptive Transfer Study

We performed adoptive transfer study as described[3]. Briefly, classical monocytes were isolated from BM by FACS sorting antibodies to CD45-APC-Cy7, CD115-APC, and Ly6C/G-PerCP-Cy5.5 (Gr1) (BD Pharmingen). Classical monocytes sorted from *Ldlr*$^{-/-}$ mice transplanted with *Rap*$^{flox/flox}$ BM or sorted from *Rap*$^{flox/flox}$ or *Mac-Rap*^{KO} mice, all fed WTD for 8 weeks, were labeled with CFSE(Life Technologies), and 10^6 cells were injected intravenously into *Ldlr*$^{-/-}$ mice transplanted with BM from either *Rap*$^{flox/flox}$ or *Mac-Rap*^{KO} mice or into *Ldlr*$^{-/-}$ mice, all fed WTD for 8 weeks. Two days after transfer, aortas of recipient mice were digested with 4U/ml liberase TH (Roche), 40U/ml DNaseI (Sigma) and 60U/ml hyaluronidase (Sigma) in PBS at 37^{o}C for 1 h. A cell suspension was obtained by mashing the aorta through a 70-μm strainer. Cells were incubated with antibodies for 30 min at 4 ^{o}C and washed twice. After washing, immunofluorescence was analyzed for CD45^{+}CD11b^{+}Gr1^{+} CFSE^{+} cells using flow cytometry. CD11b-AlexFluor647A antibody was obtained from BD Pharmingen. CFSE was from Life Technologies.

Plasma Cholesterol, Triglyceride and Lipoprotein Analysis

Blood samples were collected by tail bleeding into heparin-coated tubes. Plasma was separated by centrifugation. To assess lipoprotein cholesterol distribution by fast performance liquid chromatography (FPLC), pooled plasma was injected onto a Superose 6 10/300 GL column (Amersham Biosciences) and eluted at a constant flow rate of 500 µl/min in a buffer containing 100 mM Tris and 0.04% NaN3, pH 7.5. Fractions were assayed for cholesterol using an enzymatic kit from Wako (Cholesterol E), which was also used to

measure total plasma cholesterol. Plasma triglyceride was measured with Infinity kit (Thermo Scientific).

Lesion analysis

After the indicated period of WTD feeding, mice were sacrificed and hearts were isolated and fixed in phosphate-buffered formalin. Hearts were dehydrated, embedded in paraffin, and cross-sectioned through the aortic root. Haematoxylin-eosin and Masson's trichrome staining were performed on the sections and the average from 6 sections for each animal was used to determine lesion size. Lesion size was quantified by morphometric analysis using Image-Pro Plus software (Media Cybernetics, USA).

Oil Red O staining and analysis

After 10 weeks on WTD, mice were sacrificed and the heart was isolated. The aortic root was placed in OCT medium and was immediately frozen. Using a cryomicrotome, sections were cut serially at 8-μm intervals, starting from the aortic sinus, and mounted on slides. Oil red O staining was performed, and images were acquired. Oil red O–positive area was analyzed by applying a color threshold in ImageJ.

Blood and spleen analysis

Mouse peripheral blood was collected by tail vein puncture into heparin-coated tubes. The cells were subjected to RBC lysis and were stained using an antibody cocktail including CD45-APC-Cy7, CD115-APC, and Ly6C/G-PerCP-Cy5.5 (BD Pharmingen). Monocytes were identified as CD45+CD115+ cells and further gated as $Ly6C^{hi}$ or $Ly6C^{lo}$. Following euthanasia, spleens were dissected from mice, homogenized in PBS, and subjected to RBC lysis. The cellular mix was then stained using an antibody cocktail including CD45-APC-Cy7, CD115-APC, F4/80-PECy7, and Ly6C/G-PerCP-Cy5.5 (BD Pharmingen). Specific cell subsets were identified by flow cytometry as described above. Multiparameter analyses were performed using a LSR II flow cytometer (Becton Dickinson) with DiVa software. Data were analyzed using FlowJo software (Tree Star, Inc.).

Macrophage culture

We harvested peritoneal macrophages from mice by peritoneal lavage 3 days after intraperitoneal injection of 3% thioglycollate. The cells were cultured in DMEM supplemented with 10% fetal bovine serum as described before[2]. BM cells were collected from femurs and tibias, pooled, and differentiated *in vitro* by incubating for 7 days at 37°C in DMEM medium containing 10% FBS and 20% L929 cell-conditioned medium. Macrophages were isolated from at least three individual mice for each experiment.

Immunocytochemistry

We performed P-Stat3Ser727 immunofluorescence staining according to the online protocol from Cell Signaling Technology. The anti-P-Stat3Ser727 and anti-rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 488 Conjugate) antibodies were purchased from Cell Signaling Technology.

Immunohistochemistry

The antibody against P62 was purchased from Abcam (MA, USA). The average density (integral optical density/area) was measured with Image-Pro Plus 6.0, and the result was determined as the sum of five different fields/section. Average density was normalized to that of *Ldlr*$^{-/-}$ mice transplanted with *Raptor*$^{flox/flox}$ BM. Mac3 and CD3 antibodies were obtained from BD Bioscience (CA, USA). α-actin-FITC antibody was purchased from Sigma-Aldrich (USA). Ki67 antibody was from Vector Laboratories (MI, USA). In situ cell death detection kit (TUNEL) was purchased from Roche Applied Science (USA). Secondary antibodies were purchased from Life Technologies (NY, USA) and Cell Signaling Technology (USA). The stained sections were viewed using an Olympus IX 70 fluorescence microscope, and the images were analyzed using ImageJ

