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LOX-1 deletion and macrophage trafficking in atherosclerosis

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ABSTRACT

Background: Atherosclerosis is associated with macrophage accumulation. LOX-1 has been shown to induce macrophage attachment, and its deletion (LOX-1 knockout, KO) reduces atherosclerosis in LDLr KO mice fed a high cholesterol diet. We examined differences in macrophage trafficking in age-matched wild type, LOX-1 KO, LDLr KO, and LDLr/LOX-1 double KO mice.

Methods: Sections of aortas of mice fed high cholesterol diet were collected at weeks 0, 4, 8, 12 and 19 and analyzed by immunohistochemistry and flow cytometry.

Results: In the LDLr KO mice aorta, CD68 positivity (macrophage accumulation) increased over time up to 12 weeks, and then the accumulation fell modestly but significantly. The periaortal fat and adventitia showed more CD68 positivity than the media and intima. This pattern was also evident in the non-atherosclerotic areas. Importantly, LOX-1 KO and LDLr–LOX-1 double KO mice showed diminished CD68 positivity in comparison to wild type and LDLR KO mice, respectively. Further, macrophages from LOX-1 KO mice revealed a marked reduction in migration (vs. macrophages from wild type mice) in *in vitro* migration assay.

Conclusions: LOX-1 deletion translates into reduction in macrophage trafficking in the aorta of LDLr KO mice. Most of the macrophage trafficking appears in the subadventitial regions.

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1. Introduction

Atherosclerosis is the most common cause of cardiovascular diseases which account for a large part of healthcare burden [1,2]. Major strides have been made in understanding the pathophysiology of atherosclerosis [3,4]. It is now evident that accumulation of immune cells, especially macrophages, is a hallmark of the atherosclerotic processes. This accumulation of macrophages is predicated by lipid accumulation, particularly oxidized low density lipoprotein (ox-LDL), in the subendothelial space [3,5]. The deposition of ox-LDL leads to endothelial activation, dysfunction and injury. Prior investigations point to endothelial layer as the starting point of these processes. As one of the major receptors for oxidized low-density lipoprotein (ox-LDL), lectin-like ox-LDL scavenger receptor-1 (LOX-1), a cell surface molecule, is upregulated on the endothelial surface in atherosclerosis. Disruption of this protein has been shown to reduce the severity of atherosclerosis in mice lacking the LDL receptor (LDLr) and fed a high cholesterol diet [6].

Chronic exposure to ox-LDL has the effect of up-regulating a number of inflammatory cytokines and processes in macrophages and in other tissues [7,8]. Recently a number of groups have begun studies of perivascular tissues, such as inflammatory cells, in pursuit of factors in atherogenesis [7–11]. Other groups have already shown modifications in inflammatory response with behavior modification strategies, such as changes in diet and exercise [12,13].

This study was conducted to determine macrophage trafficking in the aortas of LDLr KO mice given high cholesterol diet and the role of LOX-1 deletion in this model.

2. Materials and methods

2.1. Animal protocol

The generation of LOX-1 KO and LOX-1/LDLr double KO mice has been described recently [6]. In brief, C57BL/6 mice (also referred to as wild-type mice) and homozygous LDLr KO mice (on C57BL/6 background) were originally obtained from Jackson Laboratories. The homozygous LOX-1 KO and LOX-1/LDLr double KO mice were backcrossed 8 times with C57BL/6 strain to replace the genetic background. C57BL/6 and homozygous LOX-1 KO, LDLr KO and LDLr/LOX-1 double KO (all on C57BL/6 background) mice were bred by brother–sister mating and housed in the breeding colony at University of Arkansas for Medical Sciences, Little Rock, Arkansas. All male animals were given a high-cholesterol diet (4% cholesterol/10% cocoa butter) for 19 weeks from the age of 6 weeks. This investigation conforms to the Guidelines for the Care

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and Use of Laboratory Animals published by the US National Institutes of Health. All experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Usage Committee.

