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Bone marrow transplantation in mice as a tool to study M2 macrophage activation in atherogenesis

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Hematopoietic Arginase 1 deficiency results in decreased leukocytosis and increased foam cell formation but does not affect atherosclerosis

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Arginase1 (Arg1), a M2 macrophage marker, plays a critical role in a number of immunological functions in macrophages, which are the main cell type facilitating atherosclerotic lesion development. Arg1 uses the substrate L-arginine to create L-ornithine, a precursor molecule required for collagen formation and vascular smooth muscle cell differentiation. By reducing L-arginine availability, Arg1 limits the production of Nitric Oxide (NO), a pro-atherogenic factor in macrophages. In endothelial cells, conversely, NO is strongly anti-atherogenic. However, until now, the role of Arg1 in atherosclerosis is largely unknown. The aim of this study is to specifically investigate the effect of Arg1 deletion in hematopoietic cells on atherosclerosis susceptibility.

LDLr KO mice were transplanted with *Arg1$^{flox/flox}$;Tie2-Cre* (Arg1 KO) bone marrow (BM) or wildtype (WT) BM. After 8 weeks recovery on chow diet, recipient mice were fed a Western-Type Diet (WTD) for 10 weeks to induce atherosclerosis. After 10 weeks WTD challenge, blood leukocyte counts were decreased by 25% (p<0.001), and spleen leukocytes were decreased by 35% (p=0.05) in LDLr KO mice transplanted with Arg1 KO BM, compared to mice transplanted with WT BM. The decrease in leukocytes was due to lower B lymphocyte counts. However, oxLDL-specific antibodies were increased in plasma of LDLr KO mice transplanted with Arg1 KO BM compared to WT BM transplanted controls, whereas oxLDL-specific IgM was not affected. On the other hand, peritoneal foam cells in Arg1 KO BM recipients were increased 3-fold ($p<0.001$) compared to WT BM recipients. No change in blood cholesterol was found. Despite changes in leukocyte counts and macrophage foam cell formation, we did not observe differences in atherosclerotic plaque size or plaque macrophage content in the aortic root. Surprisingly, there was also no difference in plaque collagen content, indicating that absence of macrophage Arg1 function does not reduce plaque stability.

Deletion of Arg1 in hematopoietic cells adversely affects blood leukocyte counts and increases foam cell formation. However, no effects on atherosclerosis could be demonstrated, indicating that hematopoietic Arg1 function is not a decisive factor in atherosclerotic plaque formation.

Introduction

Inhibition of the activity of the enzyme Arg1 is considered a promising novel therapeutic strategy for the treatment of cardiovascular disease.[1] In line, arginase inhibition by N(omega)-hydroxy-nor-l-arginine (nor-NOHA) improves endothelial function in familial hypercholesterolemia patients and reduces atherosclerotic lesion development in carotid arteries of apolipoprotein E (ApoE) knockout mice exposed to low shear stress.[2-4] Arg1 influences a number of processes implicated in the pathogenesis of atherosclerosis.[5-8] It is expressed in endothelial cells, vascular smooth muscle cells (VSMCs) and macrophages, which are all important cellular components of the atherosclerotic plaque.[1] Depending on the cell type it is expressed in, Arg1 function is expected to exert different effects on atherosclerotic plaque formation.

The primary function of Arg1 is production of urea and L-ornithine from L-arginine.[9] L-arginine, however, is also used as a substrate by the enzymes inducible- and endothelial Nitric Oxide Synthase (iNOS and eNOS) for the production of the endothelial-protective signalling molecule nitric oxide (NO).[2,10] By competition for the common substrate L-arginine, Arg1 can thus indirectly inhibit the synthesis of NO.[11,12] In line, endothelial Arg1 contributes to endothelial activation and vascular stiffness by reducing the L-arginine pool, leading to eNOS uncoupling and reduced NO production.[10,13] This results in endothelial activation and increased recruitment of immune cells to the plaque.[10,13] However, atheroprotective effects have also been described for Arg1 in

macrophages and VSMCs. By producing L-ornithine, Arg1 contributes to the synthesis of L-proline by the enzyme Ornithine Amino Transferase (OAT), which is a precursor for collagen biosynthesis. Ornithine can also be metabolised into polyamines, which leads to increased VSMC differentiation and decreased inflammation.[14-16] In agreement, lentiviral-mediated upregulation of Arg1 in a balloon-injury rabbit model inhibited plaque inflammation and augmented VSMC proliferation. Plaque size was, however, not affected.[8]

In macrophages Arg1 is found in the alternatively activated M2 cells, a macrophage subtype with an anti-inflammatory and wound healing function.[6] Downregulation of Arg1 expression and inhibition of Arg1 activity in Raw264.7 macrophages resulted in augmented LPS-induced TNF-α and IL-6 secretion.[8] On the other hand, Arg1 in macrophages suppresses Th2 dependent inflammation by dampening the production of anti-inflammatory cytokines by $CD4^+$ T cells and suppressing T-cell proliferation in mice infected with the trematode Schistosoma mansoni.[17] Differential gene expression analysis in macrophages of atherosclerosis-susceptible and -resistant rabbits suggested that high macrophage Arg1 expression was associated with low atherosclerosis susceptibility.[18] Furthermore, M2 macrophages are found predominantly in carotid plaques of asymptomatic patients that have more stable plaques,[19] indicating the positive association between macrophages of the M2 phenotype and atherosclerotic plaque stabilization. However, the functional role of macrophage Arg1 in atherosclerotic plaque development is currently still unknown.

In this study we specifically assessed the contribution of hematopoietic Arg1 to the development of atherosclerosis, by transplanting bone marrow from *Arg1$^{flox/flox}$;Tie2Cre* mice into atherosclerosis-susceptible LDL receptor knockout (LDLr KO) mice.

Material and Methods

Animals

LDLr KO mice and WT C57Bl/6 were obtained from the Jackson Laboratory and expanded at the Faculty of Science, Leiden University. *Arg1$^{flox/flox}$;Tie2Cre* (Arg1 KO) mice [20] were bred at the Faculty of Life Sciences, University of Manchester. All animal studies in the Netherlands were approved by the regulatory authority of Leiden University and carried out in compliance with the Dutch government guidelines. All animal work in the United Kingdom was performed in accordance with Home Office regulations.

mRNA Expression Analysis by Real Time PCR

Thioglycollate-elicited peritoneal macrophages (PMs) from 12-week old male C57Bl/6 mice were obtained after injection of 1 mL of 3% thioglycollate solution 5 days prior to the experiment. After adherence and washing, the macrophages were incubated with/without 10 μg/mL oxidized low density lipoprotein (oxLDL, prepared as described previously[21]) for 24 hours. After that, cells were collected for total RNA isolation.[22] Subsequently, RevertAid M-MuLV enzyme (Fermentas, Burlington, Canada) was used to transcribe RNA to cDNA. Quantitative PCR (qPCR, ABI PRISM 7500 system, Foster City, CA) was used to access the mRNA expression levels of genes interested using SYBR Green reagents (Applied Biosystems). *RPL27* and *36B4* were used as housekeeping genes.

