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Extracellular matrix protein lumican regulates inflammation in a mouse model of colitis

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Abstract

Abnormal innate immune response contributes to inflammatory bowel disease (IBD) and experimental mouse colitis. Colitis studies have focused primarily on key regulators of innate immunity, like pathogen recognition receptors and cytoplasmic mediators. Extracellular matrix (ECM) proteins are emerging as modulators of inflammatory responses by virtue of their interactions with pathogen associated molecular patterns (PAMPs), cytokines, growth factors, receptors, and ECM fragments that mimic pathogens or cytokines. The ECM proteins have not been investigated in IBD at great depth from this standpoint. We have shown previously that the ECM protein lumican modulates host sensing of bacterial lipopolysaccharides (LPS) by toll like receptor (TLR) 4, and neutrophil chemotaxis via integrins. Here we investigated the role of lumican in the development of colitis mediated by intra-rectal administration of the hapten 2-4-5, trinitrobenzene sulfonic acid (TNBS) in *Lum*^{+/+} and *Lum*^{-/-} mice. The TNBS-treated *Lum*^{+/+} mouse colons showed marked increases in CXCL1, TNF- α and neutrophil infiltration; while these responses were significantly dampened in the *Lum*^{-/-} mice. The NF- κ B transcription factor, known to regulate inflammatory genes, showed a robust increase after TNBS treatment in *Lum*^{+/+} but not in *Lum*^{-/-} colons. Also, nuclear translocation of NF- κ B was delayed in LPS-stimulated *Lum*^{-/-} primary peritoneal macrophages. Thus, the *Lum*^{-/-} mice have low innate immune and inflammatory responses, but more severe body weight loss and tissue damage, a phenomenon seen in the innate immune impaired *Tlr4*^{-/-} and *MyD88*^{-/-} mice. Therefore, lumican promotes intestinal homeostasis by aiding innate immune and inflammatory responses that are beneficial in the early stages of colitis.

Keywords

Extracellular matrix; lumican; TLR4; CD14; innate immune response; inflammation; murine colitis; neutrophils; tri-nitrobenzene sulfonic acid; dextran sodium sulfate

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Introduction

Inflammatory bowel disease (IBD), Crohn's disease and ulcerative colitis are chronic inflammatory diseases with certain common inflammatory changes but distinct etiology and pathogenesis (1,2). In Crohn's disease, transmural damage can affect any segment of the gastrointestinal tract, while ulcerative colitis manifests mucosal damage of the colon. It is now generally recognized that abnormal mucosal immune response to the commensal flora plays a major role in the etiopathology of IBD (3). In agreement with this view, IBD has been associated with changes in the toll-like receptors (TLR) and the nucleotide-binding oligomerization domain containing (Nod) pathogen recognition receptors (PRR) at the cell surface and cytoplasm, respectively (4,5). Innate immune response is the first response of host cells to pathogen associated molecular patterns (PAMPs), through TLRs and the Nod like receptors (6). Activations of these PRR ultimately lead to degradation of cytoplasmic inhibitors of NF- κ B (IkB), nuclear translocation and activation of NF- κ B and up regulation of pro-inflammatory cytokine genes (7,8). Studies investigating innate immune response have focused largely on these cell surface and cytoplasmic regulators. However, the magnitude of innate immune response is modulated by a number of other factors beyond the specific PAMPs and their host receptors. A few studies have identified extracellular matrix (ECM) components and their degradation products as having the potential to modulate innate immune response either by mimicking PAMPs, or by interacting with PAMPs or their receptors. For example, hyaluronan, an ECM glycosaminoglycan (9), and biglycan, an ECM protein, stimulate TLR 2 and 4 mediated innate immune response (10). Mindin, an ECM protein of the spondin family, recognizes PAMPs and modulates recruitment of inflammatory cells (11). However, such modulations of innate immune response by non-immune components have not been investigated in IBD.

