MACROPHAGE ARGINASE 1 DEFICIENCY RESULTS IN DECREASED LEUKOCYTOSIS AND INCREASED FOAM CELL FORMATION BUT DOES NOT AFFECT ATHEROSCLEROSIS IN LDL RECEPTOR KNOCKOUT MICE

E van Kampen[1*]
B Ren[1*]
TJC van Berkel[1]
SM Cruickshank[2]
M van Eck[1]

III

* Both Authors contributed equally to this work
1 Leiden Academic Centre for Drug Research, Cluster BioTherapeutics, Division of Biopharmaceutics, Leiden, The Netherlands
2 Manchester Immunology Group, Faculty of Life Sciences, The University of Manchester, Manchester, UK

Abstract

Arginase1 (Arg1) is an enzyme that plays a role in several pathways important for the development of atherosclerosis. 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 strong anti-atherogenic factor in endothelial cells. In macrophages, however, NO is pro-atherogenic. Furthermore, Arginase 1 is expressed by anti-inflammatory alternatively activated macrophages, and is required for cutaneous wound healing.

In order to specifically investigate the effect of Arg1 deletion in macrophages on atherosclerosis susceptibility, LDL receptor knockout (LDLr KO) mice were transplanted with bone marrow (BM) from $Arg1^{flox/flox}$ *Tie2-Cre* (Arg1 KO) donors, lacking Arg1 function in bone marrow-derived cells, or BM from wildtype controls. Blood leukocyte counts were decreased by 25% ($p<0.001$), and spleen leukocytes were decreased by 35% ($p<0.05$) in the Arg1 KO BM recipients after 10 weeks Western-type diet feeding. However, peritoneal foam cells of the Arg1 KO recipients were increased 3-fold ($p<0.001$). 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 tricuspid area of the heart. Surprisingly, there was also no difference in plaque collagen content, indicating that absence of macrophage Arg1 function does not reduce plaque stability.

In conclusion, deletion of Arg1 in bone marrow-derived cells adversely affects blood leukocyte counts and increases foam cell formation. However, no effects on atherosclerosis development could be found, indicating that myeloid cell Arg1 function is not a decisive factor in atherosclerotic plaque formation.

Introduction

Arginase 1 (Arg1) has been identified as an important enzyme in a number of processes implicated in the pathogenesis of atherosclerosis. Arg1 is a cytosolic metalloenzyme which is highly expressed in liver and kidney, where it plays an essential role in the last step of the urea cycle and the breakdown of nitrogen and ammonia to urea.[1] However, Arg1 is also expressed in endothelial cells, vascular smooth muscle cells (VSMCs) and macrophages, which are important components of the atherosclerotic plaque.[2]

Arg1 uses L-arginine to produce urea and L-ornithine, which is further broken down to L-proline by the enzyme Ornithine AminoTransferase (OAT).[3] L-arginine is also used as a substrate by the enzymes inducible- and endothelial Nitric Oxide Synthase (iNOS and eNOS) for the production of the atheroprotective signalling molecule nitric oxide (NO).[4, 5] By competition for the common substrate L-arginine, Arg1 can thus indirectly regulate the synthesis of NO. Since arginase activity is increased in animal models of atherosclerosis and augmented activity is expected to lead to NO depletion, inhibition of arginase activity is considered a promising novel therapeutic strategy for the treatment of cardiovascular disease.[2]

Depending on the cell type it is expressed in, Arg1 function is expected to exert different effects on atherosclerotic plaque formation. Endothelial Arg1 contributes to endothelial activation and vascular stiffness by reducing the L-arginine pool, leading to eNOS uncoupling and reduced NO production.[4-6] This results in endothelial activation and increased recruitment of immune cells to the plaque.[4, 6] However, Arg1 expression in macrophages and VSMCs has an anti-inflammatory effect. By producing L-ornithine Arg1 contributes to the synthesis of L-proline, which is a precursor for collagen biosynthesis. Ornithine can also be metabolised to polyamines, which leads to increased smooth muscle cell differentiation and decreased inflammation.[7-9] In line, lentiviral-mediated upregulation of Arg1 in a balloon-injury rabbit model inhibited plaque inflammation and augmented VSMC proliferation.[10] Plaque size was, however, not affected.[10]

Alternatively activated M2 macrophages express Arg1, suggesting an anti-inflammatory and wound healing role for macrophage Arg1.[11] In line, downregulation of Arg1 expression and inhibition of Arg1 activity in Raw264.7 macrophages augmented LPS-induced TNFalpha and IL-6 secretion.[10] On the other hand, Arg1 deficiency in macrophages increased the production of anti-inflammatory cytokines by CD4+ T cells and led to a reduced suppression of T-cell proliferation.[12] Differential gene expression analysis in macrophages of atherosclerosis-susceptible and -resistant rabbits suggested that high macrophage Arg1 expression was associated with low atherosclerosis susceptibility.[13] However, the functional role of Arg1 in atherosclerotic plaque development is unknown.

The current study specifically assessed the contribution of leukocyte Arg1 to the development of atherosclerosis by transplanting Arg1$^{flox/flox}$;Tie2Cre bone marrow into atherosclerosis-susceptible LDL receptor knockout (LDLr KO) mice. We hypothesize that deficiency of leukocyte Arg1 results in decreased plaque stability, as well as increased inflammation and T-cell responses.

Material and Methods

Animals

LDL receptor knockout (LDLr KO) mice were obtained from the Jackson Laboratory and bred at the Gorlaeus Laboratories in Leiden, the Netherlands. Arg1$^{flox/flox}$;Tie2Cre mice[14] 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.

Bone Marrow-Derived Macrophages

Bone marrow from 12 week old male Arg1$^{flox/flox}$;Tie2Cre mice and wildtype C57Bl/6 controls was isolated. Arg1$^{flox/flox}$;Tie2Cre (Arg1 KO) mice do not express Arg1 in the hematopoietic lineage.[14] Therefore, all bone marrow-derived cells from these animals lack Arg1 function. Arg1 KO bone marrow cells and wild type (WT) bone marrow cells were plated in DMEM/20% FCS/1% penicillin/1% streptomycin and differentiated into macrophages by addition of 30% L929 cell-conditioned media (as a source of M-CSF) for 7 days, as described previously.[15] Macrophages were incubated for 24h in the absence or presence of 100 ng/mL acetylated LDL (acLDL, prepared as described previously[16]) and subsequently lysed for mRNA extraction.

mRNA Expression Analysis by Real Time PCR

Total RNA from indicated samples was isolated using the guanidinium thiocyanate (GTC) method[17] and reverse transcribed using a RevertAid M-MuLV enzyme (Fermentas, Burlington, Canada). The mRNA expression levels were assessed by real time PCR (ABI PRISM 7500; Applied Biosystems, Foster City, CA) using SYBR Green technology (Applied Biosystems). The average of GAPDH and 36B4 expression was used as a housekeeping (HK) control.

