A Novel Role of the Lumican Core Protein in Bacterial Lipopolysaccharide-induced Innate Immune Response*

Received for publication, March 20, 2007, and in revised form, June 22, 2007. Published, JBC Papers in Press, July 5, 2007, DOI 10.1074/jbc.M702402200

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Lumican is an extracellular matrix protein modified as a proteoglycan in some tissues. The core protein with leucine-rich repeats, characteristic of the leucine-rich-repeating superfamily, binds collagen fibrils and regulates its structure. In addition, we believe that lumican sequestered in the pericellular matrix interacts with cell surface proteins for specific cellular functions. Here we show that bacterial lipopolysaccharide sensing by the Toll-like receptor 4 signaling pathway and innate immune response is regulated by lumican. Primary cultures of lumican-deficient (Lum−/−) macrophages show impaired innate immune response to lipopolysaccharides with lower induction of tumor necrosis factor α (TNFα) and interleukin-6. Macrophage response to other pathogen-associated molecular patterns is not adversely affected by lumican deficiency, suggesting a specific role for the lumican core protein in the Toll-like receptor 4 pathway. An exogenous recombinant lumican core protein increases lipopolysaccharide-mediated TNFα induction and partially rescues innate immune response in Lum−/− macrophages. We further show that the core protein binds lipopolysaccharide. Immuno precipitation of lumican from peritoneal lavage co precipitates CD14, a cell surface lipopolysaccharide-binding protein that is involved in its presentation to Toll-like receptor 4. The Lum−/− mice are hyporesponsive to lipopolysaccharide-induced septic shock, with poor induction of pro inflammatory cytokines, TNFα, and interleukins 1β and 6 in the serum. Taken together, the data indicate a novel role for lumican in the presentation of bacterial lipopolysaccharide to CD14 and host response to this bacterial endotoxin.

Innate immunity is the earliest evolved and most primitive defense mechanism that a host organism uses to detect and destroy pathogens invading tissues without extensive damage to the host barrier (1). Recent studies have led to an understanding of the elaborate host defense mechanisms that are in place at the cell surface (2) and in the cytoplasm (3). We now show that there is yet another mechanism for regulating host defense, one mediated by extracellular matrix (ECM)3 proteins such as lumican.

Innate immune response involves recognition of pathogen-associated molecular patterns (PAMPs) by pathogen recognition Toll-like receptors (TLR) present on the surface of antigen-presenting macrophages and dendritic cells (4, 5). The extracellular domain of these TLRs have leucine-rich repeat (LRR) motifs found in diverse proteins such as the ancient resistance plant proteins (R proteins), Drosophila Toll proteins (6), and mammalian ribonuclease inhibitor. These LRR motifs bind DNA, RNA, and protein ligands including those derived from invading microorganisms. In the cytoplasm, a family of cytosolic LRR proteins, the nucleotide binding oligomerization domain (NOD) proteins bind PAMPs and promote innate immune signaling (7). Ultimately, these pathways lead to the phosphorylation of IkB kinase, nuclear translocation, and the activation of NF-κB. The NF-κB transcription factor up regulates pro-inflammatory cytokines and microbicidal activities (8, 9).

Lipopolysaccharide (LPS) endotoxins from the cell wall of Gram-negative bacteria are recognized by the TLR4 signaling pathway (10). LPS sensing begins with binding of monomeric LPS to LPS-binding protein in the blood and its transfer to CD14 (11). A secreted serum protein, CD14, is linked to the cell membrane of monocytes, macrophages, and neutrophils by a glycosylphosphatidylinositol linkage. CD14 binds and transfers LPS molecules to the TLR4 transmembrane signaling complex at the cell surface (12, 13). The LPS recognition complex also requires soluble MD-2 protein, heat shock proteins, and additional factors that remain to be identified (2, 5, 14).