Transwell migration assay

We harvested BMDMs from *Raptor*$^{flox/flox}$ and *Mac-Rap*^{KO} mice as described above, seeded cells on the bottom of 24-well Transwell chamber plates (Costar), and treated cells with mmLDL (20ug/ml) with or without IL6 antibody for 24 hours on day 7. Peritoneal macrophages from wild type mice were seeded for 2 hours in regular growth media as described above. Subsequently, cells were washed and adherent macrophages were labeled with Calcein-AM (BD bioscience) for 60 min. Then, cells were washed and treated with trypsin (Sigma-Adrich) and reseeded at a density of 0.5 Mio/well on top of 5-μm polycarbonate filter inserts in the Transwell chamber plates with BMDMs and incubated for 2 hours. In CCL2-induced migration experiement, BMDMs from *Raptor*$^{flox/flox}$ and *Mac-Rap*^{KO} mice were added to upper wells, and CCL2 (50ng/ml) was used as chemoattractant for 2 and 24 hours. After this incubation period, the filters were fixed with 4% paraformaldehyde, and cells that had not migrated were removed from the upper surface of the filter by scraping with Q-Tips. The filters were then mounted onto coverslip dishes and observed by confocal microscopy (Axioskop 2 FS OT upright confocal microscope (Zeiss)) or lysed with 100μl T-PER tissue protein extraction reagent (Thermo). Fluorescence was measured at 494/517nm (Abs/Em). We quantified migrated macrophages by fluorescent intensity.

Western blot analysis

Cell lysates were prepared and analysis was performed as previously described[4] . Western blot analysis was carried out using the following primary antibodies: anti-phosphorS6 Ser240/244 (Cell Signaling Technology); anti-total S6 (Cell Signaling Technology); anti-phosho STAT3Ser727 (Cell Signaling Technology), anti-total STAT3 (Cell Signaling Technology), anti-phosphoSTAT3tyr705 (Cell Signaling Technology), anti-BCL6 (Cell Signaling Technology), and anti–β-actin (Sigma-Aldrich). Protein samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad). Blots were probed separately with antibodies as indicated in the figures. After incubation with horseradish peroxidase–conjugated secondary antibodies, proteins were visualized with SuperSignal West Pico Chemiluminescent reagents (Pierce; Thermo Scientific) on X-ray films. When comparing phosphorylated and total protein, the same membrane incubated with phospho-protein antibody was detected by stripping-reprobing with total protein antibody.

Band intensity was quantified using scanning densitometry of the autoradiogram with NIH ImageJ software (http://rsb.info.nih.gov/ij/).

Real-time quantitative PCR analysis

Total RNA was isolated using RNeasy kit (Qiagen) according to the manufacturer's instructions. 2 μg total RNA was reverse transcribed at 50°C with First Strand cDNA Synthesis Kit (Fermentas). Real-time quantitative PCR (QPCR) was performed using the Mx4000 Multiplex Quantitative PCR System with 1 cycle at 95°C for 10 minutes followed by 40 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute. The relative amounts of specific target amplicons for each primer set were estimated by a standard curve method using Mx4000 software (version 3.01; Stratagene) and were normalized to the copy number of housekeeping gene *β-actin*.

Luciferase expression constructs

For transient transfection, plasmid DNA was transfected into RAW cells using the Lipofectamine 2000 method (Invitrogen). RL-TK was cotransfected as a transfection control. WT-STAT3, STAT3Ser727 mutant (STAT3S727A) and DN-STAT3 plasmids were provided by Dr Abdelilah Soussi Gounni (University of Manitoba, Winnipeg, Canada). *Bcl6* and *Ccl2*-luciferase plasmids were purchased from Addgene (Cambridge, MA, USA). After various treatments, cells were lysed and measured by the Dual Luciferase Reporter (DLR) Assay System (Promage).

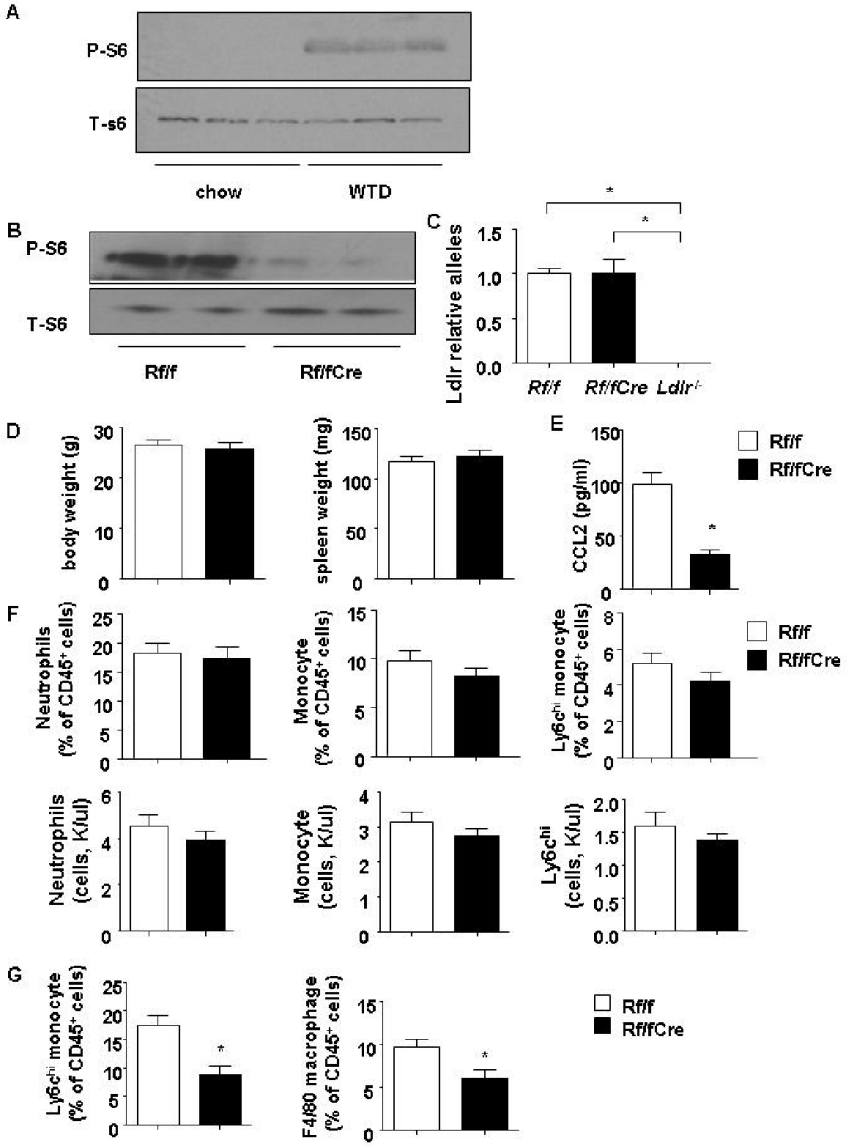
ChIP with quantitative PCR.