Bone Marrow Transplantation

Bone marrow from male Arg1$^{flox/flox}$;Tie2Cre (Arg1 KO) mice and wildtype C57Bl/6 controls (age 12 weeks) was prepared for bone marrow transplantation (BMT) to 12 weeks old female LDLr KO recipient mice. In short, $5x10^6$ bone marrow cells were injected into the tail vein of lethally irradiated recipients.[15] The mice were allowed a recovery period of 8 weeks on chow diet (RM3; Special Diet Services), after which they were fed a high-fat, high-cholesterol Western-Type Diet (WTD), containing 15% cacao butter and 0.25% cholesterol (WTD; Special Diet Services) for 10 weeks. At 18 weeks after transplantation, the mice were anaesthetized using a mix of rompun, ketamine and atropine at a lethal dose. Mice were then exsanguinated and perfused with PBS, after which organs were isolated.

Genotyping reaction

Bone marrow samples were taken from BMT recipients at 18 weeks after transplantation and used to isolate DNA. DNA samples were genotyped for the presence of Tie2 Cre (CGCATAACCAGTGAAACAGCATTGC - CCCTGTGCTCAGACAGAAATGAGA) and the successful deletion of exons 7 and 8 of the Arg1 gene (CCCCCAAAGGAAATGTAAGAA - CACTGTCTAAGCCCGAGAGTA) as published previously.[14]

Flow Cytometry Analysis and Sysmex

Blood samples, anti-coagulated with EDTA, as well as single cell suspensions of spleen, obtained using a 70µm cell strainer (734-0003, VWR), were 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 samples 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 Sysmex XT-2000iV Veterinary Haematology analyzer (Sysmex Corporation) was used to analyse leukocyte counts in spleen and blood samples and peritoneal leukocyte suspensions.

Serum cholesterol

Serum concentrations of free cholesterol were determined by enzymatic colorimetric assays with 0.048 U/mL cholesterol oxidase (228250, Calbiochem) and 0.065 U/mL peroxidase (P8375, Sigma) in reaction buffer (1.0 KPi buffer, pH=7.7 containing 0.01 M phenol, 1 mM 4-amino-antipyrine, 1% polyoxyethylene-9-laurylether, and 7.5% methanol). For the determination of total cholesterol, 0.03 U/mL cholesteryl esterase (228180, Calbiochem) was added to the reaction solution. Absorbance was read at 490 nm.

Histological Analysis of the Aortic Root

Serial sections (7 µm) of the aortic root were cut using a Leica CM3050S cryostat. The atherosclerotic plaque areas in oil red-O stained cryostat sections of the aortic root were quantified using the Leica image analysis system, consisting of a Leica DMRE microscope coupled to a video camera and Leica Qwin Imaging software (Leica Ltd, Cambridge, UK). Mean plaque area (in μm^2) was calculated from 10 consecutive oil red-O stained sections, starting at the appearance of the tricuspid valves. For the assessment of macrophage content sections were immunolabeled with MOMA-2 (Research Diagnostics Inc; dilution 1:50). The MOMA-2-positive plaque area was subsequently quantified using the Leica image analysis system and expressed as a fraction of total plaque area.

To visualize plaque collagen content a Masson Trichrome kit was used (HT15-1,4, Sigma Aldrich) consisting of Biebrich Scarlet Acid Fuchsin, Phosphotungstic Acid, Phosphomolybdic Acid and Aniline Blue. The tissue was stained according to manufacturer's instructions (Procedure HT15). As a secondary method to visualise plaque collagen, sections were stained with Picrosirius red (Direct Red 80, Sigma Aldrich), according to manufacturer's instructions.

Quantification of oxidative stress in the spleen

To quantify the presence of peroxidised lipids, a sign of oxidative stress, an MDA analysis was performed as described previously.[18] Briefly, liver and spleen samples of approximately 100mg each were homogenised and suspended in 0.2ml 1.1% phosphoric acid. Then 0.2ml of 1% thiobarbituric acid (TBA, T5500, Sigma-Aldrich) solution with 1mM butylated

hydroxytoluene (B1378, Sigma-Aldrich) was added and the solution was incubated at 100°C for 1 hour, after which butanol was added. The samples were spun down and the supernatant was measured on a plate reader at 530nm and 590nm. The 590nm readout was subtracted from the 530nm readout and the result was normalised to nmol/gram of organ weight.

Statistical Analysis

Statistically significant differences among the means of the different populations were tested using the unpaired Student's T-test (Graphpad Prism software). A Welch correction was applied to the T-test in the case of unequal variances in the dataset. The probability level (alpha) for statistical significance was set at 0.05. Results are expressed as an average ± SEM.

Results

Lipid laden Arg1 KO BMDMs have altered expression of differentiation markers, ApoE and SREBP-1 and are more likely to differentiate to foam cells

Gene expression in Arg1 KO and WT BMDMs was analysed before and after acLDL-induced lipid loading. Lipid loading led to a modest (0.85-fold, $p<0.05$) decrease in the expression of the M1 macrophage marker iNOS in WT BMDM, while the expression of the M2 marker FIZZ1 was not affected. Interestingly, a much stronger 3-fold ($p<0.01$, figure 1-A) decrease in iNOS expression was found upon incubation of BMDMs lacking Arg1 with acLDL, whereas there was a strong, 20-fold upregulation of the M2 marker FIZZ1 ($P<0.01$ figure 1-B). ABCA1 expression was increased 6-fold after lipid loading, but no effects of Arg1 deletion were observed ($P<0.05$, figure 1-C). ApoE was upregulated 1.5-fold in Arg1 KO BMDMs both before and after lipid loading ($P<0.05$, figure 1-D). Additionally, expression of SREBP-1 in WT BMDMs was decreased 2.5-fold after lipid loading, but remained unchanged in Arg1 KO BMDMs ($P<0.05$, figure 1-E). However, expression of SR-BI and LDL receptor remained unchanged (figure 1-F,G).

AcLDL-induced lipid loading resulted in increased foam cell formation in both WT BMDMs and Arg1 KO BMDMs. Interestingly, both before and after lipid loading Arg1 KO BMDMs displayed a 50% increase in foam cell differentiation compared to WT BMDMs ($P<0.05$, figure 1-H).

Generation of an atherosclerosis-prone mouse model lacking Arg1 in leukocytes

In $Arg1^{flox/flox}$;Tie2Cre mice Arg1 has been deleted in cells of the hematopoietic lineages and in endothelial cells. 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 cells of hematopoietic origin, Arg1 is expressed primarily in alternatively activated macrophages, however it can also be detected in neutrophils and innate lymphoid cells II.[14, 19-21] At 18 weeks after transplantation and after 10 weeks of WTD feeding the recipient animals were sacrificed for analysis.

