Innate immune modulation in atherosclerosis and vascular

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Chapter 6

RP105 deficiency aggravates vein graft disease and lesion instability via increased inflammation and mast cell activation

Submitted for publication

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Abstract

Aims: Venous bypass grafts are often used to bypass atherosclerotic lesions; however, the patency of these grafts is usually poor due to the development of vein graft disease. Previously, we have demonstrated that deficiency of TLR4, a key initiator of inflammatory signalling, results in a reduction of vein graft disease. As TLR4 signalling is regulated by the accessory molecule RadioProtective 105 (RP105), we aimed to investigate the effects of RP105 on vein graft disease.

Methods and results: RP105$^{-/-}$ mice and C57BL/6 mice as controls underwent vein graft surgery; interestingly, a 90% increase in vein graft lesion area was observed in RP105$^{-/-}$ mice. As lesions in vein grafts are often accompanied by superimposed atherosclerosis, we also determined the effect of RP105 on vein graft disease in a hypercholesterolemic setting. Lesion area did not differ between LDLr$^{-/-}$/RP105$^{-/-}$ mice and LDLr$^{-/-}$ mice fed a western type diet, but interestingly, we did observe a significant increase in the number of unstable lesions and intraplaque hemorrhage upon RP105 deficiency. In both experimental setups, an increase in lesional macrophages was seen. *In vitro*, RP105$^{-/-}$ smooth muscle cells and mast cells secreted increased levels of the monocyte chemoattractant CCL2. *In vivo*, the number of activated perivascular mast cells was increased in RP105$^{-/-}$ mice, as well as the amount of lesional CCL2 .

Conclusions: Together, these data indicate that aggravated vein graft disease caused by RP105 deficiency may be the result of an increased inflammatory response and exacerbated CCL2 production by both mast cells and smooth muscle cells.

Introduction

Rupture of an atherosclerotic lesion with subsequent thrombus formation may lead to distal embolization of the blood vessel, resulting in main adverse cardiovascular events[1]. Restoring blood flow to the ischemic tissue is therefore crucial, which can be accomplished by interventions such as placement of a (drug eluting) stent or a venous graft. Vein grafts are often used because of their long length, making it possible to bypass multiple atherosclerotic lesions, and their easy availability[2-4]. However, after ten years only an approximate 40% of the grafts is still patent, caused by late graft failure due to intimal hyperplasia and atherosclerosis[5-8]. It is thus of high importance to elucidate the underlying mechanisms of vein graft disease and identify new therapeutic targets to prevent vein graft failure.

Harvesting of the venous graft with subsequent placement in the arterial circulation leads to damage of the endothelium, which causes platelets to adhere[9,10]. Smooth muscle cells (SMC) then become activated and undergo a phenotypic switch. They start to migrate into the intima where they produce extracellular matrix necessary for the strength of the vein graft. However, excessive uncontrolled smooth muscle cell proliferation results in the formation of intimal hyperplasia. This process is accompanied by leukocyte influx into the vessel wall, which aggravates the inflammatory process and leads to superimposed atherosclerosis. Late vein graft failure may eventually be the result of complete occlusion caused by intimal hyperplasia or rupture of the vein graft lesion[11].

To study the complex mechanisms behind vein graft disease, as well as to explore possible treatment options, a previously described murine vein graft model has been used[12]. In this model, lesions display typical concentric hyperplasia as well as lesional disruptions with intraplaque hemorrhage, with high resemblance to the complex lesions present in human vein grafts. When vein grafts are placed in mice on a high cholesterol diet, superimposed atherosclerosis will add to the lesional burden, as illustrated by lipid depositions and foam cell accumulation[13]. Taken together, excessive SMC proliferation, lipid accumulation and an enhanced inflammatory response seem to be causing vein graft disease. Highlighting the importance of vascular inflammation in vein graft disease, we have previously shown that local silencing of Toll-like receptor 4 (TLR4) significantly reduces vessel wall thickening in these murine venous grafts[14], rendering this pathway of interest for future therapeutic interventions.

TLR4 belongs to the TLR family, a type of pattern recognition receptors, capable of inducing potent inflammatory signalling. A unique feature of TLR4, compared to other TLRs, is that is does not directly bind to its ligands. Instead, TLR4 forms a complex with the adaptor molecule MD2, which is the primary recognition site for lipopolysaccharide (LPS). The co-receptor CD14 may augment TLR4 signalling through binding of LPS and mediating its transfer to the TLR4/MD2 complex[15]. An

additional accessory molecule known to regulate TLR4 signalling is RadioProtective 105 (RP105). Similar to TLR4, RP105 forms a complex with the adaptor molecule MD1, but in contrast, the RP105/MD1 complex does not have an intracellular Toll interleukin 1 receptor (TIR) signalling domain[16]. Originally, RP105 was described as a B cell specific surface molecule, capable of enhancing cellular proliferation and activation[16]. However, RP105 was later on demonstrated to be expressed on the cell membrane of dendritic cells (DCs) and macrophages as well. In contrast to its role on the B cell, RP105 inhibits TLR4 mediated responses in DCs and macrophages, leading for instance to an aggravated inflammatory response after LPS injection in RP105 deficient mice[17].

Taking into account the profound regulatory effects of RP105 on TLR4, and the previous finding that TLR4 signalling aggravates vein graft disease, it is compelling to investigate the role of RP105 in vein graft disease. Therefore, in the current study we used the murine vein graft model to establish whether RP105 deficiency affects lesion formation. We hypothesized that lack of RP105, via increased TLR4 signalling, will result in aggravated vein graft disease. Also, we aimed to determine how RP105 affects vein graft disease in a hypercholesterolemic setting with superimposed atherosclerosis.

Material and methods

Mice

All animal experiments were approved by the animal welfare committee of the Leiden University Medical Center (approval reference number 09098 and 12153). This study was performed in compliance with Dutch government guidelines and the Directive 2010/63/EU of the European Parliament. $RP105^{-/-}$ mice were kindly provided by K. Miyake (Tokyo University, Japan) and were described previously[16]. Male wild type (WT, C57BL/6, 10-12 weeks old) (n=11) and $RP105^{-/-}$ animals (C57BL/6 background, backcrossed for more than 10 generations, 10-12 weeks old) (n=13) were bred in our facility at the Leiden University Medical Center (Leiden, The Netherlands) and during the experiment, mice were given water and chow ad libitum.

To study the effects of RP105 deficiency on vein graft disease in a hyperlipidemic setting, male $LDLr^{-/-}$ mice (n=12, 12-16 weeks old) and male $LDLr^{-/-}/RP105^{-/-}$ mice (n=12, 12-16 weeks old) were used. Mice were fed a western-type diet containing 0.25% cholesterol and 15% cacaobutter (SDS, Sussex, UK) for 4 weeks. Plasma total cholesterol levels were measured by enzymatic procedures using precipath standardized serum as an internal standard (Boehringer Mannheim, Germany).