LPS recognition by the host triggers the biosynthesis of a variety of inflammation mediators, such as tumor necrosis fac tor α (TNFα), interleukin-1β (IL-1β), IL-6, and other co-stimulatory molecules (15). TNFα is the cytokine prototype often used to assess host innate immune response. Produced locally, these innate immune response mediators help to clear infections. However, unrestricted systemic overproduction of pro-inflammatory cytokines and proteins can lead to severe sepsis, multiple organ failure, and death. Host response to infection

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3 The abbreviations used are: ECM, extracellular matrix; LPS, lipopolysaccharide; TLR, Toll-like receptor; PAMP, pathogen-associated molecular pattern; TNFα, tumor necrosis factor α; LRR, leucine-rich repeat; poly I:C, polynosinic-polycytidylic acid sodium salt; IL, interleukin; FITC, fluorescein isothiocyanate; qRT-PCR, quantitative reverse transcriptase-PCR; HEK-293, human embryonic kidney cell line 293; MEF, mouse embryonic fibroblasts; rLum, recombinant lumican; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
and bacterial endotoxins is being investigated at multiple levels to define events that lead to sepsis and septic shock. Understanding the molecular events from pathogen recognition to inflammation mediators is becoming important in the treatment of sepsis and in identifying patients at risk. For example, polymorphisms in genes encoding heat shock proteins, TNFα and IL-1 receptor antagonist, mediators of pathogen recognition and inflammation, have been linked to genetic predisposition to sepsis (16–18). Our study of an ECM protein lumican introduces a new modulator of host response to inflammation and sepsis.

Lumican is one of 12 or more related proteoglycans of the LRR protein superfamily (19–21). Lumican is expressed in a variety of stromal mesenchymal ECM of barrier tissues, such as the skin, cornea, and intestine (22, 23). We have previously investigated a structural role for lumican in binding collagen fibrils and regulating their lateral growth (24, 25). The lumican-null mice (Lum<sup>tm1/chak</sup>, referred to as Lum<sup>−/−</sup> here) have structurally abnormal collagen fibrils in the cornea, skin, and tendon and consequent functional defects such as corneal opacity and skin and tendon fragility (23, 25–27). In a keratitis model we showed that IL-1β, IL-6, and TNFα was not induced optimally in the injured cornea of Lum<sup>−/−</sup> mice. Furthermore, healing of corneal wounds was delayed compared with wild type controls. That study pointed to a functional impairment in innate immune inflammatory processes in the Lum<sup>−/−</sup> mice (28).

Here we demonstrate that lumican has a specific role in innate immune inflammatory processes in the mouse. Lumican deficient in mice are resistant to LPS-mediated septic shock and death and impaired in their ability to induce TNFα, IL-6, and other pro-inflammatory cytokines upon exposure to LPS. Consequently, this study uncovers a novel role for lumican, an ECM protein, in pathogen recognition and innate immune response, the host’s first line of defense against invading microorganisms.

**EXPERIMENTAL PROCEDURES**

**Materials**

Ultra pure *Escherichia coli* (0111:B4) LPS was purchased from List Biological Laboratories, Inc., *Salmonella typhimurium* LPS, *Staphylococcus aureus* peptidoglycan (PGN), N-acetylmuramyl-l-alanyl-d-isoglutamine hydrate (MDP), and polynosinic-polycytidylic acid sodium salt (poly I:C) were purchased from Sigma. The phosphorothioate Cpg-DNA, ODN1668 (TCCATGACGTTCCTGATGCT) and recombinant mouse TNF-α were from Operon and BIOSOURCE, respectively. Recombinant lumican (rLum) was prepared using a human lumican cDNA insert in pSecTag2 vector in HEK-293 cells (28).

