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**Title:** Damage associated molecular patterns and toll like receptors in inflammation mediated vascular remodeling : mechanistic insights and therapeutic potentials
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An unexpected intriguing effect of TLR regulator RP105 (CD180) on atherosclerosis formation with alterations on B cell activation


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Abstract

Objective In atherosclerosis Toll like Receptors are traditionally linked to effects on tissue macrophages or foam cells. RP105, a structural TLR4 homolog, is an important regulator of TLR signaling. The effects of RP105 on TLR signaling vary for different leukocyte subsets known to be involved in atherosclerosis making it unique in its role of either suppressing (in myeloid cells) or enhancing (in B cells) TLR regulated inflammation in different cell types. We aimed to identify a role of TLR accessory molecule RP105 on circulating cells in atherosclerotic plaque formation.

Approach and results Irradiated LDLr/-/- mice received RP105/-/- or wild-type bone marrow. RP105/-/- chimeras displayed a 57% reduced plaque burden. Interestingly, total and activated B-cell numbers were significantly reduced in RP105/-/- chimeras. Activation of B1 B-cells was unaltered, suggesting that RP105 deficiency only affected inflammatory B2 B-cells. IgM levels were unaltered but anti-oxLDL and anti-MDA-LDL IgG2c antibody levels were significantly lower in RP105/-/- chimeras, confirming effects on B2 B-cells rather than B1 B-cells. Moreover BAFF expression was reduced in spleens of RP105/-/- chimeras.

Conclusion RP105 deficiency on circulating cells results in an intriguing unexpected TLR-associated mechanisms that decrease atherosclerotic lesion formation with alterations on pro-inflammatory B2 B-cells.
Introduction
Atherosclerosis is characterized by infiltration of circulating leukocytes into the intimal area that initiate a local inflammatory response and subsequent atherosclerotic lesion formation\(^1\). Currently, different subtypes of leukocytes are linked to atherosclerotic lesion formation and progression. Both macrophages and T cells are well known contributors to atherosclerosis, but also dendritic cells (DCs), mast cells and B cells were more recently shown to be involved\(^2,^3\).

Toll Like Receptors (TLR) are major contributors to cardiovascular disease development\(^4^-^8\), as they initiate inflammatory responses in both immune and non-immune cells by recognizing pathogen and damage associated molecular patterns (PAMPs and DAMPs respectively) that can be up regulated upon e.g. tissue damage or cell stress\(^9^-^11\). In atherosclerosis TLR function is traditionally linked to its effect on tissue macrophages or foam cells. This mechanism is however not fully elucidated\(^12,^13\). Mice deficient for TLR4 or its downstream adaptor protein Myd88 have reduced atherosclerosis formation\(^14,^15\). TLR activation and signaling is strongly regulated by a number of accessory molecules\(^16\). Activation of TLR4 is dependent on the presence of MD2 which is capable of binding LPS and a variety of other known endogenous ligands\(^17,^18\). Next to MD2, RP105 (CD180) is an important accessory molecules acting as a regulator of TLR signalling\(^19^-^21\). RP105 consists of the same extracellular domain as TLR4, but lacks the Toll like Interleukin Receptor (TIR) domain that regulates downstream signaling and has an, for TLR family members, atypical dimeric structure\(^22\). Interestingly the role of RP105 in modulating inflammatory responses depends on the cell type making it unique in its role of either enhancing or suppressing TLR regulated inflammation in different cell types\(^23\). While it functions as an inhibitor of TLR4 signaling in myeloid cells such as DCs and macrophages, it enhances TLR4-induced activation in B cells\(^24,^25\). Both myeloid cells as well as B and T cells are considered to play a major role in atherosclerosis and these cell types can all be influenced directly or indirectly via TLR signaling and regulation\(^2\). Unlike myeloid cells, B cells do not express MD2, representing a major difference between their respective TLR4 pathways\(^25^-^27\). In MD2 deficient B cells, the RP105/MD1 complex was shown to function as MD2 on these cells\(^25\). RP105 cell surface expression is strongly influenced by MD1, a structural homolog of MD2 and capable of binding lipids in its cavity\(^28,^29\). A recent paper by Allen et al. suggested a mechanistic role for BAFF expression in B cell proliferation of RP105-/- mice\(^30\). Direct and indirect effects of TLR signaling on adaptive immunity players such as T- and B cells could play an important role in enhanced stimulation atherosclerosis development besides their traditional role on myeloid cells\(^31\). The TLR regulator RP105 may play a very important role in the complex TLR pathway mediated atherosclerosis formation by its unique function on different cell types\(^23\).