Surgical intervention

Before surgery and sacrifice, mice were anesthetized by an intraperitoneal injection with Midazolam (5 mg/kg, Roche, Woerden, The Netherlands), Domitor (0.5 mg/kg, AST Farma, Oudewater, The Netherlands) and fentanyl (0.05 mg/kg, Janssen, Beerse, Belgium). Adequacy of anaesthesia was monitored by regular visual inspection and toe pinch reflex. After surgery mice were antagonized with a subcutaneous injection of flumazenil (0.5 mg/kg, Fresenius Kabi, Schelle, Belgium) and Antisedan (2.5 mg/kg, AST farma). Buprenorphine (0.1 mg/kg, MSD animal Health, Boxmeer, The Netherlands) was given after sur-

gery to relieve pain. Vein graft surgery was performed in order to induce intimal hyperplasia in the venous grafts[12]. In brief, the carotid artery of the recipient mice was cut in the middle; both ends of the artery were everted around cuffs and ligated with 8.0 sutures. Donor littermates were anesthetized as described above, after which the vena cava was harvested and donor mice were exsanguinated. The vena cava was then sleeved over the cuffs in the recipient mice. The high blood pressure in the arterial circulation leads to endothelial damage of the vein graft, which is followed by accelerated neointimal hyperplasia. At sacrifice after 28 days, mice were exsanguinated via orbital bleeding. Vein grafts were harvested and fixated in 4% formaldehyde, dehydrated and paraffin-embedded for histology.

Histological and immunohistochemical analysis

All (immuno)-histochemical stainings and measurements were performed on six consecutive cross-sections, approximately 150 µm interspaced, of paraffin embedded vein grafts segments (5 µm thick). Hematoxylin-phloxine saffron (HPS) staining and Masson trichrome stainings were used for the measurement of vein graft lesion area and plaque dissection analysis, while collagen content was visualized with a picrosirius red staining. Composition of the vein graft lesions was further evaluated by staining for macrophages (MAC3, BD-Pharmingen, San Diego, CA, USA), smooth muscle cell actin (Sigma, Zwijndrecht, The Netherlands), CCL2 (Santa Cruz Biotechnology, Dallas, TX, USA) and CCR2 (Abcam, Cambrigde, UK). Mast cell staining was performed using an enzymatic chloroacetate esterase kit (Sigma); when granules were apparent in the vicinity of the mast cell they were scored as activated.

Plaque dissection analysis was performed over a total vein graft length of 1800 µm. The disruptions were defined as a connection or fissure between the lumen and part of the vessel wall underneath the adventitia, filled with fibrin and erythrocytes[12]. Quantification of the lesion area and immunostained positive area were performed using computer assisted software (Qwin, Leica, Cambridge, UK). In brief, the total intimal area was measured, as well as the stained area. The stained area was than calculated as a percentage of the total intimal area. All measurements were performed in a blinded manner by a single observer.

Cell culture

LDLr$^{-/-}$ and LDLr$^{-/-}$/RP105$^{-/-}$ mice were anaesthetized as described above and sacrificed via cervical dislocation, after which bone marrow (BM) suspensions were isolated by flushing the femurs and tibias with PBS. BM derived mast cells (BMMCs) were grown by culturing BM cells from LDLr$^{-/-}$ mice and LDLr$^{-/-}$/RP105$^{-/-}$ mice at a density of 0.25*10^{6} cells in RPMI containing 10% fetal bovine serum (FBS), 2 mmol/L l-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (all from PAA, Cölbe, Germany) and mIL3 supernatant (supernatant from WEHI cells overexpressing murine Interleukin (IL)-3) for 4 weeks in T175 tissue culture flasks (Greiner Bio-one, Alphen aan den Rijn, Netherlands). RAW 264.7 cells (a murine macrophage cell line) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Calf's Serum (FCS), 2 mmol/L L-glutamine, 100 U/mL penicillin and 100ug/mL streptomycin in T75 tissue culture flasks (Greiner Bio-one).

To generate bone marrow derived macrophages (BMDM), BM cells from BM of LDLr$^{-/-}$ mice and LDLr$^{-/-}$/RP105$^{-/-}$ mice were cultured for 7 days in RPMI medium supplemented with 20% fetal calf serum (FCS), 2 mmol/L l-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (all from PAA) and 30% L929 cell-conditioned medium (as the source of macrophage colony-stimulating factor (M-CSF)) in petridishes (Greiner Bio-one)[18].

Murine vSMCs explanted from aortas of RP105$^{-/-}$ mice and control mice were cultured and incubated overnight with either LPS (1 ng/mL or 3 ng/mL) or control medium (n=4). These mice were anesthetized as described above and sacrificed via cervical dislocation. IL-6, TNFα and CCL2 ELISAs were performed according to manufacturer's protocol (BD Biosciences, Breda, The Netherlands).

Macrophage activation

Bone marrow derived LDLr$^{-/-}$ and LDLr$^{-/-}$/RP105$^{-/-}$ macrophages cultured as described above, were activated with 1 ng/mL, 10 ng/mL or 50 ng/mL LPS (from E. Coli, Sigma-Aldrich) for 4 hours (n=3) at a density of 10^6 cells per well. Cells were then spun down (1500 rpm, 5 minutes) and used for RNA isolation.

Proliferation assay

To measure the effect of RP105 on macrophage proliferation, LDLr$^{-/-}$/RP105$^{-/-}$ BMDM and control LDLr$^{-/-}$ BMDM were seeded at a density of $4*10^4$ cells per well (n=4). Cells were incubated overnight with 0.5 µCi [^{3}H]thymidine (PerkinElmer, Groningen, The Netherlands) per well at 37ºC. [^{3}H]Thymidine incorporation was quantified in a liquid scintillation analyzer (Packard 1500 Tricarb, Downers Grove, IL, USA).
To investigate the effect of RP105 deficiency on mast cell proliferation, LDLr$^{-/-}$ and LDLr$^{-/-}$/RP105$^{-/-}$ BMMCs were seeded at a density of 10^4 cells per well. Cells were incubated overnight with 0.5 µCi [^{3}H]thymidine (PerkinElmer) per well. [^{3}H]Thymidine incorporation was measured as described above. The effect of RP105$^{-/-}$ mast cell supernatant on macrophage proliferation was investigated by seeding RAW cells at a density of $4*10^4$ cells per well. The next day, cells were exposed to supernatant of non-activated LDLr$^{-/-}$ or LDLr$^{-/-}$/RP105$^{-/-}$ mast cells (n=4). After overnight incubation 0.5 µCi [^{3}H]thymidine (PerkinElmer), proliferation was measured as described above.

Mast cell activation

LDLr$^{-/-}$ and LDLr$^{-/-}$/RP105$^{-/-}$ BMMCs were seeded at a density of 10^6 cells per well (n=3), after which they were stimulated with 1 ng/mL, 10 ng/mL or 100 ng/mL LPS (from E. Coli, Sigma-Aldrich, St. Louis, MO, USA). After 4 hours and 24 hours activation, cells were spun down (1500 rpm, 5 minutes) and the supernatant was collected for further analysis. ELISAs were performed according to manufacturer's protocol. Expression of the surface markers RP105 and TLR4 on control LDLr$^{-/-}$ mast cells was determined by means of FACS analysis (FACS Canto, BD Biosciences).