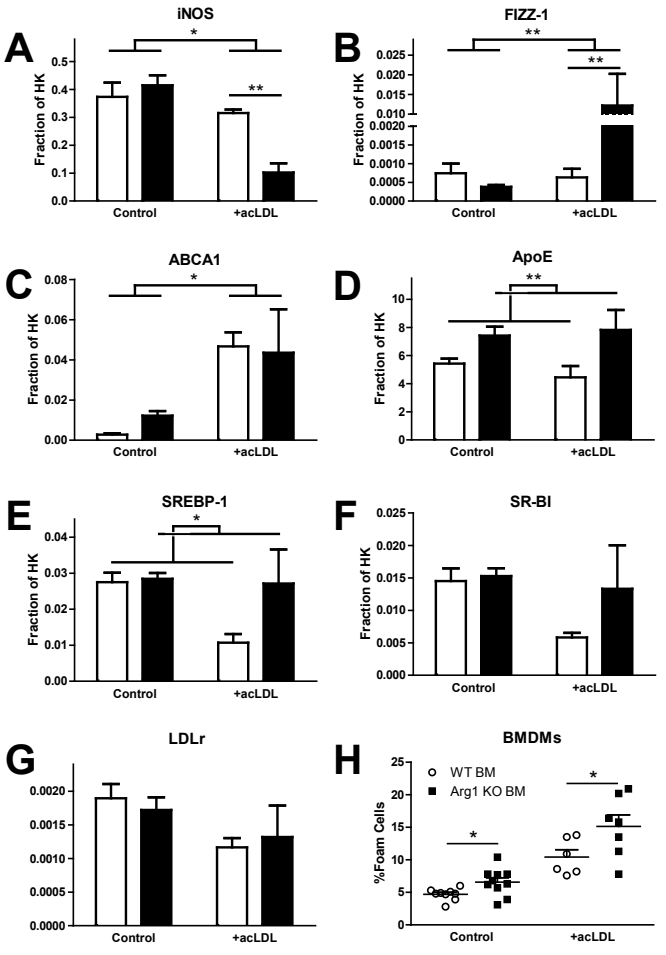

Figure 1 Effect of Arg1 deletion in bone marrow-derived macrophages (BMDMs) on gene expression and macrophage foam cell formation. A) Expression of M1 marker iNOS relative to housekeeping (HK) is decreased in Arg1 KO BMDMs (black bars) compared to WT BMDMs (white bars) after lipid loading with 100ng/ml acLDL (N=5; * P<0.05, ** P<0.01). B) Expression of M2 marker FIZZ-1 is increased in Arg1 KO BMDMS after lipid loading. (N=5; ** P<0.01, *** P<0.001). C) Expression of ABCA1 is increased in both groups after lipid loading. (N=5; * P<0.05) D) ApoE is upregulated in Arg1 KO BMDMs irrespective of lipid loading. (N=5; ** P<0.01) E) SREBP-1 expression is decreased in WT BMDMs but not in Arg1 KO BMDMs after lipid loading (N=5; P<0.05). F) No difference in expression of SR-BI could be found between Arg1 KO and WT BMDMs. G) Similarly, no differences in expression of LDLr were found between Arg1 KO and WT BMDMs. H) In increased foam cell formation in Arg1 KO BMDMs (N=8; * P<0.05). Results are expressed as mean ±SEM, significance was assessed by two way ANOVA with Bonferroni post-tests.

Genomic DNA was isolated from the bone marrow of the recipients and subjected to PCR analysis, which confirmed the presence of the Tie2-Cre transgene in the bone marrow of mice transplanted with Arg1 KO BM (figure 2-A). 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, indicating the successful disruption of Arg1 functionality in the bone marrow of the recipient mice (figure 2-B). 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.[15]

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 macrophage Arg1 functionality in the transplanted LDLr KO mice resulted in altered leukocyte numbers in the circulation or the spleen. On chow diet, no difference in total blood leukocyte numbers was detected (data not shown).

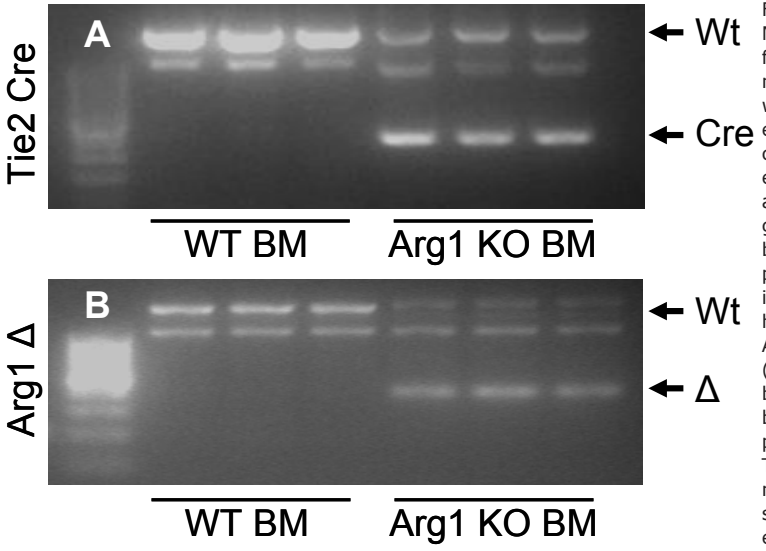

Figure 2 Bone Marrow (BM) isolated from the bone marrow recipients was genotyped for expression of Tie2 cre and the correct excision of exons 7 and 8 from the Arg1 gene. A) Genotyping by PCR shows presence of Tie2 Cre in representative mice having received the $Arg1^{flox/flox}$;Tie2Cre BM. (N=3) B) Genotyping by PCR shows positive bands for the Arg1Δ product in $Arg1^{flox/flox}$ Tie2 Cre transplanted mice, indicating successful deletion of exons 7 and 8. (N=3)

However, a reduction was found in total leukocyte counts in the blood from 15672 ±689 cells/µl in WT BM recipients to 11573 ±491 cells/µl in Arg1 KO BM recipients after 10 weeks of WTD feeding ($P<0.001$, figure 3-A). The amount of $CD11b^{+}$/$Ly6C^{hi}$ inflammatory monocytes was quantified by flow cytometric analysis, as well as the amount of $CD4^{+}$ T helper cells, $CD25^{+}$/$CD4^{+}$ activated T helper cells and $CD8^{+}$ cytotoxic T-cells. However, no difference in absolute amounts of these cell types in circulation was found (figure 3-B,C,D). 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 3-E).

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 ($P<0.05$, figure 4-A), while there were no differences in total body weight (data not shown). Correspondingly, spleens from the Arg1 KO BM recipients were found to contain 35% less splenocytes ($P=0.052$, figure 4-B). Next, splenocyte composition was assessed by flow cytometry. The absolute numbers of $CD11b^{+}$/$Ly6C^{hi}$, $CD4^{+}$, $CD8^{+}$ and $CD19^{+}$ splenocytes showed a trend towards a decrease, suggesting that the decrease in splenocyte number was not attributable to one specific cell type. Fractional analysis also showed no difference in the fraction of $CD11b^{+}$/$Ly6C^{hi}$ cells, in $CD8^{+}$ T cells and interestingly, $CD19^{+}$ B cell numbers was found (figure 4-C-E). However, a small 23% increase in $CD4^{+}$ and $CD25^{+}$/$CD4^{+}$ T cells was detected (* $P<0.05$; $ $P<0.05$, respectively. Figure 4-F).