In this study, we investigated the contribution of RP105 deficiency in atherosclerosis by re-populating lethally irradiated LDLr-/- mice with either wild-type or RP105-/- bone marrow and observed that RP105 deficiency led to an unexpected decrease in atherosclerotic plaque formation indicating a novel route via which the TLR signaling pathway affects atherosclerosis.

Material and Methods
For a detailed description of all materials and methods used, see the supplemental material, available online at http://atvb.ahajournals.org.
**Results:**

**RP105 deficiency ameliorates atherosclerosis**

To assess the role of RP105 in atherosclerosis, we generated bone marrow chimeras by transplantation of RP105-/- or wild type (WT) bone marrow cells in LDLr-/- mice. Animals were allowed to recover for 6 weeks and were subsequently fed a high fat, high cholesterol diet (Western Type Diet (WTD), 0.25% cholesterol and 15% cacao butter (SDS, Sussex, UK)). RP105 deficiency was assessed by flow cytometry on CD19+ B cells, CD11c+ Dendritic cells and CD11b+ monocytes isolated from spleen (Supplemental figure 1) Susceptibility to atherosclerosis in the proximal aortic root of LDLr-/- mice reconstituted with either WT or RP105-/- bone marrow was analyzed after 9 weeks WTD feeding. Cryostat sections of the proximal aortic root showed reduced Oil-red O staining in the RP105-/- chimera transplanted group as compared to the WT transplanted controls. (Figure 1AB). Lesion burden in the proximal aortic root was significantly decreased in the RP105-/- chimeras (230±26x10^3 μm^2 vs 131±15x10^3 μm^2, p=0.004) indicating decreased atherosclerosis formation in these mice (Figure 1C). No differences in bodyweight (supplemental figure 2) or cholesterol levels (supplemental figure 2) were observed between the WT and the RP105-/- chimeras.

**Plaque composition**

Next to the observed effects on lesion size we also assessed plaque composition. Intimal macrophage area was significantly decreased in RP105-/- chimeras (57.2±8.8x10^3 μm^2 vs 25.6±6.5x10^3 μm^2, p=0.007, Figure 1D).
Figure 1. Atherosclerotic lesion formation is attenuated in RP105-/- chimeras. Hematopoietic deficiency of RP105 in atherosclerosis and atherosclerotic plaque composition. LDLr-/- mice were irradiated and subsequently received bone marrow from either RP105-/- or wild type (WT) control mice. After recovery LDLr-/- mice received a high-fat diet for 9 weeks. A. Atherosclerotic plaques were quantified in the aortic root after Oil Red O staining. Representative pictures of Oil Red O-stained plaques from WT (A) and RP105-/- chimeras (B). Intimal lesion area was significantly decreased in RP105-/- chimeras (C). Macrophages, collagen content and T cells were visualized by MoMa, Trichrome or CD3 respectively. Macrophage content (MoMa+ intimal area (D) and relative macrophage content (E). Collagen content (F) and T cell infiltration (intimal (G) and adventitial (H)). Image acquisition was performed on a Leica DMRE microscope with Leica DC 500 camera at 50x magnification (5x/0.15 objective). T cells were counted manually at 1000x magnification (110x/1.30 oil objective). Black bars represent WT chimeras, white bars represent RP105-/- chimeras, n=16, *p<0.05, **p<0.01, ***p<0.001.

