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LOX-1 – dependent mitochondrial DNA damage and NLRP3 activation during systemic inflammation in mice

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ABSTRACT

Background: Lectin-like oxidized low-density lipoprotein scavenger receptor-1 (LOX-1) is known to be involved in many pathophysiological events, such as inflammation.
Methods: To clarify the role of LOX-1 in mtDNA damage and NLRP3 inflammasome activation, we studied wild-type (WT) and LOX-1 knockout (KO) mice given thioglycollate, an inflammatory stimulus.
Results: We observed intense inflammatory response (CD45 and CD68 expression) and mtDNA damage in spleen and kidneys of WT mice given thioglycollate. The abrogation of LOX-1 (use of LOX-1 knockout mice) reduced the inflammatory response as well as mtDNA damage ($P < 0.05$ vs. WT mice). We also observed that mice with LOX-1 deletion had markedly reduced expression of caspase-1 (P10 and P20 subunits) as well as cleaved IL-1β and IL-18. These mice also had much less mtDNA damage and only limited NLRP3 inflammasome expression.
Conclusions: These *in vivo* observations indicate that LOX-1 plays a key role in mtDNA damage which then leads to NLRP3 inflammasome activation during inflammation.

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1. Introduction

Lectin-like oxidized low-density lipoprotein scavenger receptor-1 (LOX-1) is one of the major receptors responsible for binding, internalizing and degrading ox-LDL [1,2]. Activation of LOX-1 has been known to be related to many pathophysiological events, including apoptosis, inflammation and atherogenesis [3]. As a molecular platform for immune defense, NLRP3 inflammasome triggers caspase-1, which results in the cleavage and secretion of IL-1 family cytokines, such as IL-1β and IL-18 [4].

As energy-producing organelles, mitochondria can suffer damage under oxidative stress resulting in cellular dysfunction and inflammation [5]. Similar to bacterial DNA, mitochondrial DNA (mtDNA) contains inflammatogenic unmethylated CpG motifs, whereas nuclear DNA is modified by the addition of methyl groups on certain sequences known as CpG motifs [6,7]. This differential feature allows immune cells to recognize DNA of invading bacteria.

LOX-1 has been shown to be important in inflammation and to act as a cell adhesion molecule [8,9]. In rats with endotoxin-induced inflammatory reaction, Honjo et al. [10] showed that LOX-1 antibody prevented or attenuated subsequent inflammatory response. Shin et al. [11] found that remnant lipoprotein particles stimulated NAD(P)H oxidase-dependent superoxide formation and induction of cytokines in human endothelial cell line via activation of LOX-1. However, there is paucity of data to suggest the relevance of LOX-1 in NLRP3 inflammasome activation.

We hypothesized that LOX-1 could play an important role in mtDNA damage similar to that generated by bacterial DNA during an infection and NLRP3 inflammasome activation following an inflammatory stimulus. The present study was designed to test this hypothesis.

2. Materials and methods

2.1. Animals

The generation of LOX-1 knockout (KO) mice on C57BL/6 background [also referred to as wild-type (WT) mice] has been described previously [3]. All animals were housed in the breeding colony at our institution. All experimental procedures were per-

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formed in accordance with protocols approved by the Institutional Animal Care and Usage Committee, and conform to the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health. All mice were males and used when 10 weeks old. Absence of LOX-1 in the LOX-1 KO mice was confirmed by Western blot analysis in mice spleen and kidney (Fig. 1A left). Immunohistochemistry also showed total absence of LOX-1 these tissues (Fig. 1A right).

2.2. In vivo inflammation model

Male WT and LOX-1 KO mice with similar weight were injected intraperitoneally 0.5 ml of sterile thioglycollate (4% wt/vol, BD Biosciences, Franklin Lakes, NJ), or saline (control). 72 h after injection, mice were euthanized for collection of blood and various organs.

2.3. ELISA and Western blot

IL-1β and IL-18 were measured in mice serum samples by ELISA (BD Biosciences). For Western blot, antibodies directed at caspase-1 p10, caspase-1 p20 and NLRP3 were purchased from Santa Cruz Biotechnology (Santa Cruz, Paso Robles, CA); and antibodies directed at IL-18 and IL-1β were purchased from Abcam (San Francisco, CA).