2.2. Immunohistochemistry

Aortas were harvested at 8, 12, and 19 weeks of high-cholesterol feeding. Paraffin embedded aortas from different groups of mice ($n = 3–4$ in each group) were cut into 5 μm sections placed on positively charged slides and then deparaffinized, rehydrated and antigen retrieved with citrate buffer antigen retrieval. Slides were immunoreacted with anti-CD68 (monoclonal DAKO) antibodies. CD68 positive cells were counted manually, and imaged in 6 high power fields of the adventitia and associated peri-aortal, medial and intimal regions.

2.3. Flow cytometry

Wild type, LDLr KO, LOX-1 KO and LDLr-LOX-1 double KO mice were anesthetized and their aortas were flushed by cardiac puncture with PBS containing 20 U/ml of heparin to remove blood from circulation. Harvested aortas were microdissected and digested with 125 U/ml collagenase type XI, 60 U/ml hyaluronidase type I-s, 60 U/ml DNase1, and 450 U/ml collagenase type I (Sigma–Aldrich) in PBS containing 20 mM Hepes at 37 °C for 1 h. A cell suspension was generated by pressure on the aorta through a 70-μm strainer. The cells were incubated with antibodies for 20 min at 4 °C, washed twice, and incubated with secondary antibodies for an additional 20 min. Immunofluorescence was detected by flow cytometry (FACSCalibur). Primary antibodies used were anti-CD45, CD11b and CD3 FITC and Ly6G then fluorescent-labeled secondary antibodies were added (BD Biosciences).

2.4. Migration assay

Age matched mice of wild type and LOX-1 deletion background were injected intraperitoneally with thyoglycolate media to stimulate macrophage accumulation. Mice were sacrificed, injected with sucrose solution to further encourage intraperitoneal accumulation of macrophages which were then harvested. The aspirate was spun down, and RBCs were lysed. Pooled macrophages from