Even when correcting the macrophage area for total intimal area we still observed a significant 33% decrease (27.6±3.2 vs 18.5±0.9%, p=0.039, Figure 1E and supplemental figure 2). Collagen content in the plaque was not affected (WT vs RP105-/-; 4.6±0.4 vs. 5.1±0.04 %, p=0.39, Figure 1F and supplemental figure 2). Immunohistochemical analysis of CD3 expression as a T-cell marker revealed that T-cell numbers were significantly reduced in the intimal (13.6±1.9 cells vs 4.4±0.8 cells, p=0.0001, Figure 1G and supplemental figure 2) and perivascular area (48.9±15.9 cells vs 32.0±12.4 cells, p=0.0037, Figure 1H) of the proximal aortic root in the RP105-/- chimeras.

**Decreased B-cell activation in RP105-/- chimeras**

RP105 deficiency is known to result in an increase of pro-inflammatory cytokine production by cultured myeloid cells upon LPS administration. Myeloid cells are known to be important in atherosclerosis development and contain multiple TLRs. Therefore we analyzed the activation status of splenic DC and macrophages in both chimera groups. No difference in activation status of DC, (MHC-II and CD40 expression)(Figure 2A), macrophages (CD40 expression) (figure 2B) or CD4 and CD8 T cells (Figure 2C-D) was found between the chimera groups. Since RP105 was originally described to be highly expressed on B cells, we also investigated B cells activation. Less IgM+ B cells were detected in the RP105-/- chimeras. Concomitantly, a decreased percentage of CD86+ B cells was found, indicative of a decreased number of activated B2-cells in the RP105-/- chimeras (Figure 2E). Subtype analysis showed no difference in B1-cell number or activation status (Figure 2F).

**Proliferation of splenocytes by LPS is reduced in RP105-/- chimeras**

Splenocytes from both chimera groups were stimulated ex vivo with 10 or 100ng/ml LPS and proliferation was assessed by [3H]-Thymidine incorporation. Splenocytes from the RP105-/- chimeras showed a lower degree of proliferation to LPS (proliferation index 1.9±0.5 vs. 3.0±1.6 to 10ng/ml LPS and 2.2±0.6 vs. 6.6±3.0 to 100ng/ml LPS, p<0.05) (figure 3A).

**RP105 deficient B cells have reduced proliferation and are less activated upon TLR4 stimulation**

In order to establish if the observed effect on splenocyte proliferation could be B-cell related, we isolated CD19+ B cells from WT and RP105-/- mice and exposed these cells to LPS and anti-CD40mAb to stimulate B-cell surface receptors. In agreement with earlier observations, RP105 deficient cells were less responsive to LPS (proliferation index: 22.6±3.7
vs. 58.2±7.2 to 100ng LPS, p=0.0001 and 85.7±4.7 vs. 129.7±12.8 to 1µg LPS, p=0.0007), but not to anti-CD40mAb stimulation (135.0±0.6 vs. 114.7±18.0, p=0.12, figure 3B).

The observed decrease in proliferation may reflect altered cell survival, cell cycle progression or both. Cell cycle progression was then assessed by monitoring CFSE dilution. Loss of CFSE fluorescence, indicative of cell division, was measured by flow cytometry on day 3 and was strongly reduced in the RP105-/- B cells upon LPS stimulation, but not upon anti-CD40mAb, indicating a TLR4-specific induction of cell cycle arrest (Figure 3C). B cells from RP105-/- mice also produced less IL-6 (supplemental figure 1) and showed less phenotypical activation (CD86 and CD25) upon TLR4 but not CD40 activation (supplemental figure 3).

Figure 2. Decreased B Cell activation in the spleen of RP105-/- chimeras. Splenocytes from LDLr-/- chimeras were harvested and analyzed for cell subtype and activation status by flow cytometry. Activation status of CD11c+ DCs (identified by high MHCII expression or CD40 expression) (A) and F4/80+ macrophages (identified by CD40 expression) (B). No difference in CD4 or CD8 T cell activation was observed. Activated CD4+ (C) and CD8+ T (D) cell subsets identified by low CD62L or high CD69 expression. B cells were selected as CD19 positive cells. Percentage of IgM/CD19 and CD86/CD19 positive cells (E). Percentage of B1-cells expressing IgM or CD86 (F). Black bars represent WT chimeras, white bars represent RP105-/- chimeras, n=6, *p<0.05.
Together, these results indicate that RP105 modulates B-cell function through cell surface receptor TLR4 but not CD40.