Microarray analysis

Twelve-week old female LDLr KO mice were first fed Western-type Diet (WTD; Special Diet Services) that contains 15% cacao butter and 0.25% cholesterol for a run-in period of 2 weeks before bilateral perivascular collar placement in the carotid arteries. Then the mice were challenged with WTD for another 2 weeks to induce early atherosclerotic lesion development. The carotid arteries were isolated directly after the run-in WTD period (baseline group) or 2 weeks after collar-placement (atherosclerotic plaque group) for microarray analysis as previously described.[23]

Bone Marrow Transplantation

Bone marrow from male C57Bl/6 WT controls and Arg1 KO mice (around 12 weeks old) was prepared for bone marrow transplantation (BMT) to 12 weeks old female LDLr KO recipient mice. In brief, lethally irradiated recipients received $5x10^6$ bone marrow cells via tail vein injection. The mice were allowed to recover for 8 weeks on chow diet (RM3; Special Diet Services), after which they were fed WTD to induce atherosclerosis. After the 10-week WTD challenge, the mice were anaesthetized by a lethal dose of anesthetic mixture that contained rompun, ketamine and atropine. Mice were bled and perfused with PBS, after which organs were isolated. The hematologic chimerism was confirmed in genomic DNA of recipients bone marrow using the PCR method (Supplementary Figure 1A-B).

Generation of Bone Marrow-Derived Macrophages (BMDMs)

Bone marrow from LDLr KO recipients transplanted with WT BM or Arg1 KO BM was isolated at sacrifice for the *in vitro* experiments. Bone marrow-derived macrophages were obtained as described previously.[24] Macrophages were cultured for 24 hours with or without 100µg/mL acetylated-low density lipoprotein (acLDL). The preparation of acLDL is described previously.[25] Subsequently the cells were analyzed by an automated veterinary haematology analyzer (Sysmex Corporation, XT-2000iV, Japan) for foam cell formation as described previously.[26,27] Briefly, the Sysmex XT-2000iV analyzer applies a similar principle for cell differential analysis as patented fluorescent flow cytometric analysis.[28] Laser side scatter and side fluorescence lights were used for separating cell clusters. Lipid-rich macrophages (foam cells) are larger and contain more abundant granules compared to the non-foam cells.[26,27] Thus in a differential scattergram, the lipid-rich macrophage population shifts to a larger scale on the side scatter axis and side fluorescent light axis, enabling gating of a separate, shifted population of macrophage foam cells.

Flow Cytometry Analysis and WBC Differential Analysis

Blood samples, anti-coagulated with EDTA, as well as single splenic cell suspensions, were obtained using a 70µm cell strainer (734-0003, VWR), and used for FACS analysis. Erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH=7.3) was used to lyse red blood cells in the blood and splenocyte preparations. Consecutively, the cells were analyzed on a FACS Canto II (BD Biosciences, Mountain View, CA) using the relevant FACS antibodies (all obtained from eBioscience).

An automated Haematology analyzer (XT-2000iV, Sysmex Corporation, Japan) was used to analyse leukocyte counts in spleen and blood samples. Furthermore, peritoneal leukocytes collected at sacrifice from the bone marrow transplanted animals, were analyzed for quantification of macrophage foam cells formation.

Serum Cholesterol Level Determination

Total and free cholesterol concentrations in serum were determined using an enzymatic colorimetric method as described previously.[29] Absorbance was read at 490 nm.

ELISA assay for anti-oxLDL antibodies

Copper-oxLDL was prepared as previously described.[21] The mouse immunoglobulin isotyping ELISA kit was obtained from BD Biosciences (Catalog No. 550487). HRP labelled polyclonal rabbit anti-Rat immunoglobulins (Ig) were obtained from DAKO (Product No. P045001). Total Ig and IgM antibodies against oxLDL were measured by a modified ELISA. In brief, oxLDL was coated in an ELISA plate (Corning, NY, 14831) in a concentration of 10µg/mL overnight at 4°C in coating buffer (0.42% w/v $NaHCO_3$, 0.53% Na_2CO_3, pH of 9.6). After washing with 0.05% tween-20-PBS, wells were blocked for 30 minutes in 1% BSA blocking buffer. After washing, 4uL plasma was added and plates were incubated at room temperature for an hour, then washed. For determination of total Ig, the HRP-labelled rat anti-mouse Ig antibody included in the BD ELISA kit was added. After incubation for 1 hour at room temperature and subsequent washing, the plate is ready for colour development. For the IgM determination, after incubation with IgM-specific rat anti-mouse antibody for 1 hour, wells were incubated with HRP-labeled rabbit anti-rat Ig for another hour before colour development, upon following the manufacturer's instruction. Optical density (OD) was obtained by reading the plate in a plate reader (Biotek, poowerWave 340) at 450nm and 570nm. Wavelength correction was performed by subtracting the values obtained at 570nm from the values at 450nm.

Histological Analysis of the Aortic Root

After 10-week WTD feeding, mice were sacrificed. Seven-µm serial sections of the aortic root were cut using a Leica cryostat. Oil red-O staining, MoMa2 staining and Masson's Trichrome kit were used for visualization of plaque area, macrophage positive area and collagen content respectively as described previously.[29] Quantification was performed using the Leica image analysis system (Leica Ltd, Cambridge, UK).[29]

Statistical Analysis

Student's T-test or two-way ANOVA were used to determine the statistically significant differences (Graphpad Prism software). A Welch correction was applied to the T-test in the case of unequal variances in the dataset. The statistical significance was set at 0.05. Results are shown as the mean ± SEM.

Results

Arg1 expression is induced in macrophages by oxLDL loading and in carotid arteries upon induction of collar-induced atherosclerosis

The early stages of atherosclerotic lesion development are characterized by the accumulation of lipid-laden macrophages. Therefore, first the effects of incubation of macrophages with oxLDL, and induction of collar-induced atherosclerosis in the carotid artery on the expression of Arg1 were determined. In agreement with a previous study from Gallardo-Soler *et al.*,[30] *Arg1* mRNA expression was increased 4.3-fold in thioglycollate-elicited peritoneal macrophages after 24 hours oxLDL (10µg/mL) loading (p<0.001; figure 1A) after a qPCR analysis. Oil-red O staining confirmed that after oxLDL incubation, foam cells were successfully induced (Figure 1B). Moreover, microarray analysis showed that *Arg1* expression was significantly increased in collar-induced early atherosclerotic lesions in the carotid artery of LDLr KO mice (4.5-fold, p<0.01; figure 1C).

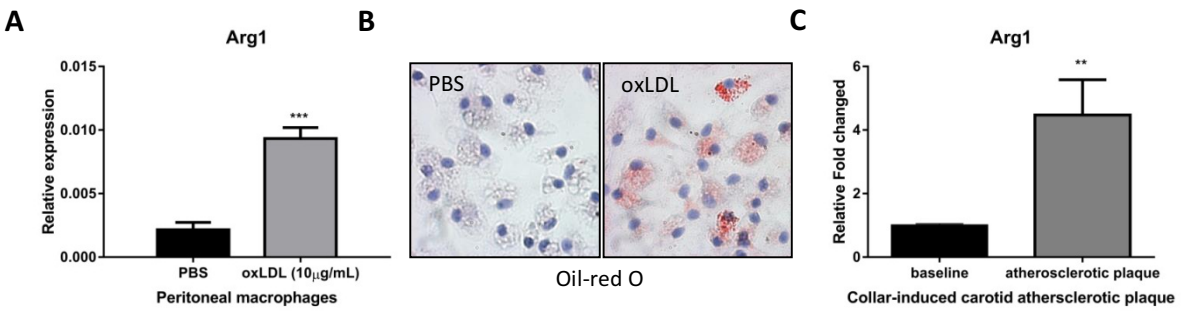

Figure 1 *Arg1* expression was induced by oxLDL loading in wild-type (WT) thioglycollate-elicited peritoneal macrophages (PMs) or Western-type diet (WTD) and collar-induced atherosclerosis in the carotid artery.
A) Thioglycollate-elicited PMs were incubated with/without 10 µg/mL oxLDL for 24 hours, and then qPCR was used for analysis of *Arg1* gene expression. (n=6). B) Oil-red O staining of PMs after incubation with/without 10 µg/mL oxLDL for 24 hours. C) LDLr KO mice were challenged with WTD 2 weeks before the collar placement (baseline). The experimental group was fed WTD for another 2 weeks after collar placement (atherosclerotic plaque). The collar-induced atherosclerotic plaques in carotid arteries of LDLr KO mice were used for microarray analysis of which the effects on*Arg1* expression are shown. (n=6). Results are expressed as mean ± SEM, significance was assessed by student T-test. **p<0.01; ***p<0.001.