**Mouse Husbandry and Experimental Treatment**

Lumican-null mice (Lum<sup>tm1/chak</sup> or Lum<sup>−/−</sup>) were generated earlier by targeted gene disruption in 129Sv/J embryonic stem cells, and a 129Sv/J male chimera was crossed directly with a CD1 female to transfer the lumican null mutation into the CD1 strain as described earlier (23). The null mutation was maintained in the CD1 strain for several years (>50 generations). All experiments investigating the effects of the null mutation were performed using gender-matched Lum<sup>+/+</sup> (or Lum<sup>+/−</sup>) and Lum<sup>−/−</sup> littermates generated by intercrossing heterozygous animals unless stated otherwise. The Lum<sup>−/−</sup> CD1 strain was also crossed to C57BL/6, and heterozygous progeny were backcrossed to the parental strain for 12 generations over the last two years for additional analysis of the Lum<sup>−/−</sup> phenotype in the C57BL/6 background. All animals were housed in the Johns Hopkins University specific pathogen-free mouse facility under conditions that were approved by the Association for Assessment and Accreditation of Laboratory Animal Care, and all animal procedures were approved by the Institutional Animal Care and Use Committee. Three different LPS doses were tested (16.7, 20, and 26 μg/g bodyweight), and the lowest dose was selected because it was sufficient to induce septic shock in wild type mice. LPS from *S. typhimurium* or *E. coli* 0111:B4 in saline or saline alone as control was administered as intraperitoneal injections. The mice were weighed daily for up to 5 days. For the cytokine analyses, the serum was harvested 32 h after treatment and stored at −20 °C until use.

**Histology**

Tissues were fixed in 10% buffered formalin overnight, paraffin-embedded, and sectioned (6 μm thick) for conventional hematoxylin and eosin staining.

**Cell Culture Techniques**

**Primary Cultures**—Mouse embryonic fibroblasts (MEFs) were derived from E14 embryos (29). The cells were allowed to attach for 6 h in Dulbecco’s modified Eagle’s medium (DMEM) F-12 plus 10% fetal bovine serum (FBS) and then transferred to DMEM F-12 plus 1% FBS. All MEF cultures were used between passages 2 and 6. To culture primary macrophages, a solution of 4% thioglycollate was injected into the peritoneal cavity (1 ml/mouse) of 6–8-week-old male mice, and the peritoneal lavage was harvested 4 days later. Cells were plated in RPMI 1640 medium and 1% fetal bovine serum at an initial density of 1.5 × 10<sup>5</sup> cells/well for 24-well plates or 5 × 10<sup>4</sup> cells/well for 96-well plates. After 24 h non-adherent cells were removed, and adherent cells were treated with specific PAMPs in fresh medium.

**Cell Lines**—HEK-293 cells (Invitrogen), 293-rLum (stable transfectants expressing recombinant lumican, rLum), and HEK-293 expressing TLR4, MD2, and CD14 (293-hTLR4/MD2-CD14, InviroGen) were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and 1% of 100× antibiotic-antimycotic (Sigma).

**Cytokine Measurements**

Selected cytokines were measured by standard sandwich enzyme linked-immunosorbent assay (ELISA) in the serum or cell culture medium. Solid phase sandwich mouse ELISA kits for TNFα and IL-6, with 3 pg/ml sensitivity, were obtained from BioSource International, Inc. Total protein concentration was determined in the CD1 strain for several years (>50 generations). All experiments investigating the effects of the null mutation were performed using gender-matched Lum<sup>+/+</sup> (or Lum<sup>+/−</sup>) and Lum<sup>−/−</sup> littermates generated by intercrossing heterozygous animals unless stated otherwise. The Lum<sup>−/−</sup> CD1 strain was also crossed to C57BL/6, and heterozygous progeny were backcrossed to the parental strain for 12 generations over the last two years for additional analysis of the Lum<sup>−/−</sup> phenotype in the C57BL/6 background. All animals were housed in the Johns Hopkins University specific pathogen-free mouse facility under conditions that were approved by the Association for Assessment and Accreditation of Laboratory Animal Care, and all animal procedures were approved by the Institutional Animal Care and Use Committee. Three different LPS doses were tested (16.7, 20, and 26 μg/g bodyweight), and the lowest dose was selected because it was sufficient to induce septic shock in wild type mice. LPS from *S. typhimurium* or *E. coli* 0111:B4 in saline or saline alone as control was administered as intraperitoneal injections. The mice were weighed daily for up to 5 days. For the cytokine analyses, the serum was harvested 32 h after treatment and stored at −20 °C until use.