**Altered levels of IgG but not IgM specific antibodies**
Plasma IgM and IgG antibody titers against oxLDL and MDA-LDL were determined at sacrifice. Ox-LDL IgG and IgG2c and MDA-LDL specific IgG2c isotype antibodies were
significantly reduced in the RP105-/- chimeras (Figure 4A/C/E). The exact role of IgG isotype antibodies is however still under debate. Interestingly we did not observe differences in oxLDL or malondialdehyde-modified LDL (MDA-LDL) specific IgM production, indicating effects of RP105 predominately on B2 but not B1a cells (Figure 4B/D). Phosphocholine (PC)-specific IgM T15/EO6, which is a B1a-cell derived natural antibody was also not different between the chimera groups, supporting the hypothesis that B1a IgM production was not altered and in line with the finding that B1-cell number and activation was not affected (Figure 4F).

Figure 4. Reduced oxLDL and MDA-LDL IgG(2c) titers in RP105-/- chimeras. Antibody titers were measured in plasma by chemiluminescent ELISA. Blood was harvested at sacrifice of the chimera hypercholesterolemic LDLr-/- mice. Total IgG against cu-oxLDL (A), IgG2c against cu-oxLDL (B), IgG2c against MDA-LDL (C), Total IgM against cu-oxLDL (D), total IgM against MDA-LDL (E) and PC-specific IgM T15/EO6 (F). Black bars represent WT chimeras, white bars represent RP105-/- chimeras and data is presented as Reflective Light Units (RLU), WT n=10, RP105-/- n=15, *p<0.05.

Decreased cytokine expression in spleens of RP105-/- chimeras.
In atherosclerosis increased expression of cytokines in the plaque usually parallels that in splenocytes. mRNA levels of IL6, IP-10, IL12 and IL10 were significantly reduced in spleens of the RP105-/- compared to WT chimeras (Figure 5A-D). Even more interesting we found a reduced expression of B-cell activating factor (BAFF) in the spleens of the RP105-/- chimeras (Figure 5E). These reductions were representative since total numbers and percentages of different leukocyte cell types in the spleen were not different between
the groups (supplemental table 1). Interestingly basal BAFF expression in splenocytes of normal WT or RP105-/- mice is much lower compared to the chimera groups receiving a western type diet. Moreover full body RP105 knockout mice on a normal diet show a trend towards higher instead of lower BAFF expression in the splenocytes (P=0.08) compared to WT mice (supplemental figure 4).

Discussion
In the present study we show that deficiency of the TLR regulator RP105 on circulating cells in LDLr-/- mice results in an unexpected 57% reduction in atherosclerotic lesion formation. This finding is quite intriguing since the hypothesis thus far was that TLR4 activation on macrophages stimulates atherosclerotic lesion development, and consequently that in the absence of the TLR4 inhibitor RP105 atherosclerotic lesion development would be enhanced. Here we show for the first time an unexpected regulation of atherosclerosis formation via TLR regulator RP105 with effects on inflammatory B2 B cells and BAFF expression but not on myeloid cells. Hereby it shows a novel manner of TLR pathway mediated atherosclerosis formation via B cell activation that was stronger than the TLR4 modulation by RP105 on macrophages.

Figure 5. Reduced cytokine expression in the spleens of RP105-/- chimeras.

