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Lipocalin 2 Deficiency Dysregulates Iron Homeostasis and Exacerbates Endotoxin-Induced Sepsis

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Various states of inflammation, including sepsis, are associated with hypoferremia, which limits iron availability to pathogens and reduces iron-mediated oxidative stress. Lipocalin 2 (Lcn2; siderocalin, 24p3) plays a central role in iron transport. Accordingly, Lcn2-deficient (Lcn2KO) mice exhibit elevated intracellular labile iron. In this study, we report that LPS induced systemic Lcn2 by 150-fold in wild-type mice at 24 h. Relative to wild-type littermates, Lcn2KO mice were markedly more sensitive to endotoxemia, exhibiting elevated indices of organ damage (transaminasemia, lactate dehydrogenase) and increased mortality. Such exacerbated endotoxemia was associated with substantially increased caspase-3 cleavage and concomitantly elevated immune cell apoptosis. Furthermore, cells from Lcn2KO mice were hyperresponsive to LPS ex vivo, exhibiting elevated cytokine secretion. Additionally, Lcn2KO mice exhibited delayed LPS-induced hypoferremia despite normal hepatic hepcidin expression and displayed decreased levels of the tissue redox state indicators cysteine and glutathione in liver and plasma. Desferroxamine, an iron chelator, significantly protects Lcn2KO mice from LPS-induced toxicity, including mortality, suggesting that Lcn2 may act as an antioxidant in vivo by regulating iron homeostasis. Thus, Lcn2-mediated regulation of labile iron protects the host against sepsis. Its small size and simple structure may make Lcn2 a deployable treatment for sepsis.

Sepsis, a catastrophically amplified and dysregulated induction of proinflammatory cytokines often eventuating in multiorgan failure and death (1), is a major public health problem resulting in >200,000 deaths in the United States (2) and a significant burden on the global healthcare system (3). Sepsis is triggered by the host response to bacterial components such as LPS, a ligand for TLR4. The death of immune cells, particularly leukocytes, via extensive apoptosis is a major contributor to significant immune suppression and mortality in septic patients (4, 5). Although sepsis is typically characterized by robust production of proinflammatory cytokines, it has been recognized that some genes induced during sepsis serve as dampening agents that seek to restore immunological equilibrium (1). Identifying such agents and defining their mechanisms of action may result in new treatment regimens for sepsis.

Sepsis is not only associated with an extreme proinflammatory response but is also accompanied by hypoferremia of inflammation, a primitive defensive mechanism to drastically reduce the concentration of circulating iron, limiting its availability to pathogens (6, 7) and also ameliorating iron-dependent oxidative stress generated via a Fenton reaction (8). Lipocalin 2 (Lcn2; human ortholog neutrophil gelatinase-associated lipocalin [NGAL]) has been shown to be dramatically upregulated in various inflammatory conditions and is considered as an acute phase protein in mice (9–12). Several in vitro studies have demonstrated that Lcn2 protects against cellular stress and exposure to H2O2 and that overexpression of Lcn2 allows cells to tolerate superphysiological iron concentrations (13–15). Under physiologic conditions Lcn2 can protect liver against iron-mediated oxidative stress. Lipocalin 2 (Lcn2) is a multifunctional protein that has been implicated in several physiologic and pathologic conditions (16–19). It has also been suggested that Lcn2 stabilizes the labile iron/siderophore complex (18, 19). Additionally, by chelating bacterial siderophores, Lcn2 exerts direct antibacterial activity and, accordingly, Lcn2-deficient (Lcn2KO) mice are sensitive to bacterial sepsis (11, 20, 21). Although such correlation could be consistent with a role for Lcn2 in driving or dampening an inflammatory response, the known bioactivities of Lcn2 led us to hypothesize that it might play a role in hypoferremia of inflammation and, thereby, the resolution of severe inflammation.

In this study, we tested the hypothesis that Lcn2 protects against severe inflammation using a murine model of LPS-induced sepsis. We observed that LPS dramatically induced Lcn2 and that Lcn2KO mice were highly sensitive to LPS-induced mortality, correlating with greater immune cell apoptosis and upregulation of proinflammatory gene expression. Such increased pathology to LPS in Lcn2KO mice is associated with both delayed hypoferremia and increased oxidative stress. Desferroxamine (DFO), an iron chelator,
offers significant protection for LPS-induced toxicity and mortality in Lcn2KO mice. Thus, our study demonstrates that Lcn2 not only protects against bacterial sepsis but also regulates host proinflammatory cytokine expression by limiting iron-mediated oxidative stress.