Arg1 deletion in bone marrow-derived cells of LDLr KO mice increases foam cell accumulation in the peritoneum in absence of effects on serum total cholesterol levels

To generate a mouse model that specifically lacks Arg1 in bone marrow-derived cells, bone marrow (BM) from *Arg1*$^{flox/flox}$*;Tie2Cre* (Arg1 KO) mice and WT controls was transplanted into LDLr KO recipients. In *Arg1*$^{flox/flox}$*;Tie2Cre* mice Arg1 has been deleted in cells of the hematopoietic lineages and in endothelial cells.[19,31]

Niese *et al.* previously showed that Arg1 deficiency does not affect bone marrow engraftment.[32] In agreement PCR analysis on genomic DNA isolated from bone marrow after 10 weeks of WTD feeding confirmed successful disruption of Arg1 functionality in the bone marrow and peritoneal cells of the LDLr KO recipient mice (supplementary figure A-C).

Differential haematology analysis was performed on peritoneal cells harvested from LDLr KO mice, transplanted with Arg1 KO or WT bone marrow after 10 weeks of WTD feeding. No difference in total number of peritoneal leukocytes (data not shown) or the percentage of macrophages within

the peritoneal leukocyte population were found between Arg1 KO BM recipients and WT BM recipients (p>0.05, figure 2A). Interestingly, a 3-fold larger fraction of foam cells in the peritoneum of Arg1 KO BM recipients was found when compared to the WT BM recipients (p<0.001, figure 2B). This increased foam cell formation could not be attributed to increased serum cholesterol levels, as no difference in serum free cholesterol (data not shown) or total cholesterol was found (p>0.05; figure 2C).

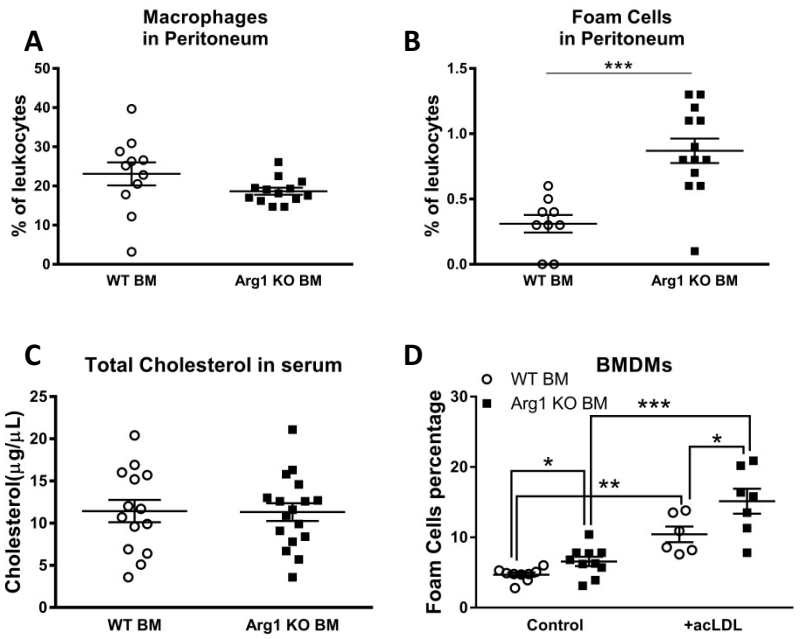

Figure 2 Increased *in vivo* peritoneal foam cell formation in LDLr KO mice transplanted with Arg1 KO BM in absence of effects on serum cholesterol and augmented *in vitro* foam cell formation in bone marrow-derived macrophages (BMDM) lacking Arg1.

Serum and peritoneal leukocytes were collected at sacrifice after 10 weeks of high-fat, high-cholesterol Western-type diet feeding. A) Macrophage percentage of total peritoneal leukocytes in BMT recipients (n=9-14). B) Foam cell percentage of total peritoneal leukocytes in the BMT recipients (n=9-14). C) Total cholesterol level in the BMT recipients (n=9-14). D) *In vitro*, BMDMs from WT BM or Arg1 KO BM recipients were incubated 24 hours in the presence or absence of 100µg/mL acLDL. Foam cell percentage was detected using a haematology analyser (n=6-10). Results are expressed as mean ± SEM, significance was assessed by student T-test or 2-way ANOVA. *p<0.05; **p<0.01; ***p<0.001.

Arg1 KO BMDMs show increased foam cell formation and tend to differentiate to an M2 macrophage phenotype upon acLDL stimulation.

BMDMs from WT and Arg1 KO mice were treated with 100µg/mL acLDL for 24 hours to gain mechanistic insight into the observed increased foam cell formation *in vivo*. AcLDL-induced lipid loading led to foam cell formation in both WT BMDMs and Arg1 KO BMDMs (4.7 ± 0.3% to 10.4 ± 1.1% for WT BMDMs, p<0.01; and 6.5 ± 0.66% to 15.1 ± 1.8% for Arg1 KO BMDMs, p<0.001, respectively; figure 2D). Interestingly, both before and after lipid loading Arg1 KO BMDMs displayed a 50% increase in foam cell formation compared to WT BMDMs (p<0.05, figure 2D). The mRNA expression of the genes related to foam cell formation was also assessed by qPCR analysis in BMDMs incubated with/without 100 µg/mL acetylated LDL for 24 hours. Upon acLDL loading, the expression of *SR-B1,* a receptor for native and modified lipoproteins, was effectively downregulated in WT BMDMs (p<0.05 as compared to non-loaded cells; figure 3A), whereas Arg1 KO BMDMs failed to downregulate *SR-B1* upon acLDL loading (p>0.05, figure 3A). No differences

were observed in *LDLr* expression between the 2 genotypes either with or without acLDL loading (p>0.05; figure 3B). Foam cell formation is determined by the balance between cholesterol uptake and synthesis on the one hand and cholesterol efflux on the other hand. Although the expression of *ABCA1*, the primary cholesterol efflux transporter, was increased in the BMDMs loaded with acLDL (WT, 15.7-fold, p<0.001; Arg1 KO, 3.7-fold, p<0.01; figure 3C), no difference in *ABCA1* expression was found upon comparison of the Arg1 KO and the WT BMDMs (p>0.05, figure 3C). Notably, expression of *SREBP1* was 2.45-fold higher in Arg1 KO BMDMs compare to WT BMDMS in response to acLDL loading, whereas no difference was found under control non-loaded conditions (Control, p>0.05; +acLDL, p<0.05; figure 3D). The difference in *SREBP1* expression between acLDL loaded Arg1 KO and WT macrophages is explained by a failure of the Arg1 KO macrophages to downregulate *SREBP-1* in response to acLDL loading (WT, p<0.01; Arg1 KO, p>0.05; figure 3D). Furthermore, the M1 and M2 markers *iNOS* and *FIZZ-1* were determined in both genotypes of BMDMs before and after acLDL loading. Under control non-loaded conditions, no differences were found in the expression of *iNOS* or *FIZZ-1*. However, in response to acLDL loading, Arg1 deficiency in BMDMs led to an M2-like phenotype, as evidenced by significantly downregulated iNOS expression (67% decrease, p<0.05; figure 3E) and extremely upregulated *FIZZ-1* expression (19-fold, p<0.001; figure 3F), whereas no such changes were observed in WT BMDMs.