mRNA expression of the cytokines IL6, IP10, IL10, p35 and p40 (subunits of IL12) in spleens. Relative expression of IL6 (A) IP-10 (B) IL-12 subunits p35 and p40 (C), IL10 (D) and BAFF (E) in WT and RP105-/- chimeras at time of sacrifice. Black bars represent WT chimeras, white bars represent RP105-/- chimeras, WT n=10, RP105-/- n=11, *p<0.05, **p<0.01, ***p<0.001.
In atherosclerosis TLR function is traditionally linked to its effect on tissue macrophages or foam cells. This mechanism is however not fully elucidated. The modulating capacities of RP105 on inflammatory responses depend on the cell type making it unique in its role of either enhancing or suppressing TLR regulated inflammation in different cell types. In our experiments we could notice effects on inflammatory B2 B cells and BAFF expression but not on myeloid cells. Although currently the role of B cells in atherosclerosis receives much attention, several issues of controversy exist\textsuperscript{32}. Interestingly the crosstalk between TLR and B cell receptor signals plays a crucial role in B cell responses to pathogens\textsuperscript{13}. B cells are considered to play a major role in the pathophysiology of autoimmune diseases\textsuperscript{34} and during the last decade a number of studies have also indicated an important role for TLRs in autoimmunity\textsuperscript{35}. Interestingly, patients with autoimmune-like diseases such as systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA) have an increased risk of atherosclerotic plaque development\textsuperscript{36}. B cells can affect atherosclerosis development via antibody production to modified LDL\textsuperscript{37}. Besides their important role in antibody production, B cells are also capable of producing cytokines and thereby can have an additional effector role to their regulating role in inflammation. The cytokine producing effector B cells are most likely derived from B2 subtype mature follicular B cells, while B1 cells are IgM antibody producing cells\textsuperscript{38}.

We now demonstrate that RP105 deficiency on hematopoietic cells reduced atherosclerotic plaque formation with reduced B cell numbers and considerably less activated B cells in the RP105-/- mice, as indicated by less CD19+ IgM+ CD86+ cells. CD86 is a B7 family member and important for co-stimulation of other cells like T cells\textsuperscript{39}. The effect of RP105 deficiency on cell number and activation was not observed on B cell subtype B1 cells (CD4-, CD5+) and we did consequently not observe effects on the levels of IgM antibodies, which are mainly produced by these cells. Our in vitro studies confirmed that RP105 affects B2 B cells via modulation of the TLR4 response. LPS, as a TLR4 cell surface stimulator, but not anti-CD40, as a CD40 cell surface stimulator, caused multiple effects on B cells. The function of RP105 is complex since it has a divergent role on myeloid and B cells\textsuperscript{16}. The differential effects of RP105 on myeloid and B cells may be related to the lack of MD2 in B cells\textsuperscript{25, 27} and the capability of RP105 to directly cause B-cell activation\textsuperscript{40}.

Previously, Ait-Oufella and co-workers showed that the atheroprotective effect of CD20+ B cell depletion may be due to effects on T cell activation. They showed this mainly depended on reduction in IgG-type antibodies and not in IgM-isotype antibody titers\textsuperscript{41}. In addition Kyaw and co-authors showed that B2 B cells may enhance atherosclerosis without presence of other inflammatory cells and independent of antibody levels\textsuperscript{42}. Both depletion studies noticed a decrease in macrophages in the plaque while Ait-Oufella et al, also observed a reduction in T-cell accumulation\textsuperscript{41}. Our data is in agreement with both studies showing a decrease in both T cells and macrophages in the plaque. More recent studies on the mechanism of B cell mediated atherosclerotic plaque formation demonstrated a role for B cell activating factor (BAFF) receptor. BAFF is required for B cell maturation and supports the survival of self reactive B cells\textsuperscript{43}. BAFF receptor deficiency in bone marrow cells resulted in reduced B2 B cells and attenuated atherosclerotic lesion development\textsuperscript{44}. Depletion of B2 B cells in BAFF receptor knockout mice inhibited lesion development by ameliorating vascular inflammation\textsuperscript{45}. In agreement with these observations, we now found reduced expression of BAFF and B2 B cell responses in the RP105-/- chimeras that had less plaque formation. Recently modulation of B cell proliferation by RP105 was mechanistically related to BAFF expression\textsuperscript{30}. In agreement we found an increase of BAFF expression in
splenocytes of full body WT and full body RP105-/- mice on a normal diet. Interestingly both chimera groups on a western type diet showed actually much higher BAFF expression. In the chimeras however BAFF expression in splenocytes of RP105-/- chimeras was significantly lower to WT chimera mice. This data suggests an association of BAFF expression with RP105 which may play an important role in effects seen on atherosclerosis in the RP105-/- chimeric LDRr-/-.