ChIP assay was performed with SimpleChIP® Enzymatic Chromatin IP Kits (cell signaling, USA) following its protocol. Briefly, cells were crosslinked with 1% formaldehyde and neutralized with 0.125 M glycine. Cell lysates were digested by micrococcal nucleasesonicated, and proteins were immunoprecipitated with antibody to STAT3 (cell signaling) or rabbit IgG (cell signaling) as a control. After complete washing, immunoprecipitated DNA was eluted in elution buffer and reverse-crosslinked overnight at 65 °C. DNA was purified and quantified by real-time PCR. Enrichment was calculated relative to input. Primers for ChIP-qPCR
Ccl2 Forward: 5-CATATTTTCCTAGAAGTCCCAGAAGC-3, *Ccl2* Reverse: 5-TGGAGGGATGATACAGATTTTTTTTC-3; *Ccnd2* Forward: 5-GAGAGCCAAACCTAAACCCTC-3, *Ccnd2* Reverse: 5-GGCCACATTGATACAGCTTTC-3; *Gapdh* Forward: 5-CTACCCAAAAGGGACACCTACA-3, *Gapdh* Reverse: 5-CATGACAACTTTGGCATTGTG-3.

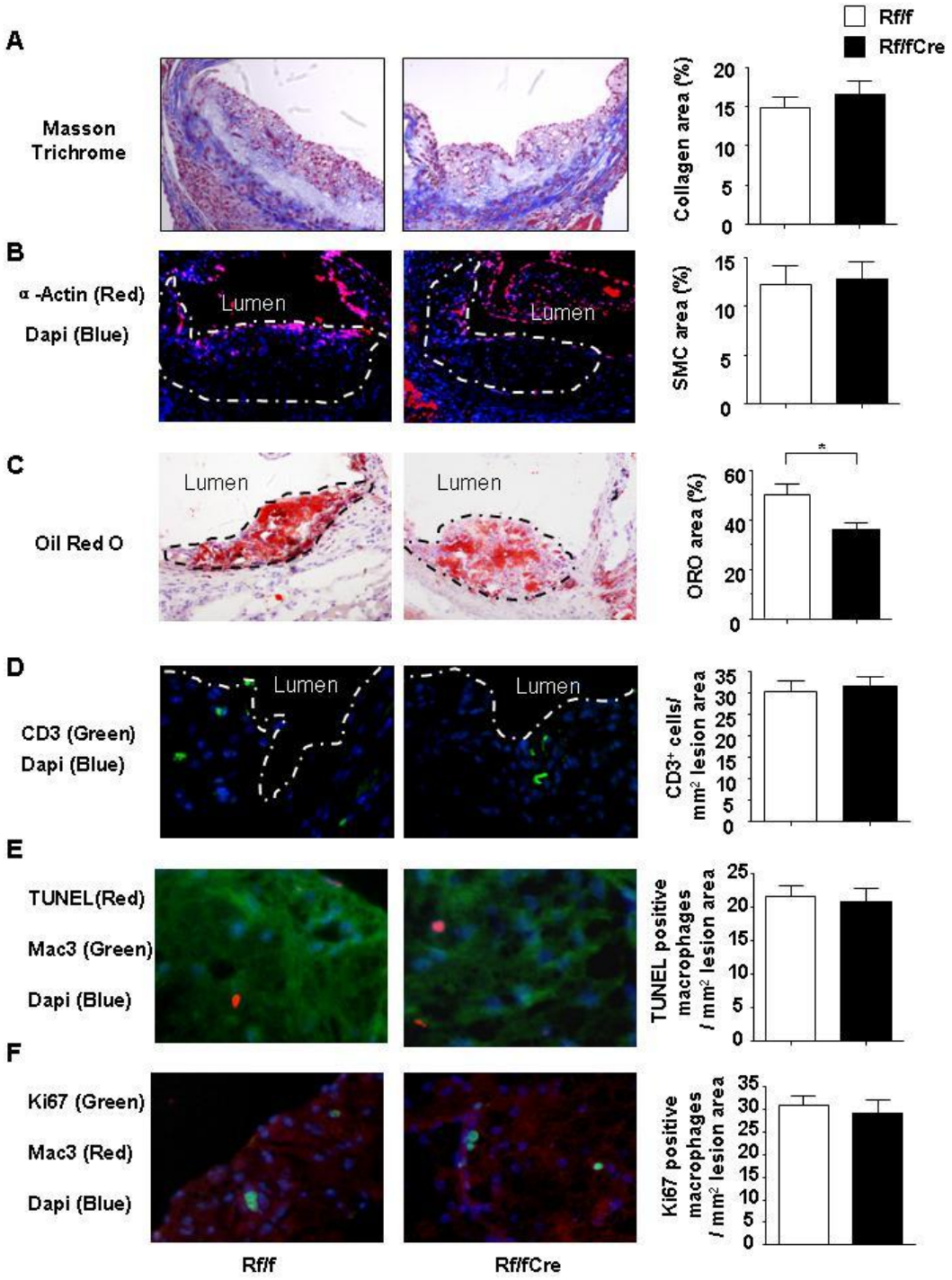
Statistics

All data are presented as means ± SEM. *n* is indicated in the figures and/or legends. The t-test was used to define differences between 2 datasets, except for atherosclerosis lesion area studies where the Mann-Whitney nonparametric test was used. To define differences between multiple datasets, one-way analysis of variance (ANOVA) was used with a Bonferroni multiple comparison post-test. The criterion for significance was set at $P<0.05$. Statistical analyses were performed using GraphPad Prism version 5.01 (San Diego, CA).

