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[Original Article]

MODULATING TOLL-LIKE RECEPTOR 4 SIGNALING PATHWAY PROTECTS MICE FROM EXPERIMENTAL COLITIS

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Abstract : Background/Aim : Several reports have indicated that environmental factors and defects in innate immunity are central to the pathogenesis of inflammatory bowel disease (IBD). Although bacteria producing lipopolysaccharide (LPS), which is a Toll-like receptor (TLR) 4 agonist, play a crucial role in the development of experimental colitis, LPS tolerance following initial exposure to LPS can result in a state of hyporesponsiveness to subsequent LPS challenge. Therefore, we initiated this study to explore the role of LPS tolerance in the development of colitis. **Methods :** Dextran sulfate sodium (DSS) colitis was induced in Balb/c mice with or without daily intraperitoneal administration of LPS. Disease activity and cytokine mRNA expression in the colon were evaluated. To confirm LPS tolerance, mouse conventional bone marrow-derived dendritic cells (BMDC) were preincubated with or without LPS, and were restimulated with LPS 24 h after first exposure. Cytokine production was measured by ELISA, and mRNA expression was evaluated by RT-PCR. Furthermore, we investigated the expression of negative regulators of LPS tolerance in BMDC. **Results :** Administration of LPS significantly suppressed colonic inflammation of DSS-induced colitis. After subsequent stimulation with LPS, TNF- α production was reduced in BMDC. IRAK-M, a negative regulator of TLR4 signaling, mRNA expression was up-regulated in LPS-treated BMDC. **Conclusion :** LPS tolerance was able to protect mice from DSS-induced colitis, and IRAK-M participated in this tolerance. Taken together, these observations suggest that loss of exposure to LPS is involved in the pathogenesis of IBD.

Key words : LPS tolerance, Toll-like receptor 4, experimental colitis

INTRODUCTION

The pathogenesis of inflammatory bowel disease (IBD) is multi-factorial, involving susceptibility genes, as well as immunological and environmental factors¹). Indeed, studies on IBD and mouse models have provided compelling evidence that bacterial flora plays a key role in the pathogenesis of the disease, as broad-spectrum antibiotics and probiotics have proven to be clinically effective in treating IBD^{2, 3}) and colitis does not occur in several mutant strains when they are maintained in a germ-free environment⁴). As a result of tolerance from con-

tinuous exposure to commensal bacteria, normal intestine does not typically exhibit inflammatory reactions. Although the mechanisms of immunological tolerance to luminal microflora remain unclear, it has been reported that the Toll-like receptor (TLR) signaling pathway activated by commensal bacteria play an important role in maintaining colonic homeostasis⁵⁻⁸).

Because TLR recognizes a variety of microbial products⁹), TLR activation pathways are the center of host defense by innate immune responses. Therefore, TLR-activated antigen presenting cells express high levels of co-stimulatory molecules and

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produce plenty of pro-inflammatory cytokines and chemokines. Contrary to this phenomenon, recent studies have demonstrated that TLR signaling in the intestine may inhibit inflammatory responses via the induction of type-1 interferon (IFN)^{10, 11}.

In enteric flora, LPS from the cell wall of gram-negative bacteria is known to activate dendritic cells (DCs)¹². For intestinal mucosa, conventional DCs are a key component in intestinal inflammation^{13, 14}. LPS is recognized by TLR4¹⁵ and produces several inflammatory cytokines, such as TNF- α and IL-6¹⁶. In the mouse colitis model, which represents human IBD, TLR4 is involved in the development of colitis¹⁷. However, DCs are the major cellular source of type-1 IFN induced by TLR signaling, and should contribute to colonic homeostasis, although the mechanism remains unclear. Several reports have suggested that negative regulation mechanisms for TLR signaling exist¹⁸ and one of these is responsible for LPS tolerance following initial exposure to LPS, resulting in a transient state of hyporesponsiveness to subsequent LPS challenge¹⁹.