Deficiency in macrophage Arg1 can result in increased production of NO, which in high levels can interact with reactive oxygen species (ROS) to cause oxidative stress.[22] Therefore, a TBARS assay was performed to quantify oxidative stress in the spleen, a macrophage-rich organ, by quantifying malondialdehyde (MDA), a by-product of oxidative stress. However, no difference in MDA concentration was found between the groups (figure 4-G).

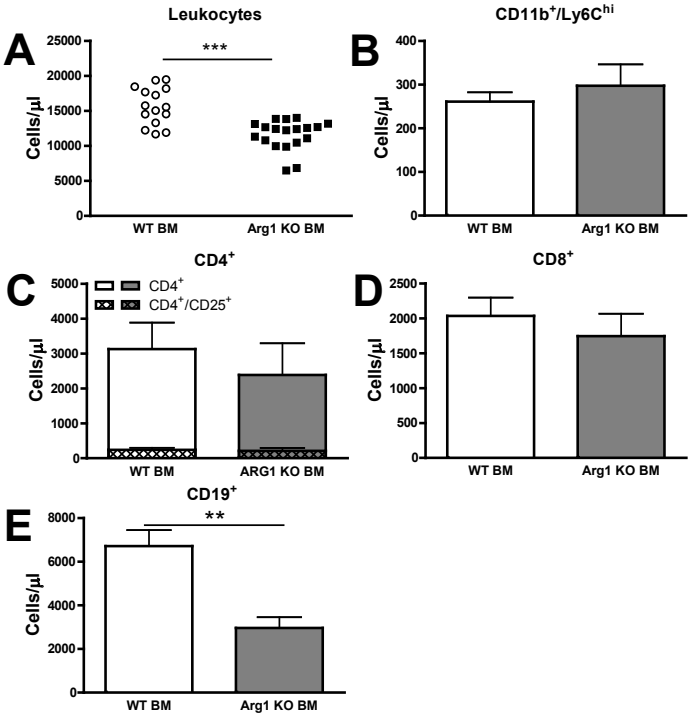

Figure 3 Increased leukocytes and lymphocytes in the blood of LDLr KO mice transplanted with Arg1 KO BM. Blood was isolated at 10 weeks feeding a high-fat, high-cholesterol Western-type diet and 18 weeks after transplantation. A) When analysed by haematology analyzer, Arg1 KO BM recipients had markedly decreased amounts of leukocytes in their circulation (*** P<0.001, N=15). B-D) Circulating leukocytes were analysed by flow cytometry for expression of several immune cell markers. No difference was found the amount of $CD11b^{+}/Ly6C^{hi}$ cells, $CD4^{+}$ cells, $CD4^{+}/CD25^{+}$ or $CD8^{+}$ cells (N=5) E) However, the amount of $CD19^{+}$ cells in circulation was decreased significantly, driving the observed decrease in total leukocytes (** P<0.01, N=5). Results are expressed as mean ±SEM, significance was assessed by student T-test.

No differences in serum total cholesterol levels, but increased foam cell accumulation in the peritoneum.

A cholesterol essay was performed on serum from bone marrow recipients after 10 weeks of WTD feeding. No difference in serum free cholesterol (data not shown) or total cholesterol was found (figure 5-A). However, analysis of peritoneal leukocytes revealed a three-fold increase in the fraction foam cells in the peritoneum (*** P<0.001, figure 5-B). This increase did not coincide with augmented total peritoneal leukocytes (data not shown) or macrophages (figure 5-C), indicating that an increased fraction of peritoneal macrophages had differentiated into foam cells.

Macrophage Arg1 deficiency affects neither atherosclerotic plaque size nor plaque composition.

At 10 weeks of WTD feeding and 18 weeks after transplantation, the tricuspid area of the aortic root was sectioned and stained with oil red-O to analyze atherosclerotic lesion development. No difference in plaque size was found between the two experimental groups (647437 ±28984 μm^2 for wildtype BM recipients vs. 633683 ±26487 μm^2 for Arg1 KO BM recipients, figure 6-A). Plaque macrophages were visualized by MoMa2 staining, and the MoMa2 positive area was corrected for total plaque size. No difference in plaque macrophage content as a fraction of total plaque size was observed (0.171 ±0.011 wildtype BM vs. 0.169 ±0.016 Arg1 KO BM, figure 6-B). Subsequently, plaque collagen was stained using a Masson Trichrome method and Picosirius Red staining, and corrected for total plaque size.