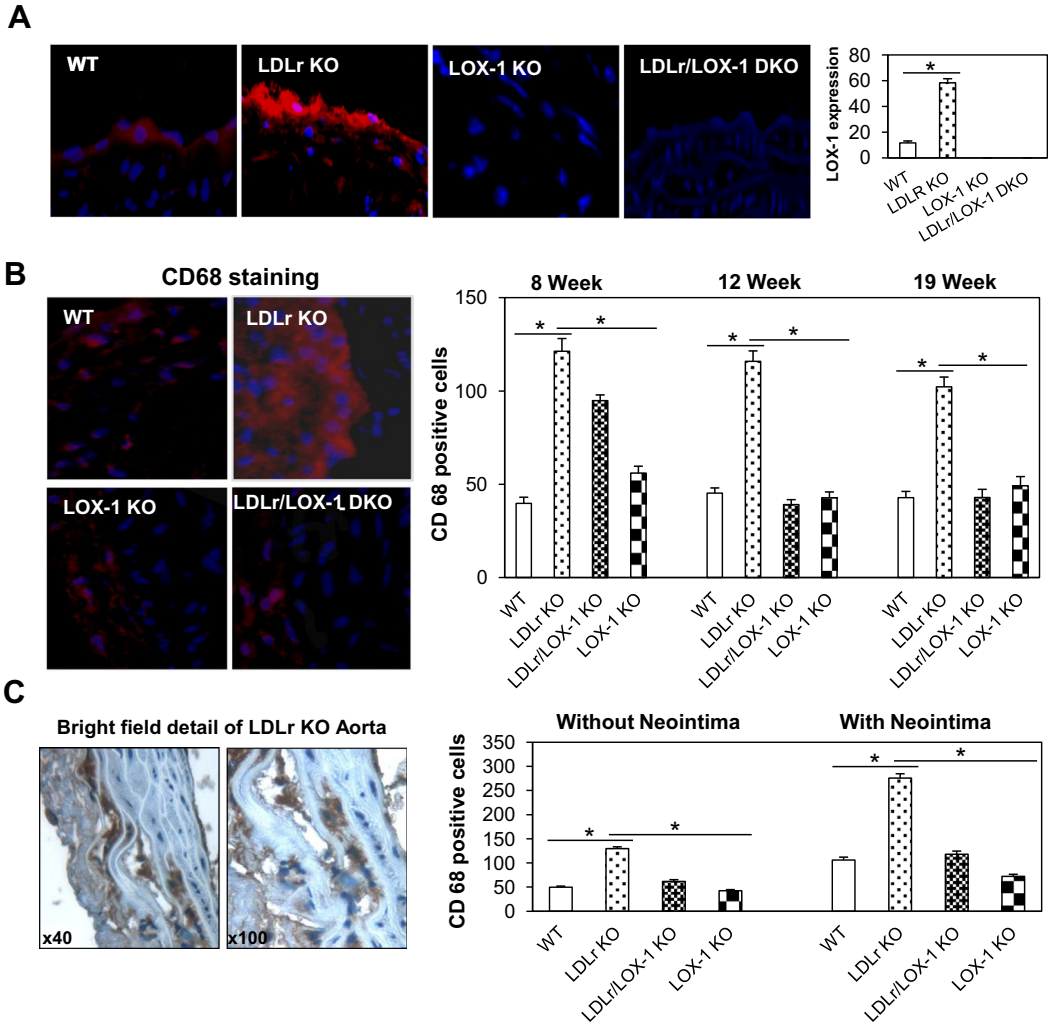

Fig. 1. CD68 immunostaining in the aortas of wild-type, LOX-1 KO, LDLr KO and LDLr/LOX-1 double KO (DKO) mice. (A) Aortic section of LDLr mice given high cholesterol diet shows over-expression of LOX-1. The expression of LOX-1 is primarily in the endothelial and intimal layers. Note that LOX-1 deletion completely eliminates LOX-1 immunostaining in the aortas of wild type and LDLr KO mice. (B) and (C). Aortic sections from LDLr mice given high cholesterol diet show markedly increased CD68 expression (representative experiments and summary of data, mean ± SD). Note that LOX-1 deletion dramatically reduces CD68 immunostaining. Lower panel – The CD68 positivity is mostly in the adventitial layers; this is evident in aortas with bright field examination with and without endothelium. Bar graphs represent data in mean ± SD based on 7 experiments, $^{*}P < 0.05$.

three mice from each background were plated in triplicate at densities of 70,000 cells per well on 8-μ migration assay chambers with f-MLP as a migration attractant [14,15]. The membrane was stained with hematoxylin, and number of migrated cells was quantified by light microscopy. Another set of mice were directly assayed by cytospin of peritoneal washes and quantification/Identification of cell populations by Giemsa staining and microscopic observation.

2.5. Statistical analysis

Data from 7 independent experiments were used for statistical analysis. Results are shown as mean ± SD. Multiple means were compared using a one-way analysis of variance (ANOVA). Paired Student's *t*-test was used to assess significant differences. *P* value <0.05 was considered significant.

3. Results

As expected, LOX-1 expression was absent in the LOX-1 KO mice (vs. wild type mice, both given high cholesterol diet). As shown earlier [6], LOX-1 expression was much higher in the aortas of LDLr KO mice as compared with wild-type mice aortas. The expression of LOX-1 was minimal in the LDLr/LOX-1 double KO mice as compared with that in the LDLr KO mice aortas (Fig. 1A).

LDLr KO mice had several-fold greater CD68 positivity (macrophage accumulation) in the aortic sections ($P < 0.01$, vs. wild-type mice), and the number of CD68 positive cells increased over time of high cholesterol diet feeding for up to 12 weeks. Importantly, aortas from LOX-1 KO and LDLr/OX-1 double KO mice at 19 weeks of high cholesterol diet feeding showed lower levels of CD68 positivity in comparison to aortas from wild type and LDLr KO mice, respectively, at 12 weeks ($P < 0.02$) (Fig. 1B).