RNA isolation, cDNA synthesis and qPCR

Guanidine thiocyanate (GTC) was used to extract total RNA from BMMCs and BMDMs[19]. RNA was reverse transcribed by M-MuLV reverse transcriptase (RevertAid, MBI Fermentas, Leon-Rot, Germany) and used for quantitative analysis of mouse genes (Supplemental Table 1) with an ABI PRISM 7700 Taqman apparatus (Applied Biosystems, Foster City, CA, USA). Murine hypoxanthine phosphoribosyltransferase (HPRT) and murine ribosomal protein 27 (RPL27) were used as standard housekeeping genes.

Statistical analysis

Data are expressed as mean±SEM. A 2-tailed Student's t-test was used to compare individual groups. Non-Gaussian distributed data were analyzed using a Mann-Whitney U test. Frequency data analysis was performed by means of the Fisher's exact test. A level of $P<0.05$ was considered significant.

Results

RP105$^{-/-}$ mice show aggravated vein graft lesion development

Vein grafts were placed in RP105$^{-/-}$ mice and control C57BL/6 mice to investigate the effect of RP105 deficiency on vein graft disease. Interestingly, lack of RP105 resulted in a 90% increased vein graft lesion area (C57BL/6: 0.36 ± 0.02 mm^2; RP105$^{-/-}$: 0.69 ± 0.12 mm^2; Figure 1A; $P<0.05$).

Plaque morphology was further analyzed by staining for macrophages, smooth muscle cells and collagen. The percentage of lesional macrophages was significantly increased in mice deficient for RP105 (C57BL/6: 2.2 ± 1.8%; RP105$^{-/-}$: 7.5 ± 0.9%; Figure 1B; P<0.05), while no changes were observed in the percentage of smooth muscle cells between the two groups (Figure 1C). Picrosirius red staining revealed a significant reduction of collagen content in the lesions of RP105$^{-/-}$ mice compared to control mice (C57BL/6: 63.0 ± 3.0%; RP105$^{-/-}$: 54.6 ± 1.6%; Figure 1D; P<0.05), which indicates that the lesional phenotype may be more unstable.

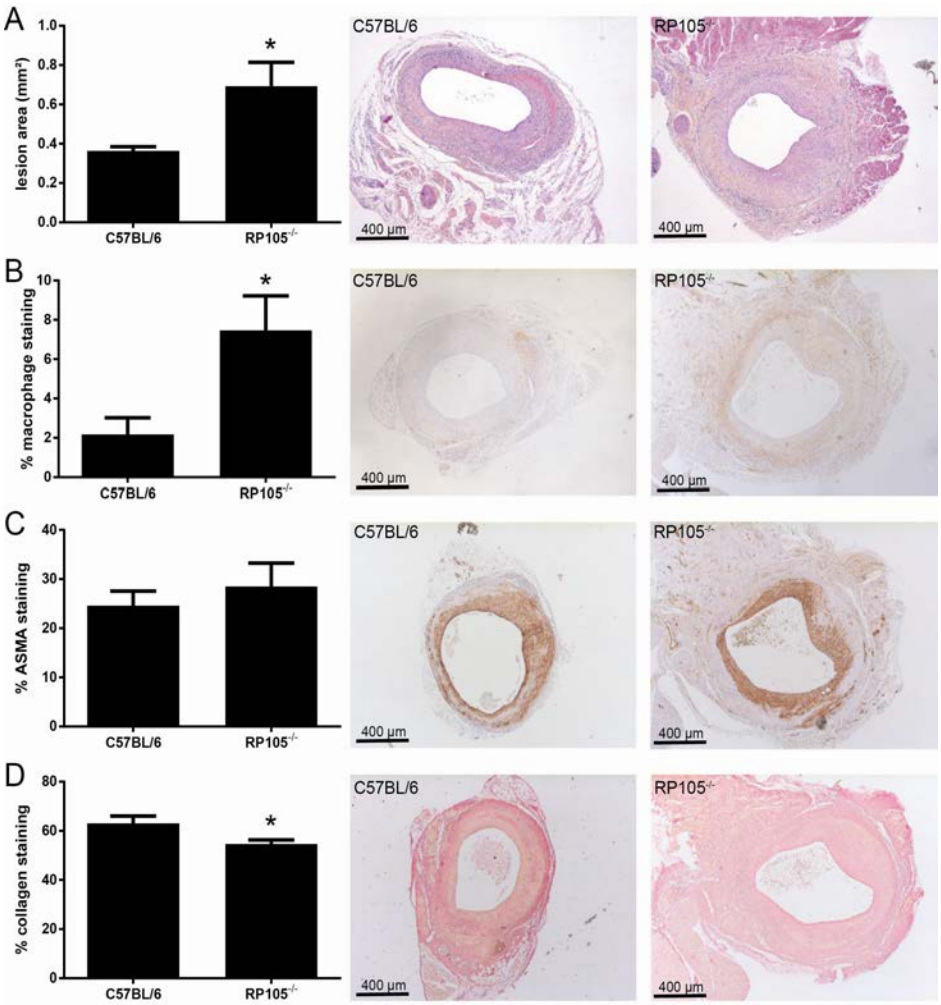

*Figure 1. Vein graft lesion area was significantly increased in RP105$^{-/-}$ mice compared to control C57BL/6 mice (A). The macrophage content, expressed as the percentage of stained area in the intimal hyperplasia, was higher in RP105$^{-/-}$ mice compared to control (B), while no changes were found in the percentage of smooth muscle cells (ASMA, alpha-smooth muscle cell actin, C). Collagen content was decreased in RP105$^{-/-}$ mice, indicative of less stable lesions (D). The micrographs show representative images of each group (50x). N=11 C57BL/6 mice/group, N=13 RP105$^{-/-}$ mice/group. *P<0.05.*

To further determine lesion stability, we analyzed sections for the presence of erythrocytes in the lesions accompanied by fibrin layers. In RP105$^{-/-}$ mice, 3 out of 13 mice displayed plaque dissections, while 0 out of 11 disruptions were observed in C57BL/6 mice.

LDLr$^{-/-}$/RP105$^{-/-}$ mice display lesions with an unstable phenotype

Next, we investigated whether high-fat diet feeding would alter the effects of RP105 deficiency on vein graft disease. LDLr$^{-/-}$/RP105$^{-/-}$ mice and LDLr$^{-/-}$ mice as controls received autologous vein grafts while fed a western-type diet for 4 weeks. No changes were detected in total bodyweight or plasma total cholesterol levels between the two groups (data not shown). In contrast to mice on chow diet, no changes in vein graft lesion area were observed in LDLr$^{-/-}$/RP105$^{-/-}$ mice compared to control LDLr$^{-/-}$ mice (LDLr$^{-/-}$: 0.59 ± 0.05 mm^2; LDLr$^{-/-}$/RP105$^{-/-}$: 0.54 ± 0.05 mm^2; Figure 2A; P=NS).