2.4. mtDNA damage analysis

Real-time quantitative PCR assay (q-PCR) was applied to assess mtDNA damage as described by Yu et al. [5]. Briefly, mtDNA and nuclear DNA (nDNA) were isolated using a genomic DNA extraction kit (QIAGEN, Chatsworth, VA), and specific primers were used to amplify a fragment of the mitochondrial and/or nDNA. Using comparative Ct method, DNA damage was quantified by comparing the relative amplification of large fragments (approximately 10 kb) of DNA from treated samples to those of controls and normalizing this to the amplification of smaller (<250 bp) fragments. DNA lesion frequencies were calculated using Poisson transformation.

mtDNA damage was also assessed as mtDNA/nDNA ratio [12]. The relative amount of mtDNA (mtDNA/nDNA ratio) was

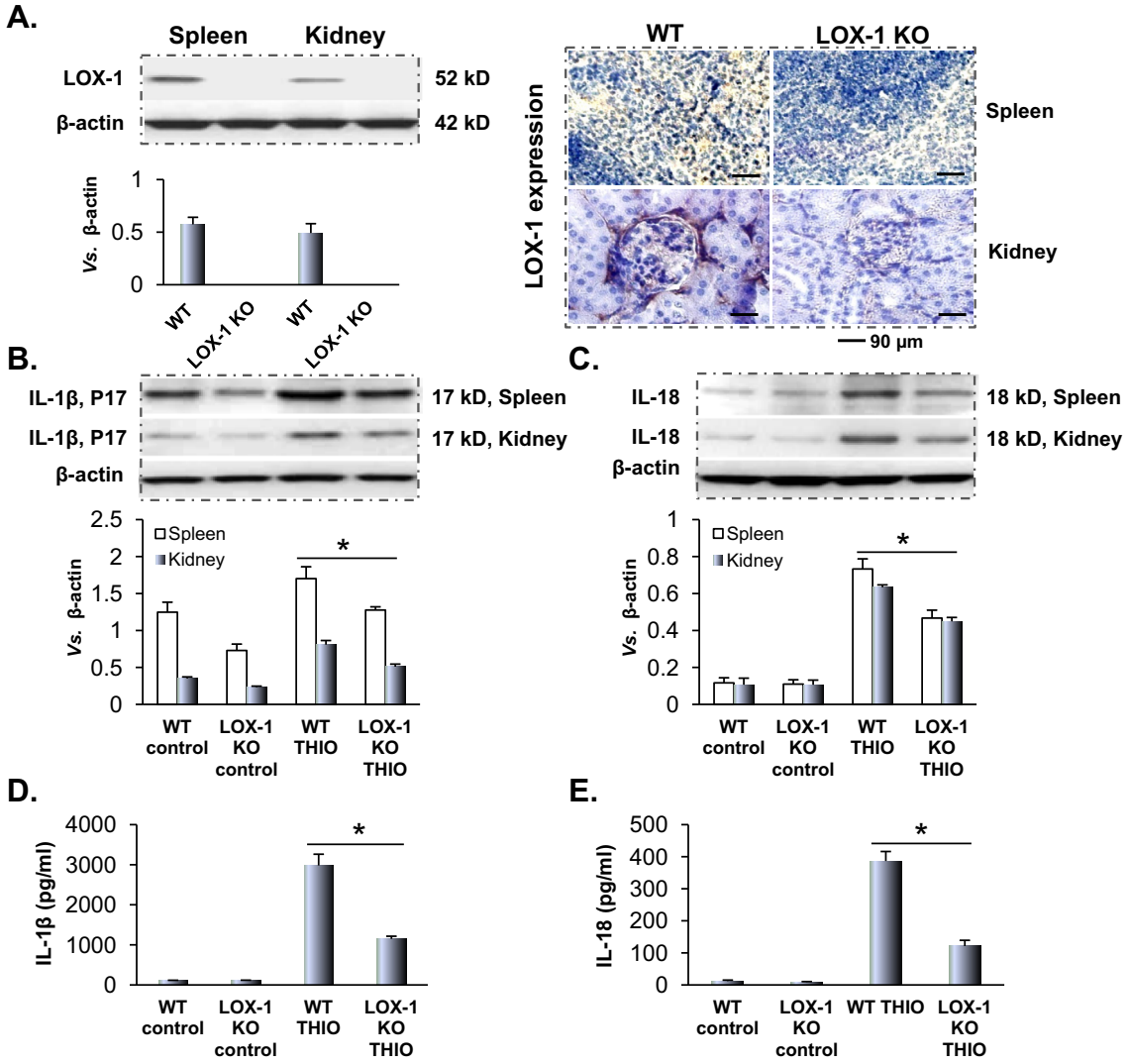

Fig. 1. LOX-1 deletion inhibits inflammatory response in mice spleen and kidney. (A) LOX-1 expression in spleen and kidney of wild-type (WT) and LOX-1 knockout (KO) mice. LOX-1 is completely missing in LOX-1 KO mice. Left panel shows absence of LOX-1 by Western blot, and the right panel shows absence of LOX-1 immunostaining in spleen and kidney. (B) and (C) Injection of thioglycollate (THIO) intraperitoneally induces expression of IL-1β and IL-18 (Western blot) in spleen and kidney tissues of WT mice, this response is significantly less in LOX-1 KO mice. (D) and (E) IL-1β and IL-18 concentrations (measurement by ELISA) in the sera of WT and LOX-1 KO mice. Bar graphs represent data in mean ± SD based on 5 independent experiments ($n = 5$), $^{*}P < 0.05$, LOX-1 KO mice vs. WT mice.

determined by q-PCR with NovaQUANT™ Human/Mouse Mitochondrial to Nuclear DNA Ratio Kit (EMD Millipore, Billerica, MA).