In a previous study, we developed a mouse model of chronic colitis by administering low doses of 2-4-5, trinitrobenzene sulfonic acid (TNBS) as weekly enemas (12). The TNBS colitis model develops transmural colonic inflammation and tissue damage as seen in Crohn's colitis. We previously investigated gene expression patterns at the early and late stages of this model. As expected, during the early stage (first six weeks) of active inflammation, several pro-inflammatory and innate immune response genes, such as *Il1b*, *Cxcl1*, several IFN- γ inducible genes and *Cd14*, were upregulated (13). Surprisingly, genes for several ECM proteins were also upregulated at this time. Among these, lumican, a leucine rich repeat ECM protein, was upregulated together with Collagen I and III. Our earlier study shows that lumican binds to bacterial lipopolysaccharides (LPS) and to CD14, the glycerol phsophatidyl inositol-linked cell surface protein that promotes host recognition of LPS through TLR 4 (14). Gene targeted lumican-null mice (*Lum*^{-/-}) are hypo-responsive to LPS mediated septic shock and produce lower levels of pro-inflammatory cytokines. At the cellular level, *Lum*^{-/-} macrophages in culture produce lower levels of proinflammatory cytokines in response to LPS. This connection between lumican and innate immune response prompted us in the current study to examine the development of colonic inflammation in the *Lum*^{-/-} mice.

Materials and Methods

Mice

Gene targeted lumican-null mice that we developed earlier and maintained in the outbred CD1 background (15) were bred into the C57BL/6J background by backcrossing for more than 12 generations. Wild type (*Lum*^{+/+}) and *Lum*^{-/-} C57BL/6J mice, 8–10 weeks of age, of both genders were used in most experiments. In a few experiments, as indicated in specific figures, the *Lum*^{+/+} and *Lum*^{-/-} mice in the CD1 background were used. The *Rag1*^{tm1Mom}

(*Rag1*^{-/-}) C57BL/6J and wildtype C57BL/6J mice at 8 weeks of age were purchased from the Jackson Laboratories.

Colitis models

Lum^{+/+} and *Lum*^{-/-} C57BL/6J mice were randomized into control and TNBS groups. The TNBS mice were sensitized with an epicutaneous application of 1% TNBS (Sigma-Aldrich) in 80% ethanol and the control group was given an epicutaneous treatment of saline (Fig. 1). Seven days after sensitization, the mice were anesthetized with avertin (350 mg/kg bodyweight) and weighed. The TNBS group received 5 µl/g body weight of 2.5% TNBS in 50% ethanol by intra-rectal administration using a syringe fitted with a 20 gauge gavage feeding needle (Fine Science Tools, 18060-20). The control group was treated in a similar manner with 5 µl/g body weight of saline or 50% ethanol. For histology and extraction of proteins, the mice were euthanized 48 hours after intra-rectal administration of TNBS or saline. The TNBS treatment described above was also used for the wild type and the *Rag1*^{-/-} C57BL/6J mice. In a few of our earlier experiments, the *Lum*^{+/+} and *Lum*^{-/-} in the CD1 background were not sensitized epicutaneously before intra-rectal administration of TNBS as described (12). To determine survival and body weight loss, the mice were weighed daily without anesthesia for 8–10 days after intra-rectal administration of TNBS. Intra-rectal administration of saline or 50% ethanol served as controls. Animals that died by 24 hours of treatment were not included in determining mortality.

To develop the dextran sulfate sodium (DSS) colitis model, *Lum*^{+/+} and *Lum*^{-/-} in the CD1 outbred strain were given 5% DSS in the drinking water. The mice were weighed daily and harvested on the 8th day.

Histology

Colon segments ~2 mm in length were taken 4 cm away from the anus and fixed in 10% buffered formalin, paraffin embedded, cut into 7 µm thick cross sections and stained with H&E. To examine thickening of the colon associated with colitis, images of H&E stained colon cross sections were captured with a Nikon Eclipse E400 microscope fitted with a DXM1200 Nikon Digital camera. The areas of colon cross sections were measured using the software NIS-Elements. An increase in cross sectional area was taken as an indication of inflammation and thickening of the colon. The pathology of the H&E stained tissue sections was scored using a scale of 0–4 as follows: No significant lesions = 0, minimal to mild inflammatory infiltrates in the lamina propria or the submucosa = 1, mild to moderate inflammatory infiltrates, edema, occasional limited focal necrosis/ulceration = 2, moderate to marked transmural inflammation, edema, necrosis affecting up to 70% of the mucosa = 3, marked transmural inflammation, edema, necrosis affecting 70–100% of the mucosa = 4.

Protein extraction and ELISA

A colon segment of approximately 2 mm length was taken from each control and experimental animal. Total protein was extracted by homogenization, followed by a brief sonication (10 seconds at 20% amplitude, 3 times) of the tissue in T-PER containing the Halt proteinase inhibitor cocktail (Thermo Scientific) and centrifuged to remove debris. Protein concentrations were estimated using the BCA Protein Assay kit (Thermo Scientific).