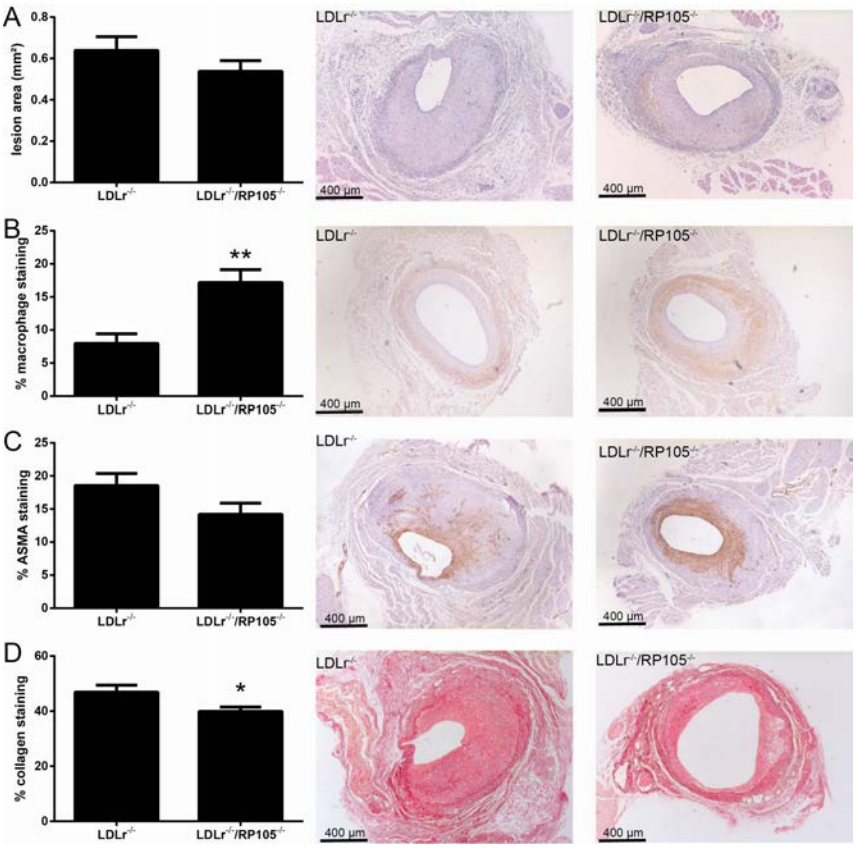

*Figure 2. No changes were observed in the vessel wall area of LDLr$^{-/-}$/RP105$^{-/-}$ mice compared to control LDLr$^{-/-}$ mice (A). Similar to mice fed a chow diet, a higher percentage of macrophages was observed in LDLr$^{-/-}$/RP105$^{-/-}$ (B). Smooth muscle cell content was unaltered between the two groups (ASMA, C) and again a decrease in lesional collagen was observed (D). The micrographs show representative images of each group (50x). N=12 LDLr$^{-/-}$ mice/group. N=12 LDLr$^{-/-}$/RP105$^{-/-}$ mice/group. *P<0.05. **P<0.01.*

Analysis of plaque morphology showed an increase in macrophage staining in lesions of LDLr$^{-/-}$/RP105$^{-/-}$ mice compared to control (LDLr$^{-/-}$: 8.0 ± 1.5%; LDLr$^{-/-}$/RP105$^{-/-}$: 15.9 ± 2.2%; Figure 2B; P<0.05). A trend towards a reduction in smooth muscle cell staining was observed in mice deficient for RP105 (P=0.069; Figure 2C), and furthermore, lesional collagen content was significantly reduced in these mice (LDLr$^{-/-}$: 46.9 ± 2.5%; LDLr$^{-/-}$/RP105$^{-/-}$: 39.9 ± 1.6%; Figure 2D; P<0.05). Interestingly, the total number of plaque dissections and intraplaque hemorrhages was profoundly increased in LDLr$^{-/-}$/RP105$^{-/-}$ mice compared to LDLr$^{-/-}$ control mice (LDLr$^{-/-}$ mice: 3 out of 12 mice; LDLr$^{-/-}$/RP105$^{-/-}$: 10 out of 12 mice; Figure 3A; P<0.05). Also, the average length of the dissections was significantly increased in the LDLr$^{-/-}$/RP105$^{-/-}$ mice (Figure 3B; P<0.05).

A

strain	*n*	*dissections*
LDLr$^{-/-}$	12	3
LDLr$^{-/-}$/RP105$^{-/-}$	12	10
		P=0.01

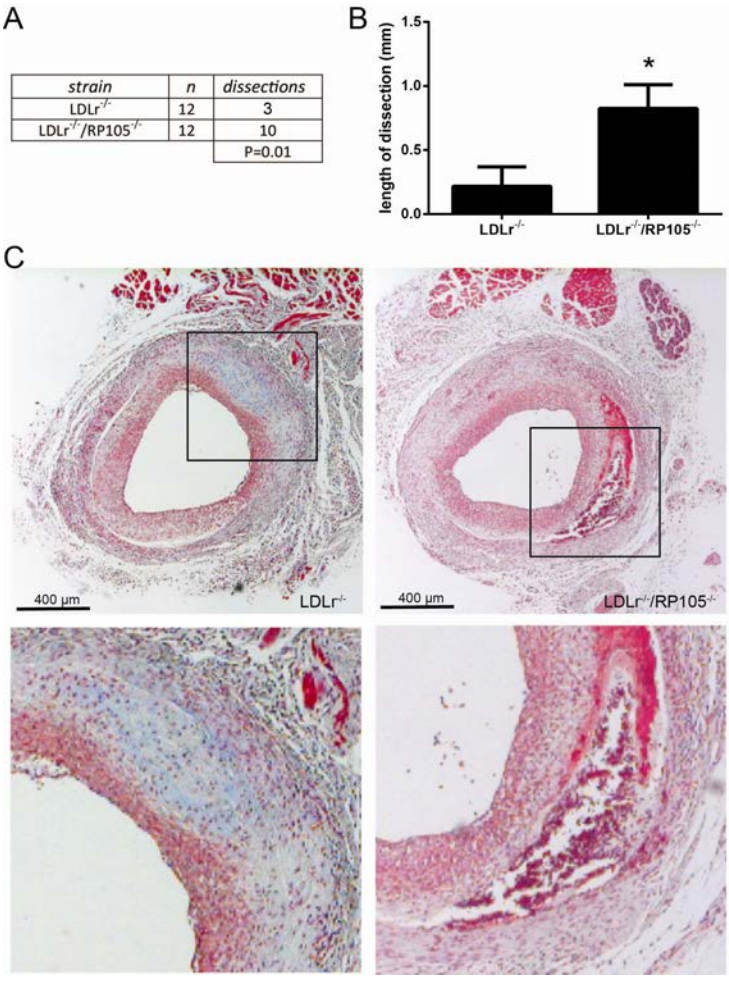

*Figure 3. The number of plaque dissections in LDLr$^{-/-}$/RP105$^{-/-}$ mice was significantly higher compared to control LDLr$^{-/-}$ mice (A). Also, the total length of plaque dissections was increased in LDLr$^{-/-}$/RP105$^{-/-}$ mice compared to control (B). The micrographs show representative images of each group (50x). N=12 LDLr$^{-/-}$ mice/group. N=12 LDLr$^{-/-}$/RP105$^{-/-}$ mice/group. *P<0.05.*

Unaltered MMP expression in RP105 deficient macrophages

As we observed a reduced collagen content in the lesions of RP105 deficient mice, and matrix metalloproteinases (MMPs) are known for its involvement in collagen homeostasis, we measured MMP expression in macrophages. However, no major changes were found in the expression of MMP2, MMP8 and MMP9 as well as in the expression of TIMP1, TIMP2 and TIMP3 at baseline or after stimulation with a concentration range of LPS in RP105$^{-/-}$ macrophages compared to control (Supplemental Figure 1).