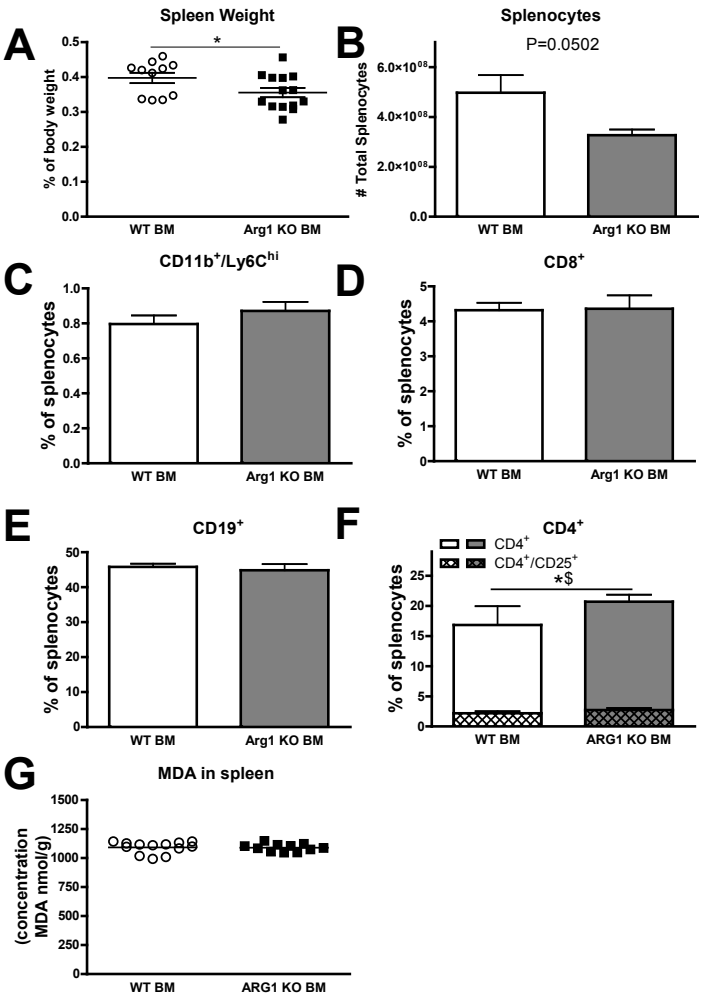

Figure 4 Decreased spleen weight in LDLr KO mice transplanted with Arg1 KO BM. Spleens were isolated at 18 weeks after transplantation and 8 weeks after feeding a high-fat, high-cholesterol Western-type diet. A) A reduction in relative spleen weight was measured in the Arg1 KO BM recipients. The readout was corrected for total body weight of the mice. (* $P<0.05$, N=12) B) This was accompanied by a reduction in total amount of splenocytes in these mice (* $P<0.05$). C) As determined by flow cytometry, the fraction of $CD11b^{+}/Ly6C^{hi}$ cells in the spleen was not different between the groups. D-E) No difference was found in the relative amount of $CD8^{+}$ and $CD19^{+}$ splenocytes. F) However, the fraction of $CD4^{+}$ and $CD4^{+}CD25^{+}$ cells in the spleen was increased. (respectively, *$P<0.05$; \$$P<0.05$) G) An MDA assay revealed no difference in oxidative stress in the spleen (N=11). Results are expressed as mean ±SEM, significance was assessed by student T-test.

A trend towards reduced plaque collagen as a fraction of total plaque size in the Arg1 KO BM recipients was found in Masson Trichrome-stained sections (0.102 ±0.009 wildtype BM vs. 0.084 ±0.004 Arg1 KO BM, P=0.06, figure 6-C). However, analysis of Picosirius Red staining did not indicate any difference between the groups (0.124 ±0.013 wildtype BM vs. 0.105 ±0.012 Arg1 KO BM, figure 6-D).

Discussion

In the current study for the first time the effects of Arg1 deletion in macrophages on macrophage function and atherosclerosis susceptibility was determined. Arg1 is highly expressed in anti-inflammatory alternatively activated macrophages. Furthermore, by depleting L-arginine, Arg1 facilitates a reduction in NO production and T-cell activation as those processes are L-arginine dependent.[12, 23] Although these processes have been described in detail, not much is known about the effect of Arg1 on other macrophage processes, such as cholesterol metabolism and foam cell formation. Bone marrow from Arg1$^{flox/flox}$;Tie2Cre (Arg1 KO) mice was used to generate Arg1 deficient BMDMs, which were subsequently loaded with acLDL. Interestingly, Arg1 KO BMDMs showed increased foam cell formation both under control conditions and after acLDL-induced lipid-loading compared to the WT BMDMs. Expression of SR-BI was investigated, as SR-BI is a receptor for modified LDL particles and might mediate uptake of acLDL in our model.[24] However, there were no differences in SR-BI expression between Arg1 KO BMDMs and WT BMDMs. Similarly, no differences were observed in LDLr expression between the 2 genotypes. 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, no difference in ABCA1 expression between the Arg1 KO and the WT BMDMs could be found. Notably, in response to acLDL loading Arg1 deficient macrophages did not down regulate expression of SREBP-1 like WT macrophages. Although gene expression analysis did not indicate a reduction in cholesterol efflux or increased uptake, augmented expression of SREBP-1 could result in increased cellular production of cholesterol and free fatty acids.[25] To this end, expression of HMG-CoA reductase, the rate limiting enzyme for cholesterol production, was measured.[26] However, no differences in expression were found.

ApoE was notably upregulated in Arg1 KO BMDMs both under control conditions and after lipid loading. Macrophage apoE contributes to only ±10% of circulating apoE levels, but local production of apoE by tissue macrophages plays an important role in atherosclerosis development.[27-30]

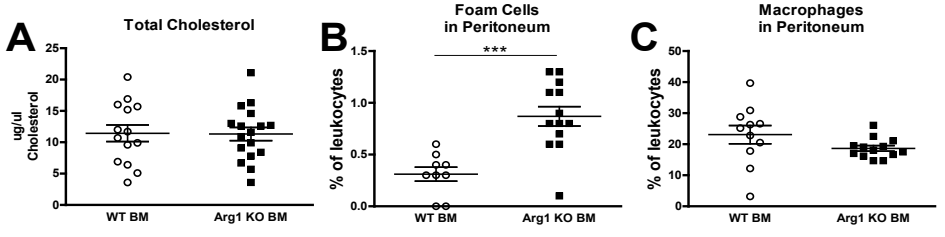

Figure 5 Increased peritoneal foam cell formation in LDLr KO mice transplanted with Arg1 KO BM in absence of effects on serum cholesterol. Blood and peritoneal leukocytes were collected at 18 weeks after BM transplantation and after 8 weeks of high-fat, high-cholesterol Western-type diet feeding. A) A cholesterol essay was performed on blood of BM recipients. No difference in total cholesterol content was observed between WT BM recipients and Arg1 KO BM recipients. (N=14) B) An increase in the amount of foam cells in the peritoneum was detected by haematology analyzer. (*** P<0.001, N=9) C) The increase in foam cells did not coincide with an increase in peritoneal macrophage content. (N=11) Results are expressed as mean ±SEM, significance was assessed by student T-test.