Additionally we found a decrease in mRNA expression of cytokines/chemokines such as IP-10 in the spleens of the RP105-/- chimeras. In atherosclerosis increased expression of cytokines in the plaque usually parallels that in splenocytes and since IP-10 is an important T-cell attractant this may partly explain differences in T-cell numbers in the plaque of the RP105-/- chimeras. In contrast hypercholesterolemic mice lacking B cells showed an increase in atherosclerosis, an effect attributed to the loss of protective natural IgM antibodies, the opposite effect of the discussed studies that depleted B cells via anti-CD20 administration. Lifelong deficiency in B cells will certainly influence (long lived) plasma cell formation and antibody production by these cells, and may very well explain the different findings in these studies. Specific depletion by anti-CD20 will spare CD20 deficient long-lived plasma cells. Interestingly autoimmune disease patients treated with anti-CD20 humanized antibody (Rituximab) show extended periods of clinical remission without reductions in antibody titers. Particularly in autoimmune settings B cells can actively promote atherosclerosis. Using Rituximab would however be a difficult therapy for CVD patients since statins may impair its effect by inducing conformational changes on CD20. This would make new therapeutic targets on B cells such as RP105 even more interesting. In addition SLE patients with a history of CVD showed elevated titers of oxLDL and malondialdehyde-modified LDL (MDA-LDL) specific IgG antibodies compared with other SLE patients without CVD or population controls. In the RP105-/- chimeras we also found a reduction in IgG2c titers against oxLDL and MDA-LDL while IgM levels against oxLDL or MDA-LDA were not altered. IgM titers specifically derived from B1a B cells were also not altered, thus confirming our results on B1 B cell number and activation and thereby indicating the cell specific effects of RP105 via TLR signaling on inflammatory type B2 B cells.

Recently new data suggests that RP105 is expressed in epididymal white adipose tissue on stromal vascular fractions and has an important role in the induction of adipose tissue inflammation. High fat diet induced obesity, adipose tissue inflammation and insulin resistance are hampered in RP105-/- mice. On long term these processes are associated with hypertension, diabetes and coronary disease. In our study we did not use full body knockout mice but performed bone marrow transfer to LDLR-/- mice. At sacrifice we did not observe any difference in body mass of LDLR-/- on a high fat diet that received RP105-/- bone marrow cells compared to mice that received WT bone marrow. Therefore effects of insulin resistance are not likely to be involved in the effects on atherosclerotic lesion formation we observed here. Nevertheless, the protective effects of RP105 deficiency on atherosclerosis in our study combined with previously published attenuation of obesity and insulin resistance by RP105 deficiency definitely point out the potential of RP105 as therapeutic target for disease processes that are considered to be responsible for the highest morbidity and mortality numbers in the western world.

In conclusion RP105 is an important TLR regulator that influences atherosclerotic plaque formation with strong effects on B2 B cells and BAFF expression without directly affecting myeloid subsets. This may have strong implications for the role of TLR signaling in athero-
sclerosis and development of novel therapeutic approaches.

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Disclosures:
None

Reference List


Significance
This is the first paper to provide evidence of RP105 involvement in atherosclerotic plaque formation with effects on B2-cells and BAFF expression. We show a previously unknown involvement of TLR signaling in B-cell regulation in atherosclerosis indicating a novel route via which the TLR signaling pathway affects atherosclerosis. These effects seem to be more pronounced than effects of RP105 deficiency on TLR signaling on monocytes/macrophages, a process thought to enhance atherosclerosis. This makes RP105 an interesting new therapeutic target and our findings not only identify a novel mediator of atherosclerotic plaque formation but may have strong implications for the understanding the underlying mechanism of TLR signaling in atherosclerosis and consequently the development of novel therapeutic approaches. This might even contribute to the explanation why patients with autoimmune diseases are prone to develop more atherosclerosis as in autoimmune settings B cells can actively promote atherosclerosis.
Online supplement: Material and methods

Animals
LDL receptor (LDLr)−/− and RP105−/− mice (on C57BL/6 background) were obtained from the local animal breeding facility. WT controls were obtained from Charles River. All experimental protocols were approved by the ethics committee for animal experiments of Leiden University Medical Center.