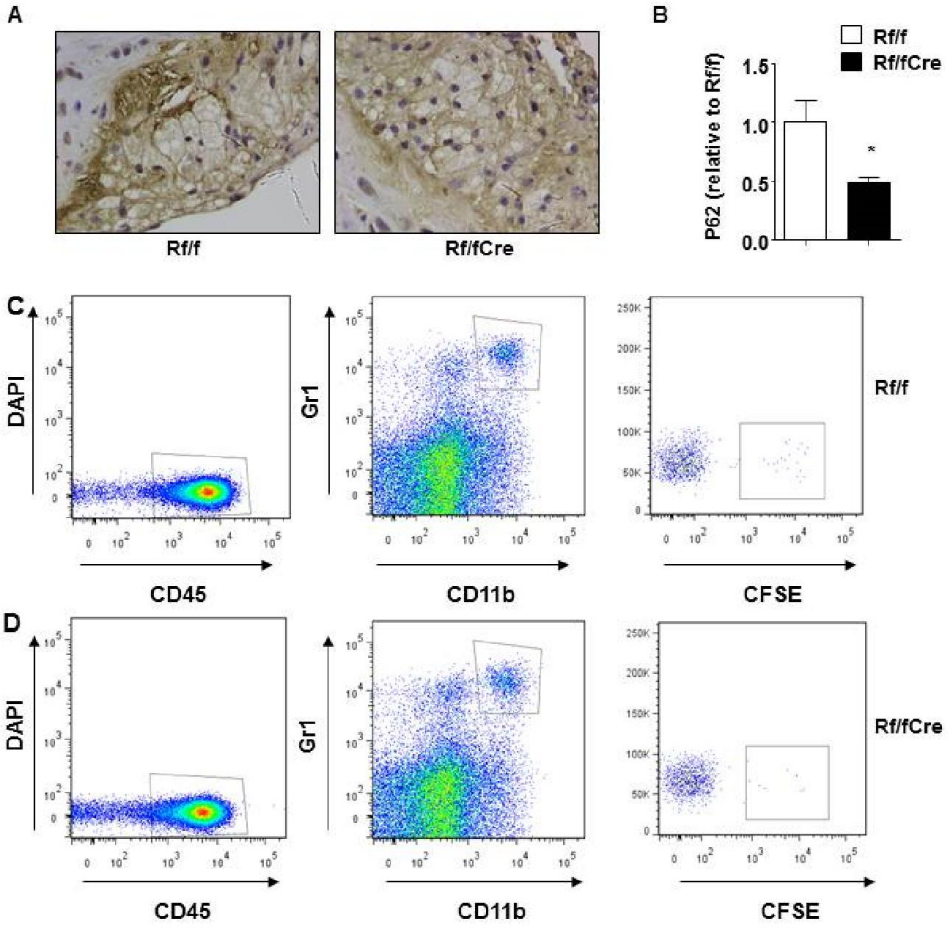
Online Figures



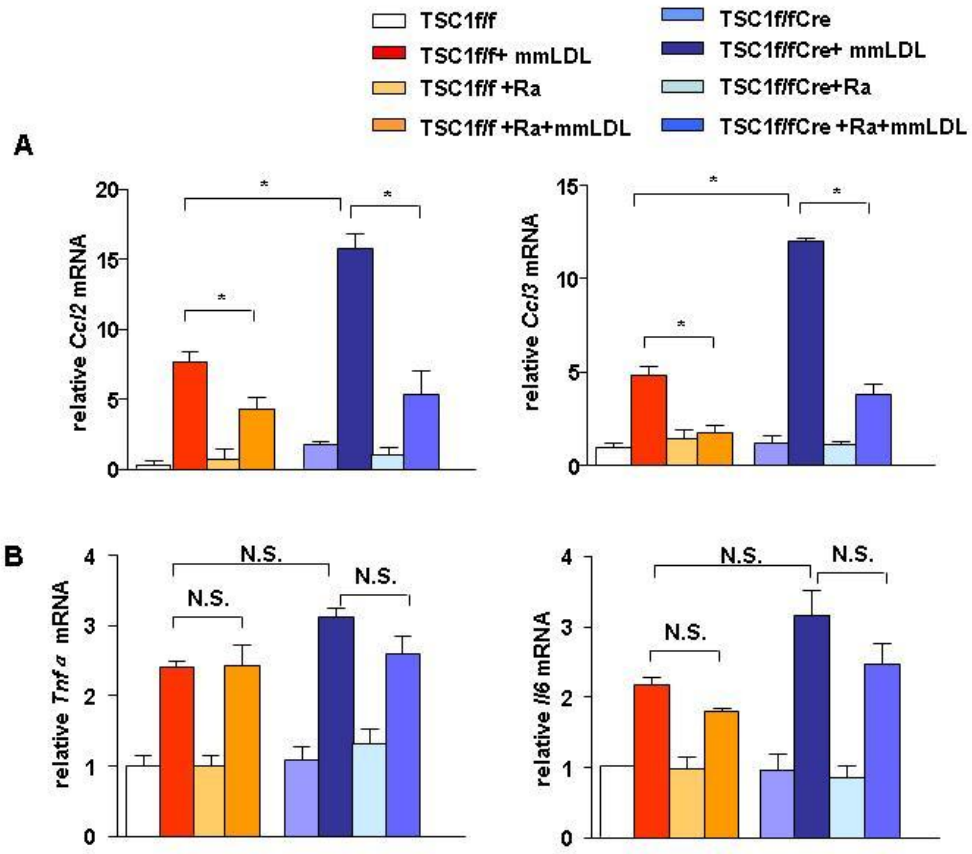
Online Figure I. Plasma CCL2 level was reduced in *Ldlr*$^{-/-}$ mice transplanted with *Mac-Rap*^{KO} bone marrow. A. Peritoneal macrophages were isolated from *Ldlr*$^{-/-}$ mice fed chow diet or WTD for 12 weeks. Phospho-S6 (P-S6) was measured by Western blot. **B.** Peritoneal macrophages were isolated from *Ldlr*$^{-/-}$ mice that were transplanted with *Raptor*$^{flox/flox}$ (*Rf/f*) or *Mac-Rap*^{KO} (*Rf/fCre*) BM and had been fed Western type diet for 10 weeks. P-S6 was measured by Western blot. **C.** Five weeks after BM transplantation, reconstitution of the BM was measured by qPCR with *Ldlr* genotyping primers on genomic DNA extracted from blood. Genomic DNA from *Ldlr*$^{-/-}$ mice was used as a control to assess the reconstitution efficiency. **D.** Body and spleen weights for the animals from **Figure 1**. n=14-15. **E.** Plasma CCL2 levels were measured by ELISA. N=5. **F.