Materials and Methods

Abs used were cyclooxygenase (COX)-2 (Cayman Chemical, Ann Arbor, MI), inducible NO synthase (iNOS; Upstate Biotechnology, Bedford, MA), and cleaved caspase-3 (Cell Signaling Technology, Danvers, MA), as well as anti–β-actin, LPS, and desferoxamine mesylate (Sigma-Aldrich, St. Louis, MO). Anti-mouse Lcn2 and all cytokine ELISA kits were from R&D Systems (Minneapolis, MN).

LPS-induced sepsis

Eight-week-old mice (both male and female) were challenged i.p. with a nonlethal dose of LPS from E. coli (InvivoGen, Grand Island, NY) for 1 h at room temperature. Immuno-blots were developed by ECL (GE Healthcare, Buckinghamshire, U.K.). For splenic cleaved-3 caspase, tissue was homogenized in RIPA buffer (Cell Signaling Technology) with protease inhibitor mixture (Roche). Total protein levels were quantitated by Bio-Rad method and 25 μg protein was loaded under denaturing conditions. Immunoblots were developed using Abs from Cell Signaling Technology as above. β-actin was probed as a loading control.

Assay of cytokines

Cytokines in serum and cell supernatants were measured by DuoSet ELISA cytokine kits according to the manufacturer’s instructions. Serum IL-1β levels were measured by ELISA using an MBL kit.

Hemolysis-free serum samples obtained by retro-orbital plexus were analyzed using automated iron and lactate dehydrogenase (LDH) biochemical assays on an Olympus AU680 chemistry analyzer (Beckman-Coulter, Brea, CA). Analysis was performed at the Comparative Clinical Pathology Services (Columbia, MO) under the supervision of a board-certified veterinary clinical pathologist (Charles E. Wiedmeyer). Serum triglycerides were measured using a kit from BioVision (Milpitas, CA).

Bone marrow-derived macrophage isolation and culture

Bone marrow-derived macrophages (BMDMs) were cultured as described by Weischenfeldt and Porse (23). Briefly, bone marrow cells were isolated and cultured in six-well plates with DMEM supplemented with 10% FBS, and 1% penicillin and streptomycin (22). Cells were cultured overnight at 37°C, washed three times with PBS to remove nonadherent cells, and stimulated with E. coli LPS (100 ng/ml) in serum-free DMEM. At various time points supernatants and lysates were collected for cytokine analysis and immunoblotting and stored at −80°C until analysis.

Measurement of apoptosis

Cleaved caspase-3 in spleen and bone marrow was analyzed by immunoblotting. TUNEL-positive cells in 10% formalin-fixed tissue were identified using TUNEL assay (Roche, Indianapolis, IN). The images were captured using a Zeiss Axioskop2 Plus (Carl Zeiss Microimaging).

Quantification of apoptotic cells by flow cytometry

The apoptotic cell population in tissue was measured as in Zhang et al. (24) with all Abs procured from eBioscience (San Diego, CA). except for Ly-6G, which was from BD Biosciences (San Jose, CA). Annexin V staining was acquired on a BD LSR II multiparameter flow cytometer and analyzed using FlowJo software (Tree Star, Ashland, OR).

Immunoblotting

Immunoblotting was performed as described in Vijay-Kumar et al. (25). Briefly, serum was diluted (1:10) in 2× Laemmli sample buffer (Bio-Rad) with 2-ME and 100 mM DTT, boiled for 5 min, immediately cooled on ice for 10 min, and subjected to SDS-PAGE on a 4–20% gel (Bio-Rad, Hercules, CA). Proteins were transferred to nitrocellulose, blocked with 5% milk for 1 h, probed with primary Ab biotinylated anti-mouse Lcn2 (0.2 μg/ml) overnight at 4°C, washed three times, and probed with streptavidin HRP (Invitrogen, Grand Island, NY) for 1 h at room temperature. Immunoblots were developed by ECL (GE Healthcare, Buckinghamshire, U.K.). For splenic cleaved-3 caspase, tissue was homogenized in RIPA buffer (Cell Signaling Technology) with protease inhibitor mixture (Roche). Total protein levels were quantitated by Bio-Rad method and 25 μg protein was loaded under denaturing conditions. Immunoblots were developed using Abs from Cell Signaling Technology as above. β-actin was probed as a loading control.

