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Chapter 7

RP105 deficiency attenuates early atherosclerosis via decreased monocyte influx in a CCR2 dependent manner

Conditionally accepted by Atherosclerosis

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Abstract

Objective: Toll-like receptor 4 (TLR4) plays a key role in inflammation and previously it was established that TLR4 deficiency attenuates atherosclerosis. Radio-Protective 105 (RP105) is a structural homolog of TLR4 and an important regulator of TLR4 signalling, suggesting that RP105 may also be an important effector in atherosclerosis. We thus aimed to determine the role of RP105 in atherosclerotic lesion development using RP105 deficient mice on an atherosclerotic background.

Methods and Results: Atherosclerosis was induced in Western-type diet fed low density lipoprotein receptor deficient ($LDLr^{-/-}$) and $LDLr/RP105$ double knockout ($LDLr^{-/-}/RP105^{-/-}$) mice by means of perivascular carotid artery collar placement. Lesion size was significantly reduced by 58% in $LDLr^{-/-}/RP105^{-/-}$ mice, and moreover, plaque macrophage content was markedly reduced by 40%. In a model of acute peritonitis, monocyte influx was almost 3-fold reduced in $LDLr^{-/-}/RP105^{-/-}$ mice, while neutrophil influx remained unaltered, suggestive of an altered migratory capacity of monocytes upon deletion of RP105. Interestingly, *in vitro* stimulation of monocytes with LPS induced a downregulation of CCR2, a chemokine receptor crucially involved in monocyte influx to atherosclerotic lesions, which was more pronounced in $LDLr^{-/-}/RP105^{-/-}$ monocytes as compared to $LDLr^{-/-}$ monocytes.

Conclusion: We here show that RP105 deficiency results in reduced early atherosclerotic plaque development with a marked decrease in lesional macrophage content, which may be due to disturbed migration of RP105 deficient monocytes resulting from CCR2 downregulation.

Introduction

Atherosclerotic lesions in the large and medium sized arteries are the major cause of cardiovascular events and complications worldwide¹. It has been well established that inflammation plays a key role in the initiation, growth and rupture of an atherosclerotic plaque²⁻⁵. Early steps in this process involve the adhesion of monocytes to dysfunctional endothelium, where they subsequently migrate into the vessel wall and take up oxidized LDL⁶. Influx of additional inflammatory cells from both the innate and adaptive immune system, such as dendritic cells, T cells and mast cells, further aggravates lesion formation^{7,8}.

Toll-like receptors (TLRs) are the primary receptors of the innate immune system: they recognize highly conserved molecular motifs called pathogen associated molecular patterns (PAMPs) as well as endogenous damage-associated molecular patterns (DAMPs)^{9,10}. TLR4, one of the most characterized receptors of the TLR family, has been subjected to extensive research regarding its role in atherosclerosis. Atherosclerosis-prone mice deficient for TLR4 develop smaller atherosclerotic lesions¹¹, while also antagonism of TLR4 results in reduced early lesion formation¹². RadioProtective 105 (RP105) is a TLR homologue capable of regulating TLR4 signalling. RP105 is structurally similar to TLR4, but lacks the intracellular Toll Interleukin Receptor (TIR) signalling domain. Originally, RP105 was described as a B cell specific molecule, able to drive cellular proliferation and to enhance B cell dependent inflammatory processes^{13,14}. However, additional research revealed that the expression of RP105 on antigen presenting cells directly mirrors that of TLR4, in which it acts as a negative regulator. Consequently, LPS injection in RP105 deficient mice results in an exaggerated inflammatory response¹⁵, and the inhibitory effect of RP105 on TLR4 responses is thought to be exerted via a direct extracellular interaction with TLR4^{16,17}.

Because of the opposite regulatory role that RP105 seems to play in different cell types, it is compelling to study the effect of RP105 deficiency in inflammatory diseases including atherosclerotic vascular remodelling. Previously, we have demonstrated that in a hyperlipidemic setting, lethally irradiated mice receiving RP105^{-/-} bone marrow display an unexpected decrease in plaque size. This reduction in atherosclerosis was mainly explained by decreased B cell activation, proliferation and immunoglobulin production¹⁸. These data would suggest that deficiency of RP105 is a novel route via which atherosclerosis can be inhibited. However, we have also shown that lack of RP105 results in increased neointima formation in a mouse model for damage induced post-interventional vascular remodelling¹⁹. Smooth muscle cells deficient for RP105 display increased proliferation following LPS stimulation, which may explain the increase in neointima formation. These opposing roles of RP105 on smooth muscle cells and B cells make it difficult to conclude on the effect of total body RP105 deficiency on atherosclerosis, since

both cell types are known to contribute to lesion formation. Therefore, it remains to be studied whether the contribution of RP105 mediated smooth muscle cell proliferation or RP105 dependent changes in leukocyte function play a more pre-dominant role in atherosclerosis.

In the current study we thus aim to investigate what the effect of total body RP105 deficiency is on atherosclerosis. Also, we determined how RP105 expression is regulated during the process of atherosclerotic lesion formation.

Material and Methods

Mouse atherosclerosis time course

All animal work was approved by the animal welfare committee of the Leiden University Medical Center and mice were bred in our facility at the Gorlaeus Laboratories (Leiden, the Netherlands). For the generation of a mouse atherosclerosis time course, male $LDLr^{-/-}$ were fed a Western type diet (0.25% cholesterol and 15% cacao butter, SDS, Sussex, UK) starting two weeks before surgery and throughout the experiment. Mice were anaesthetized by subcutaneous injection of ketamine (60 mg/kg, Eurovet Animal Health, Bladel, The Netherlands), fentanyl citrate and fluanisone (1.26 mg/kg and 2 mg/kg respectively, Janssen Animal Health, Sauterton, UK). All mice received perivascular collars around the carotid artery to induce plaque formation as previously described²⁰. In brief, a semi-constrictive collar was placed around both carotid arteries of the mice two weeks after start of the Western type diet. Disturbed flow at the proximal site of the collar results in endothelial activation with subsequent atherosclerotic lesion formation.

Gene expression profiles of carotid artery plaques in $LDLr^{-/-}$ mice were determined at 0 to 8 weeks after perivascular collar placement. Hereto, a subset of mice was sacrificed at the time points indicated below. Fixation through the left cardiac chamber was performed with phosphate-buffered saline (PBS). Subsequently, both common carotid arteries were excised and snap-frozen in liquid nitrogen for optimal RNA preservation. A schematic overview of the mouse atherosclerosis time course is depicted in Suppl. Figure 1A. For RNA isolation, two to three carotid artery segments carrying the plaque from 0, 2, 4, 6, or 8 weeks after collar placement were pooled for each sample and homogenized by grounding in liquid nitrogen with a pestle. Of each time point, three samples of pooled carotids were obtained for RNA extraction. Total RNA was extracted from the tissue homogenates using Trizol reagent according to manufacturer's instructions (Invitrogen, Breda, The Netherlands). Gene expression levels were determined using an Illumina Bead-Chip Whole Genome Microarray (ServiceXS, Leiden, The Netherlands).