Here, we present evidence that interaction between commensal bacteria and DCs inhibit colonic inflammation through modulation of TLR signaling. Our results also indicate further causes and treatment options for human IBD.

MATERIALS AND METHODS

Reagents

The following materials were obtained from commercial sources: dextran sulfate sodium (DSS, molecular weight 36-50 kDa, MP Biomedicals, Solon, OH, USA); Occult Blood Slides (Shionogi, Osaka, Japan); Purified *E. coli* LPS (InvivoGen, San Diego, CA, USA); and GM-CSF (BD Biosciences, Pharmingen, San Diego, CA, USA)

Mice

Seven- to nine-week-old female Balb/c mice were purchased from CLEA-Japan. Animals were housed under specific pathogen-free conditions. All experimental procedures were approved by the institutional committee for animal care and use of Fukushima Medical University.

Cell cultures

BMDCs were cultured from the long bones of mice in the presence of murine GM-CSF and were characterized as described previously²⁰. RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) supple-

mented with 10% FBS (JRH Bioscience, Lenexa, KS, USA), 2-ME (100 μ M), penicillin (5 U/ml) and streptomycin (50 ng/ml) was used as culture medium.

Induction and evaluation of experimental colitis

Mice were given 7% DSS. DSS was dissolved in sterile, distilled water and was provided ad libitum for 7 days⁵. Mice were then injected with sterile saline or LPS at dose of 100 ng/body intraperitoneally for 7 consecutive days. Disease activity index (DAI, combined score of weight loss and bleeding) was determined as described previously^{5, 10}. Briefly, scores were as follows: for loss in body weight, 0 = no loss, 1 = 5-10%, 2 = 10-15%, 3 = 15-20%, and 4 = over 20%; for Occult Blood Slide, 0 = no blood, 2 = positive, and 4 = gross blood.

Determination of MPO activity

Colon tissues were opened longitudinally and a 50-mg portion was homogenized in hexadecyltrimethyl-ammonium bromide (0.5%) in 50 mmol/l phosphate buffer, pH 6.0. Homogenates were sonicated for 10 seconds, frozen and thawed three times, and centrifuged for 15 min. An aliquot of the supernatant was used for determination of enzyme activity as described elsewhere^{5, 10, 21}.

Histological scoring

After 7 days of DSS administration, mice were sacrificed and the entire colon was excised, fixed with 10% Formaldehyde Solution (Wako Pure Chemical Industries Ltd., Osaka, Japan) and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin. Histological scores were assigned by experimenters blinded to sample identity. Colonic epithelial damage was scored as follows: 0 = normal; 1 = hyperproliferation, irregular crypts, and goblet cell loss; 2 = mild to moderate crypt loss (10-50%); 3 = severe crypt loss (50-90%); 4 = complete crypt loss, surface epithelium intact; 5 = small- to medium-sized ulcers (<10 crypt widths); 6 = larger ulcers (\geq 10 crypt widths). Infiltration with inflammatory cells was assigned scores separately for mucosa (0 = normal, 1 = mild, 2 = moderate, 3 = severe), submucosa (0 = normal, 1 = mild to moderate, 2 = severe) and muscle/serosa (0 = normal, 1 = moderate to severe). Scores for epithelial damage and inflammatory cell infiltration were added, resulting in a total scoring range of 0-12¹⁰.

Determination of cytokine concentrations

BMDCs (1×10^6 /ml) were stimulated with 10 ng/ml LPS. For tolerance experiments, cells were preincubated with LPS for 24 h and washed with culture medium. Cells were then stimulated with LPS for a further 24 h. ELISA kits were used to determine the levels of TNF- α , IL-6 and IL-10 (e-Bioscience, San Diego, CA, USA) in the culture supernatants according to the manufacturer's instructions.