Statistical analysis

Statistical analysis for significance (p < 0.05) was determined using a Student t test (GraphPad Prism).

Results

LPS induces Lcn2 in vivo in WT mice

To begin to investigate the role of Lcn2 in sepsis, we first analyzed the kinetics of Lcn2 secretion in response to an i.p. administered nonlethal dose of E. coli LPS (5 mg/kg body weight), a TLR4 ligand, in C57BL/6 mice. This resulted in a dramatic increase (150-fold) in serum Lcn2 that peaked at 24 h and returned to basal levels by 48 h (Fig. 1A). LPS induced Lcn2 with kinetics similar to the highly studied acute phase protein C-reactive protein in murine inflammatory models (28). Immunoblotting sera confirmed our results (Fig. 1B). Similar results were obtained by challenging WT mice with the TLR5 ligand, flagellin (data not shown).

Lcn2 deficiency sensitizes LPS-induced mortality

Lcn2 is a substantially upregulated host protein during the acute phase response not only during infection but also in sterile inflammation and aseptic diseases (29). To study the role of Lcn2 in...
sensitivity that WT mice may induce cleavage of caspase-3 at earlier time points, we monitored caspase-3 cleavage at 2, 4, 8, and 12 h in WT mice by immunoblotting, but at no time did we detect caspase-3 cleavage in the spleen (data not shown). In accordance, TUNEL assays performed on spleens showed increased numbers of apoptotic cell aggregates in LPS-treated Lcn2KO mice that were not apparent in similarly treated WT mice (Fig. 4C). Similarly, LPS induced more TUNEL-positive cells in the colon, specifically at the base of the crypts (data not shown).

Increased LPS-induced immune cell apoptosis in Lcn2KO mice

Next, to identify the immune cell populations most prone to apoptosis, we analyzed cells for annexin V staining by flow cytometry. We observed significant increases in LPS-induced apoptosis of CD4+ T cells (CD3+CD4+), neutrophils (CD11b+Ly-6G+), and dendritic cells (CD11b+CD11c+) isolated from spleens of Lcn2KO mice when compared with those derived from WT mice (Fig. 5). Analysis of bone marrow showed a similar trend of increased apoptosis in Lcn2KO neutrophils (not shown), while B cell (CD3−CD19+B220+) and macrophage (CD45+CD11b+MHC class II+F4/80+) fractions were not different between the two genotypes in both tissues assayed (data not shown).

Lcn2 deficiency potentiates LPS-induced proinflammatory gene expression

LPS is known to induce extensive production of cytokines, especially TNF-α, a main determinant of multiple organ damage and death. Furthermore, LPS induced Lcn2 robustly in WT peritoneal exudate cells (data not shown). Therefore, we studied proinflammatory cytokine response in WT and Lcn2KO mice in vivo. Lcn2KO mice exhibited significantly elevated levels of serum TNF-α and IL-18, master proinflammatory mediators that are highly linked to sepsis (Fig. 6A, 6B). Such increased cytokine production was also seen in peritoneal immune cells stimulated with LPS ex vivo. Specifically, relative to WT mice, Lcn2KO PECs exhibited greater production of KC, IL-6, and TNF-α (Fig. 6C–E). Analogous results were seen for proinflammatory enzymes. Specifically, Western blots revealed that COX-2 levels were markedly upregulated in Lcn2KO cells and only moderately upregulated in WT cells, especially at the 12 h time point. Similar results were seen for iNOS.
which was also modestly induced by LPS in WT cells but markedly induced in cells of LPS-treated Lcn2KO PECs (Fig. 6H). Such elevated proinflammatory mediators were accompanied by concomitant increases of the anti-inflammatory mediators IL-10 and secretory (s)IL-1Ra (Fig. 6F, 6G).