** Level of neutrophils, monocytes and Ly6C^{hi} monocytes in the blood of *Ldlr*$^{-/-}$-WT and *Ldlr*$^{-/-}$ *Mac-Rap*^{KO} on WTD for 10 weeks. N=10 **G.** Percentage of Ly6C^{hi} monocytes and F4/80+ macrophages in the spleens of *Ldlr*$^{-/-}$-WT and *Ldlr*$^{-/-}$ *Mac-Rap*^{KO} on WTD for 10 weeks. N=10. *P < 0.05.



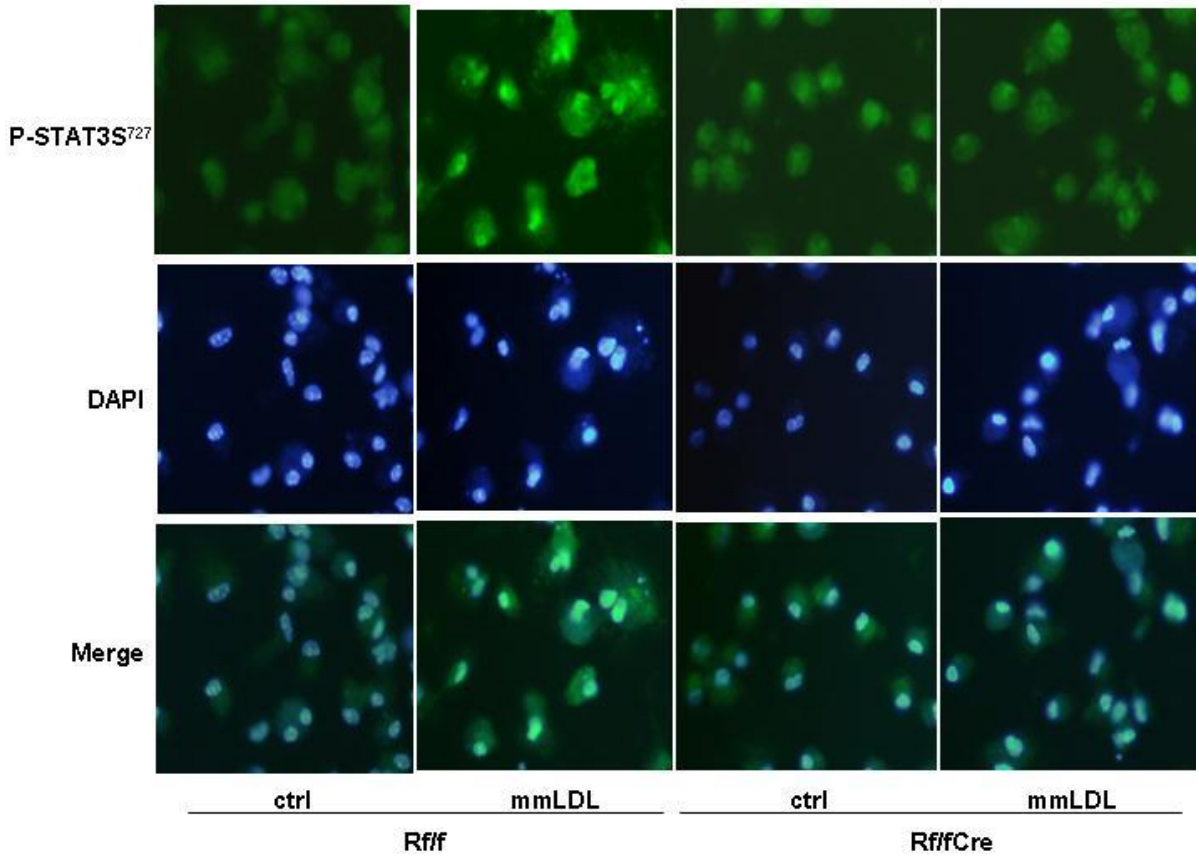
Online Figure II. Lesion characterization for collagen, smooth muscle cells, lipids, T-cells, macrophage apoptosis and macrophage proliferation. A. Sections were stained with Massion's trichrome **B.** Smooth muscle cells were stained by α-actin antibody **C.** Lipids of frozen sections were stained by Oil Red O. **A-C** data given as percentages of total lesion area. **D.** T cells were stained by CD3 antibody and values are given as number of $CD3^+$ cells/mm^2 lesion area. **E, F.** Sections were double-stained by Mac3 antibody and TUNEL reagent (**E**) or Ki67 antibody (**F**). Values represent of $TUNEL^+Mac3^+$cells/mm^2 lesion area or $Ki67^+Mac3^+$cells/mm^2 lesion area. N=5 for all experiments.



