LOX-1 Deletion Improves Neutrophil Responses, Enhances Bacterial Clearance, and Reduces Lung Injury in a Murine Polymicrobial Sepsis Model

Zhuang Wu, Tatsuya Sawamura, Anna K. Kurdowska, Hong-Long Ji, Steven Idell, and Jian Fu

Texas Lung Injury Institute, Center for Biomedical Research, University of Texas Health Science Center at Tyler, Tyler, Texas 75708, and Department of Vascular Physiology, National Cardiovascular Center Research Institute, Osaka, Japan

Received 15 December 2010/Returned for modification 3 February 2011/Accepted 3 May 2011

Inflammatory tissue injury and immunosuppression are the major causes of death in sepsis. Novel therapeutic targets that can prevent excessive inflammation and improve immune responses during sepsis could be critical for treatment of this devastating disease. LOX-1 (lectin-like oxidized low-density lipoprotein receptor-1), a membrane protein expressed in endothelial cells, has been known to mediate vascular inflammation. In the present study, we demonstrated that LOX-1 deletion markedly improved the survival rate in a murine model of polymicrobial sepsis. Wild-type (LOX-1+/+) and LOX-1 knockout (LOX-1−/−) mice were subjected to cecal ligation and puncture (CLP) to induce sepsis. LOX-1 deletion significantly reduced systemic inflammation and inflammatory lung injury during sepsis, together with decreased production of proinflammatory cytokines and reduced lung edema formation. Furthermore, LOX-1 deletion improved host immune responses after the induction of sepsis, as indicated by enhanced bacterial clearance. Interestingly, we were able to demonstrate that LOX-1 is expressed in neutrophils. LOX-1 deletion prevented neutrophil overreaction and increased neutrophil recruitment to infection sites after sepsis induction, contributing at least partly to increased immune responses in LOX-1 knockout mice. Our study results indicate that LOX-1 is an important mediator of inflammation and neutrophil dysfunction in sepsis.
Increased survival of LOX-1 knockout mice in sepsis. To investigate the potential role of LOX-1 in sepsis, we first examined the effect of LOX-1 deletion on sepsis-induced mortality after CLP surgery. LOX-1−/− mice exhibited a substantially higher survival rate than wild-type littermates (71% versus 27%) at 7 days after CLP (Fig. 1), suggesting that LOX-1 may modulate host responses and lead to increased death rates during sepsis.

LOX-1 knockout mice exhibited decreased inflammatory responses and lung injury after sepsis induction. To assess the role of LOX-1 in systemic inflammatory responses in sepsis, we examined proinflammatory cytokine production in the blood of wild-type and LOX-1 knockout mice after CLP. Blood TNF-α and IL-6 production was significantly reduced in LOX-1 knockout mice after CLP compared with the results seen with wild-type littermates (Fig. 2). Inflammatory tissue injury is a major cause of death in sepsis (43). We examined lung inflammation and injury in wild-type and LOX-1 knockout mice after CLP. Our data showed that CLP-induced TNF-α and IL-6 production and lung wet/dry ratios were significantly lower for LOX-1 knockout mice than for wild-type littermates (Fig. 3).
LOX-1 deletion enhances bacterial clearance in sepsis. We also conducted experiments to investigate whether LOX-1 is involved in the regulation of host defense. We examined the effect of LOX-1 deletion on bacterial infection during sepsis. LOX-1 knockout mice had cecal flora similar to those seen with wild-type littermates when no surgery was performed (data not shown). Interestingly, LOX-1 knockout mice exhibited decreased bacterial CFU levels in the peritoneal cavity, blood, and lung after CLP (Fig. 4) compared with wild-type littermates, suggesting that LOX-1 deletion enhances bacterial clearance during sepsis.

LOX-1 deletion increases neutrophil migration in sepsis. Neutrophils are the most abundant leukocytes in circulation and play a critical role in host defense (2). Severe sepsis has been known to cause impaired neutrophil migration to the infectious focus (1–3), which leads to immune suppression and increased mortality. To assess whether LOX-1 modulates neutrophil responses during sepsis, we examined the effects of LOX-1 deletion on neutrophil function. Blood neutrophil counts in wild-type and LOX-1 knockout mice before the surgery were not significantly different (data not shown). We found that neutrophil counts in the peritoneal cavity of LOX-1 knockout mice after CLP were significantly higher (Fig. 5A) than in that of wild-type littermates. We then conducted in vitro experiments to further assess the effects of LOX-1 deletion on neutrophil chemotaxis, phagocytosis, and bactericidal activity. We demonstrated that neutrophils isolated from wild-type mice after CLP exhibited impaired chemotaxis with respect to CXCL2 (Fig. 5B), whereas neutrophils isolated from LOX-1 knockout mice were less affected. Neutrophil phagocytosis and bactericidal activity were not significantly altered by LOX-1 deletion (Fig. 5C and D). Our data suggest that LOX-1 signaling is involved in impaired neutrophil chemotaxis during sepsis.