Macrophage proliferation rate is not altered by RP105 deficiency

To elucidate the mechanisms behind the increased percentage of lesional macrophages observed in both *in vivo* studies, we aimed to determine macrophage proliferation, since it has recently been described that local macrophage proliferation may add to the lesional burden[20]. We cultured RP105 deficient macrophages and control macrophages and determined the cellular proliferation rate, which was unaltered under basal conditions (Supplemental Figure 2).

RP105$^{-/-}$ smooth muscle cells produce increased levels of CCL2

To further examine why we observe the increased percentage of lesional macrophages in RP105 deficient mice, we determined CCL2 secretion *in vitro*, which is known to be one of the key chemokines involved in monocyte recruitment to the lesion[21]. Since the smooth muscle cell is one of the major determinants of vein graft disease, we investigated whether smooth muscle cells deficient in RP105 produce altered levels of CCL2. Indeed, primary cultured smooth muscle cells lacking RP105 were seen to secrete significantly increased CCL2 amounts after stimulation with LPS, compared to control smooth muscle cells (Figure 4; P<0.05).

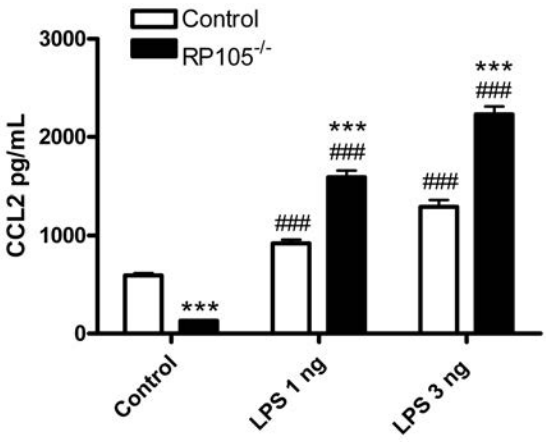

*Figure 4. After 24 hours stimulation with 1 and 3 ng/mL LPS, RP105$^{-/-}$ vascular smooth muscle cells (vSMCs) secrete dose-dependent increased levels of CCL2, which was significantly higher compared to control vSMCs (N=4). ***P<0.001 compared to control vSMCs. ###P<0.001 compared to unstimulated vSMCs.*

RP105 deficiency aggravates mast cell activation

In addition to the smooth muscle cell, we have previously shown that mast cells also play a key role in vein graft disease[22]. Moreover, mast cells can contribute to plaque destabilization, for instance via the degradation of collagen by mast cell derived tryptase and chymase[23,24]. Therefore, we aimed to determine the relevance of RP105 in mast cell function. First, we analyzed whether mast cells express RP105, which is indeed the case at both mRNA and protein level (Supplemental Figure 3A,B). Next, we examined whether RP105 plays a functional role in mast cell activation by culturing bone marrow derived mast cells from RP105 deficient mice and control mice. Mast cells were stimulated with 10 ng/mL and 100 ng/mL LPS for 4 and 24 hours. Interestingly, RP105 deficient mast cells were seen to secrete increased levels of IL-6 and TNFα compared to control mast cells, which was dose-dependent increased after 4 hours of LPS activation (Figure 5A,B). Also, CCL2 secretion of RP105$^{-/-}$ mast cells was highly increased compared to control mast cells, which may also have contributed to the increased lesional macrophages *in vivo* (Figure 5C). Furthermore, 24 hours of activation still showed a significant increase in IL-6 and CCL2 production of RP105$^{-/-}$ mast cells compared to control. These data indicate that RP105 deficiency on the mast cell results in increased activation and cytokine release.

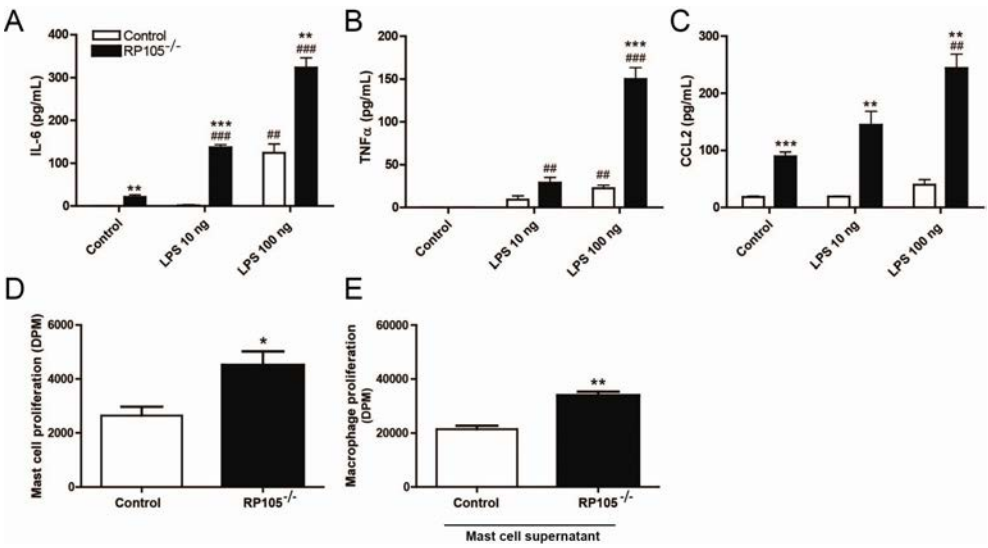

*Figure 5. After 4 hours stimulation with 10 ng/mL and 100 ng/mL LPS, bone marrow derived RP105$^{-/-}$ mast cells secreted dose-dependently increased levels of IL-6 (A), TNFα (B) and CCL2 (C), which was significantly higher compared to control mast cells (N=3). The basal proliferation rate of RP105$^{-/-}$ mast cells was seen to be significantly higher compared to control mast cells (D) (N=4). Addition of the supernatant from RP105$^{-/-}$ mast cells to macrophages resulted in increased proliferation compared to the addition of supernatant from control mast cells (E) (N=4). *P<0.05. **P<0.01. ***P<0.001 compared to control mast cells. ##P<0.01. ###P<0.001 compared to unstimulated mast cells.*

We then investigated whether cellular proliferation is altered in RP105 deficient mast cells. Interestingly, RP105 deficient mast cells show an increased proliferation rate compared to control mast cells under basal conditions (Figure 5D).
Since we observed an increased activation status of RP105$^{-/-}$ mast cells, and mast cells are known to secrete a variety of growth factors, we assessed whether the releasate of activated RP105$^{-/-}$ mast cells has differential effects on macrophages compared to control mast cells. Indeed, after adding supernatant of RP105$^{-/-}$ mast cells to macrophages, we observed an increased proliferation of macrophages compared to the addition of supernatant from control mast cells (Figure 5E).