The following ELISA kits were used to assay specific proteins and cytokines : mouse MPO kit (HK210) from Hycult Biotechnology, R&D mouse quantikine TNF-α kit (MTA00), R&D Quantikine mouse IL-4 (M4000B), R&D mouse KC/CXCL1 (MKC00B), total NF-κB p65 Sandwich ELISA kit (#7174) from Cell Signaling.

Electrophoretic mobility gel shift assay (EMSA)

NF- κ B gel binding oligonucleotides (SC2505) and mutant oligonucleotides (SC2511) were purchased from Santa Cruz and end labeled with γ^{32} P-ATP and T4 polynucleotide kinase (Fermentas). The labeled oligonucleotides were incubated with macrophage nuclear extracts and resolved by SDS PAGE in a 6% polyacrylamide gel pre-run for 20 minutes. The gels were dried and exposed to film overnight at -80°C .

Statistical analysis

Measurements taken in control and TNBS treated animals were compared using the student *t* test when results were consistent with normally distributed values. Experiments that involved unequal sample size and did not necessarily follow normal distributions were compared using the Mann-Whitney test with ≤ 0.05 considered significant. The GraphPad PRISM software (v4.02) was used to perform these analyses.

Ethical considerations

All mouse procedures were evaluated and approved by the Johns Hopkins Animal Care and Use Committee. In case of extreme lethargy and $>25\%$ loss in bodyweight after inducing colitis, the mice were taken out of the study and euthanized.

Results

Macroscopic disease and the course of colitis

TNBS treated mice of both genotypes showed signs of piloerection, lethargy and diarrhea which were not seen in the saline treated mice (data not shown). Ethanol, often used as a vehicle control for the TNBS treatment, leads to colonic tissue damage, and in our experience changes the expression of pro-inflammatory genes (13). Here we show that intra-rectal administration of ethanol leads to similar trends in body weight loss in the *Lum*^{+/+} and the *Lum*^{-/-} mice. The results further show that all ethanol treated mice and the TNBS treated *Lum*^{+/+} mice recovered their initial body weight by the sixth day after treatment (Fig. 2A). In contrast, the *Lum*^{-/-} TNBS treated mice did not recover by day 6, suggesting that the TNBS hapten had additional detrimental effects on lumican deficiency. Intra-rectal administration of saline did not lead to loss in body weight (Fig. 2B). In the same experiment, the *Lum*^{+/+} and *Lum*^{-/-} TNBS treated mice showed up to 18% loss in body weight. However, after seven to eight days the *Lum*^{+/+} TNBS group had regained most of the lost weight, while the *Lum*^{-/-} TNBS group still displayed on average an 11% body weight reduction (Fig. 2B). We also tested the TNBS colitis model in the CD1 outbred strain, and as in the C57BL/6J background, the *Lum*^{-/-} CD1 mice showed slower recovery from body weight loss (data not shown). As is typical for the TNBS model, we found an overall survival of 30% with no significant difference between *Lum*^{+/+} and *Lum*^{-/-} mice, while saline treated controls showed 100% survival (Fig. 2C). Macroscopic appearance of the colon was normal in the saline treated animals (Fig. 3 top panels), while in the TNBS treated wild type and *Lum*^{-/-} mice it was frequently swollen and bloody, with soft stool (Fig. 3 middle panels). To gauge edema and inflammation of the colon, we used two parameters: 1) cross sectional areas of H&E stained colon sections, and 2) colon to body weight ratios. The cross-sectional areas were higher for the TNBS over saline-treated controls, with the difference being more pronounced and significant ($p=0.007$) in the TNBS- *Lum*^{-/-} group over its respective saline control (Fig. 4A). The colon to body weight ratio was higher in the TNBS over saline treated mice of both genotypes. However, the increase was more pronounced in the *Lum*^{-/-} TNBS treated mice (Fig. 4B). These results suggest that colonic edema and swelling is higher in the inflamed *Lum*^{-/-} mice.

