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ORIGINAL RESEARCH

Microbiota-Inducible Innate Immune Siderophore Binding Protein Lipocalin 2 Is Critical for Intestinal Homeostasis

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SUMMARY

The current study identifies the role of lipocalin 2, a microbiota-inducible innate immune protein, in regulating intestinal homeostasis, and show that mice lacking lipocalin 2 harbor colitogenic microbiota and are susceptible to intestinal inflammatory disease.

BACKGROUND & AIMS: Lipocalin 2 (Lcn2) is a multifunctional innate immune protein whose expression closely correlates with the extent of intestinal inflammation. However, whether Lcn2 plays a role in the pathogenesis of gut inflammation is unknown. Herein, we investigated the extent to which Lcn2 regulates inflammation and gut bacterial dysbiosis in mouse models of IBD.

METHODS: Lcn2 expression was monitored in murine colitis models and upon microbiota ablation/restoration. Wild-type (WT) and *Lcn2* knockout (*Lcn2*KO) mice were analyzed for gut bacterial load, composition by 16S ribosomal RNA gene pyrosequencing, and their colitogenic potential by co-housing with interleukin (*Il*)*10*KO mice. Acute (dextran sodium sulfate) and chronic (IL10R neutralization and T-cell adoptive transfer) colitis were induced in WT and *Lcn2*KO mice with or without antibiotics.

RESULTS: Lcn2 expression was dramatically induced on inflammation and was dependent on the presence of a gut microbiota and MyD88 signaling. Use of bone marrow–chimeric mice showed that nonimmune cells are the major contributors of circulating Lcn2. *Lcn2*KO mice showed increased levels of *entA*-expressing gut bacteria burden, and, moreover, a broadly distinct bacterial community relative to WT littermates. *Lcn2*KO mice developed highly colitogenic T cells and showed exacerbated colitis on exposure to DSS or neutralization of IL10. Such exacerbated colitis could be prevented by antibiotic treatment. Moreover, exposure to the microbiota of *Lcn2*KO mice, via cohousing, resulted in severe colitis in *Il10*KO mice.

CONCLUSIONS: Lcn2 is a bacterially induced, MyD88-dependent protein that plays an important role in gut homeostasis and a pivotal role on challenge. Hence, therapeutic manipulation of Lcn2 levels may provide a strategy to help manage diseases driven by alteration of the gut microbiota. *(Cell Mol Gastroenterol Hepatol 2016;2:482–498; http://dx.doi.org/10.1016/j.jcmgh.2016.03.007)*

Keywords: Gut Microbiota; Innate Immunity; Germ-Free Mice; MyD88; Colitis; NGAL; Siderocalin; Neutrophils; IBD.

Inflammatory bowel disease (IBD) is the collective term for chronic idiopathic inflammatory diseases of the intestine, which are highly prevalent in North America and Europe.[1] The clinical symptoms of IBD may appear similar, however, its etiology and pathology may vary between IBD patients because genetics, diet, and environmental factors substantially influence the disease. Among environmental factors, gut microbiotal dysbiosis has been associated strongly with human IBD,[2] underscoring the involvement of host–microbe interactions.[3] Furthermore, IBD is associated with increased levels of a number of acute phase proteins (APPs) that drive or dampen the inflammatory response.[4] One such APP is lipocalin 2 (Lcn2, human ortholog, neutrophil gelatinase-associated lipocalin [NGAL]; also known as siderocalin, 24p3), a multifaceted

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Abbreviations used in this paper: **APP, acute phase protein; CD45, cluster of differentiation 45; cDNA, complementary DNA; CoH, co-housed; DMEM, Dulbecco's modified Eagle medium; DSS, dextran sulfate sodium; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GF, germ free; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IL, interleukin; IL10R, interleukin-10 receptor; Lcn2, lipocalin 2; Lcn2KO, lipocalin 2 deficient; LPS, lipopolysaccharide; mAb, monoclonal antibody; MPO, myeloperoxidase; MyD88, myeloid differentiation primary response gene 88; NGAL, neutrophil gelatinase-associated lipocalin; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; Rag1, recombination-activating gene 1; RBC, red blood cell; rRNA, ribosomal RNA; TLR, Toll-like receptor; TNF, tumor necrosis factor; WT, wild type.**

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antimicrobial innate immune protein whose expression is increased markedly in inflammatory diseases in both mice and human beings.[5–7] Accordingly, we have shown that fecal Lcn2 is a sensitive, broadly dynamic, noninvasive biomarker of intestinal inflammation.[8] However, several studies have shown that Lcn2 is not a mere bystander, but actively involved in several physiological and pathophysiological functions in IBD, renal diseases,[9–11] and cancer.[12,13]