LOX-1 is expressed in neutrophils. LOX-1 is a membrane protein with a single transmembrane domain (12). To explore the direct role of LOX-1 in neutrophil function, we examined whether LOX-1 is expressed in neutrophils. We detected high basal LOX-1 protein expression in neutrophils in wild-type mice (Fig. 6A and B). Interestingly, LPS challenge did not increase LOX-1 total protein expression (Fig. 6A). However, LOX-1 surface (membrane) expression, which was shown by FACS to label only cell surface LOX-1, was quickly and markedly increased after the challenge by LPS (Fig. 6B and C). LOX-1 surface expression was also upregulated by the TLR2 agonist LTA (lipoteichoic acid), but the effect of LTA was less potent than that of LPS (data not shown). Our data indicate that LOX-1 is presynthesized and stored in neutrophils and can be quickly released from the intracellular store to the cell surface after LPS challenge. Neutrophil LOX-1 expression and the specificity of LOX-1 antibodies were further confirmed using LOX-1 knockout mice. No LOX-1 protein or surface expression was observed in LOX-1 knockout neutrophils (Fig. 6A and B). We also assessed whether LOX-1 surface expression is modulated during sepsis. FACS analysis showed that LOX-1 surface expression was increased in neutrophils of wild-type mice after CLP surgery (Fig. 6D). TLR2 ̵ ̵ and TLR4 ̵ ̵ neutrophils exhibited lower LOX-1 surface expression than wild-type neutrophils after the induction of sepsis (Fig. 6B), suggesting that both TLR2 and TLR4 activation can mediate neutrophil LOX-1 surface expression during sepsis.

Fig. 4. LOX-1 deletion increases bacterial clearance in the peritoneal cavity, blood, and lung after the induction of sepsis. Bacterial CFU in mouse peritoneal cavity (A), blood (B), and lung (C) were examined in LOX-1 knockout (LOX-1 ̵ ̵) mice and wild-type (WT) mice 24 h after CLP. *, P < 0.05 versus WT. The horizontal lines represent means.

Fig. 5. Neutrophils in LOX-1 knockout mice exhibit increased peritoneal migration in sepsis. (A) At 6 h after CLP, peritoneal cells were collected. Neutrophil counts were determined by FACS analysis. Neutrophil numbers in the peritoneal cavity were significantly higher in LOX-1 knockout (LOX-1 ̵ ̵) mice. *, P < 0.01. (B) Neutrophils were isolated from whole blood 2 h after CLP. Chemotaxis of neutrophils for CXCL2 was examined. *, P < 0.01. (C) For phagocytosis assays, FITC-labeled and heat-killed E. coli bacteria were incubated with blood neutrophils isolated from LOX-1 knockout (LOX-1 ̵ ̵) or WT littermates. Following quenching of extracellular E. coli with trypan blue, uptake of E. coli was analyzed by FACS. NS, not significant (n = 8). (D) Bacterial killing by neutrophils isolated from LOX-1 knockout (LOX-1 ̵ ̵) or WT littermates was determined. NS, not significant (n = 8). PMN, polymorphonuclear leukocytes.
LOX-1 deletion inhibits neutrophil overreaction and CXCR2 downregulation in sepsis. Our finding of LOX-1 expression in neutrophils implies that LOX-1 may possess a novel function in neutrophils. To test this possibility, we examined whether LOX-1 deletion could affect neutrophil activation after the induction of sepsis. CLP induced neutrophil activation in wild-type mice, as demonstrated by increased neutrophil p38 MAPK phosphorylation and CD11b surface expression. Interestingly, neutrophil p38 MAPK phosphorylation and CD11b surface expression during sepsis were significantly lower in LOX-1 knockout mice (Fig. 7A and B), indicating that LOX-1 deletion may prevent neutrophil overreaction in sepsis.