Bone marrow transplantation
To induce bone marrow aplasia, male LDLr−/− recipient mice were exposed to a single dose of 9 Gy (0.19 Gy/min, 200 kV, 4 mA) total body irradiation using an Andrex Smart 225 Röntgen source (YXLON International) with a 6-mm aluminum filter 1 day before the transplantation. Bone marrow was isolated by flushing the femurs and tibias of RP105−/− and WT mice with PBS. Subsequently, the cell suspension was gently filtered through a 70μm cell strainer to obtain a single cell suspension (70μm pores, BD Bioscience). Irradiated recipients received 0.5×10^7 bone marrow cells by tail vein injection. Drinking water was supplied with antibiotics (83 mg/liter ciprofloxacin and 67 mg/liter polymyxin B sulfate) and 6.5 g/liter sucrose for the first three weeks after irradiation. Thereafter animals received normal drinking water ad libitum. After a six week recovery period, animals were placed on a Western-type diet containing 0.25% cholesterol and 15% cacao butter (SDS) diet for 9 wk and subsequently sacrificed. N=17 WT, N=18 RP105−/−, male mice, 12 weeks of age at start of the experiment.

Flow Cytometry
Spleens were harvested and single-cell suspensions of splenocytes were prepared by gently mincing the spleen through a cell strainer (70μm pores, BD Bioscience). Splenocytes were incubated at 4°C with erythrocyte lysis buffer (155mM NH4CL in 10mM Tris/HCL, pH 7.2) for 5 minutes. Cells were centrifuged for 5 minutes at 1500 rpm, resuspended in lysis buffer to remove residual erythrocytes. Cells were washed twice with PBS. Cell suspensions were incubated with 1% normal mouse serum in PBS and stained for the surface markers CD4, CD5, CD8, CD19, CD11c, CD40, CD62L, CD69, F4/80, IgM, MHCII(eBioscience, Vienna, Austria), at a concentration of 0.25 µg Ab/200,000 cells. 1,2,3,4 Subsequently cells were subjected to flow cytometric analysis (FACSCANTO, BD Biosciences). FACS data were analyzed with CELLQuest software (BD Biosciences).

RT-PCR
Total RNA was isolated from the spleens of the WT and RP105−/− chimeras using Tri-Reagent (Sigma-Aldrich) according to the manufacturer’s protocol. The expression levels of IL6, IP10, IL10, p35 and p40 (subunits of IL12) and BAFF were analyzed by real time polymerase chain reaction (RT-PCR) (Taqman, Applied Bioscience). The relative mRNA expression levels were determined using HPRT and RPL27 as housekeeping gene and the 2[−ΔΔC(T)] method.

Splenocyte proliferation
Splenocytes were isolated by gently squeezing the spleen over a cell strainer (70μm pores, BD Bioscience). Cells were resuspended in RPMI 1640 (supplemented with 10% FCS, 20mM L-glutamine, 100U/ml penicillin and 100 μg/mL streptomycin) and seeded at a
density of 2×10^5 cells/well in a 96 wells plate. Cells were stimulated with 10 or 100 ng/ml LPS. Cells were incubated with 0.5 μCi [3H]Thymidine during the last 16 hours of 3 days in culture. To quantify thymidine incorporation the cells was washed with PBS and lysed with 0.1M NaOH and and cell-associated radioactivity was determined by liquid scintillation counting.

**B-cell proliferation**

Single-cell suspensions of splenocytes were obtained by mincing through cell strainers, and erythrocytes were lysed with ammonium chloride solution. B cells were positively enriched by using CD19 MACS microbeads and the MACS system, according to the manufacturer’s guidelines (Miltenyi Biotec, Germany). The purity of the isolated cells was verified by flow cytometric analysis (>95% CD19). Purified B cells were cultured in IMDM medium (Invitrogen Life Technologies, Gaithersburg, MD) supplemented with 10% heat-inactivated FCS, L-glutamine, gentamicin, and 5 × 10^5 M 2-ME. B cells were seeded at 2 x 10^5 cells/well in a final volume of 200 μl in 96-well flat-bottom plates and incubated for the indicated time periods at 37°C in a humidified atmosphere containing 5% CO2. Cells were stimulated with various concentrations of either LPS (Sigma-Aldrich, St. Louis, MO) or anti-CD40 mAb (BD Pharmingen, USA).