The TNBS colitis model is considered to involve a T-cell mediated immune response in the colonic mucosa (16). However, the early acute response to the TNBS treatment may be largely innate immune-driven. To test this we used the TNBS colitis model in the *Rag1^{tm1Mom}* mice (*Rag1*^{-/-}). The *Rag1*^{-/-} mice are deficient in mature B and T cells (17) and often used as recipients in T-cell transfer colitis models (18) but not directly in TNBS colitis models. In our TNBS model weight loss in the *Rag1*^{-/-} was similar to that of the wildtype mice (Fig. 5A), and the colon, harvested 2 days after the intra-rectal TNBS treatment, appeared equally inflamed in both (Fig. 5B). Histology also indicated similar degrees of inflammation in the wild types and the *Rag1*^{-/-} mice (Fig. 5C). These results suggest that the early stage of TNBS colitis involves an innate immune inflammatory response.

Given that the TNBS colitis model as used here is primarily an injury and innate immune response, we considered if the increased morbidity in the TNBS-challenged *Lum*^{-/-} mice was entirely due to epithelial injury and delayed healing. To test this possibility we used the DSS-colitis model where inflammation is initiated by epithelial injury and loss of barrier integrity. Administration of 5% DSS in the drinking water caused diarrhea and equivalent weight loss in the *Lum*^{+/+} and the *Lum*^{-/-} mice (data not shown). This suggests that in the TNBS-colitis model the TNBS hapten itself has an additional detrimental effect in the *Lum*^{-/-} mice.

Colonic tissue damage and inflammatory cell infiltration in the TNBS treated lumican-deficient mice

Histology showed healthy tissue architecture in the saline treated mice (Fig. 6A and B). The TNBS-wild type group showed infiltration of inflammatory cells, moderate edema and ulceration (Fig. 6C). The *Lum*^{-/-} TNBS group tended to have more edema, ulceration and frequent necrosis (Fig. 6D and F). However, the histology was quite variable, with some sections showing very little inflammation and tissue damage in the TNBS-treated animals. Therefore, to compare the overall response in the *Lum*^{+/+} and the *Lum*^{-/-} TNBS challenged mice, the slides were scored using a scale of 0 to 4 (methods). The pathology scores from one set of experiments shows higher inflammation scores in a larger proportion of the TNBS challenged *Lum*^{-/-} compared to the *Lum*^{+/+} mice (Fig. 7A). Measurements of myeloperoxidase (MPO) released by inflammatory cells, taken as a measure of inflammatory cell infiltrates, showed less than normal increase in colonic extracts of the TNBS-treated *Lum*^{-/-} mice (Fig. 7B). Taken together, the results suggest increased edema, tissue damage and necrosis, but overall lower inflammatory cell influx in the *Lum*^{-/-} TNBS treated mice.

Induction of pro-inflammatory factors in inflamed colonic tissues

To further examine inflammation at the molecular level, we measured levels of selected cytokines in colonic protein extracts by ELISA. Compared to basal levels in saline-treated mice, the chemokine CXCL1/KC was markedly increased in the TNBS-treated wild type mice (12 fold over basal level, $p=0.027$). By comparison, the increase in *Lum*^{-/-} TNBS was modest (5 fold, $p=0.04$) (Fig. 8A). The pro-inflammatory cytokine TNF- α was increased significantly in the TNBS-wild type mice over saline controls, while the TNBS-treated *Lum*^{-/-} mice showed very little induction of TNF- α (Fig. 8B). We next investigated IL-4 levels as a T_H2 response-related cytokine; no significant change in IL-4 reaffirms a lack of T_H2-related immune response in this colitis model (Fig. 8C).

Since NF- κ B plays a central role in innate immune response and induction of pro-inflammatory cytokines and chemokines, we measured total NF- κ B p65 levels in colonic protein extracts of each animal using ELISA methods. While total NF- κ B p65 increased in

both genotypes after TNBS treatment, the increase in TNBS treated wild types was higher (2.5 fold) than that seen in TNBS-treated *Lum*^{-/-} mice (1.7 fold) (Fig. 9A). To further test if there is a delay in the activation of NF-κB in lumican deficiency, we performed an EMSA test on primary peritoneal macrophages. *Lum*^{+/+} and *Lum*^{-/-} macrophages in culture were exposed to LPS for different times. Nuclear extracts of these macrophages were incubated with radio-labeled NF-κB specific and nonspecific oligonucleotides and analyzed by SDSPAGE. Maximal specific binding of nuclear proteins with the target oligonucleotide occurred in extracts of *Lum*^{+/+} macrophages that were treated with LPS for 10 min. In the *Lum*^{-/-} macrophage extracts, comparable nuclear protein binding occurred after 20 min of LPS treatment. Thus nuclear localization of NF-κB in *Lum*^{-/-} peritoneal macrophages was delayed by 10 minutes compared to wild types in response to bacterial LPS treatment (Fig. 9B).