2.5. Immunohistochemical analysis

5 μ thick spleen and kidney sections were stained with different antibodies and analyzed using Mouse/Rabbit Specific HRP/DAB detection IHC kit (Abcam). Image J. v 1.46 (NIH, Bethesda, MD) was used to quantify immunohistochemical staining.

2.6. Statistical analysis

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

3. Results

3.1. IL-1β and IL-18 expression during inflammation and the effects of LOX-1 deletion

To substantiate that thioglycollate induces inflammation, IL-1β and IL-18 were measured in the spleen and kidney tissues. Western blot revealed that thioglycollate triggered expression of IL-1β and IL-18 (Fig. 1B and C). Serum levels of IL-1β and IL-18, measured by ELISA, increased several-fold following thioglycollate administration (Fig. 1D and E). Importantly, the increase in IL-1β and IL-18 expression in spleen and kidney and in the serum samples following thioglycollate was much less in the LOX-1 KO than in the WT mice (Fig. 1B through D).

3.2. Expression of CD45, CD68 and Phos-NF-κB and mtDNA damage during inflammation and the effect of LOX-1 deletion

CD45 and CD68 are considered general makers of inflammation [13]. NF-kB is an ubiquitously expressed redox-sensitive transcrip-

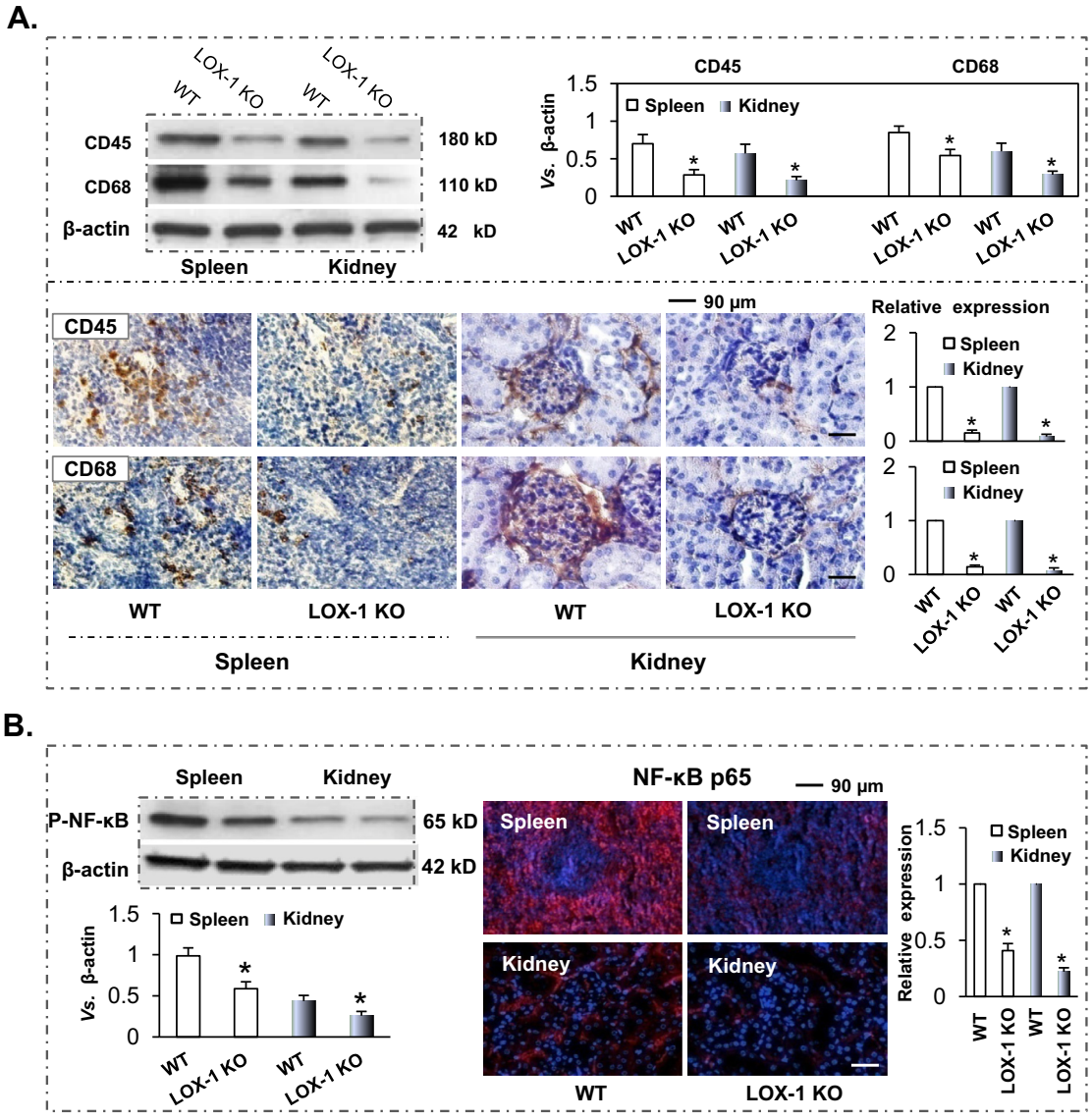

Fig. 2. LOX-1 deletion reduces inflammatory markers. LOX-1 KO and WT mice were injected with thioglycollate. (A) LOX-1 deletion inhibits the expression of CD45 and CD68, as well as Phos-NF-κB p65, in mice spleen and kidney (Western blot and immunohistochemical staining). Bar graphs on the right of panel A bottom are quantitation of immunopositivity data. (B) LOX-1 deletion inhibits the expression of Phos-NF-κB p65 in mice spleen and kidney. Bar graphs represent data in mean ± SD based on 5 experiments ($n = 5$), $^*P < 0.05$, LOX-1 KO mice vs. WT mice.

