An Unexpected Intriguing Effect of Toll-Like Receptor Regulator RP105 (CD180) on Atherosclerosis Formation With Alterations on B-Cell Activation


Objective—In atherosclerosis, Toll-like receptors (TLRs) 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 low density lipoprotein receptor deficient 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-oxidized low-density lipoprotein and anti-malondialdehyde-modified low-density lipoprotein IgG2c antibody levels were significantly lower in RP105−/− chimeras, confirming effects on B2 B cells rather than B1 B cells. Moreover, B-cell activating factor expression was reduced in spleens of RP105−/− chimeras.


Key Words: atherosclerosis ■ B lymphocytes ■ immune system ■ immunology ■ inflammation ■ Toll-like receptors

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 (TLRs) are major contributors to cardiovascular disease development4–8 because they initiate inflammatory responses in both immune and nonimmune cells by recognizing pathogen- and damage-associated molecular patterns that can be upregulated on, for example, 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 many accessory molecules.16 Activation of TLR4 is dependent on the presence of MD2 which is capable of binding lipopolysaccharide (LPS) and a variety of other known endogenous ligands.17,18 Next to MD2, RP105 (CD180) is an important accessory molecule acting as a regulator of TLR signaling.19–21 RP105 consists of the same extracellular domain as TLR4, but lacks the Toll-like interleukin (IL) receptor 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 Although 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 and 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. Unlike myeloid cells, B cells do not express MD2, representing a major difference between their respective TLR4 pathways. In MD2-deficient B cells, the RP105/MD1 complex was shown to function as MD2 on these cells. RP105 cell surface expression is strongly influenced by MD1, a structural homolog of MD2 and capable of binding lipids in its cavity. A recent article by Allen et al. suggested a mechanistic role for B-cell activating factor (BAFF) expression in B-cell proliferation of RP105−/− mice. 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. The TLR regulator RP105 may play an important role in the complex TLR pathway-mediated atherosclerosis formation by its unique function on different cell types.

In this study, we investigated the contribution of RP105 deficiency in atherosclerosis by repopulating lethally irradiated low density lipoprotein receptor deficient (LDLR−/−) mice with either wild-type (WT) 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.

### Materials and Methods

Materials and Methods are available in the online-only Supplement.

### Results

#### RP105 Deficiency Ameliorates Atherosclerosis

To assess the role of RP105 in atherosclerosis, we generated bone marrow chimeras by transplantation of RP105−/− or 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, 0.25% cholesterol, and 15% cacao butter [SDS, Sussex, United Kingdom]). RP105 deficiency was assessed by flow cytometry on CD19+ B cells, CD11c+ DCs, and CD11b+ monocytes isolated from spleen (Figure I in the online-only Data Supplement). Susceptibility to atherosclerosis in the proximal aortic root of LDLR−/− mice reconstituted with either WT or RP105−/− bone marrow was analyzed after 9 weeks of Western-type diet feeding. Cryostat sections of the proximal aortic root showed reduced Oil Red O staining in the RP105−/− chimera transplanted group as compared with the WT transplanted controls. (Figure 1A and 1B). Lesion burden in the proximal aortic root was significantly decreased in the RP105−/− chimeras (230±26×10³ μm² versus 131±15×10³ μm²; P=0.004), indicating decreased atherosclerosis formation in these mice (Figure 1C). No differences in body weight (Figure II in the online-only Data Supplement) or cholesterol levels (Figure II in the online-only Data Supplement) 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±8.8×10³ μm² versus 25.6±6.5×10³ μm²; P=0.007; Figure 1D). Even when correcting the macrophage area for total intimal area, we still observed a significant 33% decrease (27.6±3.2% versus 18.5±0.9%; P=0.039; Figure 1E and Figure II in the online-only Data Supplement). Collagen content in the plaque was not affected (WT versus RP105−/−: 4.6±0.4% versus 5.1±0.04%; P=0.39; Figure 1F and Figure II in the online-only Data Supplement). 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 versus 4.4±0.8 cells; P<0.0001; Figure 1G and Figure II in the online-only Data Supplement) and perivascular area (48.9±15.9 versus 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 proinflammatory cytokine production by cultured myeloid cells on LPS administration. Myeloid cells are known to be important in atherosclerosis development and contain multiple TLRs. Therefore, we analyzed the activation status of splenic DCs and macrophages in both chimaera groups. No difference in activation status of DCs (MHC-II and CD40 expression; Figure 2A), macrophages (CD40 expression; Figure 2B), or CD4 and CD8 T cells (Figure 2C and 2D) was found between the chimaera groups. Because RP105 was originally described to be highly expressed on B cells, we also investigated B-cell activation. Less IgM+ B cells were detected in 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 chimaera groups were stimulated ex vivo with 10 or 100 ng/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 versus 3.0±1.6 to LPS).
RP105-Deficient B Cells Have Reduced Proliferation and Are Less Activated on TLR4 Stimulation