Furthermore, NO production was determined in the culture medium of WT and Arg1 KO BMDMs, both under control conditions and after acLDL lipid loading. Two-way ANOVA showed an increased NO production in Arg1 KO BMDMs compared to the WT BMDMs (p=<0.05, figure 3G). NO production was significantly increased (1.6-fold, p<0.05, figure 3G) in Arg1 KO BMDMs, but not in WT BMDMs in response to acLDL loading, leading to 2.2-fold (p<0.01, figure 3G) higher NO concentrations in the supernatant of Arg1 KO BMDMs under these conditions.

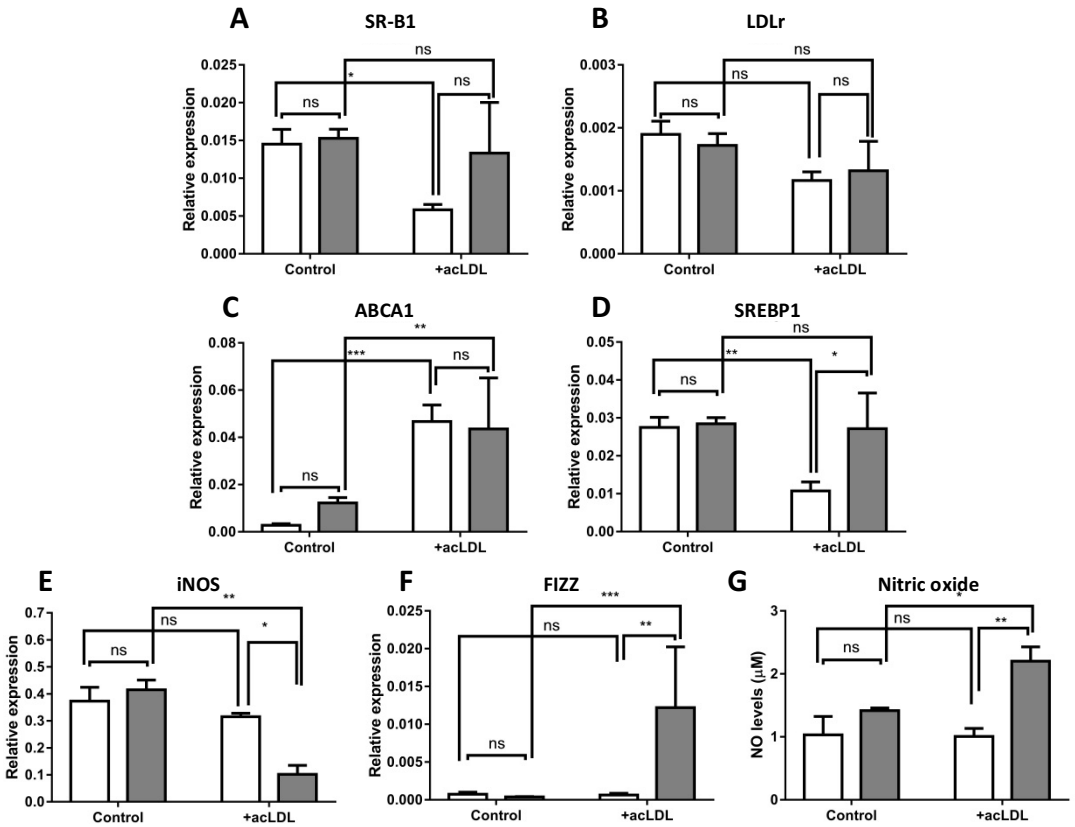

Figure 3 Gene expression and nitric oxide production in WT and Arg1 KO bone marrow-derived macrophages (BMDMs) after incubation with or without 100µg/mL acLDL for 24 hours.

Relative mRNA expression of A) *SR-B1,* B) *LDLr,* C) *ABCA1,* D) *SREBP1,* E) *iNOS*, and F) *FIZZ-1.* (Control group, n=9-12; +acLDL group, n=3-4)**.** G) Nitric oxide concentrations in the supernatant of BMDMs incubated with or without 100µg/mL acLDL 24 hours. (Control group, n=3; +acLDL group, n=4). Results are expressed as mean ± SEM, significance was assessed by student T-test or 2-way ANOVA. **p*<0.05; ***p*<0.01; ****p*<0.001.

Bone marrow Arg1 deficiency affects neither atherosclerotic plaque size nor plaque composition in LDLr KO mice.

After 10 weeks WTD feeding, the aortic root was sectioned and stained with Oil red-O to analyze atherosclerotic lesion development. Despite the observed increase in macrophage foam cell formation upon deletion of Arg1, no difference in plaque size was found between the two experimental groups (647±29 x 10^3 µm^2 for WT BM recipients vs. 634±26 x 10^3 µm^2 for Arg1 KO BM recipients, figure 4A). Macrophages in the plaque were visualized by MOMA-2 staining. No difference in plaque macrophage content as a fraction of total plaque size was observed (0.171 ±0.011 WT BM vs. 0.169 ±0.016 Arg1 KO BM, figure 4B). Collagen in the plaque was stained using a Masson's Trichrome method and Picrosirius Red staining. A trend towards a reduction in the collagen content of plaques of Arg1 KO BM recipients was found in Masson's Trichrome-stained sections (0.102 ±0.009 WT BM vs. 0.084 ±0.004 Arg1 KO BM, *p*=0.06, figure 4C). However, analysis of Picrosirius Red staining did not indicate any difference between the groups (0.124 ±0.013 WT BM vs. 0.105 ±0.012 Arg1 KO BM, figure 4D).