The peri-aortal and adventitial regions appeared to harbor more macrophages (CD68 positive cells) than the medial and intimal regions. This pattern was also evident in the non-atherosclerotic areas, and endothelium-intact and endothelium-denuded aortic tissues. These observations were confirmed on bright field examination of aortas (Fig. 1C).

The immunohistology findings of CD68 positivity was confirmed by flow cytometry analysis which showed greater number of macrophages in the LDLr KO mice than the wild-type mice aortas at all time points.

Aortas of LOX-1 KO mice showed less macrophage trafficking compared to wild type mice ($P < 0.01$) at all time points. Representative flow cytometry experiments at 12 week are shown in Fig. 2A and summary data are shown in Fig. 2B. There were fewer macrophages in LOX-1 KO mice compared to wild type mice aortas (0.16% and 0.45%, respectively, $P < 0.05$) at 8 weeks of high cholesterol diet. There were fewer macrophages in the aortas of LDLr/LOX-1 double KO compared to LDLr KO mice (0.27% vs. 1.15%, $P < 0.01$). At 12 weeks, the accumulation of macrophages in the aortas of both LDLr KO and double KO mice increased as compared to 8 weeks ($P < 0.01$). The number of macrophages continued to be significantly less in the aortas of LDLr/LOX-1 double KO mice as compared to that in the aortas of LDLr KO (0.74% vs. 5.56%, $P < 0.01$). At 19 weeks, there were fewer macrophages in aortas of LDLr KO as compared to LDLr KO mice at 12 weeks (2.6% vs. 5.56%, $P < 0.01$). However, macrophage number was still less in the aortas of LDLr–LOX-1 double KO mice as compared to LDLr KO (1.2% vs. 2.6%, $P < 0.01$).

In order to determine if macrophages express LOX-1, we performed RT-PCR analysis on isolated macrophages. Indeed, macrophages from wild-type mice expressed LOX-1, whereas macrophages from LOX-1 KO mice did not (data not shown).

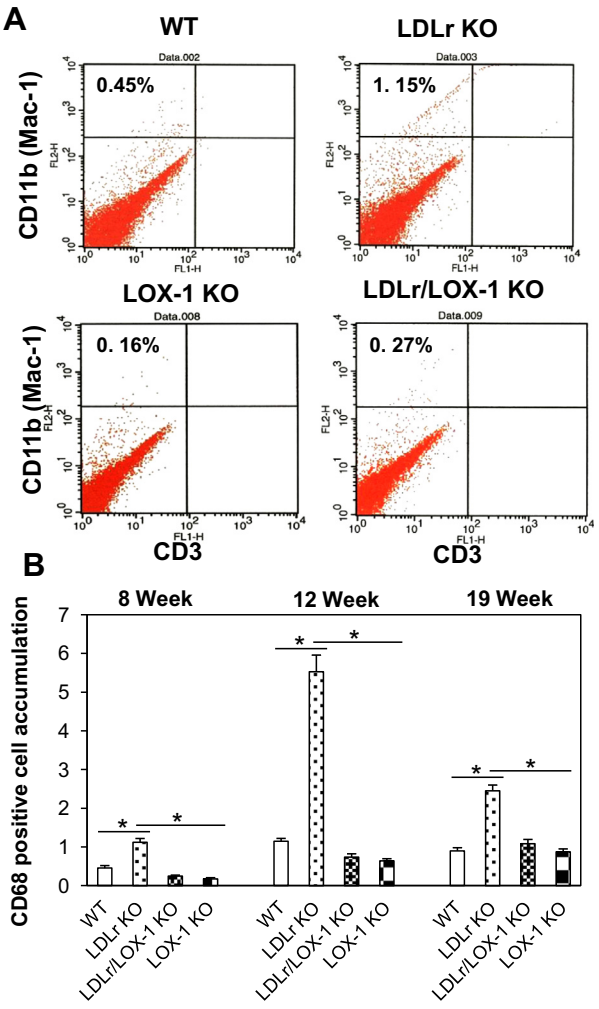

Fig. 2. CD68 positive cell accumulation. (A) A representative experiment showing SC68 positive cell accumulation. Macrophages were identified by first gating for MHC class II I-A^{b+} (not shown) and $CD11b^{+}$ cells (*y*-axis). (B) Based on flow cytometry, the CD68 positive cells increased until 12 weeks in the LDLr KO mice aortas; thereafter the CD68 positivity decreased. CD68 positive cell number was always lower in LOX-1 KO and LDLr/LOX-1 DKO mice. Bar graphs represent data in mean ± SD based on 7 experiments, $^{*}P < 0.05$.