Chemokine receptor CXCR2 is a key mediator of neutrophil migration (2, 3). Neutrophil overreaction during severe sepsis has been known to induce CXCR2 downregulation and impaired neutrophil migration (1–3). We examined whether reduced neu-
Bacterial infection and systemic inflammation during sepsis

LOX-1

↑ Systemic inflammatory response: increased TNF-α and IL-6 production

Inflammatory tissue injury

↓ Neutrophil overreaction; Downregulation of CXCR2 surface expression

Neutrophil immune suppression: Reduced chemotaxis

Mortality

FIG. 8. Schematic presentation of the role of LOX-1 in sepsis. LOX-1 signaling aggravates inflammatory responses and causes neutrophil overreaction, which leads to increased inflammatory tissue injury and neutrophil suppression and contributes to high mortality in sepsis.

trophil activation after the induction of sepsis in LOX-1 knockout mice could lead to improved CXCR2 surface expression. LOX-1 deletion prevented the downregulation of neutrophil CXCR2 surface expression during sepsis (Fig. 7C), which is consistent with the increased neutrophil migration and chemotaxis seen both in vivo and in vitro (Fig. 5), suggesting that LOX-1 deletion helps maintain a better CXCR2-mediated neutrophil response.

**DISCUSSION**

The results from our study indicate that LOX-1 signaling aggravates inflammatory responses and mediates immune suppression after the induction of sepsis (Fig. 8), likely contributing to the increased mortality seen in a mouse model of polymicrobial sepsis. Sepsis contains two pathophysiological phases, systemic inflammation and immune suppression (2, 10, 33). An ideal treatment for sepsis-induced multiorgan dysfunction would be able to prevent inflammatory tissue injury but maintain immune responses (2, 10, 33). Therefore, the finding that LOX-1 signaling modulates both the inflammatory and immune responses during sepsis is very intriguing.

Production and upregulation of proinflammatory mediators such as TNF-α and IL-6 contribute to sepsis-induced inflammatory tissue injury (17, 20). In our studies, LOX-1 deletion prevented TNF-α and IL-6 production and lung injury in sepsis. The role of LOX-1 in the development of inflammation-driving cardiovascular diseases has been well established (24). LOX-1 blockade was able to prevent proinflammatory and prooxidant responses in endothelial cells and reduce atherogenesis in mouse atherosclerosis models (18, 45). LOX-1 signaling has been shown to mediate the production of proinflammatory cytokines TNF-α and IL-1 (39). Our studies suggest that LOX-1 also plays a role in sepsis-induced inflammatory responses.

Immune suppression has been a major problem for sepsis patients (2, 25, 33). It hampers bacterial clearance and causes further exaggeration of tissue damage. Immune suppression also makes sepsis patients more susceptible to secondary infection. A high mortality rate has been reported for sepsis patients with secondary bacterial pneumonia (2, 25, 33). Sepsis has been known to suppress neutrophil function, including chemotaxis and signaling (2, 3), likely due to overreaction and exhaustion of neutrophils (1–3). Our studies showed that LOX-1 deletion prevented neutrophil overreaction in sepsis and allowed better control of neutrophil responses during sepsis, leading to increased neutrophil recruitment at the sites of infection and increased bacterial clearance.

CXCR2 surface expression in neutrophils can regulate a wide variety of cellular responses, including respiratory burst, degranulation, integrin activation, and migration (2, 3, 41). Downregulation of CXCR2 surface expression due to neutrophil overreaction during sepsis leads to impaired neutrophil migration (1–3), which is a major factor in sepsis-induced neutrophil suppression. p38 MAPK activation has been reported to mediate CXCR2 downregulation in neutrophils (19, 22, 42). LOX-1 deletion prevented p38 MAPK activation in neutrophils, possibly contributing to the inhibition of CXCR2 downregulation and improved neutrophil migration in LOX-1 knockout mice during sepsis.

Neutrophil modulation appears to involve both inflammatory and immune dysfunction in sepsis (7, 25, 33, 34). Proinflammatory cytokines and chemokines induce neutrophil activation and migration to the sites of infection (34). On the other hand, neutrophil overreaction, impaired neutrophil chemotaxis, and altered Toll-like receptor (TLR) signaling are associated with immunosuppression in sepsis (1–3). Our present study specifically examined LOX-1 expression and function in neutrophils. However, since LOX-1 is also expressed in endothelial cells and macrophages (12, 28), we cannot exclude the possibility that LOX-1 deletion may also provide the observed beneficial effects for those cells. Apparently, more studies are required to characterize the function of LOX-1 in other cell types during sepsis. Nevertheless, our studies indicate that better-controlled neutrophil responses in LOX-1 knockout mice contribute, at least in part, to the improved immune responses in sepsis. Further studies are warranted to explore the underlying mechanisms. For example, identifying potential ligands and receptors that may interact with LOX-1 in neutrophils could advance our current knowledge of neutrophil function and the pathogenesis of sepsis.