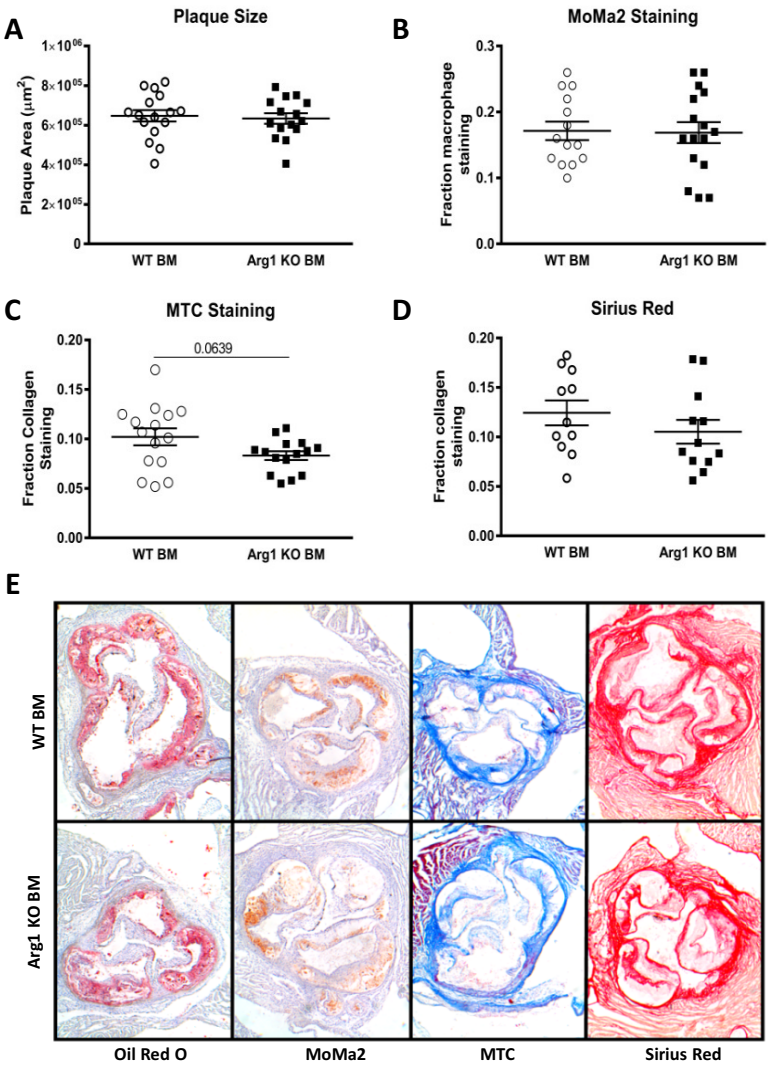

Figure 4 Deletion of Arg1 in bone marrow-derived cells does not influence atherosclerotic lesion development after 10 weeks WTD feeding.

A) Plaques were stained with Oil-Red O and plaque area was quantified. (p>0.5; WT BMT, n=16; Arg1 KO BMT, n=15). B) Plaque area stained positively with MOMA-2 antibody was measured and normalized for total lesion size. (p>0.5; WT BMT, n=14; Arg1 KO BMT, n=16). C) Plaque collagen was visualized using Masson's Trichrome (MTC) staining and plaque collagen content was analysed. (p=0.064; WT BMT, n=15; Arg1 KO BMT, n=15). D) A Sirius Red staining was performed to examine plaque collagen content further. Collagen content was analysed (p>0.5; WT BMT, n=11; Arg1 KO BMT, n=12). E) Representative images of the stainings described above. Results are expressed as mean ± SEM, significance was assessed by student's T-test.

Transplantation of Arg1 KO bone marrow into LDLr KO recipients results in reduced splenocyte and blood leukocyte counts

Flow cytometry was used to assess whether loss of Arg1 functionality in bone marrow-derived cells in the transplanted LDLr KO mice affected leukocyte numbers in the circulation or the spleen. On chow diet, no difference in total blood leukocyte numbers was detected (p>0.05, figure 5A) between the recipient mice with WT BM and the mice with Arg1 KO BM. WTD feeding for 10 weeks increased total leukocyte counts from in blood of LDLr KO mice transplanted with WT BM (chow diet: 11410 ± 708 cells/µl; WTD: 15672 ±689 cells/µl; p<0.01; figure 5A). Mice transplanted with Arg1 KO bone marrow, however, failed to show an increase in blood leukocyte counts upon WTD

feeding (chow diet: 12092 ± 906 cells/µl; WTD: 11573 ±491 cells/µl; $p>0.05$; figure 5A). Therefore, it led to lower total leukocyte counts in the blood of Arg1 KO BM recipients as compared WT BM recipients after 10 weeks of WTD feeding ($p<0.001$, figure 5A). Comparison of the subtypes of WBC, showed that, in contrast to WT BM recipients in which lymphocytes were 1.6-fold increases ($p<0.001$) after 10 weeks WTD feeding, lymphocytes did not increase in LDLr KO mice transplanted with Arg1 KO BM (Figure 5B). No differences were found in neutrophils, monocytes, and eosinophils (data not shown). Next, flow cytometric analysis was used to determine the effects on the different cellular subsets in blood at sacrifice. No differences were found in the absolute amounts of $CD11b^+$ cells, $CD11b^+/Ly6C^{hi}$ inflammatory, $CD11b^+/Ly6C^{low+med}$ patrolling monocytes and $CD11b^+/Ly6G^+$ neutrophils, nor the amounts of $CD4^+$ T helper cells, $CD25^+/CD4^+$ activated T helper cells and $CD8^+$ cytotoxic T-cells (figure 5C-H). Unexpectedly, the decrease in total blood leukocytes appeared to be driven by a 2-fold decrease in circulating $CD19^+$ B cells ($p<0.01$, figure 5I). Representative flow cytometry plots are shown in figure 5J.

At the time of sacrifice, spleens were taken and weighed. Organ weight was normalized for total body weight. A small but significant 10% decrease in spleen weight was found in LDLr KO mice transplanted with Arg1 KO BM ($p<0.05$, figure 6A), while there were no differences in total body weight (data not shown). Correspondingly, spleens from the Arg1 KO BM recipients contained 35% less splenocytes ($p=0.052$, figure 6B). Next, splenocyte composition was assessed by flow cytometry. The absolute numbers of leukocyte subtypes, including $CD11b^+$ cells, $CD11b^+/Ly6C^{hi}$ pro-inflammatory monocytes, $CD11b^+/Ly6C^{low+med}$ patrolling cells, $CD11b^+/Ly6G^+$ neutrophils, $CD19^+$ B cells and $CD8^+$ T cells in the spleen of Arg 1 KO recipients showed a significant decrease or a trend towards a decrease, suggesting that the decrease in splenocyte number was not attributable to one specific cell type ($p<0.05$, figure 6C-D). However, fractional analysis showed a 23% increase in $CD4^+$ and $CD25^+/CD4^+$ T cells in the spleen of LDLr KO mice that received Arg1 KO BM compared to the recipients that received WT BM ($*p<0.05$; $^{\$}p<0.05$, respectively. Figure 6E). Representative flow cytometry plots are shown in figure 6F.