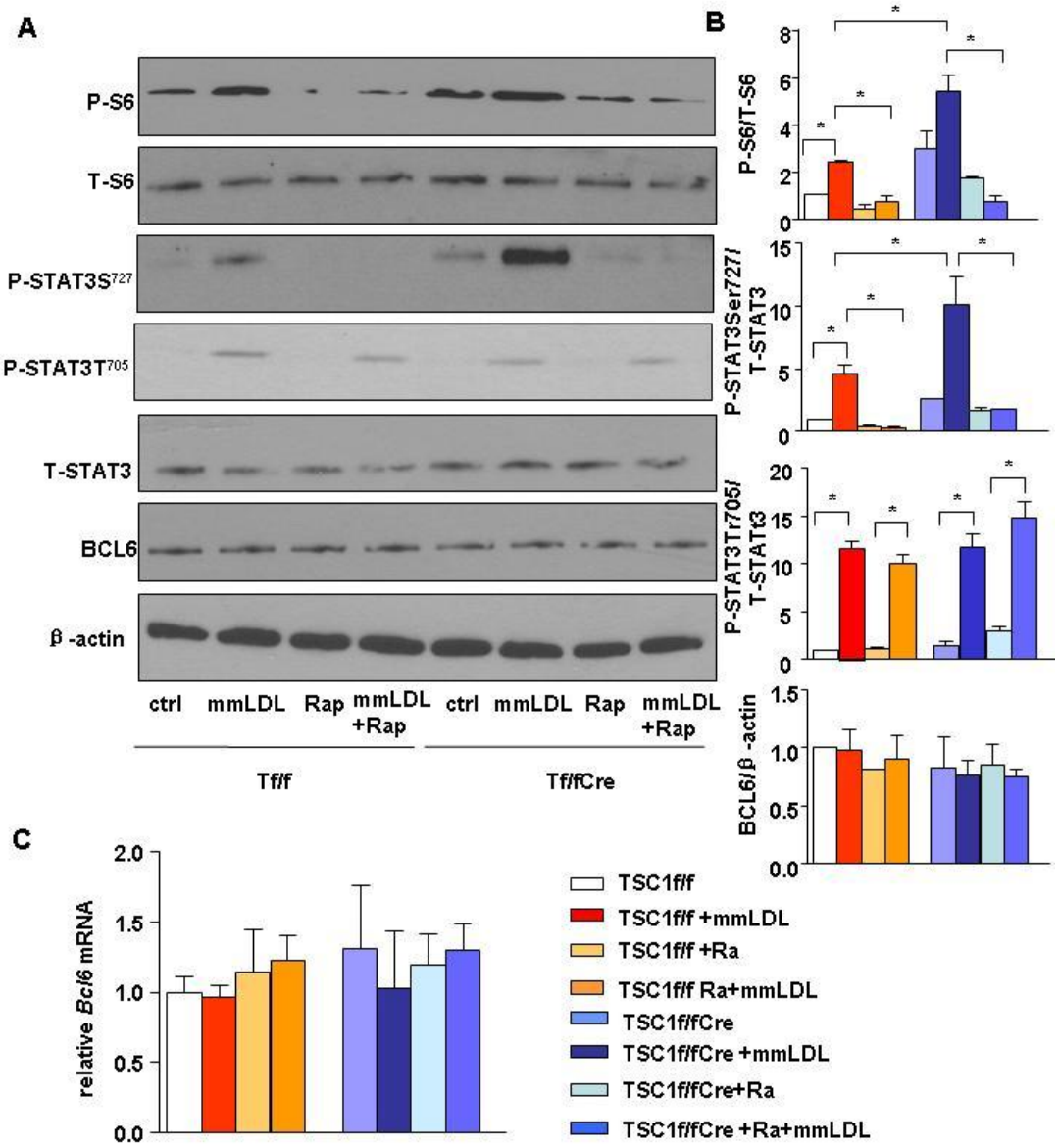
Online Figure III Lesion characterization for P62 and P-STAT3Ser727, and adoptive monocyte transplantation. A. P62 staining on atherosclerotic lesions in the aortic root. **B.** Quantification of P62 in lesion area normalized to the value of Rf/f. **C, D.** Classical monocytes of *Rap*$^{flox/flox}$ (**C**) and *Mac-Rap*^{KO} mice (**D**) were adoptively transferred into *Ldlr*$^{-/-}$ mice. Gating strategy of flow cytometry is shown. N=5