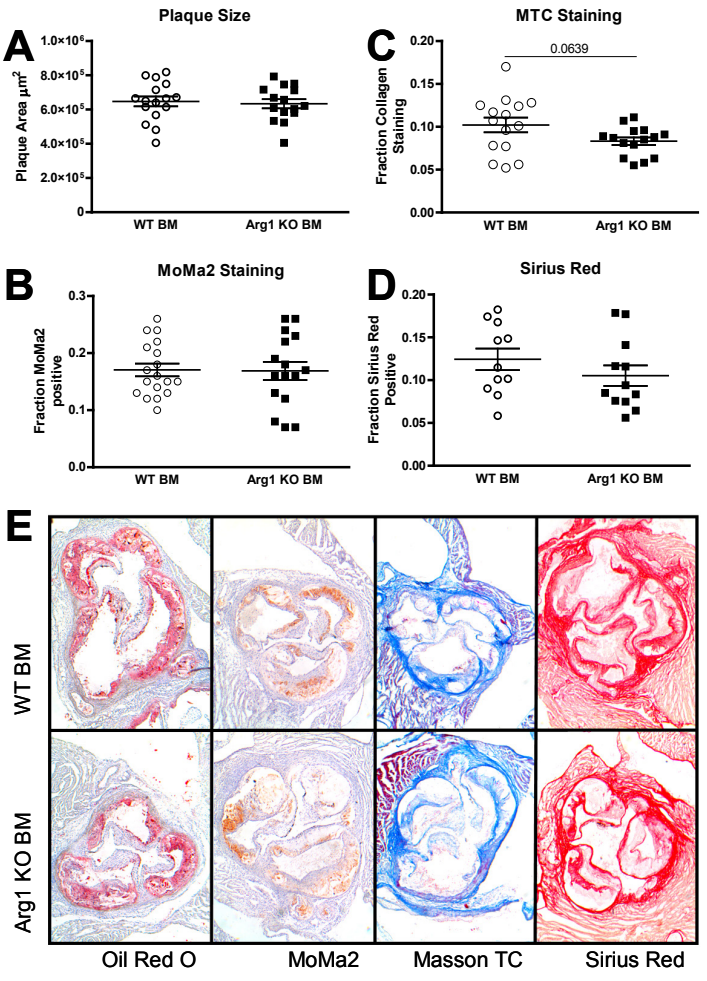

Figure 6 Deletion of Arg1 in bone marrow-derived cells does not influence atherosclerotic lesion development. A) Plaques were stained by Oil-Red O and quantified. No difference in plaque size between the groups was found. (N=15) B) Plaque area stained positively with MoMa2 antibody was measured and normalized for total lesion size. No difference in MoMa 2 positive area could be found. (N=14) C) Collagen was visualized using Masson's Trichrome Staining. A trend towards a decrease in plaque collagen content was observed. (P=0.064, N=16) D) A Sirius Red staining was performed to examine plaque collagen content further, but no difference was found between the groups. (N=12) E) Representative images of the stainings described above. Original magnification 10x. Results are expressed as mean ±SEM, significance was assessed by student T-test.

Endogenous apoE production by macrophages stimulates cellular cholesterol efflux.[31] In line, specific deletion of apoE production by bone marrow-derived cells leads to enhanced atherosclerosis susceptibility.[29, 30] Conversely, increased apoE expression is linked to a decrease in macrophage foam cell formation, indicating that the changes in apoE expression do not account for the observed increase in foam cell formation.[32] Enhanced apoE production in the arterial wall could however also affect other processes linked to atherosclerosis. Increased expression of apoE could result in increased smooth muscle cell proliferation, as well as decreased macrophage activation, including decreased production of NO and reduced expression of inflammatory factors.[33, 34]

In response to acLDL stimulation, iNOS expression was downregulated in Arg1 KO BMDMs. In addition to the observed reduction in iNOS expression, FIZZ was strongly upregulated, indicating a possible skewing towards M2 differentiation of the macrophages deficient in Arg1. 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]

Additionally, increased NO production due to Arg1 deficiency could also lead to increased foam cell formation.[37-39] In situations of low Arg1 expression, ample L-arginine substrate is available for iNOS-mediated NO production.[23] The observed down regulation of iNOS in the Arg1 deficient macrophages could thus be due to negative feedback by NO.[4, 23, 40-43]

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. Foam cell formation in the peritoneal cavity is a marker for atherosclerotic plaque development.[44, 45] However, no differences in plaque size or plaque macrophage content were detected between the LDLr KO mice transplanted with Arg1 KO or Wildtype bone marrow. In our model Arg1 was specifically deleted in bone marrow derived cells, including ILC2 cells, neutrophils and in macrophages. Neutrophils produce Arg1, potentially allowing them to modulate tissue regeneration or immune modulation.[21, 46] Classically, neutrophils in atherosclerotic lesion are considered to aggravate atherosclerosis by promoting foam cell formation, producing reactive oxygen species and eroding the fibrous cap.[47] Although the ability of neutrophils to release Arg1 into the environment indicates that they can also contribute to plaque stabilisation, the role of neutrophil Arg1 in atherosclerosis is unclear. Tie2-cre mediated Arg1 KO mice exhibit impaired cutaneous wound healing, amongst others due to impaired collagen deposition.[48] Reduced expression of Arg1 was associated with delayed wound healing, and Tie2-cre Arg1 KO mice displayed increased expression of iNOS positive macrophages in the wound.[48] The reduced collagen content in wounds of Tie2-cre Arg1 KO mice could be attributed to increased collagen degradation by neutrophils and macrophages.[48] In the atherosclerotic lesion, M1 macrophages produce Matrix Metalloproteinases (MMPs) capable of degrading extracellular matrix and reducing lesion stability.[49, 50] In contrast, Arg1 expressing M2 macrophages are associated with increased collagen production in the lesion.[51]

Ultimately, loss of Arg1 functionality did not result in a significant change in collagen content. Wang et al recently showed that increased expression of Arg1 results in increased plaque stability in balloon-injured rabbits as a consequence of augmented VSMC proliferation. In atherosclerotic plaques, VSMCs account for the majority of collagen production and Arg1 expression on VSMCs was unaffected in our model, thereby explaining the lack of effect on collagen content in the plaques.[52]

To investigate whether leukocyte 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. The $CD11b^{+}/Ly6C^{hi}$ subset of monocytes is inflammatory and pro-atherogenic, giving rise to classically activated M1 macrophages in the atherosclerotic plaque.[53, 54] However, no difference was found in the numbers of $CD11b^{+}/Ly6C^{hi}$ monocytes, in blood, spleen or peritoneum was found, between the 2 groups of bone marrow recipients. Furthermore, no difference in the amount of neutrophils in the blood was found (data not shown). 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.[12, 55] T cell counts and activation status were therefore also investigated.

Although there was no difference in the amount of $CD4^+$ cells and $CD4^+/CD25^+$ in blood, a modest increase in both subtypes was found in the spleen. The relatively modest increase in $CD4^+$ splenocytes indicates that despite its potential to deplete L-arginine, leukocyte Arg1 is not a strong regulating factor of T-cell proliferation in the spleen. Notably, a striking reduction in the amount of $CD19^+$ B cells in the circulation was found. It is not clear by what mechanism leukocyte Arg1 deficiency results in a reduction in circulating B cells, more so because the number of B cells in the spleen was unchanged. 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.[56] 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. Furthermore, ILC2 cells, which were recently shown to express Arg1, can affect B cell proliferation in the aortic wall.[57, 58] However, the effect of ILC2 cells on B cell proliferation was mediated by IL-5 production, which is not affected by lack of Arg1 expression, making it unlikely that the strong reduction in circulating B cells is due to the lack of Arg1 on ILC2 cells.[20] Whatever the mechanism behind the reduction in B cells, it is tempting to speculate that the reduced B cell numbers in the circulation counteract the pro-atherogenic effects of enhanced foam cell formation in absence of Arg1. However, B cells can be both pathological and protective in atherosclerosis, as different methods of B cell depletion have had opposite effects on atherosclerosis development.[59]