Discussion

Here we report distinct differences in the response of wild type and lumican-deficient mice to an intra-rectal challenge of TNBS that speak of weak innate immune and inflammatory responses, and increased morbidity in the absence of lumican. Lumican is an extracellular matrix proteoglycan present in large amounts in connective tissues rich in fibrillar collagens such as the intestinal submucosa (19). The lumican core protein has leucine-rich repeat (LRR) motifs, similar to those found in TLR proteins and other members of the LRR superfamily (20). Lumican deficiency disrupts collagen fibril assembly and impairs normal functions of connective tissues (15). In addition, we have shown that lumican promotes innate immune response through its interactions with the CD14-TLR4 pathway and neutrophil migration by interacting with β2 integrin, a subunit of the neutrophil migration receptor MAC1 or CD11b/CD18 (14,21). We also know that lumican expression is induced in epithelial cells after injury (22), and epithelial wound healing in the cornea is delayed in *Lum*^{-/-} mice (23,24). The emerging role of lumican, and possibly some of the other members of this group of ECM proteins, is one of modulating innate immune signals during inflammation and wound repair and maintaining extracellular matrix homeostasis.

We used the TNBS-colitis model to follow disease manifestation and inflammatory responses during acute and active inflammation of the colon. MPO measurements from colonic protein extracts suggest very little influx of neutrophils in the TNBS-treated *Lum*^{-/-} mice. CXCL1/KC was induced in all TNBS-colons, but the increase in *Lum*^{-/-} mice was markedly lower than that seen in the *Lum*^{+/+} mice. Mouse KC, a functional equivalent of human IL-8, is a strong chemoattractant for neutrophils; its weaker induction in *Lum*^{-/-} TNBS treated colons is consistent with low MPO measurements in these animals. The pro-inflammatory cytokine TNF-α was also not induced to significant levels in the *Lum*^{-/-} TNBS treated colons. Lower inductions of KC and TNF-α in the *Lum*^{-/-} mice suggest weak innate immune response from epithelial cells and macrophages in the colon. An ELISA that detects the p65 subunit of NF-κB showed lower levels of this subunit in the *Lum*^{-/-} compared to *Lum*^{+/+} TNBS treated colons. By gel shift assays we found that in *Lum*^{-/-} primary peritoneal macrophage cultures there was a delay in nuclear localization of NF-κB in response to an LPS challenge. The NF-κB transcription factor is a common switch that responds to several cell surface and cytoplasmic pathogen recognition receptors, including TLR4, and mediates the upregulation of many inflammatory genes (25). NF-κB blockade *in vivo* has been shown to effectively ameliorate inflammation in the TNBS mouse colitis model (26). In a previous study we used this NF-κB blockade treatment in the chronic TNBS colitis model and found effective suppression of *Cxcl1* (13). Lumican was also inhibited by NF-κB blockade indicating that its regulation closely mimics that of the inflammatory genes, rather than ECM collagens (13). Our observations in the current study further support the idea that lumican has an inflammation-regulatory role in colitis. The

Lum^{-/-} mice show subtle but clearly discernible differences in overall disease response that relate to the differences seen in innate immune signals at the molecular level. While there is no dramatic difference in mortality between wild type and *Lum*^{-/-} mice challenged with TNBS, the *Lum*^{-/-} mice show significantly slower recovery of lost body weight. Histology of the inflamed colons, on average, show more edema and tissue damage in the *Lum*^{-/-} TNBS colon. Also, there were more instances of necrosis in the *Lum*^{-/-} TNBS colon that could be due to increased bacterial colonization of the colon.

The TNBS colitis first tested in rats (27) and then adapted in the mouse (16,28) is considered to be driven by T-cell mediated immune response as TNBS haptensates host proteins and makes them immunogenic. However, response to the TNBS-ETOH challenge also involves an innate immune response that in the short term can produce pro-inflammatory cytokines and an inflammatory response without significant antigen presentation to T cells and the induction of T_H1 cells. Thus, in the *Rag1*^{-/-} mice, deficient in mature T and B lymphocytes, we also detected wild type-like inflammatory response within 6 days after TNBS treatment. The DSS colitis model, which is T-cell independent and mediated by epithelial injury and inflammatory cell infiltration, has been shown by others to be equally effective in the *Rag1*^{-/-} mice (29). We tested the DSS colitis model in the *Lum*^{-/-} mice, and compared to the wild type mice, found no difference in disease manifestation. It appears that the DSS-mediated epithelial injury and healing may not be affected by lumican-deficiency. In contrast, the TNBS-haptensated host proteins seem to be stimulating an early innate immune response that is affected by lumican deficiency.