For colitis experiments, serum cytokine levels of TNF- α and IL-6 on day 7 were determined in naive and LPS-injected mice using ELISA kits according to the manufacturer's instructions.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

For gene induction, BMDCs (1×10^6 /ml) were stimulated with 10 ng/ml LPS for 24 h, following subsequent stimulation with same dose of LPS for 4 h. Total RNAs were isolated from BMDCs or from 100 μ g of colonic sections of mice from DSS-induced colitis. RNA was reverse transcribed to single-stranded cDNA using Random Primers, dNTP Mixture (TAKARA SHUZO Co., Ltd., Shiga, Japan) and RNasin[®] Ribonuclease Inhibitor (Promega, Madison, WI, USA), according to the manufacturer's protocol. The cDNA was used for semiquantitative analysis by PCR. The following sets of PCR primers were used:

TNF- α (sense) 5'-ATCAGTTCTATGGCCCAG ACCCTCACA-3', (antisense) 5'-TCACAGAGCAAT GACTCCAAAGTAGAG-3'; IL-6 (sense) 5'-ATGA AGTTCCTCTCTGCAAGAGACT-3', (antisense) 5'-CACTAGGTTTGCCGAGTAGATCTC-3'; IL-10 (sense) 5'-ACCTGGTAGAAGTGATGCCCCAG GCA-3', (antisense) 5'-CTATGCAGTTGATGAAG ATGTCAA-3'; IRAK-M (sense) 5'-TCTTCAGG TGTCCCTTCTCCACTGT-3', (antisense) 5'-CCTCT TCTCCATTGGCTTGCTCCCAT-3'; SOCS1 (sense) 5'-CACTCACTTCCGCACCTTCC-3', (antisense) 5'-CAGCCGGTCAGATCTGGAAG-3'; TOLLIP (sense) 5'-CAAGAATCCCCGCTGGAAT AAG-3', (antisense) 5'-ATGGCTTTCAGGTCCTC CTCGC-3'; mTLR4 (sense) 5'-TGCCTTCACTAC AGAGACTTTATTCTT-3', (antisense) 5'-TCAGGT CCAAGTTGCCGTTTCTTGTTTC-3'; and internal control β -actin (sense) 5'-GCTACAGCTTACCAC CACAG-3', (antisense) 5'-GGTCTTTACGGATGTC AACGTC-3'. PCR consisted of one cycle at 95°C for 15 min and 94°C for 30 s, followed by PCR amplification at 57°C for IL-6, mTLR4 and TOLLIP, or at

59°C for TNF- α , SOCS1 and IRAK-M, for 30 s for denaturation, 72°C for 1 min for annealing and 72°C for 10 min for extension. PCR products were separated on 2.0% agarose gels, visualized by ethidium bromide staining and analyzed with a gel-imaging system.

Statistical analysis

Data are expressed as means \pm SD. Statistical analysis for significant differences was performed using Student's *t* test for unpaired data. *p* values of less than 0.05 were considered significant.