Importantly, LOX-1 mice had an 8-fold reduction in migration in response to f-MLP when compared to wild type mice (Fig. 3).

4. Discussion

Macrophage migration and activation have traditionally been investigated in response to infection and scavenger functions. More recently, macrophage trafficking has been a focus of the process of atherogenesis [16]. Macrophages express a number of receptors for ox-LDL and internalize ox-LDL and transform into foam cells- a hallmark of atherosclerosis [17]. Although most attempts to modify cardiovascular risk has thus far hinged on lipid, blood sugar and blood pressure control, immune modulation may be a reasonable approach to limit atherogenesis [16]. Although accumulation of inflammatory and immune cells may reflect a response to tissue injury caused by hemodynamic stress and endothelial injury, accumulation of macrophages within the vessels may well be a contributor to initiation of atherosclerosis and its progression [2]. Our data suggests that macrophage trafficking starts early in atherosclerosis and goes on for at least for 19 weeks in the LDLr KO mice. Complementary to this data, others have

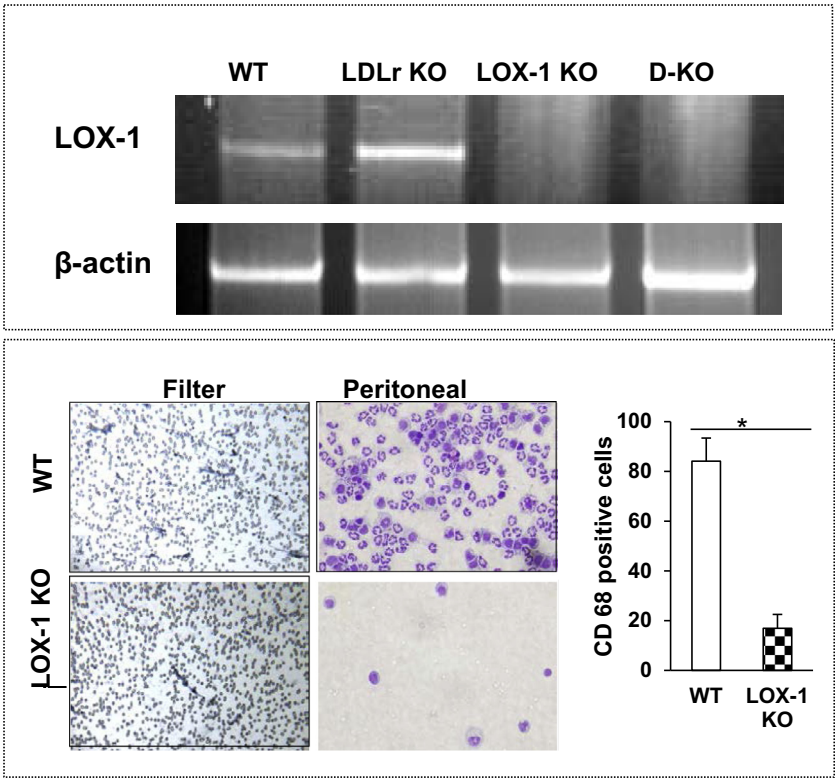

Fig. 3. Macrophage migration in wild type (WT) and LOX-1 KO mice in the peritoneal cavity as well as their migration across the filter in response to f-MLP. In keeping with data shown in Figs. 2 and 3, there is much less CD68 positivity in the peritoneal cavity as well as across the filter in response to the chemoattractant f-MLP. Bar graphs represent data in mean ± SD based on 5 experiments, $^{*}P < 0.05$.

identified other immune populations within "non-inflamed" vascular tissues [11]. However, we were unable to find significant number of lymphocytes in the LDLr KO mice aorta, either by flow cytometry or immunohistochemistry.