Among its diverse functions, one well-established role of Lcn2 is to inhibit bacterial proliferation by chelating bacterial siderophores such as enterobactin, thus limiting iron acquisition.[14] However, Lcn2 alone cannot bind to ferric (Fe^{+3}) iron; instead, it requires the aid of small chemical molecules such as mammalian siderophore 2,5 dihyoxybenzoic acid or catechol to bind and transport iron in and outside of the cell.[15–17] A recent report showed that Lcn2 also can complex with Fe^{+3}-bound epigallocatechin gallate, a polyphenol from green tea.[16] Aside from its role in iron homeostasis, Lcn2 also is involved in establishing systemic hypoferremia during inflammation,[13,18] inducing epithelial cell secretion of chemokine interleukin (IL)8,[19,20] mediating tolerance to cellular iron overload,[21] and, furthermore, serves as a survival factor for epithelial cells.[22] Previously, we and Flo et al[23] have shown that Lcn2-knockout (*Lcn2*KO) mice are highly susceptible to lipopolysaccharide (LPS)- and bacterial-induced sepsis, respectively.[18] More recently, Allred et al[24] showed that Lcn2 limits the deleterious health effects induced by synthetic radionuclides by mediating their physiological transport via secondary ligand-based sequestration mechanisms. Altogether, these findings indicate broad and distinct physiological functions of Lcn2.

Neutrophils, the first responder cell type to most inflammation-inducing injuries, constitutively express and store Lcn2 within their specific granules. Lcn2 is indispensible for neutrophil infiltration, adhesion, and function.[25,26] Recently, we showed that Lcn2 preserves the bactericidal activity of neutrophil-derived host immune protein myeloperoxidase (MPO) by preventing its inactivation by bacterial siderophore enterobactin.[27] Lcn2 also is known to deactivate and modulate polarization of macrophages,[28] which are one of the most abundant leukocytes in the intestinal mucosa. In addition, Lcn2 facilitates mucosal regeneration[25,26] by promoting cell migration[29] and by forming heterodimers with matrix metalloproteinase-9, a metalloproteinase involved in tissue repair.[30] Although it is known that Lcn2 levels increased by several log orders of magnitude in various models of inflammation,[11,23] including murine colitis[7,8,31] and human IBD,[6,32,33] the role of Lcn2 in such inflammatory states is not known.

We hypothesized that Lcn2, which regulates gut bacterial growth, systemic inflammation, and mucosal repair, may play a key role in the gut homeostasis and IBD pathogenesis. Herein, we studied the basic mechanisms that regulate Lcn2 expression and, moreover, its role in intestinal pathobiology. We report that Lcn2 expression is regulated by microbiota-induced MyD88 signaling, which allows for rapid robust induction of this protein upon a range of challenges. Such Lcn2 expression helps keep microbiota in check, and consequently protects the intestine against colitis in multiple murine models.

Materials and Methods

Reagents and Antibodies

Reagent grade dextran sulfate sodium salt (DSS) (reagent grade; molecular weight, 36–50 kilodaltons; catalog number 160110) was purchased from MP Biomedicals (Solon, OH). Duoset enzyme-linked immunosorbent assay (ELISA) kits for mouse and human Lcn2 and keratinocyte-derived chemokine were obtained from R&D Systems (Minneapolis, MN). α-Hu-Lcn2 SYBR Green mix and the qScript complementary DNA (cDNA) synthesis kit were procured from Quanta BioSciences (Gaithersburg, MD). Guaiacol (2-methoxyphenol) was obtained from Alfa Aesar (Ward Hill, MA). MPO was procured from Sigma (St. Louis, MO). Ly6G antibody was purchased from Abcam (Cambridge, MA). Rat anti-mouse IL10 receptor (IgG_1) monoclonal antibody (αIL10R) and isotype control antibody (rat anti-mouse IgG1) were procured from BioXcell (West Lebanon, NH). Anti-mouse cluster of differentiation 45 (CD45)–Alexa Fluor 594 and CD326 monoclonal antibodies were procured from BioLegend (San Diego, CA). RegIIIγ and Ang4 antibodies were a kind gift from Dr Lora Hooper (UT Southwestern Medical Center, Dallas, TX). All other fine chemicals used in the present study were reagent grade and procured from Sigma.

Mice

*Lcn2*KO mice were originally generated by Dr Shizuo Akira (Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Japan.) on a C57BL/6 background and were obtained via Dr Kelly Smith (University of Washington, Seattle, WA). The offspring were cross-bred to obtain 4 possible genetic variants in the litter. Wild-type (WT) littermates of the respective gene (ie, *Lcn2*, *Il10*, myeloid differentiation primary response gene 88 [*Myd88*], or Toll-like receptor 5 [*Tlr5*]) KOs were used as controls throughout the study. All mice used in the present study were bred and maintained in specific pathogen–free conditions at Emory University and Georgia State University (Atlanta, GA), and The Pennsylvania State University (State College, PA). The institutional animal ethical committees at Emory University, Georgia State University, and The Pennsylvania State University approved the animal experiments.

Intestinal Epithelial Cell Culture

HT-29 human intestinal epithelial cells were cultured on 24-well plates in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin.[34] The epithelial cells were cultured on collagen-coated filters and then the polarized epithelial cells were stimulated with flagellin basolaterally in serum-free media. After 24 hours, basolateral and apical supernatants were collected separately and assayed for NGAL secretion via ELISA.

Immunoblotting

Neutrophils, obtained from 4 different individuals, were lysed in 0.5 mL RIPA buffer (Cell Signaling Technology, Inc,