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

References

1. Getz GS, Reardon CA. Arginine/arginase no no no. *Arterioscler Thromb Vasc Biol*. 2006;26:237-239
2. Pernow J, Jung C. Arginase as a potential target in the treatment of cardiovascular disease: Reversal of arginine steal? *Cardiovasc Res*. 2013;98:334-343
3. Durante W. Role of arginase in vessel wall remodeling. *Front Immunol*. 2013;4:111
4. Berkowitz DE, White R, Li D, Minhas KM, Cernetich A, Kim S, Burke S, Shoukas AA, Nyhan D, Champion HC, Hare JM. Arginase reciprocally regulates nitric oxide synthase activity and contributes to endothelial dysfunction in aging blood vessels. *Circulation*. 2003;108:2000-2006
5. Gao X, Xu X, Belmadani S, Park Y, Tang Z, Feldman AM, Chilian WM, Zhang C. Tnf-alpha contributes to endothelial dysfunction by upregulating arginase in ischemia/reperfusion injury. *Arterioscler Thromb Vasc Biol*. 2007;27:1269-1275
6. White AR, Ryoo S, Li D, Champion HC, Steppan J, Wang D, Nyhan D, Shoukas AA, Hare JM, Berkowitz DE. Knockdown of arginase i restores no signaling in the vasculature of old rats. *Hypertension*. 2006;47:245-251
7. Durante W, Liao L, Peyton KJ, Schafer AI. Thrombin stimulates vascular smooth muscle cell polyamine synthesis by inducing cationic amino acid transporter and ornithine decarboxylase gene expression. *Circ Res*. 1998;83:217-223
8. Durante W, Liao L, Reyna SV, Peyton KJ, Schafer AI. Physiological cyclic stretch directs l-arginine transport and metabolism to collagen synthesis in vascular smooth muscle. *Faseb J*. 2000;14:1775-1783
9. Zhang M, Caragine T, Wang H, Cohen PS, Botchkina G, Soda K, Bianchi M, Ulrich P, Cerami A, Sherry B, Tracey KJ. Spermine inhibits proinflammatory cytokine synthesis in human mononuclear cells: A counterregulatory mechanism that restrains the immune response. *J Exp Med*. 1997;185:1759-1768
10. Wang XP, Zhang W, Liu XQ, Wang WK, Yan F, Dong WQ, Zhang Y, Zhang MX. Arginase i enhances atherosclerotic plaque stabilization by inhibiting inflammation and promoting smooth muscle cell proliferation. *Eur Heart J*. 2013
11. Khallou-Laschet J, Varthaman A, Fornasa G, Compain C, Gaston AT, Clement M, Dussiot M, Levillain O, Graff-Dubois S, Nicoletti A, Caligiuri G. Macrophage plasticity in experimental atherosclerosis. *PLoS One*. 2010;5:e8852
12. Pesce JT, Ramalingam TR, Mentink-Kane MM, Wilson MS, El Kasmi KC, Smith AM, Thompson RW, Cheever AW, Murray PJ, Wynn TA. Arginase-1-expressing macrophages suppress th2 cytokine-driven inflammation and fibrosis. *PLoS Pathog*. 2009;5:e1000371
13. Teupser D, Burkhardt R, Wilfert W, Haffner I, Nebendahl K, Thiery J. Identification of macrophage arginase i as a new candidate gene of atherosclerosis resistance. *Arterioscler Thromb Vasc Biol*. 2006;26:365-371
14. El Kasmi KC, Qualls JE, Pesce JT, Smith AM, Thompson RW, Henao-Tamayo M, Basaraba RJ, Konig T, Schleicher U, Koo MS, Kaplan G, Fitzgerald KA, Tuomanen EI, Orme IM, Kanneganti TD, Bogdan C, Wynn TA, Murray PJ. Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. *Nat Immunol*. 2008;9:1399-1406
15. Van Eck M, Bos IS, Hildebrand RB, Van Rij BT, Van Berkel TJ. Dual role for scavenger receptor class b, type i on bone marrow-derived cells in atherosclerotic lesion development. *Am J Pathol*. 2004;165:785-794
16. Basu SK, Goldstein JL, Anderson GW, Brown MS. Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. *Proc Natl Acad Sci U S A*. 1976;73:3178-3182
17. Chomczynski P, Sacchi N. Single-step method of rna isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem*. 1987;162:156-159
18. Mihara M, Uchiyama M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal Biochem*. 1978;86:271-278
19. Bronte V, Zanovello P. Regulation of immune responses by l-arginine metabolism. *Nat Rev Immunol*. 2005;5:641-654
20. Bando JK, Nussbaum JC, Liang HE, Locksley RM. Type 2 innate lymphoid cells constitutively express arginase-i in the naive and inflamed lung. *J Leukoc Biol*. 2013;94:877-884
21. Jacobsen LC, Theilgaard-Monch K, Christensen EI, Borregaard N. Arginase 1 is expressed in myelocytes/metamyelocytes and localized in gelatinase granules of human neutrophils. *Blood*. 2007;109:3084-3087
22. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A*. 1990;87:1620-1624
23. Chang CI, Liao JC, Kuo L. Arginase modulates nitric oxide production in activated macrophages. *Am*