Large to medium size arteries have a tiered structure consisting of the intimal lining the lumen with endothelial cells, media consisting of proliferating smooth muscle cells and adventitia with a large number of fat cells [18]. The endothelial layer expresses an array of functions, particularly upon exposure to ox-LDL when it develops pro-thrombotic and pro-inflammatory properties and loses it potential to generate nitric oxide. LOX-1 expression/activation on endothelial lining upon exposure to ox-LDL or during hypercholesterolemia induces all of these properties on the endothelium [3]. The deletion of LOX-1 restores nitric oxide-dependent vasodilatation and reduces the pro-inflammatory potential [6]. The present study shows over-expression of LOX-1 on the endothelial cell lining as well as in the sub-endothelial layers in the LDLr KO mice given high cholesterol diet. LOX-1 deletion, as expected, reduced LOX-1 expression in the LDLr KO mice.

Further, the LOX-1 KO mice aorta showed fewer macrophages than the wild-type mice aorta. The LDLr/LOX-1 double KO mice aorta also exhibited reduced amount of CD68 positive cells as compared with LDLr KO mice aorta. These data suggest that macrophage accumulation is modulated by the presence of LOX-1, and point to a pivotal role of LOX-1 as an immune modulatory protein. Our data are in keeping with the results of a study that showed diminished CD68 expression in the aorta of LDLr/LOX-1 double KO mice. Furthermore, LOX-1 is known to operate as a leukocyte adhesion molecule that assists migration of leukocytes [19]. Therefore, the reduction of monocyte/macrophage migration in our study may well have been mediated by LOX-1 activation. This phenomenon is likely to be mediated by LOX-1 expressed on monocytes/macrophages rather than on endothelial cells.

The present study provides new information that most of the CD68 positivity was in the adventitial layers and the peri-aortal fat. Most investigators have focused on the subintimal and endothelial components of the vessel wall in atherosclerosis [20]. Some have even suggested that the outer layer of the artery is a bystander in this process [19]. However, others have considered the subadventitial layers as powerful contributor to the atherosclerotic process [21,22]. Moos et al. [23] showed that adventitia is a major site of inflammatory cell accumulation in standard chow-fed Apo-E null mice. It is conceivable that the inflammatory/immune cells are attracted to the outer layers of the arterial wall by exaggerated expression of chemoattractants and chemokines, or some yet unknown factors in the adventitial peri-aortal fat; however, the differential expression of these factors in different layers of the arterial wall following high cholesterol diet administration remains undefined. There are some, but conflicting, data on the adventitial origin of vasa vasorum which presumably may transport monocytes/macrophages to the arterial wall.

The results of immunostaining were confirmed by flow cytometry data. An interesting aspect of the study that became evident on immunohistochemistry as well as flow cytometry was that the macrophage accumulation in the aortic wall progressed to week 12, and thereafter seemed to stabilize or decrease. This relative reduction in macrophage accumulation has been reported in at least one earlier study [24]. The precise basis of reduction in inflammatory cells in atherosclerotic artery in not known, but may be due to deposition of collagen in the vessel wall as the plaque matures thus leaving only a limited space for macrophages to accumulate [24–26]. This will need to be confirmed by specific examination of the arterial wall for collagen deposition.

In conclusion, this study provides new data on macrophage trafficking in the aortas of LDLr KO mice given high cholesterol diet. This trafficking appears to preferentially take place in the

adventitial layers. Importantly, macrophage trafficking appears to decline as the plaque matures. Blockade of macrophage trafficking in the aortas of wild type and LDLr KO mice by LOX-1 deletion suggests that LOX-1 expression and its upregulation by high cholesterol diet are key events in this process.

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