In the *Lum*^{-/-} TNBS treated mice cytokine induction and inflammatory cell infiltration was reduced, yet disease severity in terms of weight loss and tissue damage was elevated. A more extreme version of reduced inflammatory response but aggravated disease is seen in the *Tlr4*^{-/-} and *MyD88*^{-/-} mice. These mutant mice show poor neutrophil infiltration, aggravated commensal gram-negative bacteria in the lamina propria and overall increased disease (30). In the same vein, *MyD88*^{-/-} mice showed increased bacterial burden in a *Citrobacter rodentium* infection model (31). TLR4 and MYD88 dependent innate immune responses play a central role in epithelial and mucosal health (32–34). The exact protective mechanism is not well understood, but a combination of processes is credited. First, TLR activation by commensal bacteria promotes epithelial integrity and homeostasis. TLR mediated activation of pro-inflammatory cytokines help epithelial survival and cell migration for efficacious healing of wounds. Second, infiltrating neutrophils and macrophages are protective by restricting microbial (commensal and pathogenic) overgrowth.

Innumerable studies of IBD point to an etiopathogenesis of dysregulated immune response and altered response to environmental and microbial factors, with the concept of altered host innate immune response gaining significant traction (1,3,35). NOD2/CARD15, a key regulator of innate immune response has been established as a Crohn's disease susceptibility gene; TLR4 and CD14 have also been associated with IBD genetics. Investigations of the major innate immune signaling nodes will no doubt continue to elucidate IBD pathogenesis and significant susceptibility genes. The ECM genes may modify IBD pathogenesis to affect disease severity and possibly susceptibility. ECM proteins bind growth factors, cytokines and cell surface receptors, and can either enhance or attenuate signals (24,36,37). Clearly, the absence of lumican in the mouse affects the outcome of colitis in a small but significant way. Other related ECM proteins that may have similar functions are fibromodulin, decorin and biglycan. We believe that as more extracellular matrix proteins become better characterized, many will be identified as modifiers of key innate immune and inflammatory genes and as having an effect on IBD pathogenesis.

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FIGURE 1.

TNBS Colitis model. The mice were pre-sensitized by epicutaneous application of 1% TNBS in 80% ethanol. Seven days later 5 μ l/g body weight of 2.5% TNBS in 50% ethanol was administered intra-rectally under anesthesia. The mice were euthanized 48 hours later for tissue harvest, or followed up to 8 days after TNBS treatment for daily body weight measurements.

FIGURE 2.

Response to intra-rectal TNBS treatment. **A.** *Lum*^{+/+} and *Lum*^{-/-} in the C57BL/6J mice were challenged with 2.5% TNBS in 50% ethanol (ETOH) or ETOH alone. Change in body weight was expressed as a percent weight change relative to their weight on day 0. The *Lum*^{-/-} TNBS challenged mice failed to recover the lost body weight, whereas the ETOH challenged *Lum*^{-/-} and the wild type TNBS and ETOH treated mice recovered by day 6. **B.** TNBS colitis using saline treatment as a control. The saline treated mice did not lose weight while the TNBS treated mice lost weight. **C.** Survival presented as a Kaplan Meier survival plot. *Lum*^{+/+} and *Lum*^{-/-} C57BL/6J mice challenged with or without TNBS as in Figure 1, and observed for survival for 8 days. There was no significant difference in survival between the two genotypes. The data shown here is one of two representative experiments.

FIGURE 3.

Macroscopic appearance of the colon in TNBS-treated *Lum*^{+/+} and *Lum*^{-/-} C57BL/6J mice.

The colons were harvested 48 hours after intra-rectal TNBS administration and photographed. Saline treated colons (top two panels) showed no inflammation. TNBS treated colons with mild inflammation (middle two panels) appeared bloody in areas (arrows), while colons showing significant inflammation (bottom panels) were macroscopically thickened and necrotic (arrow heads). The highly inflamed colons were seen more frequently in the *Lum*^{+/+} TNBS-treated mice. This is one of three representative experiments.

FIGURE 4.