Atherosclerosis in $LDLr^{-/-}/RP105^{-/-}$ versus $LDLr^{-/-}$ mice

$RP105^{-/-}$ mice were kindly provided by K. Miyake (Tokyo University, Japan) and were described previously²¹. Male $LDLr^{-/-}$ (n=12) and male $LDLr^{-/-}/RP105^{-/-}$ mice ($LDLr^{-/-}$ background, backcrossed for more than 10 generations) (n=12) bred in our laboratory (Gorlaeus Laboratories), were fed a Western type diet throughout the experiment. After two weeks of diet feeding, body weight was measured and plasma total cholesterol levels were determined by incubation with 0.025 U/ml cholesterol oxidase (Sigma, Zwijndrecht, The Netherlands) and 0.065 U/ml peroxidase and 15 µg/mL cholesterol esterase (Roche Diagnostics, Mannheim, Germany) in polyoxyethylene-9-lauryl ether, and 7.5% methanol). Precipath was used as an internal standard (standardized serum; Boehringer, Mannheim, Germany) and absorbance was read at 490 nm. At that time-point, mice were anaesthetized and collars were placed as described in the previous section, after which lesions were allowed to develop for another four weeks (schematic overview in Suppl. Figure 1B). At sacrifice, four weeks after collar placement, *in situ* fixation through the left cardiac

chamber was performed, after which carotid artery lesion size and morphology were analyzed.

Histology and morphometry

Paraffin embedded carotid plaques were cut in sections of 5 μm thick and stained with hematoxylin-phloxine-saffron (HPS) to determine lesion size. (Immuno)histochemical staining were performed for macrophages (MAC3;1:200;BD-Pharmingen, San Diego, USA), alpha smooth muscle cell actin (1:1000;Sigma), collagen (picosirius red staining) and CCR2 (1:400;Abcam, Cambridge, UK). An enzymatic staining (CAE, Sigma) was used for visualizing mast cells.

Morphometric analysis (Leica Qwin image-analysis software) was performed at site of maximal stenosis. (Immuno)histochemical stainings were quantified by computer assisted analysis (Leica, Qwin, Cambridge, UK) and expressed as the percentage of positive stained area of the total intimal area. Perivascular mast cells were counted manually and scored as either resting when all granula were inside the cell, or activated when granula were deposited in the tissue surrounding the mast cell.

Flow cytometry

At sacrifice, blood was collected and peritoneal cells were isolated ($n=5/\text{group}$) by flushing the peritoneal cavity with 10 mL PBS. Erythrocytes were removed using an erythrocyte lysis buffer (pH 7.3). Blood cells were stained with CD11b;Ly6C;Ly6G to determine the amount of neutrophils and monocytes. CD4;CD8;CD25;CD19 antibodies were used to detect T and B cells. Peritoneal cells were stained to analyze B cells (CD19⁺), B cell subsets (B1 cells: CD5⁺;CD4⁺;IgM⁺, and B2 cells: IgM⁺;IgD⁺, both calculated as a percentage of CD19⁺ cells), and mast cells (CD117;IgE;FcγR;FcεRI). FACS analysis was performed on a FACSCantoII (BD-Biosciences, San Jose, USA) and data were analyzed using FACSDiva software.

Serum immunoglobulin detection and MCP-1 levels

IgM, IgG1 and IgG2a levels in serum were detected with the use of a mouse immunoglobulin isotyping ELISA kit according to manufacturer's protocol (BD Biosciences). Total IgE in serum was determined by a mouse IgE quantitative ELISA (Bethyl Laboratories, Montgomery, USA). To detect MCP-1 in serum an ELISA was performed according to manufacturer's protocol (BD-Biosciences).

In vivo influx to peritoneum

LDLr^{-/-}/RP105^{-/-} and LDLr^{-/-} mice ($n=5/\text{group}$) were injected intra-peritoneal with 1 mL 3% Brewer's thioglycollate (Difco) or 1 mL PBS as a control. Five days after injection the peritoneal cavity was flushed with 10 mL PBS, cells were centrifuged for 5 minutes at 1500 rpm and resuspended in 1 mL PBS. Flow cytometry was used to analyze monocyte numbers (CD11b;Ly6G;Ly6C), monocyte chemokine receptor expression (CD11b;CCR2;CX3CR1) and macrophages numbers (F4/80;CD40;CD80). FACS analysis was performed on a FACSCantoII (BD-Biosciences) and data were analyzed using FACSDiva software.

Cell culture

Bone marrow (BM) derived monocytes were isolated and differentiated as described previously²². In brief, BM was isolated by flushing femurs and tibias with PBS. Cells were cultured for 5 days in RPMI 1640 medium supplemented with 10% FCS, 2 mmol/L l-glutamine, 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin and 20 ng/mL recombinant murine M-CSF (eBioscience, San Diego, USA) to generate BM-derived monocytes. After 5 days, non-adherent cells were harvested and analyzed by FACS for CD11b and Ly6C expression to determine monocyte purity, which reached approximately 84%.

BM derived monocytes (10^6 cells) were stimulated with LPS (1 or 10 ng/mL, from E. Coli, Sigma) or control medium overnight at 37°C, after which cells were harvested for RNA isolation.

For FACS analysis of CCR2 and CCR5 expression on monocytes, cells were stimulated for 2 hours with LPS

(1 or 10 ng /mL) and stained with antibodies for CD11b;Ly6C;CCR2;CCR5.

RNA isolation and cDNA synthesis

RNA was isolated using a standard TRIzol-chloroform extraction protocol after which RNA was examined by nanodrop (Nanodrop® Technologies). Relative quantitative mRNA PCR was performed on reverse transcribed cDNA using Taqman gene expression assays and qPCRs were run on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, USA). The relative expression of CCR2, CCR5, CCR1 and CX3CR1 was determined using the murine housekeeping genes HPRT, RPL27, B-actin (Suppl. table 1).

Statistical analysis

Data are expressed as mean \pm SEM. A 2-tailed Student's t-test was used to compare individual groups. The *in vivo* influx study was analyzed by performing a one-way ANOVA, followed by a Tukey's multiple comparison test. Non-parametric data were analyzed using a Mann-Whitney U test. $P < 0.05$ was considered significant.

Results

RP105 deficiency results in reduced plaque size

During atherosclerosis, RP105 was seen to be significantly upregulated during early lesion development, which directly mirrors the upregulation of TLR4 expression (Suppl. Figure 2A). In order to investigate the effect of RP105 deficiency on early atherosclerosis, LDLr^{-/-}/RP105^{-/-} mice and LDLr^{-/-} mice on a high fat diet received perivascular collars around the carotid arteries. Weight and cholesterol levels did not differ between the groups (Figure 1A). FACS analysis of the blood at sacrifice revealed no changes in the percentage of monocytes (CD11b⁺Ly6G^{low} cells), neutrophils (CD11b⁺Ly6G^{high} cells) or in the percentage of B cells (CD19⁺ cells) (Figure 1B).