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on-γ, and granulocyte-macrophage colony-stimulating factor from the serum. We acquired 100 assay points per cytokine for each sample using the Luminex® 100 IS System. Results are presented as median fluorescence intensity. Recombinant cytokines were used as standard controls.

**Quantitative RT-PCR (qRT-PCR)**

Total RNA was extracted using TRIzol reagent (Invitrogen) from peritoneal macrophages and MEFs. The expression of lumican and glyceraldehyde-3-phosphate dehydrogenase (Gapdh, an internal reference gene) was determined by qRT-PCR using iQ™SYBR Green Supermix kit (Bio-Rad). The threshold cycle difference, ΔCt, was defined as Ct_{Gapdh} − Ct_{Lum} and relative expression was defined as 2^{ΔCt}. The following primers were used: for Lum, 5′-TCGAGCTGTCTCTCCTCTAT-3′ (forward) and 5′-TGGTCCAGGATCTTGACAGA-3′ (reverse); for Gapdh, 5′-TTGTCTCTGCGACCTCA-3′ (forward) and 5′-CCTGTT-GCTTAGCCGTATT-3′ (reverse).

**Binding of rLum to Lum−/− Macrophages**

Peritoneal macrophages (1 × 10^7 cells/ml) were incubated with rLum (20 μg/ml) or bovine serum albumin (as a control) at room temperature for 1 h, washed 3 times with phosphate-buffered saline and incubated with 1 μg 3,3′-dithiobis sulfosuccinimidyl propionate at room temperature for 2 h. The presence of rLum in the cell extract (M-Per lysis buffer, Pierce) was quantified by ELISA. Macrophage extracts of rLum-treated and appropriate control cells were added to wells precoated with a goat polyclonal anti-lumican antibody (Santa Cruz Biotechnology) and with a rabbit pre-immune antibody to determine the amount of rLum retained in wells as previously described (28).

**LPS Binding Assay**

Corning Costar 96-well plates (polystyrene, with black walls, Fisher) were coated with 100 μl of 1 μg/ml rLum in 0.1 M NaHCO_3 and 2.5 mM Na_2CO_3, pH 9.6, overnight at 4 °C. After 3 washes with phosphate-buffered saline containing 0.2% Tween, rLum-coated and uncoated wells were blocked with 3% bovine serum albumin in phosphate-buffered saline for 2 h at room temperature. FITC-labeled E. coli LPS (Sigma-Aldrich) at 0.0625–1 μg/ml in Hanks’ balance salt solution (100 μl/well) was added and incubated for 1 h at room temperature followed by 3 washes. Fluorescence was measured by a SpectraMax M2 microplate reader (Molecular Devices) with 485 nm for excitation and 525 nm for emission wavelengths. Experiments were replicated twice, and results are shown as relative fluorescence units normalized to a set of reference wells.

**Lumican-CD14 Interactions**

Conditioned medium from 293-rLum and 293-hTLR4/MD2-CD14 were used in ex vivo pulldown assays to determine binding between rLum and the LPS receptor complex. The 293-rLum and 293-hTLR4/MD2-CD14 cells were co-cultured (2.5 × 10^6 cells/ml) for 24 h. Individual cell types were grown separately as controls. The medium was harvested and incubated with Probond™ nickel-resin (Invitrogen) equilibrated under native conditions with gentle agitation for 1 h at 4 °C to allow for specific binding of rLum to the resin. The resin was washed with native wash buffer (50 mM Na_2PO_4, 0.5 mM NaCl). Resin-bound proteins were eluted using increasing concentrations of imidazole, pH 6.0 (50–250 mM). Eluted samples were resolved by 10% SDS-PAGE, transferred by electroblotting onto nitrocellulose, and analyzed by immunoblotting using anti-lumican (29), anti-TLR4, anti-CD14 (Abcam) primary antibodies, horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibody, and the ECL (Pierce) detection kit.