J Physiol. 1998;274:H342-348
24. Krieger M, Herz J. Structures and functions of multiligand lipoprotein receptors: Macrophage scavenger receptors and ldl receptor-related protein (lrp). *Annu Rev Biochem*. 1994;63:601-637
25. Kim HJ, Takahashi M, Ezaki O. Fish oil feeding decreases mature sterol regulatory element-binding protein 1 (srebp-1) by down-regulation of srebp-1c mrna in mouse liver. A possible mechanism for down-regulation of lipogenic enzyme mrnas. *J Biol Chem*. 1999;274:25892-25898
26. Pfohl M, Naoumova RP, Klass C, Knisel W, Jakober B, Risler T, Thompson GR. Acute and chronic effects on cholesterol biosynthesis of ldl-apheresis with or without concomitant hmg-coa reductase inhibitor therapy. *J Lipid Res*. 1994;35:1946-1955
27. Boisvert WA, Spangenberg J, Curtiss LK. Treatment of severe hypercholesterolemia in apolipoprotein e-deficient mice by bone marrow transplantation. *J Clin Invest*. 1995;96:1118-1124
28. Van Eck M, Herijgers N, Yates J, Pearce NJ, Hoogerbrugge PM, Groot PH, Van Berkel TJ. Bone marrow transplantation in apolipoprotein e-deficient mice. Effect of apoe gene dosage on serum lipid concentrations, (beta)vldl catabolism, and atherosclerosis. *Arterioscler Thromb Vasc Biol*. 1997;17:3117-3126
29. Van Eck M, Herijgers N, Vidgeon-Hart M, Pearce NJ, Hoogerbrugge PM, Groot PH, Van Berkel TJ. Accelerated atherosclerosis in c57bl/6 mice transplanted with apoe-deficient bone marrow. *Atherosclerosis*. 2000;150:71-80
30. Fazio S, Babaev VR, Burleigh ME, Major AS, Hasty AH, Linton MF. Physiological expression of macrophage apoe in the artery wall reduces atherosclerosis in severely hyperlipidemic mice. *J Lipid Res*. 2002;43:1602-1609
31. Langer C, Huang Y, Cullen P, Wiesenhutter B, Mahley RW, Assmann G, von Eckardstein A. Endogenous apolipoprotein e modulates cholesterol efflux and cholesteryl ester hydrolysis mediated by high-density lipoprotein-3 and lipid-free apolipoproteins in mouse peritoneal macrophages. *J Mol Med (Berl)*. 2000;78:217-227
32. Hasty AH, Linton MF, Brandt SJ, Babaev VR, Gleaves LA, Fazio S. Retroviral gene therapy in apoe-deficient mice: Apoe expression in the artery wall reduces early foam cell lesion formation. *Circulation*. 1999;99:2571-2576
33. Laskowitz DT, Thekdi AD, Thekdi SD, Han SK, Myers JK, Pizzo SV, Bennett ER. Downregulation of microglial activation by apolipoprotein e and apoe-mimetic peptides. *Exp Neurol*. 2001;167:74-85
34. Zhang H, Wu LM, Wu J. Cross-talk between apolipoprotein e and cytokines. *Mediators Inflamm*. 2011;2011:949072
35. Oh J, Riek AE, Weng S, Petty M, Kim D, Colonna M, Cella M, Bernal-Mizrachi C. Endoplasmic reticulum stress controls m2 macrophage differentiation and foam cell formation. *J Biol Chem*. 2012;287:11629-11641
36. Chu EM, Tai DC, Beer JL, Hill JS. Macrophage heterogeneity and cholesterol homeostasis: Classically-activated macrophages are associated with reduced cholesterol accumulation following treatment with oxidized ldl. *Biochim Biophys Acta*. 2013;1831:378-386
37. Buttery LD, Springall DR, Chester AH, Evans TJ, Standfield EN, Parums DV, Yacoub MH, Polak JM. Inducible nitric oxide synthase is present within human atherosclerotic lesions and promotes the formation and activity of peroxynitrite. *Lab Invest*. 1996;75:77-85
38. Leeuwenburgh C, Hardy MM, Hazen SL, Wagner P, Oh-ishi S, Steinbrecher UP, Heinecke JW. Reactive nitrogen intermediates promote low density lipoprotein oxidation in human atherosclerotic intima. *J Biol Chem*. 1997;272:1433-1436
39. Stocker R, Keaney JF, Jr. Role of oxidative modifications in atherosclerosis. *Physiol Rev*. 2004;84:1381-1478
40. Gobert AP, Daulouede S, Lepoivre M, Boucher JL, Bouteille B, Buguet A, Cespuglio R, Veyret B, Vincendeau P. L-arginine availability modulates local nitric oxide production and parasite killing in experimental trypanosomiasis. *Infect Immun*. 2000;68:4653-4657
41. Han YJ, Kwon YG, Chung HT, Lee SK, Simmons RL, Billiar TR, Kim YM. Antioxidant enzymes suppress nitric oxide production through the inhibition of nf-kappa b activation: Role of h(2) o(2) and nitric oxide in inducible nitric oxide synthase expression in macrophages. *Nitric Oxide*. 2001;5:504-513
42. Woo A, Shin W, Cuong TD, Min B, Lee JH, Jeon BH, Ryoo S. Arginase inhibition by piceatannol-3'-o-beta-d-glucopyranoside improves endothelial dysfunction via activation of endothelial nitric oxide synthase in apoe-null mice fed a high-cholesterol diet. *Int J Mol Med*. 2013;31:803-810
43. Kleinert H, Schwarz PM, Forstermann U. Regulation of the expression of inducible nitric oxide synthase. *Biol Chem*. 2003;384:1343-1364
44. Li AC, Binder CJ, Gutierrez A, Brown KK, Plotkin CR, Pattison JW, Valledor AF, Davis RA, Willson TM, Witztum JL, Palinski W, Glass CK. Differential inhibition of macrophage foam-cell formation and atherosclerosis in mice by pparalpha, beta/delta, and gamma. *J Clin Invest*. 2004;114:1564-1576
45. Yvan-Charvet L, Ranalletta M, Wang N, Han S, Terasaka N, Li R, Welch C, Tall AR. Combined deficiency of abca1 and abcg1 promotes foam cell accumulation and accelerates atherosclerosis in

mice. *J Clin Invest*. 2007;117:3900-3908
46. Munder M, Mollinedo F, Calafat J, Canchado J, Gil-Lamaignere C, Fuentes JM, Luckner C, Doschko G, Soler G, Eichmann K, Muller FM, Ho AD, Goerner M, Modolell M. Arginase i is constitutively expressed in human granulocytes and participates in fungicidal activity. *Blood*. 2005;105:2549-2556
47. Soehnlein O. Multiple roles for neutrophils in atherosclerosis. *Circ Res*. 2012;110:875-888
48. Campbell L, Saville CR, Murray PJ, Cruickshank SM, Hardman MJ. Local arginase 1 activity is required for cutaneous wound healing. *J Invest Dermatol*. 2013;133:2461-2470
49. Gough PJ, Gomez IG, Wille PT, Raines EW. Macrophage expression of active mmp-9 induces acute plaque disruption in apoe-deficient mice. *J Clin Invest*. 2006;116:59-69
50. Johnson JL, George SJ, Newby AC, Jackson CL. Divergent effects of matrix metalloproteinases 3, 7, 9, and 12 on atherosclerotic plaque stability in mouse brachiocephalic arteries. *Proc Natl Acad Sci U S A*. 2005;102:15575-15580
51. Stoger JL, Gijbels MJ, van der Velden S, Manca M, van der Loos CM, Biessen EA, Daemen MJ, Lutgens E, de Winther MP. Distribution of macrophage polarization markers in human atherosclerosis. *Atherosclerosis*. 2012;225:461-468
52. Newby AC, Zaltsman AB. Fibrous cap formation or destruction--the critical importance of vascular smooth muscle cell proliferation, migration and matrix formation. *Cardiovasc Res*. 1999;41:345-360
53. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity*. 2003;19:71-82
54. Swirski FK, Libby P, Aikawa E, Alcaide P, Luscinskas FW, Weissleder R, Pittet MJ. Ly-6chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. *J Clin Invest*. 2007;117:195-205
55. Choi BS, Martinez-Falero IC, Corset C, Munder M, Modolell M, Muller I, Kropf P. Differential impact of l-arginine deprivation on the activation and effector functions of t cells and macrophages. *J Leukoc Biol*. 2009;85:268-277
56. de Jonge WJ, Kwikkers KL, te Velde AA, van Deventer SJ, Nolte MA, Mebius RE, Ruijter JM, Lamers MC, Lamers WH. Arginine deficiency affects early b cell maturation and lymphoid organ development in transgenic mice. *J Clin Invest*. 2002;110:1539-1548
57. Perry HM, Oldham SN, Fahl SP, Que X, Gonen A, Harmon DB, Tsimikas S, Witztum JL, Bender TP, McNamara CA. Helix-loop-helix factor inhibitor of differentiation 3 regulates interleukin-5 expression and b-1a b cell proliferation. *Arterioscler Thromb Vasc Biol*. 2013;33:2771-2779
58. Tedgui A, Ait-Oufella H. Innate lymphoid cells: New players in atherosclerosis? *Arterioscler Thromb Vasc Biol*. 2013;33:2697-2698
59. Nilsson J, Fredrikson GN. The b cell in atherosclerosis: Teaming up with the bad guys? *Clin Chem*. 2010;56:1789-1791