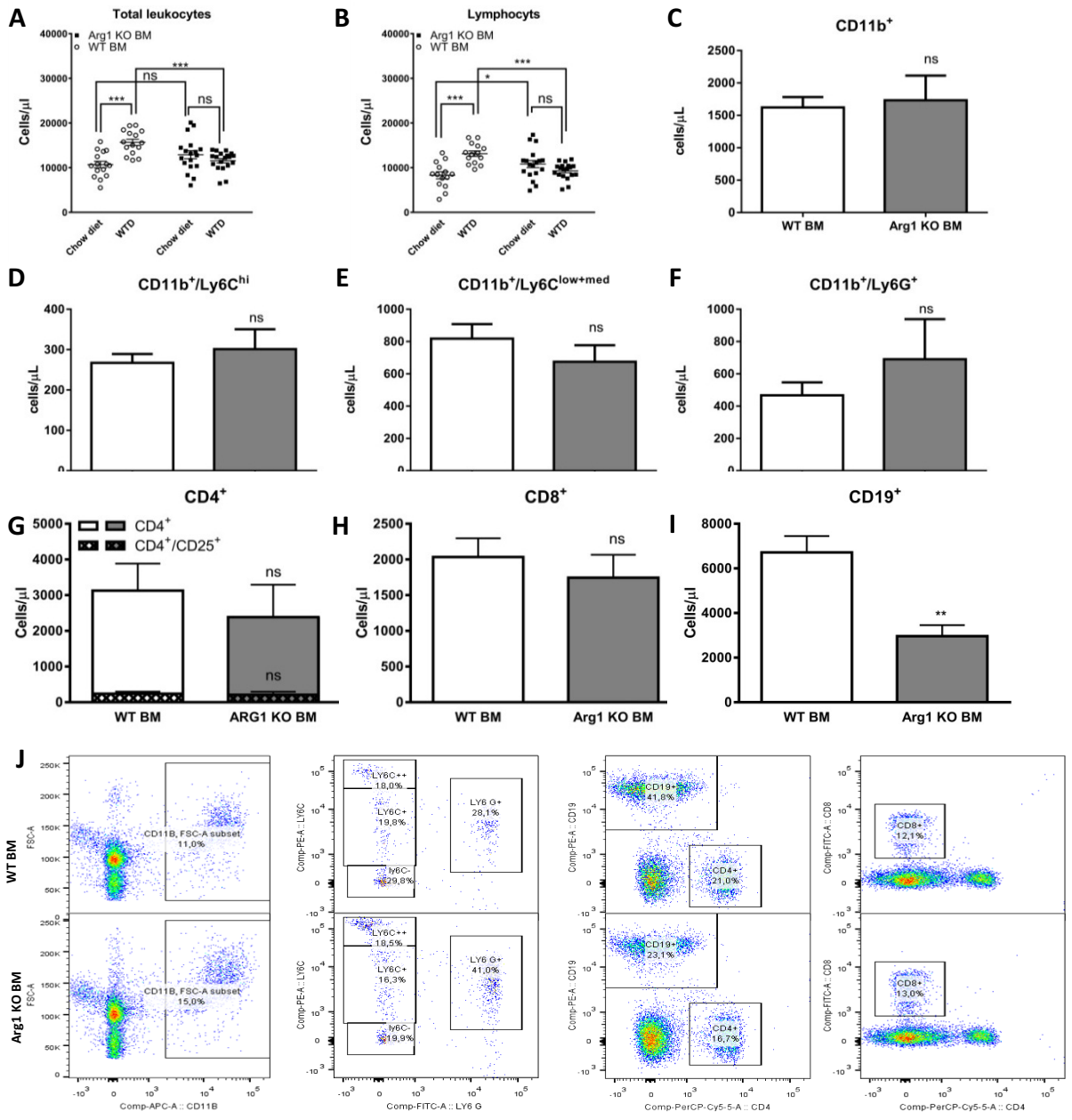

Figure 5 Decreased leukocytes in blood of LDLr KO mice transplanted with Arg1 KO BM after 10 weeks feeding a high-fat, high-cholesterol Western-type diet (WTD).

After BMT, recipient mice were fed chow diet for 8 weeks (shown as "chow diet" in figures) before the switch to WTD for an additional 10 weeks (shown as "WTD" in figures) to induce atherosclerosis development. Circulating A) leukocytes and B) lymphocytes on chow diet shown at 8 weeks after transplantation and after 10 weeks on WTD at 18 weeks after transplantation. (n=15-19). B) Circulating lymphocytes shown at 8 weeks after transplantation on chow diet and 10 weeks on WTD. (n=15-19). C-I) Circulating leukocytes were analyzed by flow cytometry for the expression of several immune cell markers. (n=5). I) Total $CD19^+$ cells in circulation (n=5). J) Representative flow cytometry plots. Results are expressed as mean ± SEM, significance was assessed by student T-test or 2-way ANOVA. *p<0.05; **p<0.01; ***p<0.001; ns, non-significant.

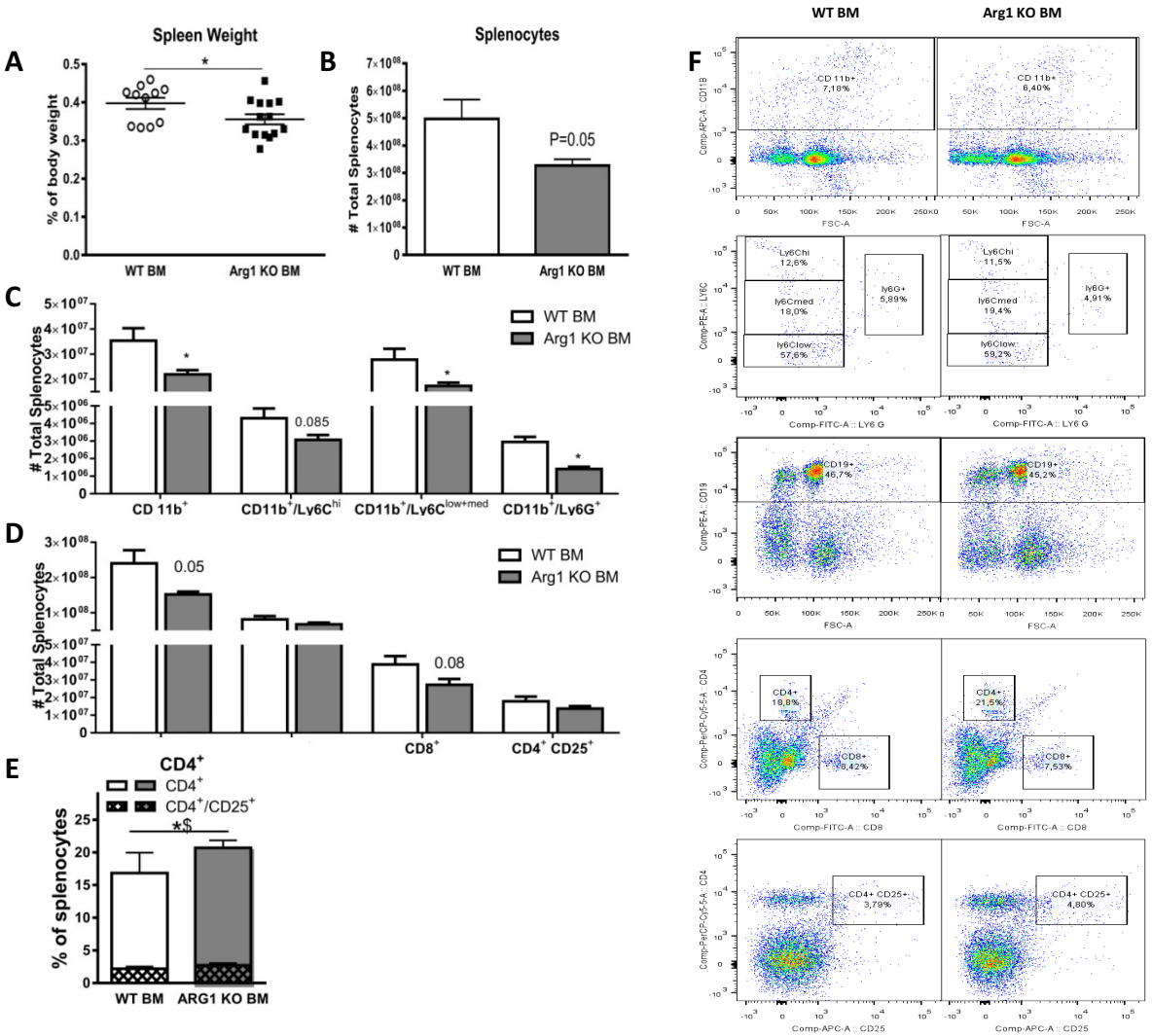

Figure 6 Decreased spleen weight in LDLr KO mice transplanted with Arg1 KO BM.