**Immunoprecipitation**

Elicited primary macrophages were harvested from the peritoneal lavage of wild type mice, and total protein was extracted using the M-PER extraction kit (Pierce). A preparation of protein A-agarose beads (Amersham Biosciences) was used for immunoprecipitating lumican using rabbit anti-lumican or goat anti-lumican as described previously (28). As a positive control, CD14 was immunoprecipitated with an anti-CD 14 antibody (ab25090, Abcam) and with a rabbit pre-immune serum as a negative control. The immunoprecipitate was resolved by SDS-PAGE, and CD14 was detected by immunoblotting (BAF982, R&D Systems).

**Statistical Methods**

To assess the significance of the differences between two groups we used unpaired, 2-tailed Student’s t test with the assumptions of equal variance. We considered a p value ≤ 0.05 as statistically significant. A log rank test (Graphpad Prism software) was used to compare survival differences in Lum+/+ and Lum−/− mice after LPS treatment.

**RESULTS**

Lum−/− Mice Are Hypo-responsive to Bacterial LPS—To investigate the effects of lumican deficiency on innate immune functions, we tested the response of Lum−/− mice to LPS-induced septic shock. After testing three different doses, we selected a dose of 16.7 μg/g of body weight LPS since it was sufficient to induce septic shock and death in Lum+/+ mice. Seven-week-old Lum−/− and Lum+/+ littermates were given a single intraperitoneal injection of E. coli or S. typhimurium LPS. Within 24–36 h, Lum+/+ appeared visibly sick, showing piloerection, hunching, closed eyes, and lethargy. On the other hand, Lum−/− mice appeared healthy with little sign of distress (data not shown). Approximately 50–70% of Lum+/+ mice died 3 days after E. coli or S. typhimurium LPS injection (Fig. 1A). In contrast, only 10–25% of Lum−/− mice died at day 3, showing overall higher survival. A log rank test indicated that the survival trend between Lum−/− and Lum+/+ mice treated with E. coli or S. typhimurium LPS was significantly different (p = 0.039). E. coli LPS in general caused higher lethality in both Lum+/+ and Lum−/− mice. None of the saline-injected control mice died during the study (not shown).

Mice were harvested at 32 h after LPS to assess immune response. This time point was selected since significant numbers of animals in the wild type group died after this point, and
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Inflammatory cytokines, TNFα and IL-6 (Fig. 3), was measured by ELISA. The LPS dose-dependent increase in TNFα was significantly lower in Lum−/− macrophages compared with wild type macrophages (Fig. 3A). The temporal induction of TNFα and IL-6 was further measured; TNFα was induced within 1 h and continued to increase after 4 h of LPS treatment (Fig. 3B). IL-6 increased 4 h after LPS treatment in Lum−/− macrophages (Fig. 3C). The timing of TNFα and IL-6 induction in the Lum−/− cells was similar to Lum+/+. However, the extent of induction at the later time points in particular was significantly lower in the Lum−/− cells.

We further tested innate immune functions of Lum−/− mice bred into a second genetic background, C57BL/6J. Macrophages from Lum−/− C57BL/6J also showed significantly lower induction (p < 0.05) of TNFα in response to E. coli LPS (Fig. 3D).