Next, to examine the extent to which hyperresponsiveness of Lcn2KO PECs resulted from loss of Lcn2 in these cells per se or was an indirect consequence of a Lcn2-deficient environment, we compared responses of naive BMDMs that had been cultured from WT and Lcn2KO mice. First, we verified that WT BMDMs induced Lcn2 in response to LPS (data not shown). Similar to PECs, BMDMs from Lcn2KO mice exhibited greater LPS-induced cytokine secretion relative to BMDMs from WT mice. Such elevated cytokine secretion was seen for both proinflammatory cytokines (IL-1β, IL-6, and KC) and the anti-inflammatory cytokine (sIL-1Ra) at both 100 and 500 ng/ml concentrations of LPS (Supplemental Fig. 1), suggesting the increased septic response observed in Lcn2KO mice was not due to a broad defect in counterregulatory mediators but rather reflects an important direct role for Lcn2 in containing sepsis.
Delayed LPS-induced hypoferremia in Lcn2KO mice

Inflammatory responses are accompanied by hypoferremia of inflammation, a primitive host protective mechanism that limits the iron available to invading pathogens and that can participate in a Fenton reaction, exacerbating oxidative stress (7, 33). Lcn2 is known to chelate iron and play a role in its transport (16, 17). Thus, we reasoned that loss of Lcn2 may disrupt iron transport while rendering intracellular siderophore-bound iron more labile (18, 19), thus mediating the high toxicity to LPS seen in Lcn2KO mice. Measurement of serum iron revealed a modest increase in basal iron levels in Lcn2KO mice relative to WT littermates. Moreover, Lcn2KO mice exhibited only modest hypoferremia in response to LPS, ∼25% by 4 h, whereas iron levels dropped by >50% in WT mice. At later times, serum iron levels fell in Lcn2KO mice such that they were similar to those of WT mice from 6 to 24 h after LPS treatment (Fig. 7). Interestingly, there was no significant

FIGURE 5. Increased LPS-induced immune cell apoptosis in Lcn2KO mice. WT or Lcn2KO mice (n = 3–6) were given either PBS or LPS (5 mg/kg body weight) and after 24 h (%) apoptotic cells were measured in the spleen by flow cytometry. (A) CD3+CD4+ T cells. (B) CD11b+Ly-6G+ neutrophils. (C) CD11b+CD11c+ dendritic cells. *p < 0.05.

FIGURE 6. Lcn2 deficiency potentiates LPS-induced proinflammatory gene expression. Mice (n = 3–6) were given either PBS or LPS (5 mg/kg body weight) and, after 6 h, serum was analyzed for (A) TNF-α and (B) IL-18 (pg/ml). (C–H) PECs stimulated in triplicate with either PBS or LPS (100 ng/ml) in serum-free media. Supernatants were collected at 12 and 24 h and analyzed for cytokines by ELISA or lysates subjected to immunoblotting for COX2 and iNOS. (C) KC, (D) IL-6, (E) TNF-α, (F) IL-10, (G) sIL-1Ra (expressed in ng/mg protein). (H) Immunoblot showing iNOS and COX2 expression in PEC lysates. (A) and (B) and (C)–(H) are representatives of two and three independent experiments, respectively. *p < 0.05.
difference in basal or LPS-induced hepatic hepcidin, a major hypoferremic hormone, in WT and Lcn2KO mice (Supplemental Fig. 2A).

Increased LPS-induced oxidative stress in Lcn2KO mice

Lcn2 not only transports and stabilizes iron but also regulates the intracellular iron concentration. Nairz et al. (34) demonstrated that Lcn2KO mice have increased cellular iron levels. We hypothesized that such defective iron homeostasis in Lcn2KO mice may render them more susceptible to oxidative stress via participation of iron in the Fenton reaction. To study this, we analyzed GSH and cysteine, as well as GSSG and CySS, in plasma and liver as indicators of redox state, itself an important determinant of cytokine expression as changes in cysteine and GSH metabolism modulate inflammation (35). As shown in Fig. 8A and 8B, Lcn2KO mice exhibited significantly elevated levels of CySS and GSSG in the liver 24 h after LPS administration when compared with similarly treated WT mice. Similar, but more modest, elevations in CySS and GSSG were observed in plasma (data not shown). Next, we analyzed expression of the antioxidant enzymes SOD2 and Gpx1 in livers of WT and Lcn2KO mice. Whereas WT mouse livers upregulated SOD2 expression by 10-fold 6 h after LPS with expression returning to basal levels by 24 h, Lcn2KO mice exhibited significantly less SOD2 expression when compared with WT mice despite decreased levels of tissue redox state indicators (Fig. 8C). No significant differences were observed in Gpx levels between the two strains (Supplemental Fig. 2B).