tion factor regulating various biological functions, including mitochondrial damage and activation of NLRP3 inflammasome [2,6,14]. As shown in Fig. 2A and B, we observed that CD45, CD68 and Phos-NF-kB signals were very prominent in both spleen and kidney of WT mice given thioglycollate. The expression of CD45, CD68 and Phos-NF-κB p65 was much less in the LOX-1 KO mice despite thioglycollate administration. Tissue immunohistochemistry confirmed the results of Western blot analysis. These observations indicate that LOX-1 deletion attenuates inflammatory response and activation of NF-kB.

Next, we used q-PCR to assess mtDNA damage. DNA lesions by oxidative damage (e.g. strand breaks, base modifications, and abasic sites) can block polymerase progression during polymerase chain reaction. Therefore, total mtDNA damage can be assessed by comparing the amplification of a large segment of mtDNA with amplification of a short target to control for mtDNA copy number [5]. As shown in Fig. 3A, mtDNA damage was very prominent following thioglycollate administration in both spleen and kidney of WT mice. Of note, spleen and kidney tissues from LOX-1 KO mice had much less mtDNA damage, indicating that LOX-1 activation is responsible, at least in part, for mtDNA during inflammation.mtDNA content reflects a fine balance between mitochondrial dysfunction and mitochondria-nuclear cross talk. Hence, we evaluated the ratio of mtDNA and nuclear DNA (nDNA) copy numbers (mtDNA/nDNA) as another indicator of mtDNA damage. As shown in Fig. 3B, the mtDNA/nDNA ratios were higher in LOX-1 KO mice than in WT mice (in both spleen and kidney), indicating that LOX-1 deletion protects mtDNA from damage during the inflammatory cascade.

3.3. Expression of caspase-1 and NLRP3 during inflammation and the effect of LOX-1 deletion

Caspase-1 activation is a key event in the innate immune response to infectious and noxious stimuli, and promotes the cleavage and secretion of the pro-inflammatory cytokines IL-1β