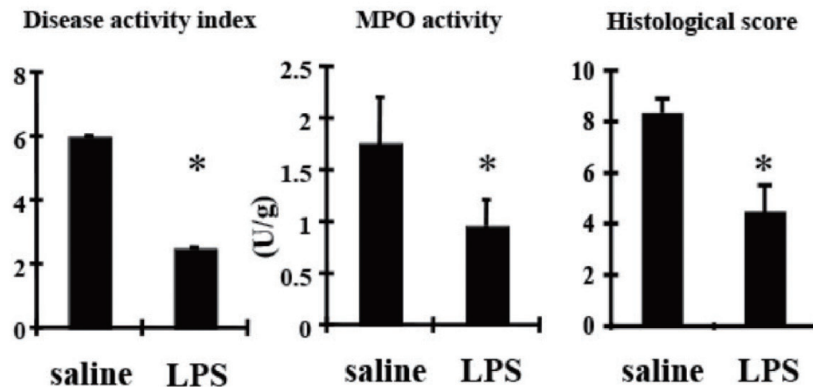
RESULTS

Repeated administration of LPS protects against DSS-induced colitis

We initially determined the protective role of a single administration of LPS. As expected, there were no inhibitory effects in DAI or MPO activity (data not shown). We also evaluated the effective dose of LPS and found that systemic administration of 100 ng of LPS resulted in protection against DSS-induced colitis. Previous studies demonstrated that the severity of colonic inflammation induced by DSS is dependent on dose and lot number of DSS, and mouse strain^{4, 21}. Balb/c mice are relatively resistant to DSS water. Therefore, we initiated this experiment using 7% DSS water and injected 100 ng of LPS intraperitoneally into DSS-induced colitis mice for 7 consecutive days. The repeated administration of LPS attenuated the severity of DSS-induced colitis, as reflected in DAI, colonic MPO activity and histological score (Fig. 1A). On microscopy, the extensive superficial ulceration and mucosal inflammatory reactions induced by DSS were abolished in mice treated with LPS, although minimal inflammatory reactions were observed (Fig. 1B).

In order to evaluate whether the protective effects of repeated LPS administration on DSS-induced colitis are due to the cytokine balance induced by LPS, we examined serum levels of IL-6 and TNF- α in mice on day 7 after DSS induction. We were unable to detect these cytokines in mice treated with either LPS or saline (data not shown). Levels of mRNA of several pro-inflammatory cytokines and anti-inflammatory cytokines in the affected colon of day 7 were also analyzed by RT-PCR. As shown in Fig. 2, administration of DSS induced TLR4 mRNA expression in the colon with or without LPS treatment, suggesting that

A.



B.

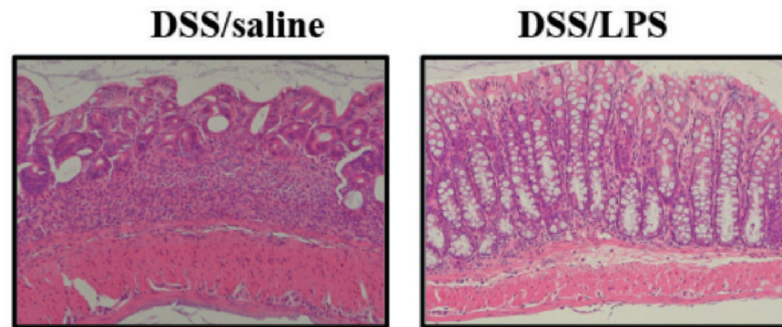


Fig. 1. Repeated administration of LPS protected against DSS colitis.

A : LPS administration significantly protected colonic inflammation in DSS-induced colitis, showed lower DAI (6.0 ± 0.1 vs. 2.5 ± 0.1), MPO activity (1.7 ± 0.4 vs. 0.9 ± 0.2) and histological scores (8.3 ± 0.5 vs. 4.5 ± 1.0) than saline-administered mice (saline vs. LPS, $n=8$ in each group ; $p<0.05$).

B : The extensive superficial ulceration with mucosal inflammatory reactions, such as colonic wall thickness and accumulation of inflammatory cells, induced by DSS was abolished in mice treated with LPS.

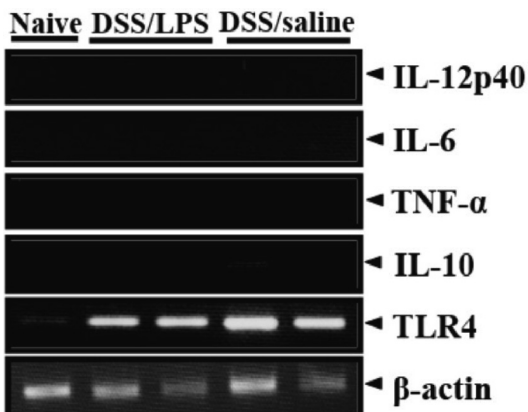


Fig. 2. Gene induction in colon of DSS-induced colitis.