DFO rescues LPS-induced toxicity in Lcn2KO mice

DFO is a siderophore-based iron chelator routinely used clinically to treat iron overload. DFO-bound iron is metabolically inactive and unable to participate in a Fenton reaction, thus limiting free radical generation (36–38). To study our hypothesis that delayed LPS-induced hypoferremia, as well as increased intracellular labile iron, in Lcn2KO mice may render them more susceptible to LPS-induced septic shock, we pretreated Lcn2KO mice with DFO (100 μM) i.p. 1 h before LPS (5 mg/kg body weight) administration. Control mice were given PBS. Such pretreatment of Lcn2KO mice with DFO resulted in significant reduction of cleaved caspase-3 in the spleen (Fig. 9A) and systemic proinflammatory gene expression. Specifically, in DFO-pretreated Lcn2KO mice, LPS-induced IL-6 and KC were reduced by >50% at 4 h relative to Lcn2KO mice pretreated with PBS. Additionally, whereas at 24 h cytokine levels remained elevated in PBS-pretreated LPS-challenged Lcn2KO mice, they were undetectable in DFO-pretreated Lcn2KO mice (Fig. 9B, 9C). Furthermore, DFO pretreatment substantially reduced LPS-induced AST and ALT, markers of liver and multiple organ damage, respectively (Fig. 9D, 9E). Finally, to test whether DFO-mediated reduction in cleaved caspase-3 and serum inflammatory markers would protect against LPS-induced mortality, we challenged Lcn2KO mice with a high dose of LPS (20 mg/kg body weight) 1 h after DFO administration. Lcn2KO mice that were pretreated with DFO still exhibited symptoms of sepsis initially but recovered and survived (70%), whereas Lcn2KO mice given PBS succumbed to death within 7 d (Fig. 9F). Collectively, our studies demonstrate that Lcn2 is a host protective factor against LPS-induced toxicity (Fig. 10).

Discussion

Lcn2 is an acute phase protein known to be highly upregulated during inflammatory conditions such as sepsis. Induced acute phase proteins may amplify or dampen the inflammatory response. For instance, HMGB1 and SAA are known to amplify the inflammatory response by activating TLR4. However, the role of Lcn2 in sepsis is not known. In this study we demonstrate that Lcn2 is highly upregulated in WT mice in response to the TLR4 ligand, LPS. In Lcn2KO mice, LPS-induced sepsis resulted in increased toxicity as...
accessed by immune cell apoptosis, proinflammatory cytokine gene expression, systemic organ damage markers, and mortality. Furthermore, we demonstrated that Lcn2KO mice exhibited delayed hypoferremia of inflammation at the onset of inflammation, which eventuated in increased oxidative stress. Whereas delayed hypoferremia was most prominent at 4 h, elevated oxidative stress was only observed at later time points (24 h). This temporal difference likely reflects that the major nonprotein thiol and cellular reductant, GSH, is generally present in high concentrations (39), likely high enough to protect from oxidative damage for a limited period, thus manifesting in depletion of GSH stores only at 24 h (39). Importantly, reducing levels of free iron with the iron chelator DFO substantially ameliorated LPS-induced toxicity and mortality.

Sepsis is characterized by an exacerbated host proinflammatory response and elevated immune cell apoptosis eventuating in immunosuppression. The increased sensitivity of Lcn2-deficient cells to LPS suggests that Lcn2 may negatively regulate proinflammatory gene expression. Our results also support the previous study that used siderophore/iron-free recombinant Lcn2 to substantially reduce LPS-induced proinflammatory cytokines in macrophages (40). However, it is not clear at present whether the highly upregulated Lcn2 observed during inflammatory conditions is apo-Lcn2 (iron- and siderophore-free) or whether it is bound to a siderophore, another aspect of Lcn2 biology that warrants further investigation. It would be reasonable to predict that mammalian-derived Lcn2 would be in a siderophore-containing form, as Lcn2 can only bind iron through the action of a mammalian siderophore such as catechol or 2,5-dihydroxybenzoic acid (19). Exploring the role of apo- and holo-Lcn2 (siderophore- or siderophore- and iron-bound) during inflammatory conditions, their targets and molecular fates could reveal key insights regarding Lcn2 signaling and iron homeostasis. However, current technology does not easily permit in-depth study of the iron binding states of Lcn in vivo.