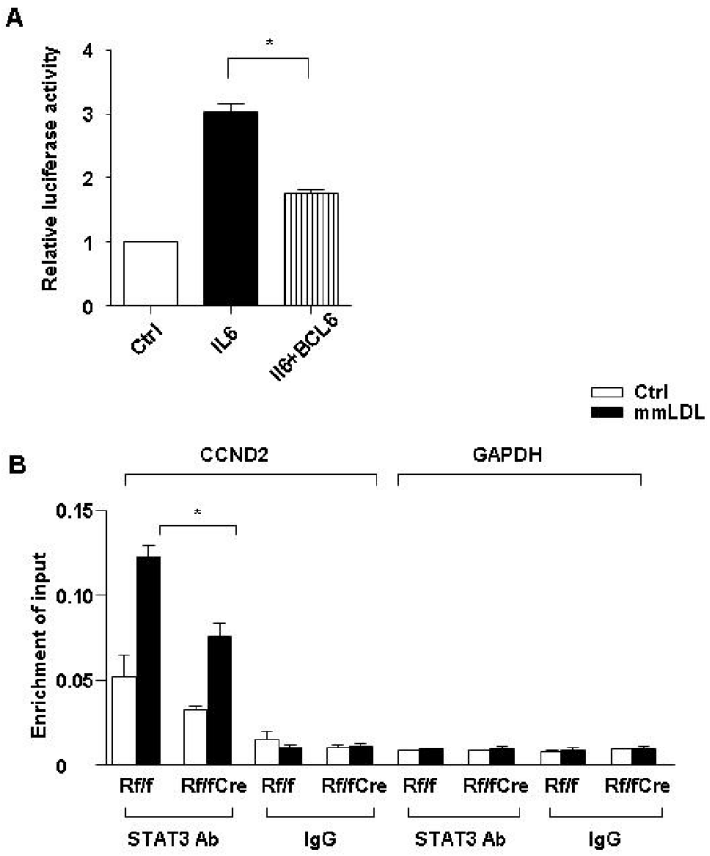
Online Figure IV. mmLDL mediated inflammatory factor production in BMDMs from *Tsc1*^{floxflox} and *Mac-Tsc1*^{KO} mice. A, B, BMDMs from *Tsc1*^{floxflox} (*Tf/f*) and *Mac-Tsc1*^{KO} mice (*Tf/fCre*) were treated with mmLDL (50 μg/ml) for 2 hours with or without rapamycin (100 nM) pretreatment for 30 min. Expression levels of *Ccl2*, *Ccl3, Tnfα* and *Il6* expression were measured by quantitative PCR and normalized to expression levels observed without treatment. *P<0.05. N=3. N.S. not significant.



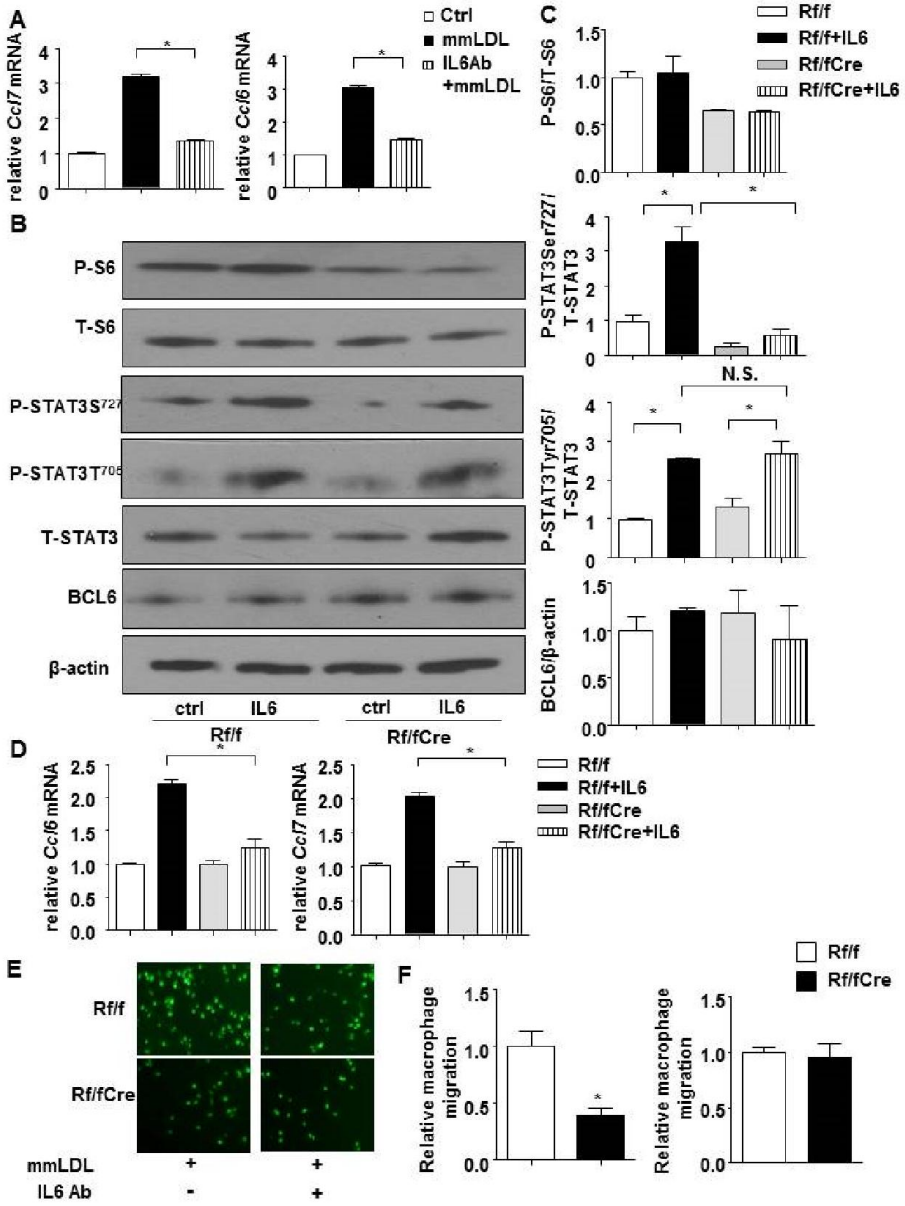
Online Figure V. Raptor deficiency decreased STAT3Ser727 phosphorylation induced by mmLDL. Immunocytochemical analysis of STAT3Ser727 localization in BMDM cells from *Rf/f* and *Rf/fCre* mice 1 hour after mmLDL treatment. Representative images are shown.