Analysis of plaque size, using hematoxylin-phloxine-saffron (HPS) stained sections, revealed a marked decrease in atherosclerotic lesion formation in the RP105 deficient mice (LDLr^{-/-}: $20.66 \pm 4.7 \times 10^3 \mu\text{m}^2$; LDLr^{-/-}/RP105^{-/-}: $8.7 \pm 2.3 \times 10^3 \mu\text{m}^2$; $P < 0.05$; Figure 1C). Medial thickness remained unaltered between the two groups (LDLr^{-/-}: $55.0 \pm 2.8 \times 10^3 \mu\text{m}^2$; LDLr^{-/-}/RP105^{-/-}: $49.6 \pm 3.6 \times 10^3 \mu\text{m}^2$; $P = 0.25$).

Lesional macrophages are decreased in LDLr^{-/-}/RP105^{-/-} mice

Plaque composition was further analyzed by staining for smooth muscle cells, collagen content, mast cells, and macrophages. The percentage of intimal smooth muscle cells of total plaque area was not affected by RP105 deficiency (LDLr^{-/-}: $11.1\% \pm 2.6\%$; LDLr^{-/-}/RP105^{-/-}: $18.9\% \pm 3.8\%$; Figure 2A) and also, Sirius red staining revealed no differences in relative collagen content (LDLr^{-/-}: $16.2\% \pm 1.9\%$; LDLr^{-/-}/RP105^{-/-}: $17.8\% \pm 1.6\%$; Figure 2B). The number of mast cells in the perivascular tissue and their activation status, as measured by the amount

of granules present in the vicinity of the cell, did not differ between the groups (Figure 2C,D). However, we observed a profound 40% decrease in lesional macrophage content (as percentage of total plaque area, LDLr^{-/-}: 26.0%±3.7%; LDLr^{-/-}/RP105^{-/-}: 15.6%±2.3%; P<0.05; Figure 2E). Interestingly, RP105 expression in the atherosclerosis time course was seen to increase mostly during the first two weeks after collar placement, which was highly comparable to the expression patterns of monocyte/macrophage markers during lesion development (Suppl. Figure 2B), suggesting that intra-lesional RP105 is predominantly monocyte/macrophage derived.

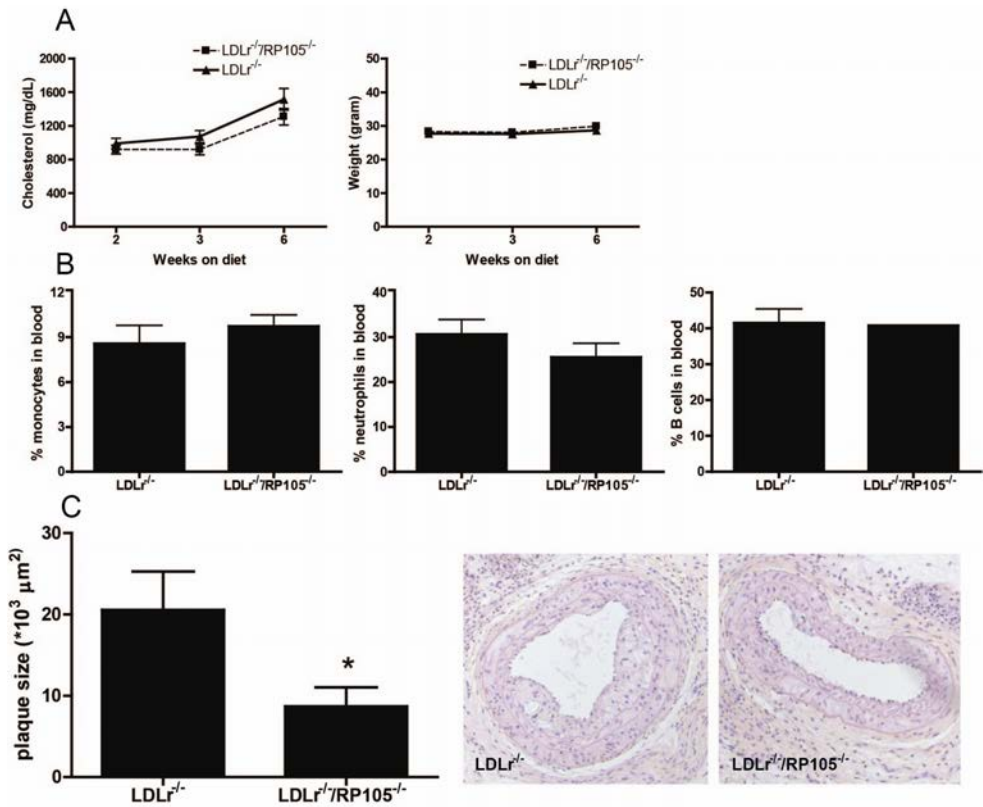


Figure 1. RP105 deficiency results in reduced plaque size. After 2, 3 and 6 weeks of diet, no changes were seen in cholesterol levels and weight between LDLr^{-/-}/RP105^{-/-} mice and LDLr^{-/-} control mice (A). FACS analysis of the blood at time of sacrifice revealed no changes in monocytes (CD11b⁺;Ly6G^{low}), neutrophils (CD11b⁺;Ly6G^{high}) or B cells (CD19⁺) between LDLr^{-/-}/RP105^{-/-} mice and control LDLr^{-/-} mice (B). Plaque size was significantly reduced in LDLr^{-/-}/RP105^{-/-} mice compared to LDLr^{-/-} mice (C). Micrographs show representative images of each group (100x). N=12 mice/group. *P<0.05. A 2-tailed Student's t-test was used to compare individual groups.