Total RNAs were isolated from 100 μ g of colonic sections of mice from DSS-induced colitis and RT-PCR was obtained. There were no differences in IL-12p40, IL-6, TNF- α , IL-10 and TLR4 mRNA expression in colons of DSS-administered mice treated with or without LPS.

commensal bacteria produce LPS, which is an agonist of TLR4, and that this affects development of DSS-induced colitis. In contrast, we did not detect gene induction of either pro-inflammatory cytokines, such as IL-12p40, IL-6 and TNF- α , or anti-inflammatory cytokines, such as IL-10, in either group in later phases of colon inflammation.

LPS treatment induces tolerance to subsequent stimulation with LPS in BMDCs

In order to further evaluate the potential effects of LPS on DSS-induced colitis *in vitro*, we prepared conventional BMDCs, as DCs are a key component in intestinal inflammation and colonic innate immune responses^{13, 14}. LPS is associated with a phenomenon known as LPS tolerance, in which initial exposure to LPS induces hyporesponsiveness to subsequent LPS challenge¹⁹. Therefore, to confirm this phenomenon, BMDCs were preincubated with or

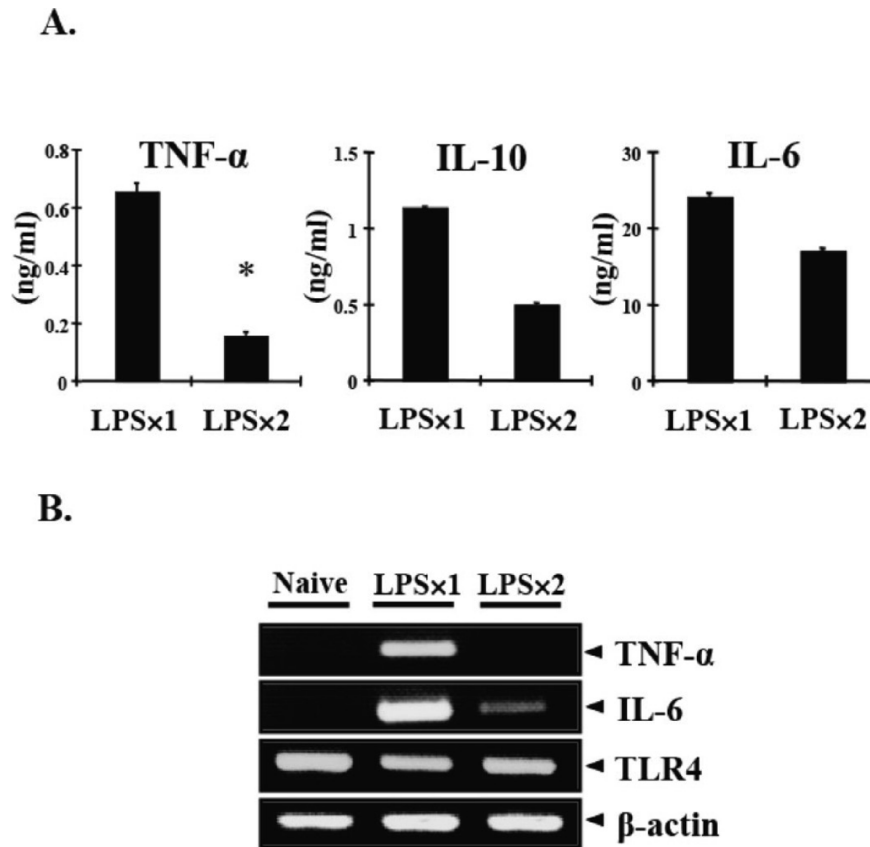


Fig. 3. Cytokine production and mRNA expression in BMDCs. DCs were stimulated with the indicated concentrations of LPS.

A : After second stimulation with LPS, TNF- α production (0.65 ± 0.02 vs. 0.16 ± 0.01) was significantly lower, which agreed with previous reports on LPS tolerance. IL-6 (1.14 ± 0.01 vs. 0.5 ± 0.02) and IL-10 (24.3 ± 1.0 vs. 17.2 ± 0.5) production were also decreased, but there were no significant differences (LPSx1 vs. LPSx2, data represent mean \pm SD from 1 experiment out of 3 performed ; *denotes $p < 0.05$).