**REFERENCES**

1. Alves-Filho, J. C., A. de Freitas, M. Russo, and F. Q. Cunha. 2006. Toll-like receptor 4 signaling leads to neutrophil migration impairment in polymicrobial sepsis. Crit. Care Med. 34:461–470.
2. Alves-Filho, J. C., A. de Freitas, F. Spiller, F. O. Souto, and F. Q. Cunha. 2008. The role of neutrophils in severe sepsis. Shock 30(Suppl. 1):3–9.
3. Aomatsu, K., et al. 2008. Toll-like receptor agonists stimulate human neutrophil migration via activation of mitogen-activated protein kinases. Immunology 123:171–180.
4. Apte, R. N., et al. 2006. The involvement of IL-1 in tumorigenesis, tumor invasiveness, metastasis and tumor-host interactions. Cancer Metastasis Rev. 25:387–408.
5. Bosio, R., C. Bossemeyer-Pourie, N. Steinckwich, C. Dournon, and O. Nusse. 2004. Mouse bone marrow contains large numbers of functionally competent neutrophils. J. Leukoc. Biol. 75:604–611.
6. Buras, J. A., B. Holzmann, and M. Sitkovsky. 2005. Animal models of sepsis: setting the stage. Nat. Rev. Drug Discov. 4:854–865.
7. Craig, A., J. Mai, S. Cai, and S. Jeyaseelan. 2009. Neutrophil recruitment to the lungs during bacterial pneumonia. Infect. Immun. 77:568–575.
8. Decker, T. 2004. Sepsis: avoiding its deadly toll. J. Clin. Invest. 113:1387–1389.
9. de Jong, H. K., T. van der Poll, and W. J. Wiersinga. 2010. The systemic pro-inflammatory response in sepsis. J. Innate Immun. 2:422–430.
11. Deng, J. C., et al. 2006. Sepsis-induced suppression of lung innate immunity is mediated by IRAK-M. J. Clin. Invest. 116:2532–2542.
12. Dunn, S., et al. 2008. The lectin-like oxidized low-density-lipoprotein receptor: a pro-inflammatory factor in vascular disease. Biochem. J. 409:349–355.
13. Everhart, M. B., et al. 2006. Duration and intensity of NF-kappaB activity determine the severity of endotoxin-induced acute lung injury. J. Immunol. 176:4995–5005.
14. Fu, J., A. P. Naren, X. Gao, G. U. Ahmmed, and A. B. Malik. 2005. Protease-activated receptor-1 activation of endothelial cells induces protein kinase Calpha-dependent phosphorylation of syntaxin 4 and Munc18c: role in signaling p-selectin expression. J. Biol. Chem. 280:3178–3184.
15. Gao, H., S. K. Leaver, A. Burke-Gaffney, and S. J. Finney. 2008. Severe sepsis and Toll-like receptors. Semin. Immunopathol. 30:29–40.
16. Gao, X. P., et al. 2007. Blockade of class IA phosphoinositide 3-kinase in neutrophils prevents NADPH oxidase activation- and adhesion-dependent inflammation. J. Biol. Chem. 282:6116–6125.
17. Goodman, R. B., J. Fugin, J. S. Lee, and M. A. Matthey. 2003. Cytokine-mediated inflammation in acute lung injury. Cytokine Growth Factor Rev. 14:523–535.
18. Hu, C., et al. 2007. LOX-1 deletion alters signals of myocardial remodeling immediately after ischemia-reperfusion. Cardiovasc. Res. 76:292–302.
19. Juffermans, N. P., et al. 2000. Expression of the chemokine receptors CXCR1 and CXCR2 on granulocytes in human endotoxemia and tuberculosis: involvement of the p38 mitogen-activated protein kinase pathway. J. Infect. Dis. 182:890–894.
20. Kamochi, M., et al. 1999. P-selectin and ICAM-1 mediate endotoxin-induced neutrophil recruitment and injury to the lung and liver. Am. J. Physiol. 277:L1301–L1319.
21. Kang, J. L., et al. 2001. Genistein prevents nuclear factor-kappa B activation and acute lung injury induced by lipopolysaccharide. Am. J. Respir. Crit. Care Med. 164:2206–2212.
22. Khandaker, M. H., et al. 1998. CXCR1 and CXCR2 are rapidly down-modulated by bacterial endotoxin through a unique agonist-independent, kinase-dependent mechanism. J. Immunol. 161:1930–1938.
23. Koh, A. Y., G. P. Priebe, C. Ray, N. Van Rooijen, and G. B. Pier. 2009. Inescapable need for neutrophils as mediators of cellular innate immunity to acute Staphylococcus aureus pneumonia. Infect. Immun. 77:5300–5310.