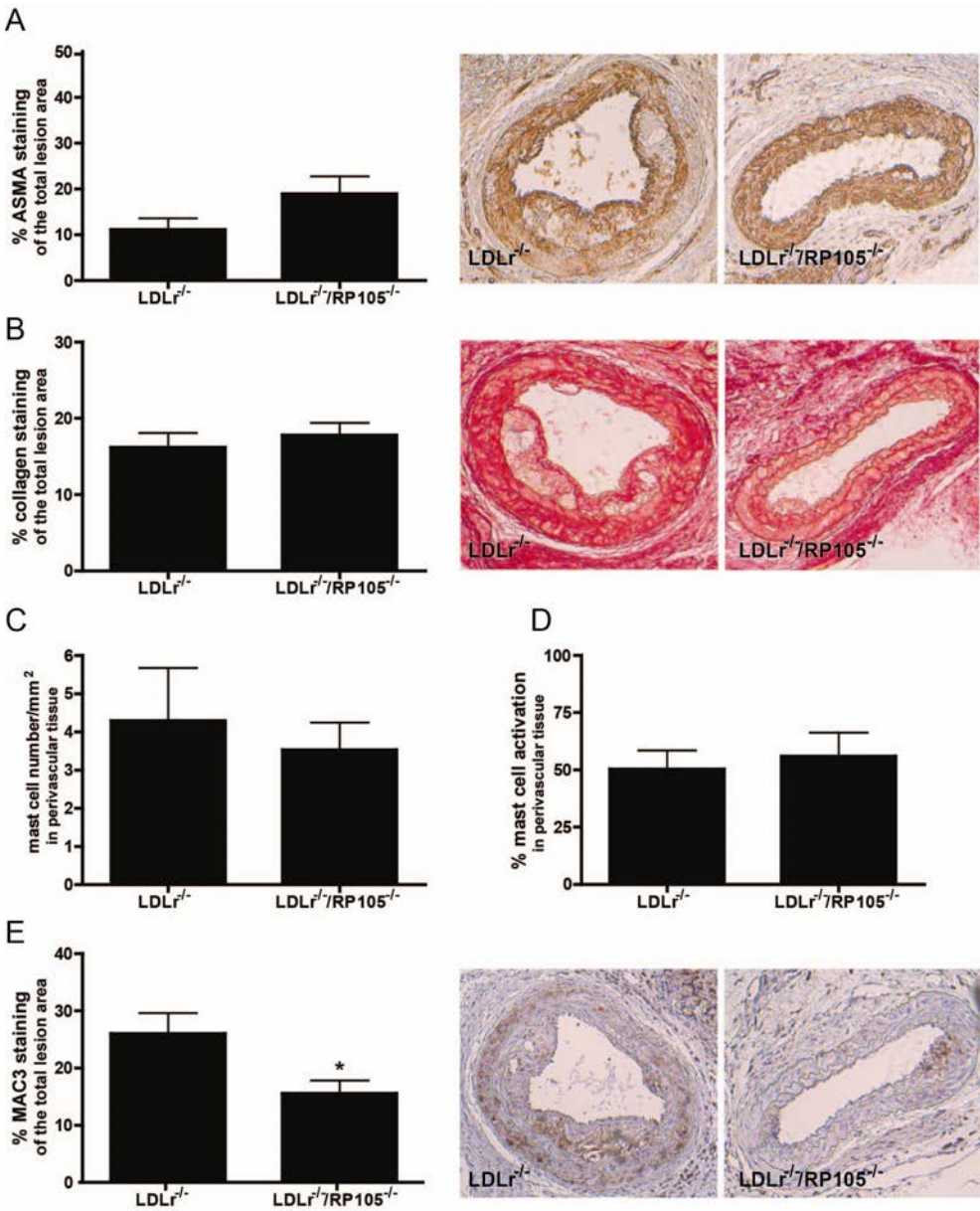


Figure 2. Lesional macrophages are decreased in LDLr^{-/-}/RP105^{-/-} mice. Plaque morphology was analyzed by staining for macrophages, collagen, smooth muscle cells and mast cells. No differences were found between the percentage of smooth muscle cells (ASMA, A), collagen content (Sirius Red, B), or perivascular mast cell numbers and activation status (C,D). Macrophage content in LDLr^{-/-}/RP105^{-/-} mice was significantly reduced compared to LDLr^{-/-} mice (MAC3, E). Micrographs show representative images of both groups (100x). N=12 mice/group. *P<0.05. A 2-tailed Student's t-test was used to compare individual groups.

Decrease in peritoneal B cells and reduction in plasma IgE and IgM levels

Since RP105 deficiency has been described to affect B cell activation and proliferation²³, we further analyzed the percentage and subpopulations of B cells in the peritoneum by FACS analysis. The percentage of B cells (CD19⁺ cells) was significantly decreased in mice deficient for RP105 (LDLr^{-/-}: 68.1%±3.6%; LDLr^{-/-}/RP105^{-/-}: 51.4%±4.1%; P<0.05; Figure 3A). B cell subsets, determined as a percentage of the total B cell population, did not differ between the groups (Figure 3B,C). Also, mast cell content did not differ (Figure 3D). RP105 deficiency markedly reduced plasma IgE and IgM levels, while not affecting plasma IgG1 or IgG2a concentrations (Figure 3E-H).

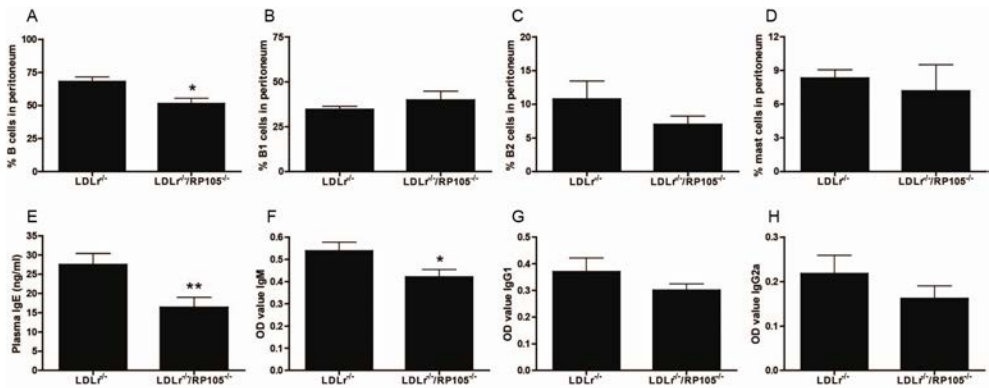


Figure 3. Decrease in peritoneal B cells and reduction in plasma IgE and IgM levels. B cells in the peritoneum (CD19⁺) were significantly decreased in LDLr^{-/-}/RP105^{-/-} mice compared to LDLr^{-/-} mice (A). No changes were observed between the percentage of peritoneal B1 cells (percentage of CD5⁺CD4⁺IgM⁺ cells within CD19⁺ cells) (B) and B2 cells (percentage of IgM⁺IgD⁺ cells within CD19⁺ cells) (C). The percentage of mast cells in the peritoneum did not differ between the two groups (D). A significant reduction of plasma IgE (E) and IgM (F) was detected in LDLr^{-/-}/RP105^{-/-} mice at time of sacrifice, while no changes were seen in IgG1 (G) or IgG2a (H) levels. N=12 mice/group. *P<0.05, **P<0.01. A 2-tailed Student's t-test was used to compare individual groups.

In vivo migration is impaired in RP105 deficient monocytes

In order to elucidate the mechanism behind the decreased amount of lesional macrophages found *in vivo*, we aimed to investigate whether monocyte migration was disturbed in mice lacking RP105. Monocyte influx to the peritoneum was measured after thioglycollate injection in LDLr^{-/-} and LDLr^{-/-}/RP105^{-/-} mice by staining for CD11b⁺Ly6G⁻ cells. Interestingly, monocyte migration in RP105 deficient mice is markedly disturbed compared to LDLr^{-/-} mice as revealed by FACS analysis (LDLr^{-/-}: 31.3±1.3 fold increased influx; LDLr^{-/-}/RP105^{-/-}: 11.9±3.7 fold increased influx; P<0.001; Figure 4A). Concomitantly, a decrease in the percentage of CCR2 positive monocytes was observed in LDLr^{-/-}/RP105^{-/-} mice P<0.05; Figure 4B). No changes were detected in neutrophil influx (Figure 4C).