Spleens were isolated at 10 weeks after feeding a high-fat, high-cholesterol Western-type diet. A) A reduction in relative spleen weight was measured in the Arg1 KO BM recipients. The readout was corrected for the total body weight of the mice (**p*<0.05; WT BMT, n=11; Arg1 KO BMT, n=14). B) Reduction observed in the total amount of splenocytes in the Arg1 KO BM recipients (*p*=0.05; WT BMT, n=5; Arg1 KO BMT, n=5). C) Flow cytometry analysis of splenocytes showing that the amount of $CD11b^+$, $CD11b^+/Ly6C^{hi}$, $CD11b^+/Ly6C^{low+med}$ monocytes and $CD11b^+/Ly6G^+$ neutrophils were significantly decreased or trended to a decrease, in spleens of Arg1 KO recipients (**p*<0.05; WT BMT, n=5; Arg1 KO BMT, n=5). D-E) A trend to a decrease, in the amount of $CD19^+$ and $CD8^+$ splenocytes. The fraction of $CD4^+$ and $CD4^+CD25^+$ cells in the spleen was increased (**p*<0.05; $*p*<0.05, respectively; WT BMT, n=5; Arg1 KO BMT, n=5), though the total amount of $CD4^+$ and $CD4^+CD25^+$ cells in the spleen was unchanged (*p*>0.05; WT BMT, n=5; Arg1 KO BMT, n=5). F) Representative flow cytometry plots. Results are expressed as mean ± SEM, significance was assessed by student T-test. **p*<0.05; $*p*<0.05.

Arg1 deficiency in the bone marrow of LDLr KO mice induces circulating oxLDL-specific antibody levels.

The total level of ox-LDL specific immunoglobulins (Ig) and its isotype IgM were determined in plasma of the LDLr KO mice transplanted with WT or Arg1 KO BM after 10 weeks WTD challenge. As shown in figure 7, deletion of Arg1 in the bone marrow of LDLr KO mice led to increased total oxLDL-specific Ig levels (1.2-fold, $p<0.05$ compared to the WT BM LDLr KO recipients after 10 weeks WTD challenge, which was not driven by IgM.

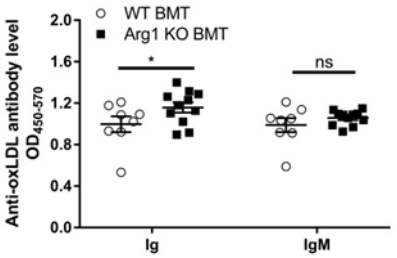

Figure 7 Increased oxLDL-specific antibody levels in LDLr KO mice transplanted with Arg1 KO BM after 10 weeks WTD feeding.

Total oxLDL-specific antibody and isotype IgM levels were assessed using ELISA. Results are expressed as mean ± SEM, significance was determined by student's T-test. *$p<0.05$ as compared between LDLr KO recipient with WT BM and recipients with Arg1 KO BM in the level of total oxLDL-Specific antibody (Ig). ns, non-significant.

Discussion

In the current study we determined for the first time the effects of Arg1 deletion in bone marrow-derived cells on macrophage foam cell formation and atherosclerosis susceptibility. We show that *Arg1* expression is increased in elicited peritoneal macrophages upon oxLDL-induced lipid loading and during the development of early murine atherosclerotic lesions in the carotid-artery. In agreement, Gallardo-Soler, Alejandro, *et al.*, previously demonstrated that *Arg1* expression is highly induced in bone marrow-derived macrophage foam cells upon oxLDL or acLDL stimulation.[30] Inhibition of PPAR-γ/δ suppresses the lipid-induced increase in macrophage Arg1 expression,[30] indicating that *Arg1* expression is increased during macrophage foam cell formation probably due to PPAR-γ/δ activation. Combined, these results indicate that Arg1 might participate in macrophage foam cell formation, thereby influencing atherosclerosis development. However, so far there had been no reports on the effects of Arg1 function on macrophage foam cell formation. Interestingly, Arg1 KO BMDMs showed increased foam cell formation both under control conditions and after acLDL-induced lipid-loading compared to the WT BMDMs. Genes involved in foam cell formation were investigated to gain insight into the mechanism behind the enhanced foam cell formation in absence of Arg1. No difference was found in the expression of *ABCA1*, the primary cholesterol export transporter, between WT and Arg1 KO macrophages. In response to acLDL loading, Arg1 deficient macrophages however did display an impaired suppression of *SREBP1* and *SR-B1* compared to WT macrophages. SREBP1 is an important regulator of cellular lipid biosynthesis,[33] while *SR-B1* facilitates the uptake of native and modified lipoproteins.[34] Enhanced foam cell formation of macrophages lacking Arg1 might thus be explained by defective suppression of cellular lipid biosynthesis and modified LDL uptake. Moreover, upon lipid loading, macrophages lacking Arg1 seem more likely to skew towards the

M2 phenotype, as evidenced by the significantly downregulated M1 marker *iNOS* and strongly upregulated M2 marker *FIZZ-1* expression in Arg1 KO macrophages. M2 macrophages exhibit increased cholesterol loading by oxLDL compared to M1 macrophages, providing one possible mechanism for the increased foam cell formation in our model.[35,36] Collectively, these findings suggest a link between Arg1 expression and macrophage foam cell formation. As Arg1 and iNOS compete for the common substrate L-arginine, macrophage Arg1 deficiency indirectly leads to enhanced synthesis of NO upon lipid loading. The observed downregulation of iNOS in the Arg1 deficient macrophages could thus be due to negative feedback by NO.[10,37-41] Notably, in agreement with our findings, excess NO has recently been shown to induce oxLDL-induced macrophage foam cell formation by inhibition of the LXR-ABCA1-cholesterol efflux axis.[42]

Next we investigated whether the observed increased foam cell formation *in vitro* also translated into increased macrophage foam cell formation *in vivo* and augmented atherosclerosis susceptibility. Hereto, bone marrow from *Arg1$^{flox/flox}$;Tie2Cre* mice was transplanted into LDLr KO mice, a commonly used model to study atherosclerosis. We decided to use *Arg1$^{flox/flox}$;Tie2Cre* mice as donors as the Tie2-cre deleter has previously been shown to lead to a complete ablation of Arg1 activity in macrophages, while in *Arg1$^{flox/flox}$;LysMCre* only ~80% reduction in macrophage Arg1 activity was achieved.[20] In line with the observed increase in lipid accumulation in Arg1 KO macrophages *in vitro*, foam cell formation in the peritoneal cavity of LDLr KO mice transplanted with Arg1 KO bone marrow was increased after 10 weeks WTD feeding.

Foam cell formation in the peritoneal cavity is a marker for atherosclerotic plaque development.[43,44] However, no differences in plaque size or plaque macrophage content were detected between the LDLr KO mice transplanted with Arg1 KO or WT bone marrow. Increased expression of Arg1 in balloon-injured rabbits results in augmented plaque stability as a consequence of enhanced VSMC proliferation.[8] Moreover, *Arg1$^{flox/flox}$;Tie2Cre* (Arg1 KO) mice exhibit impaired cutaneous wound healing, to some extent due to impaired collagen deposition.[43] The reduced collagen content in wounds of *Arg1$^{flox/flox}$;Tie2Cre* (Arg1 KO) mice was attributed to increased collagen degradation by neutrophils and macrophages lacking Arg1.[45] In atherosclerosis, collagen deposition provides stability to the atherosclerotic plaque. However, no significant effect on the collagen-content of atherosclerotic plaques of LDLr KO mice transplanted with Arg1 KO bone marrow was found. In the aortic root lesions, smooth muscle cells, the main producers of collagen deposited in atherosclerotic lesions,[46] were not affected by deletion of Arg1 in bone marrow-derived cells.[47] Conclusively, deletion of Arg1 in bone marrow-derived cells does not affect atherosclerosis susceptibility despite a clear increase in macrophage foam cell formation, indicating other compensatory mechanisms.