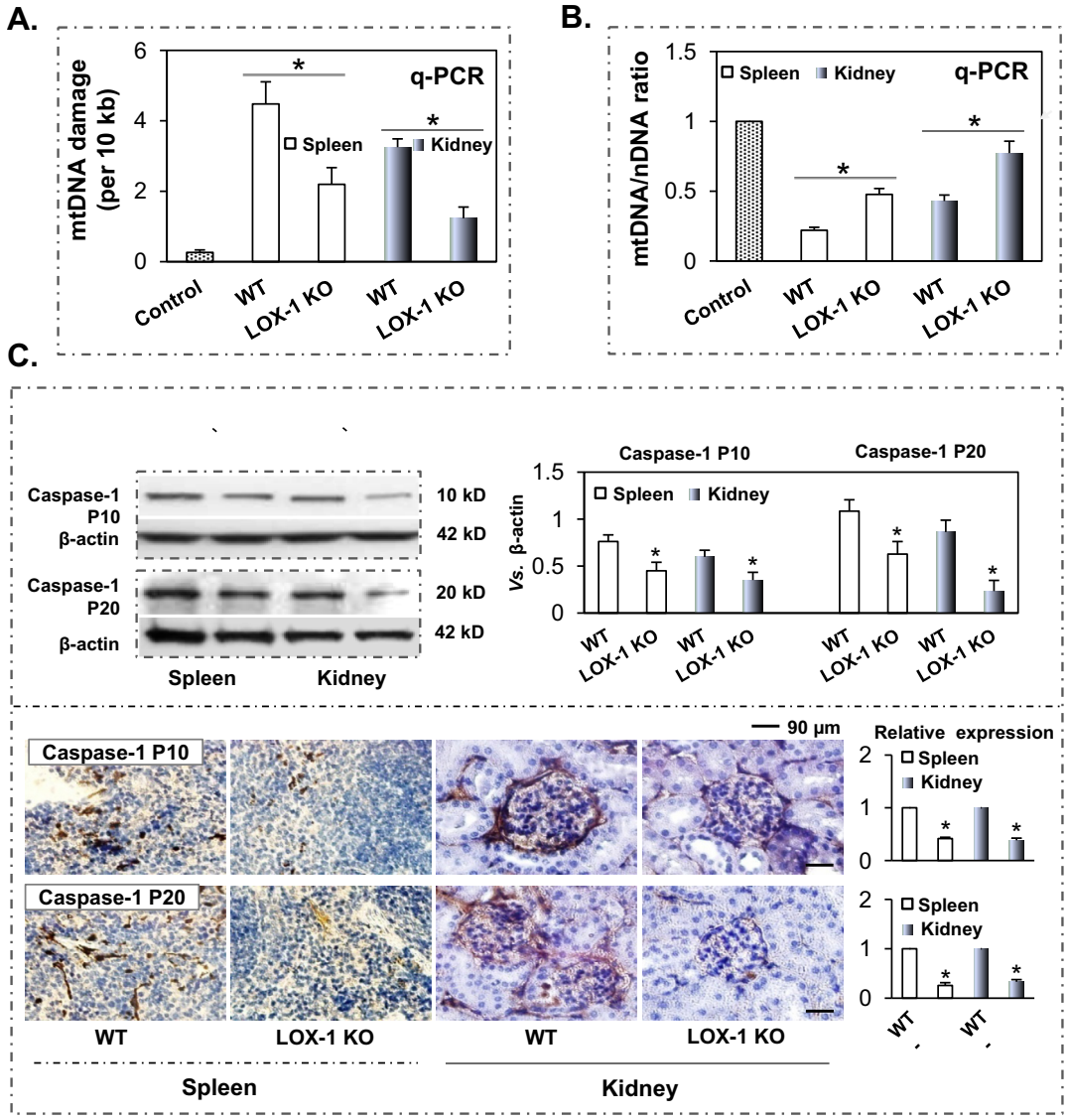

Fig. 3. LOX-1 deletion inhibits mtDNA damage and expression of caspase-1 subunits P10 and P20. (A) LOX-1 deletion inhibits mtDNA damage quantitated by q-PCR. (B) mtDNA/nDNA ratio confirms the results shown in panel A. (C) LOX-1 deletion inhibits thioglycollate-induced caspase-1 P10 and P20 subunits. Bar graphs on the right of upper panel are quantitation of Western blot data. Bar graphs on the right of lower panel are quantitation of immunopositivity data. Bar graphs represent data in mean ± SD based on 5 experiments ($n = 5$), $^*P < 0.05$, LOX-1 KO mice vs. WT mice.