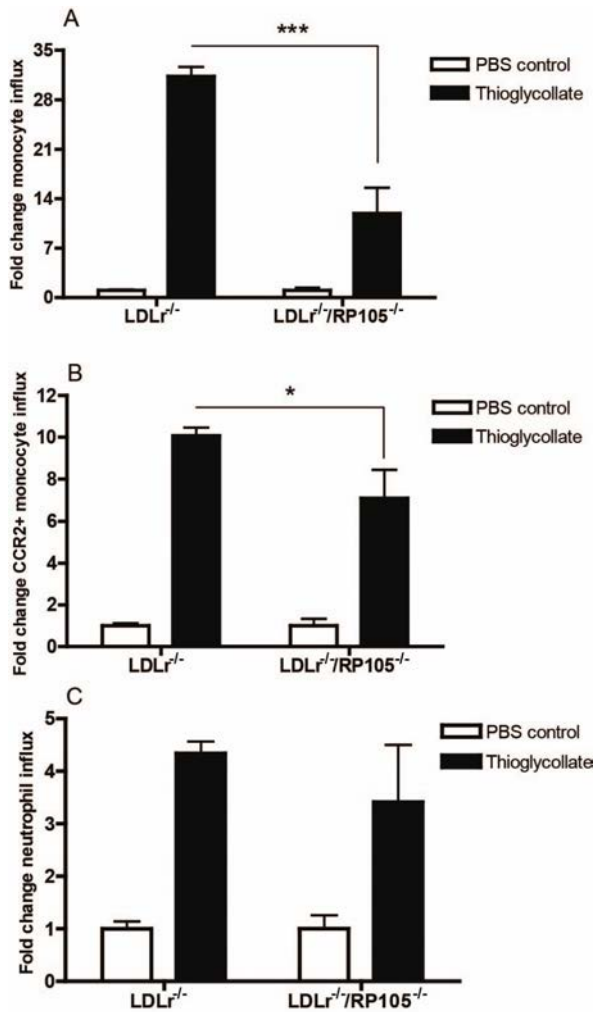


Figure 4. In vivo migration is impaired in *LDLr*^{-/-}/*RP105*^{-/-} monocytes. FACS analysis showed a significant reduction of monocyte influx to the peritoneum in *LDLr*^{-/-}/*RP105*^{-/-} mice compared to *LDLr*^{-/-} mice after intra-peritoneal injection of thioglycollate, depicted as a fold change compared to PBS injection (A). Also, decreased influx of CCR2⁺ monocytes was observed in *LDLr*^{-/-}/*RP105*^{-/-} mice (B). No changes were found in the relative influx of neutrophils (C). N=5 mice/group. *P<0.05 compared to thioglycollate treated *LDLr*^{-/-} mice. ***P<0.001 compared to thioglycollate treated *LDLr*^{-/-} mice. A one-way ANOVA was performed, followed by a Tukey's multiple comparison test.

Decrease of CCR2⁺ *LDLr*^{-/-}/*RP105*^{-/-} monocytes after LPS activation *in vitro*

To further investigate the effects of *RP105* deficiency on monocyte migration we observed *in vivo*, we determined the expression of chemokine receptors CCR2 and CCR5 on bone marrow derived monocytes *in vitro*. FACS analysis showed an increased percentage of CCR2⁺ positive *LDLr*^{-/-}/*RP105*^{-/-} monocytes compared to *LDLr*^{-/-} monocytes when unstimulated. However, after stimulation with 1 or 10 ng LPS, a significant dose-dependent decrease in the percentage of CCR2⁺ cells was observed in the *LDLr*^{-/-}/*RP105*^{-/-} monocyte population, which did not occur in *LDLr*^{-/-} monocytes (Figure 5A). Moreover, after stimulation with 10 ng LPS, the percentage of CCR2⁺ *LDLr*^{-/-}/*RP105*^{-/-} monocytes was significantly reduced compared to *LDLr*^{-/-} monocytes. The percentage of CCR5 positive *LDLr*^{-/-}/*RP105*^{-/-} monocytes was increased compared to monocytes from *LDLr*^{-/-} mice, also after stimulation

with 1 ng LPS. However 10 ng LPS stimulation did not result in changes between the two groups (Figure 5B).

Expression of CCR2 at mRNA level after LPS stimulation showed a decrease in both $LDLr^{-/-}$ and $LDLr^{-/-}/RP105^{-/-}$ monocytes, however this decrease was more pronounced in $LDLr^{-/-}/RP105^{-/-}$ monocytes (Figure 5C). After stimulation with 1 ng LPS, CCR2 expression was significantly decreased in $LDLr^{-/-}/RP105^{-/-}$ monocytes compared to $LDLr^{-/-}$ monocytes. Stimulation with 10 ng LPS resulted in such a strong reduction of CCR2 expression that no significant differences were detectable any more between the two groups. We have measured the expression of CCR1, CCR5 and CX3CR1 in *in vitro* cultured $LDLr^{-/-}$ and $LDLr^{-/-}/RP105^{-/-}$ monocytes as well, which did not show a similar downregulation after LPS stimulation as CCR2 (Suppl. Figure 3). CX3CR1 gene expression is somewhat reduced in $LDLr^{-/-}/RP105^{-/-}$ monocytes, suggesting that RP105 deficiency may affect this chemokine receptor as well, however not to the extent of CCR2.