Tie2 is expressed in all hematopoietic lineage cells.[20] Hence, in our bone marrow transplantation model using the *Arg1$^{flox/flox}$;Tie2Cre* mice as donors, Arg1 is not only deleted in macrophages, but in all hematopoietic cells.[48] For long it has been thought that Arg1 is only expressed in the myeloid lineage and not in the lymphoid lineages.[49] Indeed Arg1 is expressed primarily in anti-inflammatory alternatively activated macrophages, however it can also be detected in neutrophils,[50,51] and innate lymphoid cells II (ILC2).[52]

To investigate whether bone marrow Arg1 deficiency had any atheroprotective effects to counteract the observed increase in foam cell formation, the leukocytes in blood and spleen, the major hematopoietic organ and an important reservoir for monocytes, were analysed in the BM transplanted mice. In agreement with previous studies by Niese *et al.*,[32] deletion of Arg1 in bone

marrow-derived cells did not affect circulating leukocytes on regular chow diet. Hypercholesterolemia is commonly known to induce leukocytosis in animal models.[53,54] and humans.[55,56] Upon challenge with a high fat/high cholesterol WTD, LDLr KO mice transplanted with Arg1 KO bone marrow failed to increase leukocyte counts in the circulation. The inflammatory and pro-atherogenic $CD11b^{+}/Ly6C^{hi}$ subset of monocytes gives rise to classically activated M1 macrophages in the atherosclerotic plaque.[57,58] However, no difference was found in the numbers of $CD11b^{+}/Ly6C^{hi}$ monocytes in blood, nor in the amount of circulating neutrophils between the 2 groups of bone marrow recipients after 10 weeks WTD challenge. Hypercholesterolemia-induced monocytosis and neutrophilia was thus not affected in the blood by deletion of Arg1 in bone marrow. In addition to myeloid cell mobilization from bone marrow, high fat diet-induced inflammatory conditions like atherosclerosis could also induce hematopoietic stem and progenitor cells (HSPCs) settlement in the spleen and leading to local production of monocytes and neutrophils.[59,60] Spleen-derived monocytes and neutrophils eventually infiltrate into the growing atherosclerotic lesion, giving rise to foam cell formation and pro-inflammatory cytokine production.[60] Notably, 30% of the total number of aortic monocytes were spleen-derived $Ly\text{-}6C^{hi}$ monocytes, which are also reported to contribute to foam cell formation in the lesions.[60] Arg1 depletion in bone marrow leads to a significant reduction of the $CD11b^{+}$ cells, $CD11b^{+}/ly6C^{low+med}$ monocytes and neutrophils in the spleen of LDLr KO mice and a tendency towards a decrease in pro-inflammatory $CD11b^{+}/Ly6C^{hi}$ monocytes. This might provide an atheroprotective mechanism counteracting the increased foam cell in LDLr KO mice transplanted with Arg1 KO BM.

As L-arginine is required for $CD4^{+}$ T-cell function and maturation, Arg1-mediated depletion of L-arginine by leukocytes results in decreased T cell proliferation.[17,61] T cell counts and activation status were therefore also investigated. However, there was no difference in the amount of $CD4^{+}$ cells and $CD4^{+}/CD25^{+}$ in blood and spleen, although a modest fractional increase in both subtypes was found in the spleen. This indicates that leukocyte Arg1 is not a strong regulating factor of T-cell proliferation in the spleen. Notably, strikingly lower amounts of $CD19^{+}$ B cells were found in both the circulation and the spleen of Arg1 KO transplanted LDLr KO mice as compared to WT transplanted animals after 10 weeks WTD challenge, explaining the decrease in total blood leukocyte counts. L-arginine is an essential amino acid for B cell maturation in the bone marrow and arginase-mediated L-arginine depletion leads to reduced B cell emigration from the bone marrow and reduced B cell numbers in the spleen and lymph nodes.[62] If anything, leukocyte Arg1 deletion is thus anticipated to enhance B cell emigration from bone marrow, which clearly cannot explain the reduced B cell numbers in blood. B cells, as the antibody producing cells of the immune system, play an important role in atherosclerosis. Anti-oxLDL antibodies, especially IgM anti-oxLDL, are inversely related to atherosclerotic plaque size in experimental studies.[63] Therefore, oxLDL antibody levels were determined in plasma obtained from recipient mice after 10 weeks WTD feeding. To our surprise, despite the reduced numbers of B cells in the LDLr KO recipients transplanted with Arg1 KO BM, total oxLDL-specific antibody levels were increased. OxLDL-specific IgM that is suggested to be atheroprotective,[63] however, was not changed. Although in clinical cardiovascular disease diverging results have been described on the association between oxLDL-specific antibodies and atherosclerosis, animal studies consistently suggest an atheroprotective role for oxLDL antibodies.[64] Whatever the mechanism behind the reduction in B cells and increased circulating levels of oxLDL-specific antibodies, it might counteract the pro-atherogenic effects of enhanced foam cell formation in the absence of Arg1.

We conclude that despite leading to an increase in foam cell formation and a decrease in circulating B cells, Arg1 deficiency in bone marrow-derived cells does not significantly affect atherosclerotic plaque development.

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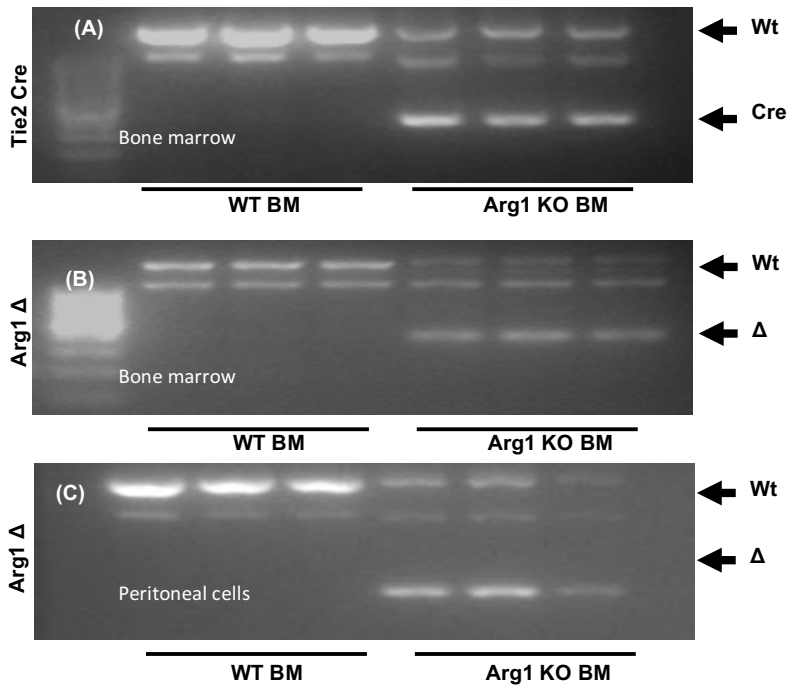
Supplementary material

Genotyping of expression of Tie2Cre and the correct excision of exons 7 and 8 from the Arg1 gene. At 18 weeks after transplantation and after 10 weeks of WTD feeding the recipient animals were sacrificed and genomic DNA was isolated from the bone marrow and peritoneal cavity of the recipients and subjected to genotyping PCR analysis. A) Genotyping by PCR shows presence of Tie2Cre in representative mice having received the Arg1flox/flox;Tie2Cre BM. B-C)The presence of the Arg1Δ construct, i.e. the successful Cre-mediated excision of exons 7 and 8 from the Arg1 gene, was also detected using a set of 3 primers. Genotyping by PCR shows positive bands for the Arg1Δ product in Arg1flox/flox Tie2Cre transplanted mice, indicating successful deletion of exons 7 and 8 in bone marrow and peritoneal cells. A faint band indicating the presence of WT DNA can still be seen, this is in accordance with previous studies where a transplantation efficiency of 95% was demonstrated[24].



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