Increased perivascular mast cell numbers and activation status in vivo
Considering the profound effects of RP105 deficiency on the mast cell found *in vitro*, we aimed to determine whether mast cell numbers and activation status *in vivo* are altered as well. Indeed, the average number of peri-adventitial mast cells was increased in RP105$^{-/-}$ mice compared to control mice (C57BL/6: 0.67 ± 0.28 mast cells/mm^2; RP105$^{-/-}$: 3.24 ± 0.98 mast cells/mm^2; Figure 6A; $P<0.05$). Moreover, in RP105$^{-/-}$ mice the average number of activated mast cells was significantly higher (C57BL/6: 0.29 ± 0.12 activated mast cells/mm^2; RP105$^{-/-}$: 1.80 ± 0.60 activated mast cells/mm^2; Figure 6B; $P<0.05$).
In LDLr$^{-/-}$ mice, basal mast cells levels were increased already upon western type diet feeding compared to C57BL/6 controls (5.2 ± 0.5 mast cells/mm^2), which was further increased in mice lacking RP105, however this did not reach significance (7.4 ± 1.0 mast cells/mm^2; Figure 6C; $P=0.062$). Also, the number of activated mast cells showed a trend towards an increase in LDLr$^{-/-}$/RP105$^{-/-}$ mice compared to control (LDLr$^{-/-}$: 3.0 ± 0.3 mast cells/mm^2; LDLr$^{-/-}$/RP105$^{-/-}$: 4.1 ± 0.5 mast cells/mm^2; Figure 6D; $P=0.057$).

Increased CCL2 expression in vivo
Taking into consideration the increased amount of CCL2 secreted *in vitro* by both RP105 deficient smooth muscle cells and mast cells, we aimed to determine whether lesional CCL2 expression was increased in RP105$^{-/-}$ mice as well. Intriguingly, the CCL2 positive area in the lesions was significantly increased in RP105$^{-/-}$ mice compared to control mice (C57BL/6: 0.03 ± 0.007 mm^2; RP105$^{-/-}$: 0.7 ± 0.008 mm^2; Figure 7A; $P<0.01$). Also, the lesional CCL2 positive area in hypercholesterolemic LDLr$^{-/-}$/RP105$^{-/-}$ mice was profoundly increased compared to control mice (LDLr$^{-/-}$: 0.08 ± 0.01 mm^2; LDLr$^{-/-}$/RP105$^{-/-}$: 0.3 ± 0.05 mm^2; Figure 7B; $P<0.001$). The higher expression of CCL2 in vein graft lesions of RP105 deficient mice may thus explain the increased macrophage content observed in these lesions. The expression of lesional CCR2, the receptor for CCL2 remained unaltered in both experimental groups (Figure 7C,D).

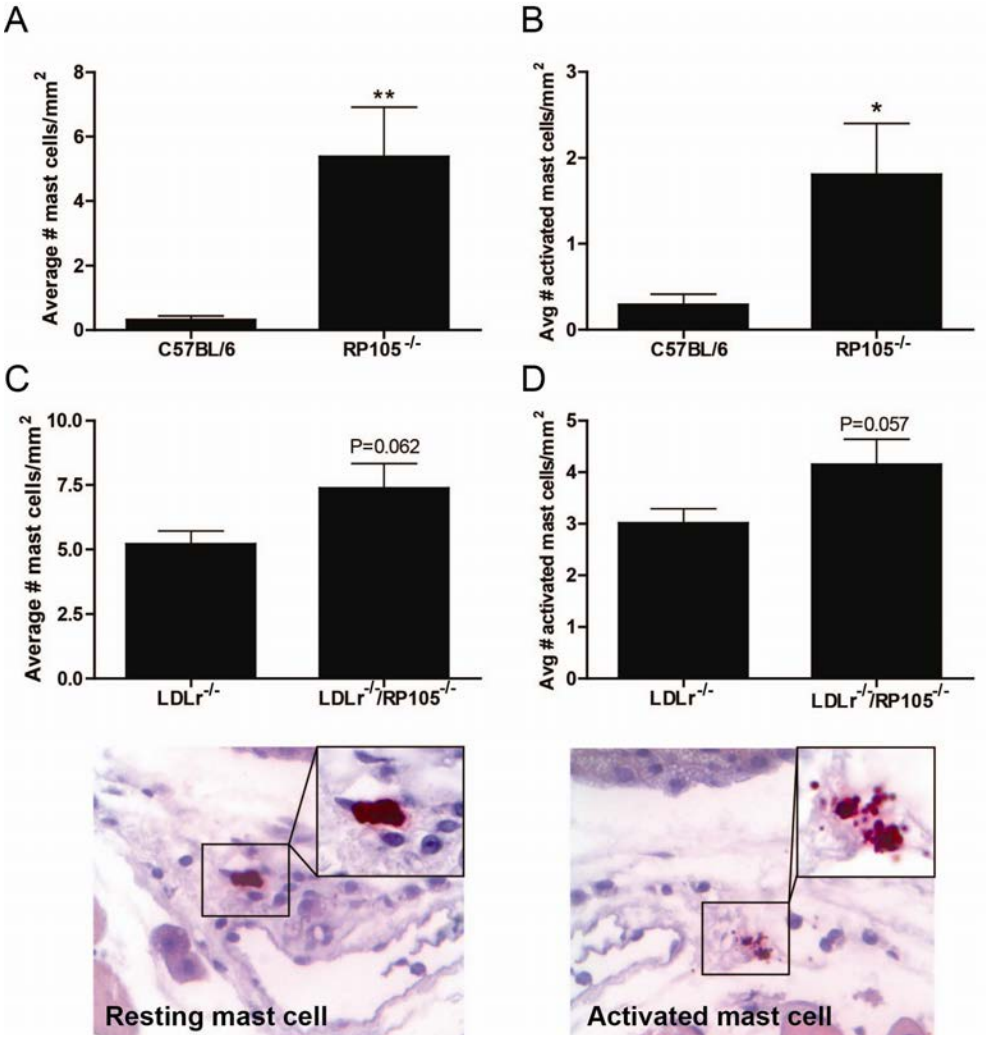

*Figure 6. The amount of perivascular mast cells was markedly increased in the perivascular tissue from vein grafts of RP105 deficient mice compared to control C57BL/6 mice (A). Also, the number of activated mast cells was significantly higher in RP105$^{-/-}$ mice (B). Mast cell numbers, as well as the amount of activated mast cells, showed a trend towards an increase in LDLr$^{-/-}$/RP105$^{-/-}$ mice fed a western type diet compared to LDLr$^{-/-}$ mice (C,D). Micrographs show representative pictures of perivascular mast cells in a resting state (left panel) and in an activated state (right panel), with granules clearly surrounding the mast cell N=11 C57BL/6 mice/group, N=13 RP105$^{-/-}$ mice/group, N=12 LDLr$^{-/-}$ mice/group. N=12 LDLr$^{-/-}$/RP105$^{-/-}$ mice/group. (1000x). *P<0.05. **P<0.01.*