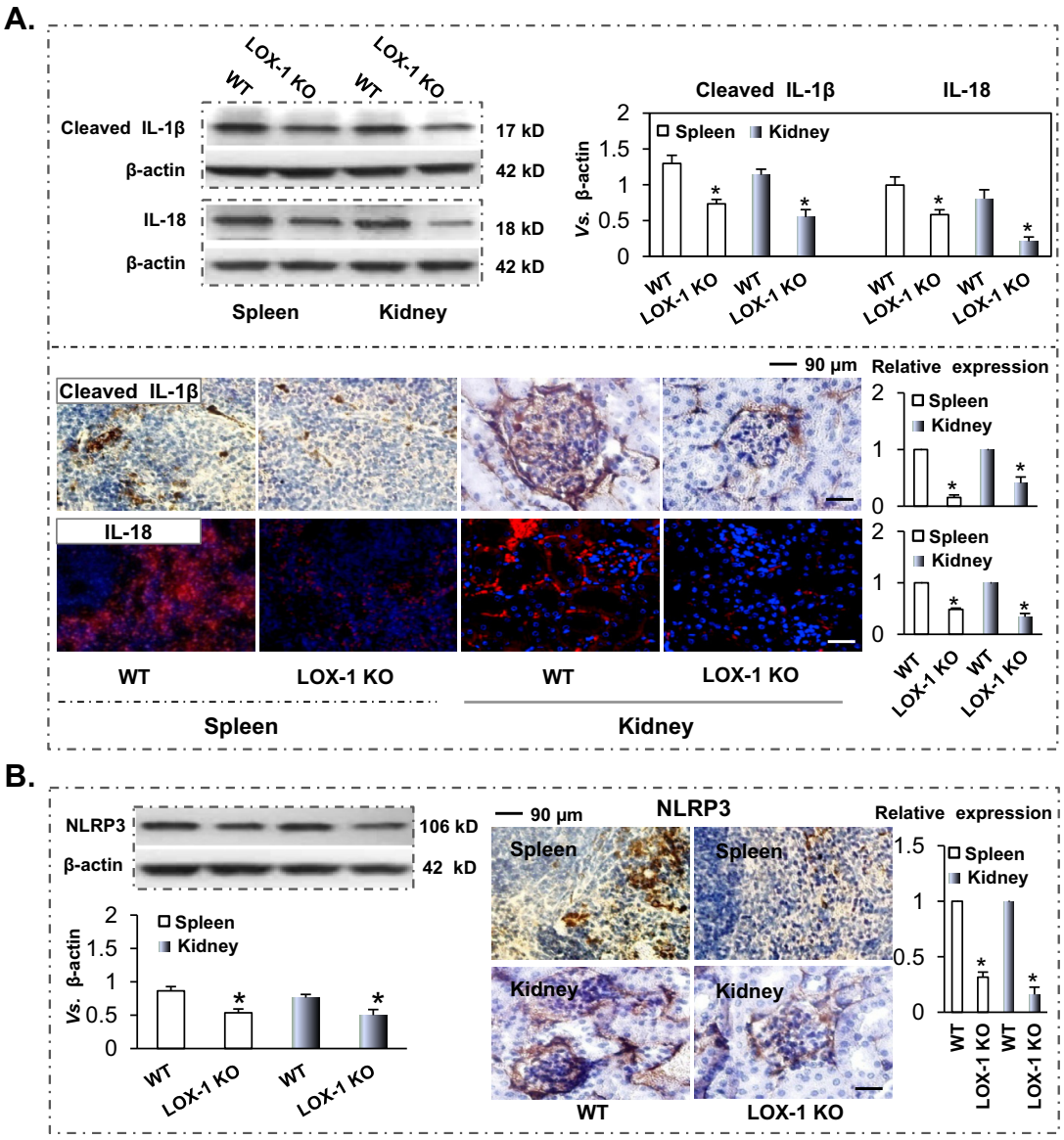

Fig. 4. LOX-1 deletion inhibits expression of cleaved IL-1β and IL-18 and NLRP3 inflammasome. (A) LOX-1 deletion reduces expression of cleaved IL-1β and IL-18 (B) LOX-1 deletion reduces expression of NLRP3 inflammasome. Measurements by Western blot and immunohistochemical staining in spleen and kidney of WT and LOX-1 KO mice given thioglycollate. Bar graphs represent data in mean ± SD based on 5 experiments ($n = 5$), $^{*}P < 0.05$, LOX-1 KO mice vs. WT mice.

and IL-18 [4,15]. Autoproteolytic separation of its p10 and p20 subunits is considered a pivotal step in caspase-1 activation [15]. Indeed, we observed intense signal for both P10 and P20 components of caspase-1 in the spleen and kidney of WT mice given thioglycollate (Fig. 3C); the cleavage products IL-1β and IL-18 were also very prominent (Fig. 4A). Importantly, LOX-1 deletion reduced the expression of caspase-1 P10 and P20 components as well as cleaved IL-1β and IL-18 (Fig. 3C and 4A). Of note, the results of Western blot and immunohistochemical analysis were congruent.

Next, we examined if LOX-1 regulates the expression of the NLRP3 inflammasome formation during inflammation. As shown in Fig. 4B, both Western blot and immunohistochemical staining indicated that LOX-1 deletion inhibited NLRP3 inflammasome expression by about 40%. These changes were noted in spleen as well as in kidney, and the magnitude of change was similar.

4. Discussion

As an innate proteolytic complex, NLRP3 inflammasome is known to be activated by a variety of danger signals released from damaged and dying cells, such as ROS, extracellular ATP, damaged mtDNA and oxidized lipids [14,15]. Damaged mtDNA in turn impairs the function of mitochondria, and promotes inflammation, apoptosis, autophagy and cell senescence [6,16]. LOX-1 is emerging as a potent pro-inflammatory regulator [2,3,6]. Based on the findings in the present study, we suggest that LOX-1-mtDNA damage axis plays an important role in the activation of NLRP3 inflammasome that regulates the processing and secretion of pro-IL-1β and pro-IL-18 into mature and active cytokines.

As an inflammation stimulus, thioglycollate induces LDL oxidation and, ox-LDL is a potent stimulus for LOX-1 expression [17–19], suggesting a potent link between LOX-1 and inflammation. In the

present study, we show that LOX-1 activation plays an important role in thioglycollate-induced inflammation in mice tissues. Systemic inflammatory response was evidenced by a marked increase in serum levels of IL-1β and IL-18. The levels of both IL-1β and IL-18 were much lower in the LOX-1 KO mice. Further, we found that LOX-1 deletion inhibited the expression of CD45 and CD68, well-recognized inflammatory markers [13]. These changes in response to thioglycollate were seen in different organs (spleen and kidney) and LOX-1 deletion reduced thioglycollate-mediated changes in spleen as well as in kidney.