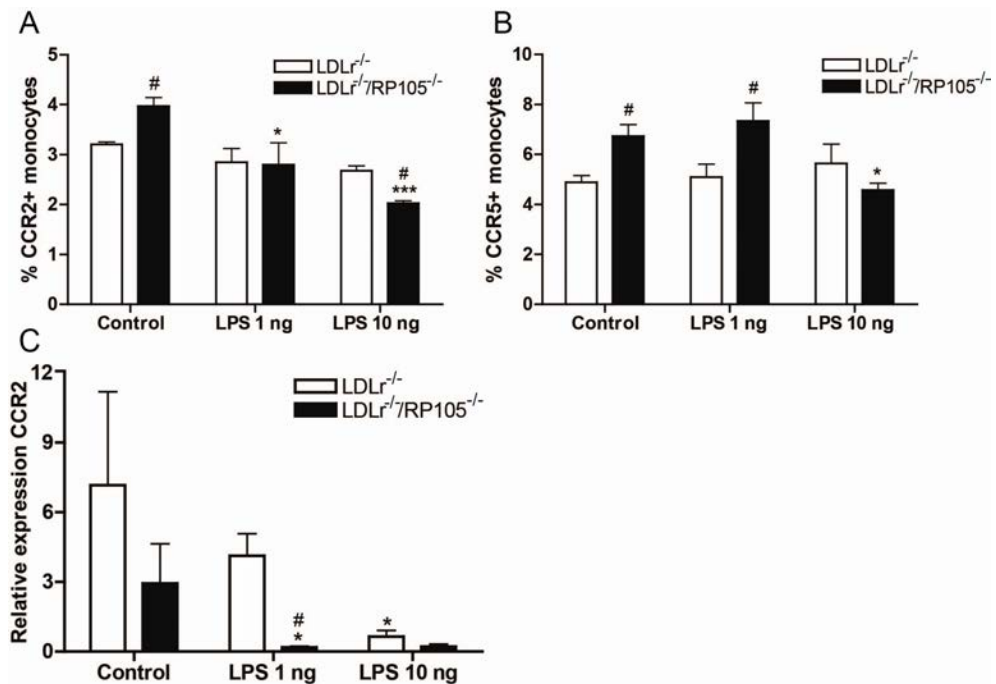


Figure 5. Decrease in CCR2⁺ $LDLr^{-/-}/RP105^{-/-}$ monocytes after LPS activation *in vitro*. FACS analysis of *in vitro* cultured $LDLr^{-/-}/RP105^{-/-}$ monocytes stimulated with 1 or 10 ng LPS revealed a significant dose-dependent decrease in the percentage of CCR2⁺ RP105 deficient monocytes. After stimulation with 10 ng LPS, the percentage of CCR2⁺ monocytes was significantly reduced in $LDLr^{-/-}/RP105^{-/-}$ monocytes compared to control $LDLr^{-/-}$ monocytes (A). The percentage of CCR5⁺ $LDLr^{-/-}/RP105^{-/-}$ monocytes was increased compared to $LDLr^{-/-}$ monocytes in the unstimulated control and after stimulation with 1 ng LPS stimulation, 10 ng LPS stimulation did not result in changes between the two groups (B). LPS stimulation decreased the relative CCR2 expression in both $LDLr^{-/-}$ and $LDLr^{-/-}/RP105^{-/-}$ monocytes; however, after stimulation with 1 ng LPS, CCR2 expression was significantly decreased in $LDLr^{-/-}/RP105^{-/-}$ monocytes compared to $LDLr^{-/-}$ monocytes (C). $N=4$. # $P<0.05$ compared to $LDLr^{-/-}$ monocytes; * $P<0.05$ compared to unstimulated monocytes; *** $P<0.001$ compared to unstimulated monocytes. A 2-tailed Student's *t*-test was used to compare individual groups.

CCR2 expression in atherosclerotic plaques

Atherosclerotic plaques of LDLr^{-/-} and LDLr^{-/-}/RP105^{-/-} mice were stained for CCR2 to determine whether expression of this chemokine receptor within the lesion is altered by RP105 deficiency. No significant differences were observed in CCR2 staining (as percentage of intimal area) between the LDLr^{-/-}/RP105^{-/-} and LDLr^{-/-} mice (LDLr^{-/-}: 29.9%±4.2%; LDLr^{-/-}/RP105^{-/-}: 21.6%±2.5%; P=0.1; Suppl. Figure 4). To determine whether RP105 deficiency affected circulating levels of MCP-1, one of the most important ligands for CCR2, we analyzed plasma MCP-1 levels in LDLr^{-/-} and LDLr^{-/-}/RP105^{-/-} mice at time of sacrifice. We did not observe any changes between the two groups (Suppl. Figure 5).

Discussion

The current study is the first to demonstrate that expression of RP105 is upregulated early in atherogenesis, as shown in the time course experiment, and that total body RP105 deficiency attenuates early atherosclerotic lesion formation via decreased monocyte influx. Also, we show that LPS stimulation of RP105 deficient monocytes results in a downregulation of the chemokine receptor CCR2, which may be the cause of the observed reduction in migratory capacity of RP105 deficient monocytes.

In the current study, the most prominent finding is a 40% reduction in lesional macrophage content in LDLr^{-/-}/RP105^{-/-} mice resulting in reduced lesion development as compared to LDLr^{-/-} controls, fuelling the hypothesis that a disturbed monocyte influx into the vessel wall underlies the reduction in atherosclerosis in LDLr^{-/-}/RP105^{-/-} mice. Indeed, influx of LDLr^{-/-}/RP105^{-/-} monocytes into the peritoneum was significantly decreased compared to LDLr^{-/-} monocytes. *In vitro*, the percentage of CCR2⁺ LDLr^{-/-}/RP105^{-/-} monocytes was significantly reduced after LPS stimulation, while the relative expression of CCR2 on LDLr^{-/-}/RP105^{-/-}, as compared to LDLr^{-/-} monocytes, was also significantly reduced. These results indicate that CCR2 may be one of the chemokine receptors involved in decreased monocyte migration in LDLr^{-/-}/RP105^{-/-} mice, which leads to the decrease in early lesion formation with reduced macrophage content observed *in vivo*^{24,25}. In addition, future research may point to regulation of other chemokine receptors in RP105 deficient monocytes as well.

Previously, we have shown that lethally irradiated atherosclerosis prone LDLr^{-/-} mice receiving RP105^{-/-} bone marrow display reduced lesion development, in which the atheroprotective effects were attributed to a reduction in B cell numbers, and in particular of B2 cells¹⁸. However, in that study, leukocyte specific RP105 deficiency was investigated, thereby excluding effects that RP105 deficiency may have on non-myeloid cells, such as on vascular smooth muscle cells. In fact, it

has also been previously established that RP105 deficiency results in increased neointima formation via enhanced smooth muscle cell proliferation¹⁹. Therefore, in the current study, we aimed to investigate total body RP105 deficiency, in order to take into account the effects of both myeloid and non-myeloid deletion of RP105. Our results indicate that smooth muscle cell derived RP105 does not significantly impact atherosclerotic lesion development, as smooth muscle cell and collagen staining failed to show any significant changes between plaques of LDLr^{-/-} and LDLr^{-/-}/RP105^{-/-} mice. Similar to the previous study we observed a decrease in B cells; however, this was not as prominent and the ratio between B cell subsets remained unaltered. This reduction of B cells observed in the current study is reflected by decreased levels of total plasma IgE and IgM. Previous studies have shown that B cells are hardly present within the atherosclerotic plaque or perivascular tissue²⁶, suggesting that effects B cells can have on the plaque may be predominantly systemically or indirect. For example, it has been described that IgM specific for oxLDL may be atheroprotective by limiting accumulation of apoptotic cells and inhibiting inflammatory gene expression²⁷. In the present study we measured only total IgM, instead of oxLDL specific IgM and therefore, in this experimental setup, it remains unclear whether the observed reduction of total plasma IgM contributed to lesion formation.

Influx of monocytes at predisposed areas into the vessel wall is considered an important initial step in atherosclerosis³⁻⁵. Suppression of monocyte recruitment in atherogenesis has been shown to result in reduced plaque size and altered plaque composition, however, these effects were not seen when monocytes were reduced in late stage atherosclerosis, stressing the importance of monocyte influx in early atherogenesis²⁸. Intriguingly, we have recently found that RP105 deficient monocytes display signs of increased activation and disturbed migration towards the adductor muscle in hind limb ischemia²⁹. This led to the hypothesis that disturbed monocyte migration into the vessel wall in LDLr^{-/-}/RP105^{-/-} mice may be the cause of decreased atherosclerotic lesion formation observed in the current study. Monocyte migration occurs via the well-known chemokine receptors CCR1, CCR2, CCR5 and CX3CR1^{16,30}. Already 15 years ago it has been shown that lesion development in apoE^{-/-} mice deficient for the C-C chemokine receptor CCR2 is markedly decreased, demonstrating an important role of CCR2 in atherosclerosis^{25,26,31,32}. Interestingly, activation of monocytes via the TLR4 route has been shown to downregulate chemokine receptors, in particular CCR2³³⁻³⁶. In the current study, we have also demonstrated that stimulation of monocytes with LPS, the most important activator of TLR4, decreases both the percentage of CCR2 positive monocytes as well as the relative expression of CCR2. Intriguingly, this effect was more pronounced in monocytes deficient for RP105. RP105 is thought to act as a negative regulator in dendritic cells and macrophages via direct interaction with TLR4 on the cell membrane¹⁶. RP105 expression has shown to inhibit

TLR4-driven NF- κ B transactivation and IL-8 production by HEK293, while lack of RP105 results in increased activation and cytokine release³⁷. Therefore, we speculate that the more pronounced decrease of CCR2 in RP105 deficient monocytes may be due to increased signalling via the TLR4 route.