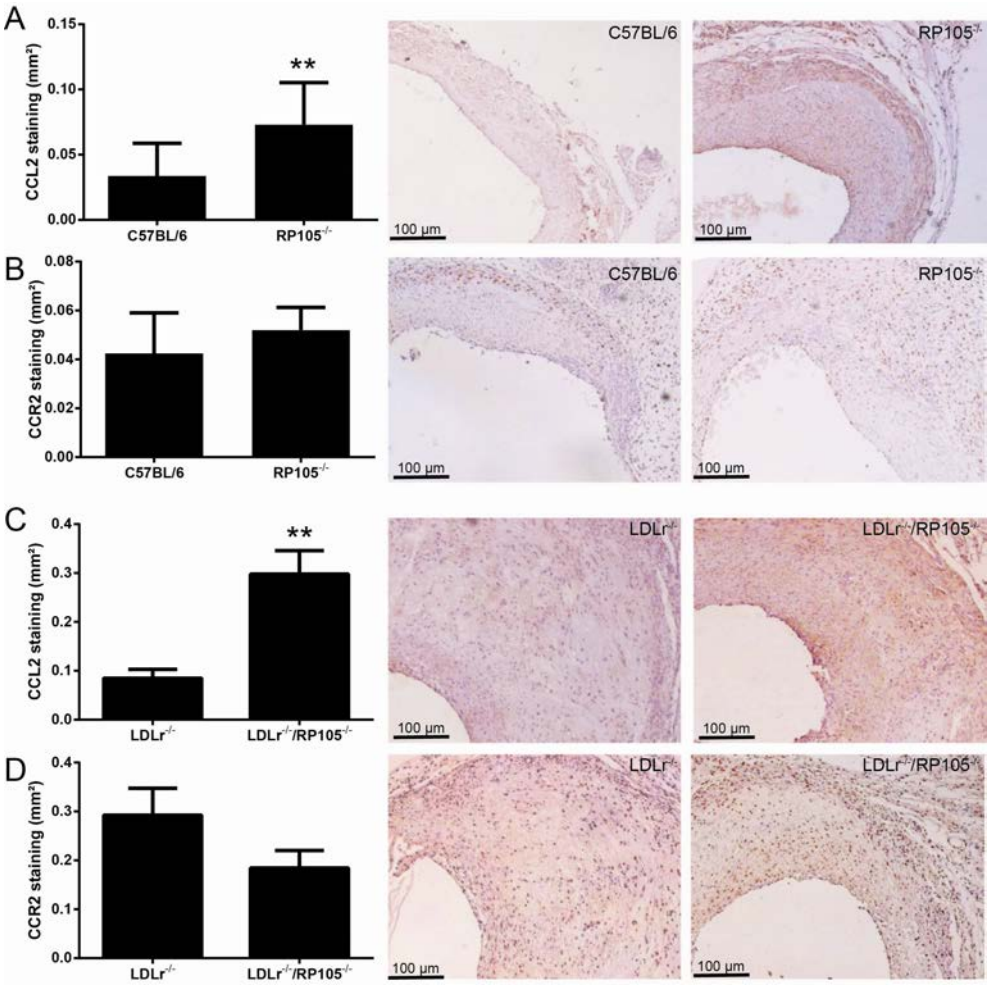

*Figure 7. In vivo CCL2 and CCR2 staining. Immunohistochemical stainings revealed a significant increase in the CCL2 positive area in the lesions of RP105$^{-/-}$ mice compared to control C57BL/6 mice (A). Also, the lesional CCL2 area was profoundly increased in hypercholesterolemic LDLr$^{-/-}$/RP105$^{-/-}$ mice compared to control LDLr$^{-/-}$ mice (C). No differences were observed in lesional CCR2 expression in both experimental groups (B,D). N=11 C57BL/6 mice/group, N=13 RP105$^{-/-}$ mice/group, N=12 LDLr$^{-/-}$ mice/group. N=12 LDLr$^{-/-}$/RP105$^{-/-}$ mice/group. **P<0.01.*

Discussion

In the current study we demonstrate that lack of the regulatory molecule RP105 results in a marked increase in vein graft lesion area. Furthermore, we show that in a hypercholesterolemic setting lesions display an increase in plaque dissections and plaque destabilization. In both studies decreased lesional collagen content was observed while the macrophage content was enhanced. Our *in vitro* data

demonstrate that RP105 deficient smooth muscle cells as well as RP105$^{-/-}$ mast cells secrete excessive levels of CCL2 compared to control cells, which may contribute to the increase of macrophages in the plaque. Moreover, we are the first to demonstrate that RP105 plays a functional role in mast cell activation and proliferation, possibly aggravating lesion destabilization.

Mast cells are potent inflammatory cells which have previously been implicated in vein graft disease and plaque destabilization[23,24,25]. They exert their detrimental effects through the secretion of specific proteases, tryptase and chymase, histamine, VEGF and a variety of cytokines and chemokines, which all contribute to increased inflammation[26]. Activation of mast cells may be induced by a range of mediators, such as IgE; neuropeptides[27]; complement components C5a and C3a[22]; or via Toll-like receptors, in particular TLR4[28]. Whether TLR4 signalling may actually lead to mast cell degranulation is still under debate, however, it is well established that TLR4 activation results in increased proinflammatory cytokine secretion[29]. RP105 is known to inhibit TLR4 signalling in macrophages and dendritic cells; however up to date evidence describing a role for RP105 in mast cells is lacking. As expected, we found a dose-dependent increase of CCL2, IL-6 and TNFα after stimulation of mast cells with LPS. Interestingly, this increase was significantly higher in RP105 deficient mast cells, indicating that these cells, upon activation, secrete excessive amounts of cytokines in its surroundings. We now thus show that also on the mast cell, RP105 has an inhibitory effect and consequently, lack of RP105 leads to increased activation. Therefore, we postulate that the increase in vessel wall thickening observed in RP105$^{-/-}$ mice on a chow diet may at least partly be caused by aggravated mast cell activation. This is in line with previous findings, in which we also show that perivascular mast cell activation by a dinitrophenyl hapten results in exacerbated vein graft thickening[22]. Also, mast cell derived tryptase and chymase have previously been shown to increase collagen degradation[24], which thus may have contributed to the decreased lesional collagen content observed in the current study.