To establish whether 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-CD40 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 versus 58.2±7.2 to 100 ng LPS; \( P = 0.0001 \) and 85.7±4.7 versus 129.7±12.8 to 1 \( \mu \)g LPS; \( P = 0.0007 \)) but not to anti-CD40 monoclonal antibody stimulation (135.0±0.6 versus 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 carboxyfluorescein succinimidyl ester dilution. Loss of carboxyfluorescein succinimidyl ester fluorescence, indicative of cell division, was measured by flow cytometry on day 3 and was strongly reduced in the RP105−/− B cells on LPS stimulation, but not on anti-CD40 monoclonal antibody, indicating a TLR4-specific induction of cell cycle arrest (Figure 3C). B cells from RP105−/− mice also produced less IL-6 (Figure I in the online-only Data Supplement) and showed less phenotypic activation (CD86 and CD25) on TLR4 but not on CD40 activation (Figure III in the online-only Data Supplement). 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 oxidized low-density lipoprotein (oxLDL) and malondialdehyde-modified LDL (MDA-LDL) were determined at euthanasia. OxLDL IgG and IgG2c and MDA-LDL–specific IgG2c isotype antibodies were significantly reduced in the RP105−/− chimeras (Figure 4A, 4C, and 4E). The exact role of IgG isotype antibodies is however still under debate.32 Interestingly, we did not observe differences in oxLDL or MDA-LDL–specific IgM production, indicating effects of RP105 predominately on B2 but not B1a cells. (Figure 4B and 4D). Phosphocholine-specific IgM T15/
of different leukocyte cell types in the spleen were not different between the groups (Table I in the online-only Data Supplement). Interestingly, basal BAFF expression in splenocytes of normal WT or RP105−/− mice was much lower compared with the chimera groups receiving a Western-type diet. Moreover, full-body RP105 knockout mice on a normal diet show a trend toward higher instead of lower BAFF expression in the splenocytes (P = 0.08) compared with WT mice (Figure IV in the online-only Data Supplement).

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 because 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.

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.32 Interestingly, the cross-talk between TLR and B-cell receptor signals plays a crucial role in B-cell responses to pathogens.13 B cells are considered to play a major role in the pathophysiology of autoimmune diseases,34 and during the last decade, several studies have also indicated an important role for TLRs in autoimmunity.35 Interestingly, patients with autoimmune-like diseases, such as systemic lupus erythematosus or rheumatoid arthritis, have an increased risk of atherosclerotic plaque development.36 B cells can affect atherosclerosis development via antibody production to modified LDL.37 Besides their important role in antibody production, B cells are also capable of producing cytokines and thereby can have an effector role in addition to their regulating role in inflammation. The cytokine-producing effector B cells are most likely derived from B2 subtype mature follicular B cells, whereas B1 cells are IgM antibody–producing cells.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 costimulation of other cells such as T cells.39 The effect of RP105 deficiency on cell number and activation was not observed on B-cell subtype B1 cells (CD4−, CD5+) and consequently, we did not observe effects on the levels of IgM.

Decreased Cytokine Expression in Splenes of RP105−/− Chimeras

In atherosclerosis, increased expression of cytokines in the plaque usually parallels that in splenocytes.33 mRNA levels of IL-6, interferon gamma-induced protein 10, IL-12, and IL-10 were significantly reduced in spleens of the RP105−/− compared with WT chimeras (Figure 5A–5D). Even more interesting, we found a reduced expression of BAFF in the spleens of the RP105−/− chimeras (Figure 5E). These reductions were representative because total numbers and percentages

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 2. Decreased B-cell activation in the spleen of RP105−/− chimeras. Splenocytes from low density lipoprotein receptor deficient (LDLr−/−) chimeras were harvested and analyzed for cell subtype and activation status by flow cytometry. Activation status of CD11c+ dendritic cells (DCs; identified by high MHC-II 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+ cells. Percentage of IgM/CD19+ (E) cell subsets identified by low CD62L or high CD69 expression. B cells were selected as CD19+ cells. Percentage of IgM/CD19+ (E) cell subsets identified by low CD62L or high CD69 expression. B cells were selected as CD19+ cells.
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 because it has a divergent role on myeloid and B cells.16 The differential effects of RP105 on myeloid and B cells may be related to the lack of MD2 in B cells 25,27 and the capability of RP105 to directly cause B-cell activation.40 Previously, Ait-Oufella et al 41 showed that the atheroprotective effect of CD20+ B-cell depletion may be attributable to effects on T-cell activation. They showed this mainly depended on reduction in IgG-type antibodies and not in IgM isotype antibody titers.41 In addition, Kyaw et al42 showed that B2 B cells may enhance atherosclerosis without presence of other inflammatory cells and independent of antibody levels. Both depletion studies noticed a decrease in macrophages in the plaque, whereas Ait-Oufella et al43 observed a reduction in T-cell accumulation. Our data are 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 BAFF receptor. BAFF is required for B-cell maturation and supports the survival of self-reactive B cells.43 BAFF receptor deficiency in bone marrow cells resulted in reduced B2 B cells and attenuated atherosclerotic lesion development.44 Depletion of B2 B cells in BAFF receptor knockout mice inhibited lesion development by ameliorating vascular inflammation.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.46 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 bone marrow transfer experiment, however, BAFF expression was significantly lower in splenocytes of RP105−/− chimeras compared to the expression in WT chimera splenocytes. These data suggest an association of BAFF expression with RP105 which may play an important role in effects seen on atherosclerosis in the RP105−/− chimeric LDLr−/−.