CCR2 staining in the atherosclerotic plaques was not significantly reduced in LDLr^{-/-}/RP105^{-/-} mice as compared to LDLr^{-/-} mice, despite changes in CCR2 expression in LDLr^{-/-}/RP105^{-/-} monocytes. Apart from the difficulty to detect subtle differences in protein expression levels by immunohistochemistry, this may also be due to the fact that monocytes, once arrived in the plaque, differentiate into macrophages and thereby alter expression of receptors, and in particular of chemokine receptors³⁸. CCR2 expression may thus be altered on LDLr^{-/-}/RP105^{-/-} monocytes, but upon arrival in the vessel wall and subsequent differentiation, these changes in chemokine receptor expression become less pronounced. Further research is aimed at the elucidation of mechanisms causing disturbed CCR2 expression upon RP105 deficiency in atherosclerosis and other inflammatory disorders.

In conclusion, we here show that LDLr^{-/-}/RP105^{-/-} mice develop reduced early atherosclerotic plaques with a marked decrease in lesional macrophages. Our data suggest that this is due to disturbed migration of RP105 deficient monocytes, caused by a downregulation of CCR2.

Conclusions

In this study, we found a significant upregulation of RP105 expression in early atherogenesis. Total body RP105 deficiency in LDLr^{-/-}/RP105^{-/-} mice resulted in a marked reduction of atherosclerotic lesion size, compared to control LDLr^{-/-} mice. No changes were observed in the smooth muscle cell percentage in the plaque; however, macrophage content was significantly reduced in LDLr^{-/-}/RP105^{-/-} mice. We deliver *in vivo* experimental proof that monocyte migration is reduced in RP105 deficient mice, which may explain the reduction in lesional macrophages. Furthermore, *in vitro* investigations show that this decreased migratory capacity may be due to a more pronounced downregulation of CCR2 in LDLr^{-/-}/RP105^{-/-} monocytes compared to LDLr^{-/-} monocytes, after LPS stimulation. These data may thus provide a novel mechanism via which RP105 deficiency may attenuate atherosclerosis.

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Supplemental Material

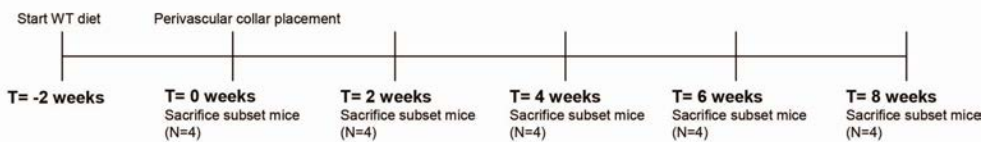
Supplemental table 1.

| Gene | Forward primer | Reversed primer |
|----------------|---------------------------|----------------------------|
| CCR2 | CCTTGGGAATGAGTAAGTGTGA | TGGAGAGATACCTTCGGAAGTCT |
| CCR5 | AATTCTTTGGACTGAATAAGTCA | TGGATCGGGTATAGACTGAGCTT |
| CCR1 | CAATCAGTGTGAGCAGAGTAAGCA | CACAACAGTGGGTGTAGGCAA |
| CX3CR1 | AAGTTCCCTTCCCATCTGCT | CAAAATTCTCTAGATCCAGTTCAGG |
| RPL27 | CGCCAAGCGATCCAAGATCAAGTCC | AGCTGGGTCCCCTGAACACATCCTTG |
| HPRT | TTGCTCGAGATGTCATGAAGGA | AGCAGGTCAGCAAAGAACTTATAG |
| β -actin | CGCCAAGCGATCCAAGATCAAGTCC | AGCTGGGTCCCCTGAACACATCCTTG |

Supplemental figure 1. (A) Schematic overview of the atherosclerosis time course used for RNA analysis (N=4 mice/group). **(B)** Schematic overview of the atherosclerosis study induced in $LDLr^{-/-}/RP105^{-/-}$ versus $LDLr^{-/-}$ mice (N=12 mice/group).

