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

Baoyan Ren [a, *, 1], Erik Van Kampen [a, 1], Theo J.C. Van Berkel [a], Sheena M. Cruickshank [b], Miranda Van Eck [a]

[a] Division of Biopharmaceutics, Cluster BioTherapeutics, Leiden Academic Centre for Drug Research, Leiden University, Leiden, The Netherlands
[b] Manchester Immunology Group, Faculty of Life Sciences, The University of Manchester, Manchester, UK

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ABSTRACT

Background and aims: Arginase1 (Arg1), an 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.

Methods: Ldlr KO mice were transplanted with *Arg1$^{flox/flox}$;Tie2-Cre* (Arg1 KO) bone marrow (BM) or wildtype (WT) BM. After 8 weeks of recovery on chow diet, recipients mice were fed a Western-Type Diet (WTD) for 10 weeks to induce atherosclerosis.

Results: After 10-week 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.

Conclusions: 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.

1. Introduction

Inhibition of the activity of the enzyme arginase 1 (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.

* Corresponding author. Division of Biopharmaceutics, Leiden Academic Centre for Drug Research, Gorlaeus Laboratories EE1.13, Einsteinweg 55, 2333 CC Leiden, The Netherlands.
E-mail address: b.ren@lacdr.leidenuniv.nl (B. Ren).
[1] These authors contributed equally to this work.

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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.

2. Materials and methods

2.1. 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.

2.2. mRNA expression analysis by real time PCR

Thioglycollate-elicited peritoneal macrophages (PMs) from 12-week old male C57Bl/6 mice was 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 h. 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.

2.3. 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].

2.4. 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 5×10^6 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 anesthetic mixture that contains 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 recipient bone marrow using the PCR method (Supplementary Fig. 1A and B).

2.5. 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 h with or without 100 μg/mL acetylated-low density lipoprotein (acLDL). The preparation of acLDL is described previously [25]. Subsequently the cells were analysed 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 gaiting of a separate, shifted population of macrophage foam cells.

2.6. Flow cytometry analysis and WBC differential analysis

Blood samples, anti-coagulated with EDTA, as well as single splenic cell suspensions, were obtained using a 70 μm 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 analysed on a FACS Canto II (BD Biosciences, Mountain View, CA) using the relevant FACS antibodies (all obtained from eBioscience).