In addition, we found a decrease in mRNA expression of cytokines/chemokines, such as interferon gamma-induced protein 10, in the spleens of the RP105−/− chimeras. In atherosclerosis,
increased expression of cytokines in the plaque usually parallels that in splenocytes, and because interferon gamma-induced protein 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, and the opposite effect of the discussed studies that depleted B cells in WT chimeras hypercholesterolemic LDLr−/− mice. Total IgG against copper oxidized-low density lipoprotein (cu-oxLDL) titers were measured in plasma by chemiluminescent ELISA. Blood was harvested at euthanasia of WT: n=10; RP105−/−: n=15; *P<0.05; **P<0.01; ***P<0.001.

Figure 4. Reduced oxidized low-density lipoprotein (oxLDL) and malondialdehyde-modified LDL (MDA-LDL) IgG2c titers in RP105−/− chimeras. Antibody titers were measured in plasma by chemiluminescent ELISA. Blood was harvested at euthanasia of WT: n=10; RP105−/−: n=15; *P<0.05.

Figure 5. Reduced cytokine expression in the spleens of RP105−/− chimeras. mRNA expression of the cytokines interleukin (IL)-6, interferon gamma-induced protein 10 (IP-10), IL-10, and IL-12 subunits p35 and p40 (subunits of IL-12) in spleens. Relative expression of IL-6 (A), IP-10 (B), IL-12 subunits p35 and p40 (C), IL-10 (D), and B-cell activating factor (BAFF; E) in wild-type (WT) and RP105−/− chimeras at time of euthanasia. Black bars represent WT chimeras, and white bars represent RP105−/− chimeras, and data are presented as reflective light units (RLU). WT: n=10; RP105−/−: n=15; *P<0.05.

Recently, new data suggest that RP105 is expressed in epitidymal 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. In the RP105−/− chimeras, we also found a reduction in IgG2c titers against oxLDL and MDA-LDL, whereas IgM levels against oxLDL or MDA-LDL 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.

On long term, patients with autoimmune disease 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 patients with cardiovascular disease because 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, patients with systemic lupus erythematosus with a history of cardiovascular disease showed elevated titers of oxLDL and MDA-LDL–specific IgG antibodies compared with other patients with systemic lupus erythematosus without cardiovascular disease or population controls. In the RP105−/− chimeras, we did not use full-body knockout mice but performed bone marrow transfer to LDLr−/− mice. At euthanasia, we did not observe any difference in body mass of LDLr−/− mice. At euthanasia, we did not observe any difference in body mass of LDLr−/− mice.
received RP105−/− bone marrow cells compared with 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 atherosclerosis and development of novel therapeutic approaches.

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Disclosures

None.

References


**Significance**

This is the first article to provide evidence of RP105 involvement in atherosclerotic plaque formation with effects on B2 cells and B-cell activating factor expression. We show a previously unknown involvement of Toll-like receptor signaling in B-cell regulation in atherosclerosis indicating a novel route via which the Toll-like receptor signaling pathway affects atherosclerosis. These effects seem to be more pronounced than effects of RP105 deficiency on Toll-like receptor 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 also may have strong implications for understanding the underlying mechanism of Toll-like receptor 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.
An Unexpected Intriguing Effect of Toll-Like Receptor Regulator RP105 (CD180) on Atherosclerosis Formation With Alterations on B-Cell Activation

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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 70\(\mu\)m cell strainer to obtain a single cell suspension (70\(\mu\)m pores, BD Bioscience). Irradiated recipients received 0.5\(\times\)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. 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 $2[-\Delta\DeltaC(T)]$ 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 2x10^5 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 x 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 × 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 described^{32, 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 DMRE 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.

**References**


Supplemental Files:

Supplemental table I: Cellular composition of the spleens of WT and RP105\(^{-}\)/- chimeras

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Supplemental figure I

A

% RP-105+ splenocytes

WT

RP105−/−

***

B

% RP-105+ CD19+ B cells

WT

RP105−/−

***

C

% RP105+ CD11c+ Dendritic cells

WT

RP105−/−

*

D

% RP105+ F4/80+ macrophages

WT

RP105−/−

**
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 II

Cholesterol levels (A) and bodyweight (B) for the WT and the RP105<sup>−/−</sup> LDLR<sup>−/−</sup> chimeras. IL6 production by B cells from RP105<sup>−/−</sup> and WT mice stimulated with LPS (B). Representative pictures of MoMa2 macrophage, collagen and CD3 T Cell staining in
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.
Supplemental figure III
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.

**Supplemental figure IV**

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