**Cell cycle analysis**

Cell cycle progression was analyzed by flow cytometry using CFSE. B cells (1 x 10^7) were washed three times with PBS, and subsequently CFSE was added to a final concentration of 5 μM in PBS. After 10 min at 37°C, labeling was stopped by adding 10% FCS-containing IMDM and cells were washed twice. CFSE-labeled cells were cultured, as described above, with 1 μg/ml LPS or anti-CD40 for 3 days.

**ELISA assays**

ELISA assays were performed with cell free supernatant using commercial available kits following the instructions of the manufacturer (BD Biosciences). Specific antibody titers to given antigens in plasma were determined by chemiluminescent ELISA, as previously described32, 33

**Histological analysis**

Cryostat sections of the aortic root (10 μm) were collected and stained with Oil-red-O to determine lesion size. Macrophages were visualized immunohistochemically with an antibody directed against a macrophage specific antigen (MOMA-2, monoclonal rat IgG2b, dilution 1:50; Serotec, Oxford, UK). Goat anti-rat IgG-AP (dilution 1:100; Sigma, St. Louis, MO) was used as secondary antibody and NBT-BCIP (Dako, Glostrup, Denmark) as enzyme substrates. Masson’s trichrome staining (Sigma) was used to visualize collagen (blue staining). T cells were visualized immunohistochemically with an antibody directed against CD3 (CD3). Goat anti-rabbit (dilution 1:100; Sigma-Aldrich) was used as secondary antibody and Novared (Dako) as enzyme substrates. Histological analysis was performed at room temperature, by an independent operator (blinded to specimen identity) using Leica DMRLE Microscope equipped with a Leica DC 500 camera and with Qwin quantification software (Leica, Rijswijk, the Netherlands).
**Statistics**

Data are expressed as mean±SEM. A two-tailed Student’s T-test was used to compare individual groups. Nonparametric data were analyzed using a Mann-Whitney U test. A level of P<0.05 was considered significant.


Supplemental table 1: Cellular composition of the spleens of WT and RP105−/− chimeras

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RP105 expression levels on total splenocytes (A), CD19+ B Cells (B), CD11c+ Dendritic Cells (C) and F4/80+ macrophages (D) in WT and RP105−/− LDLR−/− chimeras (A). Black bars represent WT chimeras, white bars represent RP105−/− chimeras. *p<0.05, **p<0.01, ***p<0.001. n=6 mice per group.
Supplemental figure 2 Cholesterol levels (A) and bodyweight (B) for the WT and the RP105-/- LDLR-/- chimeras. IL6 production by B cells from RP105-/- and WT mice stimulated with LPS (B). Representative pictures of MoMa2 macrophage, collagen and CD3 T Cell staining in 5 WT and RP105-/- chimeras (D). Black bars represent WT chimeras, white bars represent RP105-/- chimeras. **p<0.01. Average of 3 experiments n=4 mice per experiment.
Number of activated B cells from RP105-/- and WT mice stimulated with either LPS or anti-CD40. B cell activation measured by the number of CD19/CD86 and CD19/CD25 positive cells. After LPS stimulation; CD19/CD86 cells: 87.6% (WT) vs 73.6% (RP105-/-) positive cells. CD19/CD25 cells: 71.8% (WT) vs 55.7% (RP105-/-) positive cells after LPS stimulation. After anti-CD40 stimulation; CD19/CD86: 71.3% (WT) vs 68.2% (RP105-/-) positive cells. CD19/CD25: 56.5% (WT) vs 54.4% (RP105-/-) positive cells.

Relative BAFF expression in splenocytes from WT and RP105-/- mice. (N=5 per group, p=0.08)