Because lumican deficiency reduced response to LPS, we questioned if exogenous lumican would restore LPS sensitivity to Lum−/− macrophages. We tested our hypothesis by measuring LPS-mediated TNFα induction in macrophages isolated from lumican-deficient (Lum−/−) and lumican-expressing (Lum+/+) C57BL/6J mice. The addition of exogenous recombinant lumican core protein (rLum) showed a consistent increase in TNFα induction in Lum−/− macrophages (Fig. 3E). However, in Lum+/+ macrophages, excess rLum (2–4 μg) had an inhibitory effect on TNFα induction by LPS. This could be due to a competition between endogenous lumican and exoge-
Lumican is a Stress-response Protein—We next tested if lumican itself behaves as a stress-response gene. Lum (lumican gene) expression was measured by qRT-PCR in total RNA isolated from MEFs treated with LPS or IL-1β as pro-inflammatory signals and transforming growth factor-β as an immunosuppressive signal. The results show that Lum was induced by LPS (Fig. 5A) in fibroblasts and at a much lower level in primary macrophages (Fig. 5B). Lum expression was also induced by pro-inflammatory IL-1β (Fig. 5C) but inhibited by immunosuppressive transforming growth factor-β (Fig. 5D) in a dose-dependent manner. Thus, lumican is a new member of the arsenal of host proteins that are induced during innate immune responses.

We hypothesize that lumican sequestered in the pericellular ECM of macrophages modulates the LPS-TLR4 signaling pathway. Consistent with our hypothesis is the finding of an earlier study that showed specific binding of lumican to surfaces of macrophages (31). We further tested if rLum was able to bind lumican-deficient macrophages (Fig. 6). Lum−/− macrophage cell suspensions were incubated with rLum and treated with a cross-linker to stabilize protein-protein interactions at the cell surface. ELISA tests were used to measure macrophage-bound rLum. Lum−/− macrophages not treated with rLum showed no rLum reactivity as expected. Lum−/− macrophages incubated with rLum showed a statistically significant increase in rLum immunoreactivity.
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FIGURE 4. Response to a panel of PAMPs. A, Lum+/+ and Lum−/− macrophages were treated with 10 µg/ml peptidoglycan (PGN), 10 µg/ml polyinosinic-polycytidylic acid (poly I:C), or 10 ng/ml LPS for 4 h. B, macrophages were treated with increasing doses of CpG-DNA or 10 ng/ml N-acetylmuramyl-L-alanyl-d-isoglutamine hydrate (MDP, muramyl dipeptide). ELISA measurements of LPS-mediated TNFα induction (mean ± S.E., n = 3) indicated significantly lower amounts in the Lum−/− macrophages compared with Lum+/+ macrophages. *p < 0.05 (two-tailed Student’s t test).

DISCUSSION

We report a novel role for the ECM protein, lumican, in promoting recognition of bacterial LPS and host innate immune response. Lumican is better known as a member of the small leucine-rich repeat proteoglycans that bind collagen and modify the structure of collagen-rich connective tissues (33). Lumican-deficient mice are impaired in inducing pro-inflammatory cytokines in response to an intraperitoneal injection of LPS. Several pro-inflammatory cytokines, including those associated with Th1 T cell functions, are induced in wild type Lum+/+ mice but not induced optimally in Lum−/− mice exposed to LPS. Lower production of pro-inflammatory cytokines may explain the higher survival of Lum−/− mice challenged with LPS, at a dose that would otherwise lead to septic shock and death as in Lum+/+ mice.

Isolated Lum−/− macrophages show lower induction of pro-inflammatory TNFα and IL-6 in response to LPS. Moreover, exogenous recombinant lumican is able to partially restore LPS sensitivity and TNFα induction in Lum−/− primary macrophages, indicating a role for lumican in this pathway. Activation of the NF-κB transcription factor is a major route to TLR-mediated induction of pro-inflammatory genes. We found NF-κB activation in Lum−/− bone marrow-derived macrophages to be delayed in comparison with Lum+/+ cells (data not shown).
Our study suggests that impaired innate immune response to LPS in *Lum*−/− mice may be due to a direct involvement of lumican in LPS sensing. Earlier studies established that lumican binds macrophages (31), whereas our current study shows that rLum is also able to bind *Lum*−/− macrophages. Our results further indicate binding of recombinant lumican with LPS. Importantly, immunoprecipitation of lumican from peritoneal lavage co-precipitates CD14, indicating lumican–CD14 interactions in vivo. Based on these observations, we propose that lumican is present in the pericellular matrix of macrophages, from where it is able to regulate LPS presentation and TLR4 signaling. Although macrophages stimulated with LPS express lumican, it is markedly lower than that produced by fibroblasts. Therefore, the major source of lumican in the macrophage pericellular matrix may be mesenchymal fibroblasts.