In LDLr$^{-/-}$/RP105$^{-/-}$ mice fed a western type diet, we observed an increase in plaque dissections, however, we did not observe any effects on vessel wall thickening, which was accompanied by less pronounced effects on mast cell activation. Previously, we have seen that upon western type diet feeding, vascular mast cell activation is enhanced (unpublished data). Indeed, control LDLr$^{-/-}$ mice fed a western type diet already displayed higher numbers of activated mast cells, compared to control C57BL/6 mice on a chow diet. Therefore, the additive inflammatory effect of RP105 deficiency on mast cells in mice receiving a high cholesterol diet may be less pronounced.

Previously, we have investigated the effects of RP105 in other mouse models of vascular remodelling as well. Besides vein graft surgery, balloon angioplasty with

stent placement is used for the treatment of atherosclerosis; however in-stent restenosis impairs the success rate of this operation[30]. In a murine mouse model for restenosis we have shown that RP105 deficiency results in increased neo-intima formation[31], which is in line with the observed effects on vein graft disease in the current study. Conversely, in a setting of atherosclerosis, mice on a western type diet lacking RP105 on their myeloid cells develop reduced atherosclerotic lesions due to changes in B cells[32]. Also, total body RP105 deficiency was seen to reduce atherosclerosis and decrease lesional macrophage content, induced by a reduced monocyte influx (unpublished data). These disturbances in monocyte migration were also observed in RP105$^{-/-}$ mice with hind limb ischemia[33]. The fact that we observe an increase in lesional macrophages may be explained by the profound increase in CCL2 secretion by both RP105$^{-/-}$ smooth muscle cells and mast cells, thus resulting in increased monocyte recruitment to the lesion. Indeed, CCL2 expression in the vein graft lesions of mice deficient for RP105 was significantly increased compared to controls. As vein graft lesions are highly enriched in smooth muscle cells compared to atherosclerotic plaques, the effect of smooth muscle cell derived products, such as CCL2, is more prominent in vein graft disease as compared to atherosclerosis. These contrasting effects highlight the difference in underlying mechanisms of diet-induced atherosclerotic lesion development versus restenosis and vein graft disease, the latter two in which smooth muscle cells play a dominant role.

In summary, the current study is the first to demonstrate that lack of RP105 results in increased lesion area in murine vein grafts. In a hypercholesterolemic setting, RP105 deficiency resulted in an increase of unstable lesions and a higher number of plaque dissections accompanied by intraplaque hemorrhage. *In vitro* investigations suggest that this may be caused by excessive CCL2 secretion by RP105$^{-/-}$ smooth muscle cells, as well as by an increased inflammatory and proliferative phenotype of RP105$^{-/-}$ mast cells.

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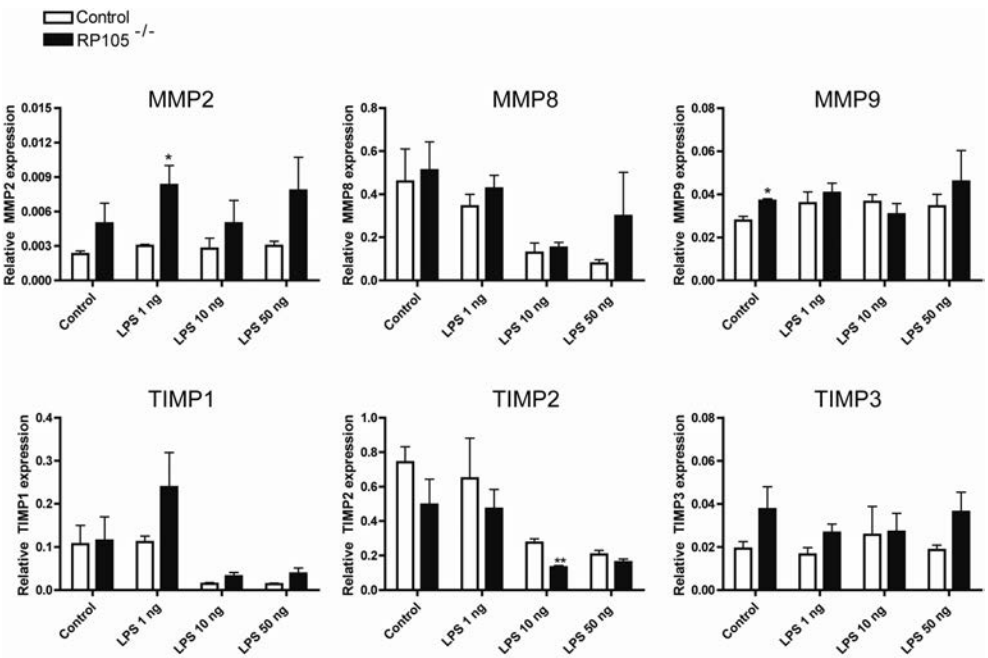
Supplemental data

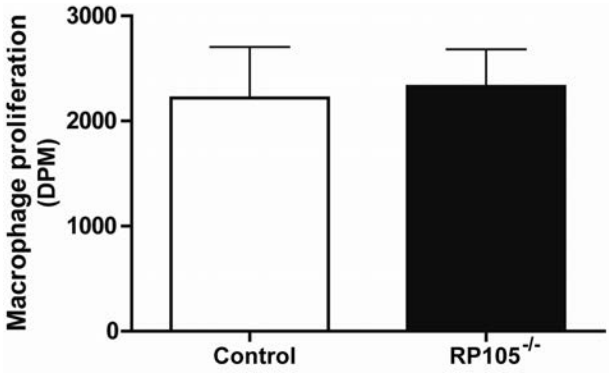
Supplemental table 1. List of primers used for the *in vitro* experiments.

Gene	Forward primer	Reversed primer
RP105	ACCATTCAAAACACGACCTTCAGCAGA	GGGGATTTGCGGTTAGTACAAGTGTGT
TLR4	CCAATTTTTCAGAACTTCAGTGGCTGG	TTGAGAGGTGGTGTAAGCCATGC
MMP2	CCGAGGACTATGACCGGGATA	GGGCACCTTCTGAATTTCCA
MMP8	TGACCTCAATTTCATATCTCTGTTCTG	TCATAGCCACTTAGAGCCCAGTACT
MMP9	CCCTGGAACTCACACGACATCTTC	CTCATTTTGGAAACTCACACGCCAG
TIMP1	ACACCCCAGTCATGGAAAGC	CTTAGGCGGCCCGTGAT
TIMP2	GTTTATCTACACGGCCCCCTCTT	ATCTTGCCATCTCCTTCTGCCTT
TIMP3	ACTGTGCAACTTTGTGGAGAGGT	GAGACACTCATTCTTGGAGGTCA
HPRT	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
β-actin	CGCCAAGCGATCCAAGATCAAGTCC	AGCTGGGTCCCTGAACACATCCTTG

Supplemental Figure 1. Relative expression of MMPs and TIMPs in RP105 deficient and control BM derived macrophages after stimulation with 1 ng, 10 ng or 50 ng LPS, or with control medium.



Supplemental Figure 2. The cellular proliferation rate of control and RP105 deficient BM derived macrophages is unaltered under basal conditions.



Supplemental Figure 3. Bone marrow derived mast cells express RP105 and TLR4 on mRNA (A) and protein level (B).