Once inflammation sets in, it activates the transcription factor NF-κB that further triggers mtDNA damage [6]. In keeping with this concept, we found increase in Phos-NF-kB in the tissues of WT mice given thioglycollate. Again, LOX-1 deletion significantly reduced the expression of this redox-sensitive transcription factor.

The enhanced expression of NLPR3 related signaling (caspase-1 activation and cleavage products IL-1β and IL-18) seen in the spleen and kidney of thioglycollate-treated mice may be a response to damaged mtDNA [5]. This phenomenon appears dependent on LOX-1 expression and activation, as suggested earlier [6]. This relationship was confirmed *in vivo* by the observation that LOX-1 deletion reduced the extent of damage to mtDNA in spleen and kidney of mice given thioglycollate; simultaneously, there was a marked reduction in the expression NLRP3 inflammasome. These data suggest a role of LOX-1 in mtDNA damage and NLRP3 expression, possibly via inhibition of NF-κB activation, during inflammation.

In these studies, we examined the role of LOX-1 in mtDNA damage and NLRP3 inflammasome expression in two different tissues – kidney and spleen. These two organs play vastly different roles in inflammatory states. Kidneys serve homeostatic functions such as the regulation of electrolytes, maintenance of acid–base balance, and regulation of blood pressure. Hu et al. [20] demonstrated a role for LOX-1 activation in injury to renal glomeruli and tubules during sustained hypertension. Lu et al. [21] observed intense LOX-1 expression and oxidant stress in kidneys in association with cytokine surge in systemic circulation during sustained myocardial ischemia. Dominguez et al. [22] showed that kidneys of obese rats develop intense oxidative stress, leukocyte infiltration, depressed mitochondrial enzyme level and function, and peritubular fibrosis, and LOX-1 antibody ameliorates many of these changes. These observations suggest that LOX-1 may be relevant at least in part in renal injury induced by inflammatory states as diverse as hypertension, myocardial ischemia and metabolic syndrome. Spleen on the other hand is a part of the mononuclear phagocyte system, and plays an important role in immune response through humoral and cell-mediated pathways. Graham et al. [23] found increased expression of LOX-1 as well as for pro-inflammatory cytokines IL-6, TNF-α, IL-1β, and IFN-γ in splenic T-lymphocytes isolated mice fed a high fat diet. However, there is paucity of studies linking LOX-1 to splenic function. Our study is probably the first to demonstrate a direct role for LOX-1 in mtDNA damage and NLRP3 inflammasome activation in kidney as well as spleen during systemic inflammatory state.

A host of disease states are associated with intense inflammatory reaction, and are dependent, at least in part, on LOX-1 activation [24,25]. From the data presented here, we conclude that in systemic inflammatory states, LOX-1 activation may underlie NF-κB phosphorylation, mtDNA damage and NLRP3 inflammasome activation. This is followed by the expression of caspase-1 P10/20, and cleaved IL-1β and IL-18, the major pro-inflammatory proteins. Our studies in mice missing LOX-1 clearly showing blockade or significant attenuation of these events, suggest that LOX-1 is upstream of mtDNA damage-NLRP3 inflammasome activation during systemic inflammation.

5. Disclosures

None.