A proteoglycan in the cornea, lumican appears as a glycoprotein without its keratan sulfate glycosaminoglycan side chains in most other tissues (36). Therefore, its innate immune function is likely to reside in the core protein and not the keratan sulfate side chains. This is further supported by the fact that in our LPS binding assays and the functional rescue studies, the rLum used was a glycoprotein and not a proteoglycan.

FIGURE 6. Exogenous rLum binds to *Lum*−/− macrophages. *Lum*−/− macrophage suspensions were incubated for 1 h with rLum or bovine serum albumin (BSA, control) at room temperature. Macrophage-bound rLum in the cell lysates was determined by ELISA (mean ± S.E., *n* = 3). Microtiter wells were coated with an anti-rLum antibody. *Lum*−/− macrophages not treated with rLum showed background levels of immunopositive reactions as expected, whereas cells incubated with rLum showed rLum-immunopositivity, indicating the presence of macrophage-bound rLum. *, *p* < 0.05 (2-tailed Student’s *t* test).

FIGURE 7. Lumican binds LPS. A, binding of LPS to rLum was tested in a solid-phase binding assay. rLum-coated wells showed dose-dependent binding to increasing amounts of FITC-LPS, measured as relative fluorescence units (RFU). B, binding of rLum to FITC-LPS (60 ng/ml) and its competitive inhibition by 1000 ng/ml unlabeled LPS or 20 ng/ml soluble CD14.

FIGURE 8. Lumican–CD14 interactions. A–D, interactions between rLum and CD14, TLR4, MD2 expressed in HEK-293 cells was tested using a pulldown assay. rLum was pulled down via its His6 tag using a nickel (ProBond, Invitrogen) resin. Resin-bound rLum was specifically eluted at 150–175 mM imidazole concentration as shown by immunoblotting with anti-lumican (A). The blot was stripped (B) and re-probed with anti-CD14 showing retention and co-elution of CD14 with rLum (C). The CD14, TLR4, and MD2 complex without rLum showed no specific binding of CD14 to the resin (D). E, elicited peritoneal macrophage extracts were used to immunoprecipitate (IP) lumican using two separate anti-lumican primary antibodies (IPLum1 and IPLum2). The IPLum samples were analyzed for the presence of CD14 by immunoblotting with an anti-CD14 antibody. The results show the presence of CD14 in the lumican immunoprecipitate samples. Immunoprecipitation (IP) carried out with a pre-immune antiserum served as a negative control, and IP with an anti-CD14 antibody was used as a positive control for CD14. W. blot, Western blot.
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not yet been resolved, the crystal structure for decorin, a lumican-like proteoglycan, shows certain overall structural similarity with the horseshoe-shaped structure of CD14 and TLR3 (40, 41).