Increased thickening of TNBS treated colons. **A.** Cross-sectional areas of colons, taken as a measure of colon thickening and inflammation for *Lum*^{+/+} and *Lum*^{-/-} C57BL/6J mice treated with TNBS. Colon cross-sectional areas were increased significantly in *Lum*^{-/-} TNBS treated mice compared to saline controls. * $p=0.02$ (Mann-Whitney test). The data shown is one of two representative experiments. **B.** Increased colon to body weight ratio in *Lum*^{+/+} and *Lum*^{-/-} CD1 mice treated with TNBS. The increase in colon to body weight ratio was significantly increased in TNBS challenged *Lum*^{+/+} ($p=0.005$) and *Lum*^{-/-} ($p=0.0002$) mice compared to their saline treated controls. * A p value ≤ 0.05 was considered statistically significant based on Mann-Whitney test. Representative data from two similar experiments is shown here.

FIGURE 5.

TNBS colitis in the $Rag1^{-/-}$ mice. **A.** The mice were given an intra-rectal treatment of TNBS or saline and weighed daily. Weight loss in the TNBS treated wild type and the $Rag1^{-/-}$ was similar. **B.** Macroscopic appearance of the colon. The colon appeared equally inflamed in the wild type and the $Rag1^{-/-}$ mice. **C.** Histology. Colonic sections of the TNBS treated $Rag1^{-/-}$ mice showed signs of inflammation that were comparable to those seen in the wild type. The submucosa was inflamed and edematous and inflammatory cells were visible in the submucosa and the lamina propria.

FIGURE 6. Histology of colon cross sections. **A.** *Lum*^{+/+} and **B.** *Lum*^{-/-} saline and TNBS treated mice. **C.** *Lum*^{+/+} and **D.** *Lum*^{-/-} TNBS treated colons under low magnification show inflammation of the submucosa (SM) and some inflammatory cell infiltrates in the lamina propria (LP). Necrosis and tissue damage is shown under high magnification in **E.** *Lum*^{+/+} and **F.** *Lum*^{-/-} TNBS treated mice.

FIGURE 7.

Reduced influx of neutrophils in *Lum*^{-/-} mice compared to *Lum*^{+/+} TNBS treated colons but increased overall tissue damage. **A.** Tiled bar graph showing percent of animals with 0–4 inflammation scores (Methods). In this experimental set there were no mice that displayed scores of 3 or 4. Overall, the *Lum*^{-/-} TNBS challenged group showed more animals with an inflammation score of 2. **B.** Measurements of MPO by ELISA to assess neutrophil infiltration after TNBS challenge. TNBS treated *Lum*^{-/-} showed no significant increases in MPO, whereas *Lum*^{+/+} TNBS treated colons showed significant increases (**p*=0.048, Mann-Whitney test).

FIGURE 8.

Cytokine levels in colonic protein extracts. **A.** CXCL1/KC was increased significantly ($p=0.01$) in colonic extracts of *Lum*^{+/+} TNBS-treated mice compared to saline treated controls. The *Lum*^{-/-} mice showed little increase in KC after TNBS treatment. **B.** TNF α levels were increased significantly ($p=0.012$) in the *Lum*^{+/+} TNBS mice, while there was no significant increase in TNF- α in *Lum*^{-/-} TNBS compared to saline-treated controls. **C.** IL-4 levels showed no significant increase after TNBS treatment in the *Lum*^{+/+} or the *Lum*^{-/-} mice. * A p value ≤ 0.05 was considered statistically significant based on Mann-Whitney test. The result shown is one of two similar experiments.

FIGURE 9.

Poor NF-κB response in the absence of lumican. **A.** Total NF-κB level was assessed in colonic extracts by ELISA measurements of the p65 subunit. The increase in total NF-κB in TNBS treated mice of both genotypes was significant ($p \leq 0.05$) compared to the saline controls. However, the increase was much lower in the *Lum*^{-/-} (1.7X) compared to that seen in the *Lum*^{+/+} (2.5X) mice. **B.** Delayed nuclear localization of NF-κB in peritoneal macrophages of *Lum*^{-/-} mice compared to *Lum*^{+/+} after exposure to 10 ng/ml LPS. Nuclear extracts were tested by EMSA (Methods). *Lum*^{-/-} peritoneal macrophages show maximal NF-κB binding after 20 min LPS exposure, while this was achieved by 10 min in *Lum*^{+/+} macrophages.