24. Li, X., et al. 2002. Statins mediate oxidized high-density lipoprotein-mediated adhesion molecule expression in human coronary artery endothelial cells: role of LOX-1. J. Pharmacol. Exp. Ther. 302:601–605.
25. Lyn-Kew, K., and T. J. Standiford. 2008. Immunosuppression in sepsis. Curr. Pharm. Des. 14:1870–1881.
26. Matsunaga, T., S. Hakari, I. Koyama, T. Harada, and T. Komoda. 2003. NF-kappa B activation in endothelial cells treated with oxidized high-density lipoprotein. Biochem. Biophys. Res. Commun. 303:313–319.
27. McGovern, N. N., et al. 2011. Hypoxia selectively inhibits respiratory burst activity and killing of Staphylococcus aureus in human neutrophils. J. Immunol. 186:453–463.
28. Mehta, J. L., J. Chen, P. L. Hermonat, F. Romeo, and G. Novelli. 2006. Lectin-like, oxidized low-density lipoprotein receptor-1 (LOX-1): a critical player in the development of atherosclerosis and related disorders. Cardiovasc. Res. 69:36–45.
29. Mehta, J. L., et al. 2007. Deletion of LOX-1 reduces atherogenesis in LDLR knockout mice fed high cholesterol diet. Circ. Res. 100:1634–1642.
30. Moreno, S. E., et al. 2006. IL-12, but not IL-18, is critical to neutrophil activation and resistance to polymicrobial sepsis induced by cecal ligation and puncture. J. Immunol. 177:3218–3224.
31. Muller Kobold, A. C., et al. 2000. Leukocyte activation in sepsis: correlations with disease state and mortality. Intensive Care Med. 26:883–892.
32. Oka, K., et al. 1998. Lectin-like oxidized low-density lipoprotein receptor 1 mediates phagocytosis of aged/apoptotic cells in endothelial cells. Proc. Natl. Acad. Sci. U. S. A. 95:9535–9540.
33. Reddy, R. C., G. H. Chen, P. K. Tekchandani, and T. J. Standiford. 2001. Sepsis-induced immunosuppression: from bad to worse. Immunol. Res. 24:273–287.
34. Reddy, R. C., and T. J. Standiford. 2010. Effects of sepsis on neutrophil chemotaxis. Curr. Opin. Hematol. 17:18–24.
35. Rencz, L., et al. 2006. Matrix metalloproteinase-9 deficiency impairs host defense against abdominal sepsis. J. Immunol. 176:3735–3741.
36. Shih, H. H., et al. 2009. CRP is a novel ligand for the oxidized LDL receptor LOX-1. Am. J. Physiol. Heart Circ. Physiol. 296:H1643–H1650.
37. Shimaoa, T., et al. 2001. Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) supports cell adhesion to fibronectin. FEBS Lett. 504:65–68.
38. Shimaoa, T., et al. 2001. LOX-1 supports adhesion of Gram-positive and Gram-negative bacteria. J. Immunol. 166:5108–5114.
39. Shin, H. K., Y. K. Kim, K. Y. Kim, J. H. Lee, and K. W. Hong. 2004. 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 109:1022–1028.
40. Tavares-Murta, B. M., et al. 2002. Failure of neutrophil chemotactic function in septic patients. Crit. Care Med. 30:1056–1061.
41. Tsai, W. C., et al. 2000. CXC chemokine receptor CXCR2 is essential for protective innate host response in murine Psuedomonas aeruginosa pneumonia. Infect. Immun. 78:5300–5310.
42. van den Blink, B., et al. 2004. P38 mitogen activated protein kinase is involved in the downregulation of granulocyte CXC chemokine receptors 1 and 2 during human endotoxemia. J. Clin. Immunol. 24:37–41.
43. van der Poll, T., and J. C. Meijers. 2010. Systemic inflammatory response syndrome and compensatory anti-inflammatory response syndrome in sepsis. J. Innate Immun. 2:379–380.
44. Van Zillje, J. A., and C. A. Lowell. 2009. Neutrophil-specific deletion of Syk kinase results in reduced host defense to bacterial infection. Blood 114:4871–4882.
45. Xu, X., X. Gao, B. J. Potter, J. M. Cao, and C. Zhang. 2007. Anti-LOX-1 rescues endothelial function in coronary arterioles in atherosclerotic ApoE knockout mice. Arterioscler. Thromb. Vasc. Biol. 27:871–877.

Editor: J. N. Weiser