Immune cell apoptosis is a major factor underlying the increased mortality seen in septic patients (5). The increased immune cell apoptosis observed in Lcn2KO mice may contribute to the high mortality of the mice. Furthermore, Lcn2-deficient cells not only exhibited elevated proinflammatory cytokines but also substantially upregulated anti-inflammatory molecules (IL-10 and sIL-1Ra), which may also contribute to immunosuppression, a phenomenon known as compensatory anti-inflammatory response syndrome (41). Additionally, apoptotic immune cells appeared as aggregates in the spleens of Lcn2KO mice, suggesting the possibility that impaired autophagy results in a delay in the clearance of apoptotic cells contributing to the leakage of cellular debris, including many activators of innate immunity (danger-associated molecular patterns such as Hsp70 and HMGB1), which would further amplify the proinflammatory response (42).

Furthermore, our study clearly demonstrates that Lcn2 facilitates LPS-induced hypoferremia at early time points, allowing the host to control iron equilibrium based on inflammatory conditions. It is likely that Lcn2KO mice with significantly elevated proinflammatory cytokines accompanied with modestly elevated basal systemic iron levels may further aggravate LPS-induced toxicity in these mice. It is also possible that elevated concentrations of intracellular “labile” iron (34) in Lcn2KO mice may readily participate in a Fenton reaction, potentiating LPS-induced toxicity.
(18, 19). The substantial reduction of LPS-induced caspase-3 cleavage, organ damage markers, and proinflammatory cytokines by DFO suggests that dysregulated iron homeostasis plays a role in LPS-induced toxicity in Lcn2KO mice.

Macrophages play a central role in sepsis via secretion of proinflammatory cytokines, particularly TNF-α, and also by regulating iron homeostasis (43). The secretion of proinflammatory cytokines in response to LPS is NF-κB dependent (44, 45). It has been shown that treatment of cultured hepatic macrophages with 1,2-dimethyl-3-hydroxyprid-4-one, a lipophilic iron chelator, blocked LPS-induced NF-κB activation and proinflammatory TNF-α and IL-6 expression (46). Furthermore, Darshan et al. (47) demonstrated that 6 h pretreatment of RAW264.7 macrophages with DFO inhibited LPS-induced IL-6 in vitro and LPS-induced TNF-α and IL-6 were significantly lower in rats on an iron-deficient diet. Similarly, DFO treatment reduced LPS-induced mortality in mice (48). Iron supplementation increases proinflammatory cytokines in IL-10KO mice, which are prone to develop spontaneous colitis (49). Furthermore, Sindirilru et al. (50) have shown that in humans with chronic venous leg ulcers, iron-loaded macrophages exhibit increased proinflammatory cytokines. This study also showed that coadministering iron/dextran delays wound healing whereas DFO improves wound healing. Taken together, these studies demonstrate that iron homeostasis plays a crucial role not only in ROS generation but also in host proinflammatory gene expression. Accordingly, neutralization of LPS-induced toxicity by DFO in Lcn2KO mice strongly supports previous work, which demonstrated elevated intracellular labile iron levels in Lcn2KO mice (34). Thus, elevated Lcn2 during sepsis serves to induce hypoferremia and stabilize iron, which can limit oxidative stress, cellular apoptosis, and proinflammatory gene expression as shown schematically in Fig. 10.

That dramatic induction of Lcn2 protects mice against catastrophic inflammation suggests the possibility that Lcn2, and its human ortholog NGAL, may have therapeutic potential in the treatment of septic shock. Indeed, our results suggest that Lcn2 not only exerts antiseptic activity via bacterial siderophore sequestration (51), but it also acts as an anti-inflammatory agent by regulating hypoferremia of inflammation, which rescues host immune cell apoptosis/proinflammatory gene expression by maintaining thiol/disulfide redox homeostasis. The fact that NGAL is not an acute phase protein in humans (52) and thus does not exhibit the thiol/disulfide redox homeostasis. The fact that NGAL is not an acute phase protein in humans (52) and thus does not exhibit the thiol/disulfide redox homeostasis.

References