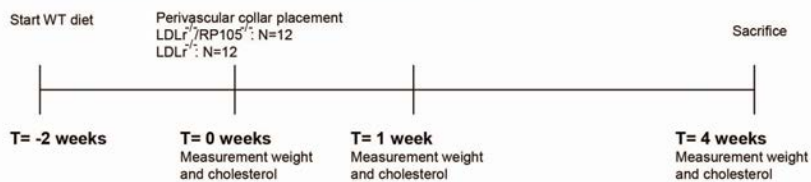
A

Atherosclerosis time course RNA analysis $LDLr^{-/-}$ mice

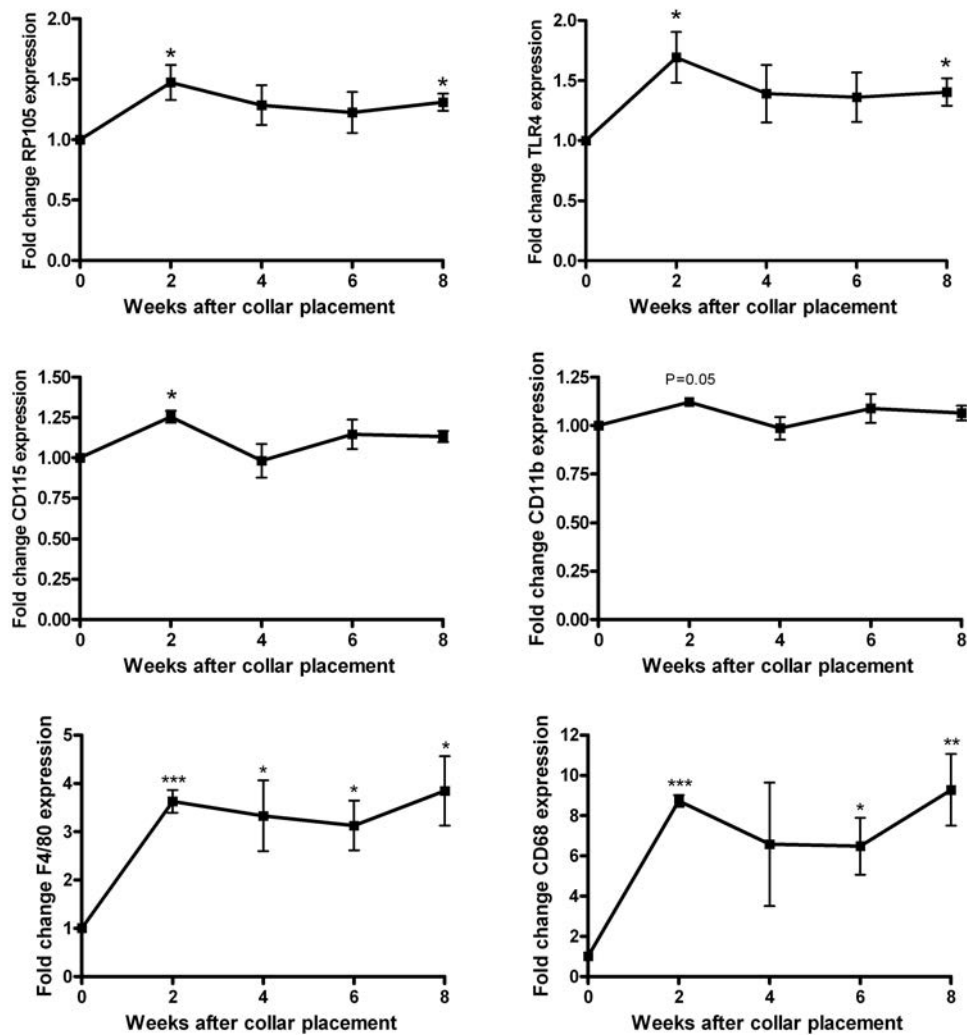


B

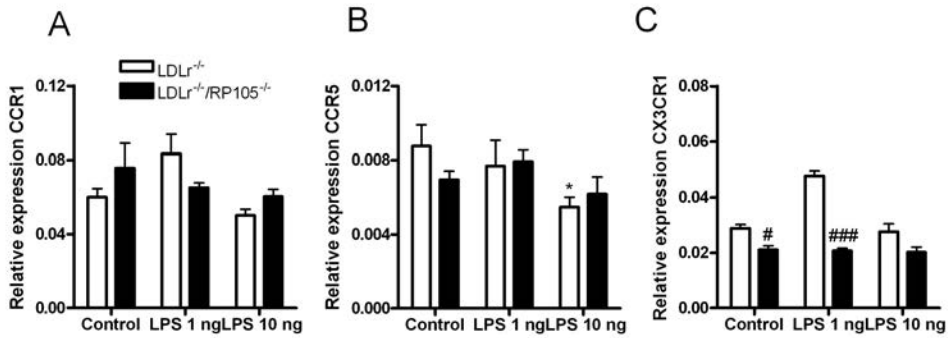
Atherosclerosis in $LDLr^{-/-}/RP105^{-/-}$ versus $LDLr^{-/-}$ mice



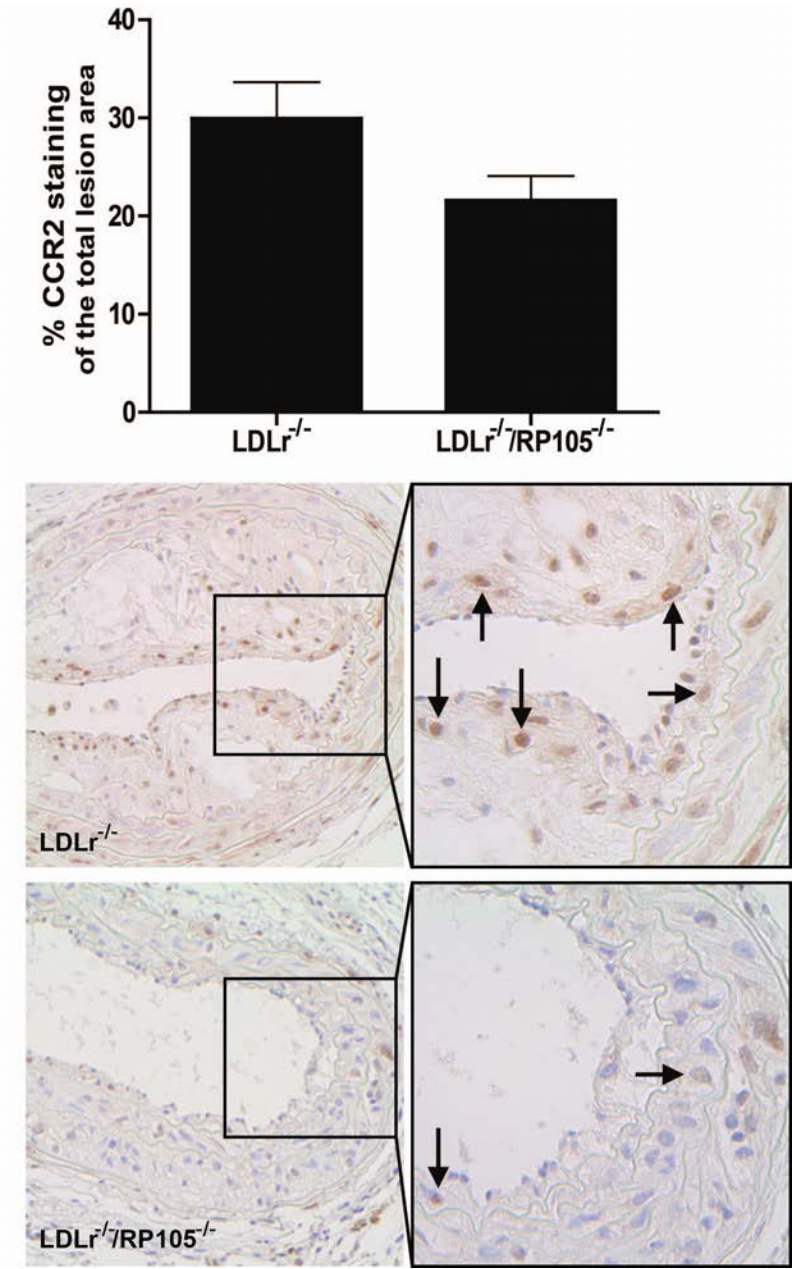
Supplemental Figure 2. (A) Fold change in expression of RP105 en TLR4 compared to 0 weeks of diet. (B) Fold change in expression of monocyte/macrophage markers CD115, CD11b, F4/80 and CD68 compared to 0 weeks of diet. *P<0.05, **P<0.01, ***P<0.0001.



Supplemental figure 3. (A) CCR1 expression remained unaltered after stimulation with 1 ng and 10 ng LPS in both $LDLr^{-/-}$ and $LDLr^{-/-}/RP105^{-/-}$ monocytes. (B) The relative expression of CCR5 was slightly reduced in $LDLr^{-/-}$ monocytes after stimulation with 10 ng LPS, however, no significant changes were found between $LDLr^{-/-}$ and $LDLr^{-/-}/RP105^{-/-}$ monocytes. (C) The CX3CR1 expression in $LDLr^{-/-}$ monocytes was increased after stimulation with 1 ng LPS, while no changes were observed in CX3CR1 expression when comparing unstimulated $LDLr^{-/-}/RP105^{-/-}$ monocytes with LPS stimulated $LDLr^{-/-}/RP105^{-/-}$ monocytes. N=4. # $P<0.05$ compared to $LDLr^{-/-}$ monocytes; ### $P<0.001$ compared to $LDLr^{-/-}$ monocytes; *** $P<0.001$ compared to unstimulated monocytes. A 2-tailed Student's t-test was used to compare individual groups.



Supplemental figure 4. CCR2 protein expression was measured as the percentage of staining in the intimal area. The micrographs below show representative images of CCR2 staining in both groups (200x). Cells positive for CCR2 are indicated with arrows in the enlarged micrographs (400x). N=12 mice/group. A 2-tailed Student's t-test was used to compare individual groups.



Supplemental figure 5. Plasma MCP-1 levels, as measured by means of ELISA, did not differ between $LDLr^{-/-}/RP105^{-/-}$ mice and control $LDLr^{-/-}$ mice. N=12 mice/group. A 2-tailed Student's t-test was used to compare individual groups.