If lumican, present in most barrier ECM tissues, serves as a PAMP recognition protein in the ECM, we speculated if other members of this group of ECM proteins and proteoglycans have similar roles. Thus far, cell surface LRR proteins (TLRs, CD14) and those of the cytoplasm (nucleotide binding oligomerization domain) have been primarily associated with regulating innate immune response. Mindin, an ECM protein of the spondin family, was recently identified as an ECM regulator of pathogen-recognition, and Mindin-deficient mice are hypo-responsive to a variety of PAMPs (34). Biglycan is the only other lumican-like proteoglycan that was reported to regulate TLR4- and TLR2-mediated innate immune response (35). Biglycan-deficient mice are hypo-responsive to LPS (35). However, unlike lumican, biglycan was able to induce innate immune response by itself in the absence of PAMPs and, thus, was described as “analogous to PAMPs” in that study. We interpret lumican serving as a PAMP recognition protein rather than a PAMP analog for the following reasons. 1) Lumican and the PAMP recognition receptors share structural similarities; 2) recombinate lumican binds LPS; 3) unlike biglycan, lumican in the absence of LPS does not induce TNFα in macrophages. A potential outcome of impaired LPS response in the lumican−/− mice is poor host defense against bacterial infection. To test this possibility, we challenged lumican+/+ and lumican−/− mice with live S. typhimurium (2 × 10⁶ bacteria per animal). After 5 days of infection, bacterial yield from the spleen and the liver of infected lumican−/− mice was marginally higher than that of lumican+/+ mice (data not shown). Also, induction of TNFα in the serum of these animals was not significantly different (data not shown). There may be several explanations for not seeing a marked reduction in innate immune response to whole bacteria in the lumican−/− mice. First, lumican may be one of several extracellular modulators of the TLR4 pathway. Challenge with the whole bacteria may resemble exposure to high levels of LPS, induction of multiple pathways, and high innate immune response. Second, bacterial internalization can trigger multiple inflammatory pathways (42). The impact of CD14 deficiency on innate immune response is understandably higher than that of lumican deficiency, CD14 being the common cell surface protein that receives LPS and transfers it to the TLR4 signaling complex. Thus, CD14-null mouse macrophages show an almost complete lack of innate immune response to E. coli LPS, but response to whole E. coli was marginally hindered (42). In contrast, the CD14-null mice were susceptible to Acinetobacter baumannii, an opportunistic bacterial pathogen associated with nosocomial pneumonia, as shown in another study (43). In the same vein, virulent Gram-negative bacterial infections of lumican−/− mice other than the S. typhimurium infection tested in our study may be more harmful.

The biological significance of lumican-mediated recognition of monomeric LPS may be to enhance host sensitivity, as suggested for CD14, allowing heightened but regulated host response to trace levels of pathogen. Impaired innate immune response in lumican−/− mice clearly impacts the outcome of localized injury and inflammation in these animals, underscoring a need for lumican in innate immune response to bring on the “good” side of inflammation (44, 45). For example, innate immune functions are compromised in MyD88 and TLR2- and TLR4-deficient mice. In localized injury models, these animals show poor recruitment of inflammatory cells. However, instead of dampened inflammation, these models manifest exacerbated tissue damage and worse disease outcome (44, 46). Similarly we previously reported in the lumican−/− mice, corneal wounds exposed to LPS show poor induction of pro-inflammatory cytokines and reduced influx of inflammatory cells compared with the wild type mice. Despite lower inflammation, repair of injured corneas is delayed in lumican−/− mice (28). Therefore, in addition to heightening LPS sensitivity, lumican may be required to maintain basal innate immune functions, important to epithelial integrity and repair (44, 46, 47).

Our study shows that host innate immune response is modulated by lumican, an ECM protein of the LRR superfamily. Investigations of other LRR members of this family of proteins and proteoglycans will determine whether ECM-mediated modulation of innate immune response is a major route of host defense against microbial factors. This study identifies ECM lumican as a modulator of inflammation and septic shock, providing a potential candidate gene for predisposition to sepsis and chronic inflammation, and a target for therapeutic interventions.

Acknowledgments—We thank Drs. Claudio Fiocchi (The Cleveland Clinic Foundation), Akhilesh Pandey, Abdel Hamad, and Mark Solski (Johns Hopkins University) for helpful discussions and Lindsey Savino and Jennifer Sipes (Hopkins Digestive Disease Basic Research Development Center, National Institutes of Health Grant DK64388) for technical assistance.

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SEPTEMBER 7, 2007•VOLUME 282•NUMBER 36•JOURNAL OF BIOLOGICAL CHEMISTRY 26417