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References

[1] T. Sawamura, N. Kume, T. Aoyama, et al., An endothelial receptor for oxidized low-density lipoprotein, Nature 386 (1997) 73–77.
[2] J. Chen, J.L. Mehta, N. Haider, et al., Role of caspases in ox-LDL-induced apoptotic cascade in human coronary artery endothelial cells, Circ. Res. 94 (2004) 370–376.
[3] J.L. Mehta, N. Sanada, C.P. Hu, et al., Deletion of LOX-1 reduces atherogenesis in LDLR knockout mice fed high cholesterol diet, Circ. Res. 100 (2007) 1634–1642.
[4] J.R. Lukens, J.M. Gross, T.D. Kanneganti, IL-1 family cytokines trigger sterile inflammatory disease, Front. Immunol. 3 (2012) 315.
[5] E. Yu, P.A. Calvert, J.R. Mercer, et al., Mitochondrial DNA damage can promote atherosclerosis independently of reactive oxygen species through effects on smooth muscle cells and monocytes and correlates with higher-risk plaques in humans, Circulation 128 (2013) 702–712.
[6] Z. Ding, S. Liu, X. Wang, et al., Oxidant stress in mitochondrial DNA damage, autophagy and inflammation in atherosclerosis, Sci. Rep. 3 (2013) 1077.
[7] T. Oka, S. Hikoso, O. Yamaguchi, et al., Mitochondrial DNA that escapes from autophagy causes inflammation and heart failure, Nature 485 (2012) 251–255.
[8] Z. Ding, X. Wang, L. Schnackenberg, et al., Regulation of autophagy and apoptosis in response to ox-LDL in vascular smooth muscle cells, and the modulatory effects of the microRNA hsa-let-7g, Int. J. Cardiol. 168 (2013) 1378–1385.
[9] Z. Ding, S. Liu, X. Wang, Y. Dai, LOX-1, mtDNA damage, and NLRP3 inflammasome activation in macrophages: implications in atherogenesis, Cardiovasc. Res. (2014). Epub ahead of print.
[10] M. Honjo, K. Nakamura, K. Yamashiro, et al., Lectin-like oxidized LDL receptor-1 is a cell-adhesion molecule involved in endotoxin-induced inflammation, Proc. Natl. Acad. Sci. 100 (2003) 1274–1279.
[11] H.K. Shin, Y.K. Kim, K.Y. Kim, et al., Remnant lipoprotein particles induce apoptosis in endothelial cells by NAD(P)H oxidase-mediated production of superoxide and cytokines via lectin-like oxidized low-density lipoprotein receptor-1 activation: prevention by cilostazol, Circulation 109 (2004) 1022–1028.
[12] M.R. Urschel, K.M. O'Brien, High mitochondrial densities in the hearts of Antarctic icefishes are maintained by an increase in mitochondrial size rather than mitochondrial biogenesis, J. Exp. Biol. 211 (2008) 2638–2646.
[13] M.P. Ramprasad, V. Terpstra, N. Kondratenko, O. Quehenberger, D. Steinberg, Cell surface expression of mouse macrosialin and human CD68 and their role as macrophage receptors for oxidized low density lipoprotein, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 14833–14838.
[14] H. Xiao, M. Lu, T.Y. Lin, et al., Sterol regulatory element binding protein 2 activation of NLRP3 inflammasome in endothelium mediates hemodynamic-induced atherosclerosis susceptibility, Circulation 128 (2013) 632–642.
[15] G. Licandro, H. Ling Khor, O. Beretta, et al., The NLRP3 inflammasome affects DNA damage responses after oxidative and genotoxic stress in dendritic cells, Eur. J. Immunol. 43 (2013) 2126–2137.
[16] Y. Kurihara, T. Kanki, Y. Aoki, et al., Mitophagy plays an essential role in reducing mitochondrial production of reactive oxygen species and mutation of mitochondrial DNA by maintaining mitochondrial quantity and quality in yeast, J. Biol. Chem. 287 (2012) 3265–3272.
[17] J. Lu, S. Mitra, X. Wang, M. Khaidakov, J.L. Mehta, Oxidative stress and lectin-like ox-LDL-receptor LOX-1 in atherogenesis and tumorigenesis, Antioxid. Redox Signal. 15 (2011) 2301–2333.
[18] D. Li, J.L. Mehta, Intracellular signaling of LOX-1 in endothelial cell apoptosis, Circ. Res. 104 (2009) 566–568.
[19] K. Kaneko, M. Sakai, T. Matsumura, et al., Group-II phospholipase A(2) enhances oxidized low density lipoprotein-induced macrophage growth through enhancement of GM-CSF release, Atherosclerosis 153 (2000) 37–46.
[20] C. Hu, B.Y. Kang, J. Megyesi, et al., Deletion of LOX-1 attenuates renal injury following angiotensin II infusion, Kidney Int. 76 (2009) 521–527.

[21] J. Lu, X. Wang, W. Wang, et al., Abrogation of lectin-like oxidized LDL receptor-1 attenuates acute myocardial ischemia-induced renal dysfunction by modulating systemic and local inflammation, Kidney Int. 82 (2012) 436–444.
[22] J.H. Dominguez, J.L. Mehta, D. Li, et al., Anti-LOX-1 therapy in rats with diabetes and dyslipidemia: ablation of renal vascular and epithelial manifestations, Am. J. Physiol. Renal Physiol. 294 (2008) F110–119.
[23] L.S. Graham, Y. Tintut, F. Parhami, et al., Bone density and hyperlipidemia: the T-lymphocyte connection, J. Bone Miner. Res. 25 (2010) 2460–2469.
[24] Z. Ding, S. Liu, C. Sun, et al., Concentration polarization of ox-LDL activates autophagy and apoptosis via regulating LOX-1 expression, Sci. Rep. 3 (2013) 2091.
[25] Z. Ding, S. Liu, X. Wang, et al., Lectin-like ox-LDL receptor-1 (LOX-1) – Toll-like receptor 4 (TLR4) interaction and autophagy in CATH.a differentiated cells exposed to angiotensin II, Mol. Neurobiol. (2014). Epub ahead of print.