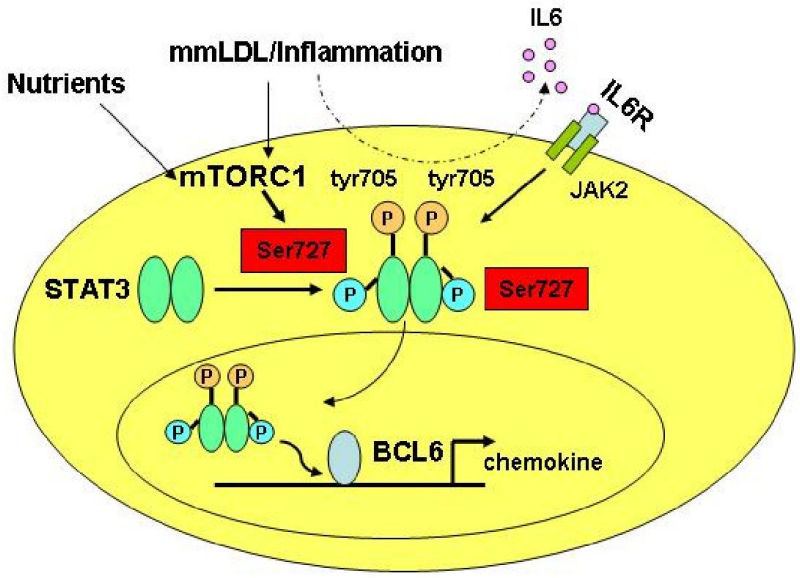
Online Figure VI. mmLDL mediated inflammatory factor production in BMDMs from *Tsc1*^{floxflox} and *Mac-Tsc1*^{KO} mice. A. BMDMs from *Tsc1*^{floxflox} (*Tf/f*) and *Mac-Tsc1*^{KO} mice (*Tf/fCre*) were treated with mmLDL (50 μg/ml) for 2 hours. Western blot analysis of S6 and STAT3 phosphorylation, and BCL6 expression. β-Actin was used as an internal control. P-, phosphorylated; T-, total. **B.** Quantification of protein levels **C.** *Bcl6* gene expression in BMDMs of *Tf/f* and *Tf/fCre* mice 2 hours after mmLDL (50 μg/ml) treatment. *P<0.05. N=3.



Online Figure VII. Mechanism of CCl2 regulation by Raptor. A. A *Ccl2* promoter was co-transfected with control or *Bcl6* plasmids into RAW cells which were harvested 24 hours after IL6 (20ng/ml) or vehicle treatment. N=3 **B.** ChIP-qPCR analysis of STAT3 binding on the *Ccnd2* and *Gapdh* genomic locis in BMDMs from *Rf/f* or *Rf/fCre* mice with or without mmLDL treatment. The fold enrichment of *Ccnd2* (positive control) and *Gapdh* (negative control) locus was determined by qPCR and calculated as percentage of input. Data are from three independent experiments. * P<0.05



Online Figure VIII. Effect of IL-6 on mmLDL induced inflammatory gene expression in *Rf/f* and *Rf/fCre* macrophages. A. Expression levels of *Ccl7* and *Ccl6* were measured by quantitative PCR in wild type BMDMs 2 hours after mmLDL(50 μg/ml) treatment with a 1 hour rat IgG against IL6 or isotype control (0.5 μg/ml) pretreatment and normalized to Ctrl. **B.** Western blot analysis of S6 and STAT3 phosphorylation and BCL6 expression. β-Actin was used as internal control. P-, phosphorylated; T-, total. **C.** Quantification of protein levels. N=3, *P < 0.05. N.S., not significant. **D.** BMDMs from *Rf/f* and *Rf/fCre* mice were treated with IL6 (20ng/ml) for 2 hours. Expression levels of *Ccl7* and *Ccl6* were measured by quantitative PCR and normalized to expression levels observed without treatment. *P<0.05. N=3. **E.** The representative images from Figure 5C are shown. **F.** Macrophage migration assay was performed in which BMDMs from *Rf/f* and *Rf/fCre* mice were added to upper wells, and CCL2 (50ng/ml) was used as chemoattractant for 2 (left) and 24 (right) hours. The migrated macrophages were quantified by fluorescence spectroscopy. Values were normalized to *Rf/f*. N=3.



Online Figure IX. A schematic of the function of mTORC1 in the development of atherosclerosis. Decreased phosphorylation of STAT3 at Ser727 by suppression of mTORC1 reduced STAT3 transcriptional activity and the antagonist effect on repression of chemokine gene expression by BCL6, which attenuated the pro-inflammation effect of IL6 in atherosclerotic environment. As a result, raptor knockout reduced the development atherosclerosis.

References

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