B : Induction of TLR4 mRNA was noted, while TNF- α and IL-6 mRNA expression was reduced after second stimulation with LPS.

without LPS, and were restimulated with LPS 24 h after the first stimulation. After second stimulation with LPS, TNF- α production decreased, which corresponded to previous reports on LPS tolerance^{19, 22}, while IL-6 and IL-10 production did not significantly vary (Fig. 3A).

We further analyzed TNF- α , IL-6 and TLR4 mRNA expression in response to LPS. As TLR4 is constitutively expressed on cell surfaces, there were no differences in induction of TLR4 mRNA, whereas TNF- α and IL-6 mRNA expression were reduced on second stimulation with LPS (Fig. 3B). Overall, these findings indicate a protective role of repeated administration of LPS in DSS-induced colitis, and this protection is, at least in part, the result of LPS tolerance in stimulated DCs.

IRAK-M participates in LPS tolerance

Although the molecular mechanisms of LPS tolerance are poorly understood, several negative reg-

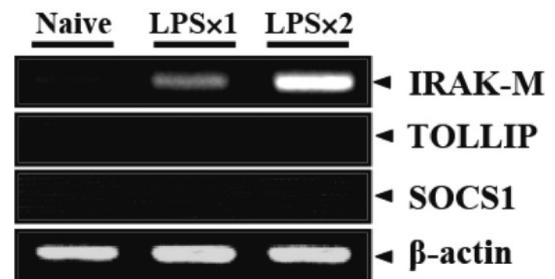


Fig. 4. mRNA expression of negative regulators in TLR4 signaling were analyzed by RT-PCR after stimulation with LPS. Only IRAK-M expression increased after second stimulation with LPS.

ulators of TLR4 signaling have been implicated¹⁸. One of the mediators of LPS tolerance is the TLR signaling pathway inhibitor IRAK-M²³, which is a member of the IL-1 receptor-associated kinases (IRAK) family of adaptor molecules²⁴. Therefore, we verified by RT-PCR whether several negative regulators, including IRAK-M, are induced

in BMDCs after repeated stimulation with LPS. As shown in Fig. 4, only IRAK-M was induced after second stimulation of LPS. This observation suggests that IRAK-M plays a key role in protecting against intestinal inflammation by mediating LPS tolerance in the TLR4 signaling pathway.

DISCUSSION

In this study, we demonstrated that frequent administration of LPS, which modulates the TLR4 signaling pathway, protected mice from DSS-induced colitis. Several reports in human disease and experimental animal models led to the hypothesis that environmental factors and defects of innate immunity are central to the pathogenesis of IBD¹. To explore this issue, we selected DSS-induced colitis, as it is a model of disrupted epithelial barrier function and results in increased exposure of lamina propria innate immune elements to luminal microbes²⁵. Unfortunately, there is no animal model that completely recapitulates its cause and manifestations. Although mechanisms of DSS-induced colitis remain unclear, this will be the best model for investigating the interaction of innate immune function and microbial clearance.

TLR activation is a double-edged sword¹⁸. It is essential for provoking the innate response and enhancing adaptive immunity against pathogens, and is even involved in the pathogenesis of autoimmune and infectious diseases²⁶⁻²⁸. In contrast, it is now widely accepted that intestinal homeostasis is primarily maintained via TLR signaling, despite the constant exposure to various microbes^{5,6,8}. In enteric flora, LPS from the cell walls of gram-negative bacteria is well known to activate DCs. Excess activation of DCs by LPS typically leads to endotoxin shock and serious systemic disorders with a high mortality rate in human and experimental animals^{29,30}, whereas normal intestine exposed to LPS does not exhibit endotoxin shock. As suggested in this study, LPS tolerance is one of the mechanisms controlling the development of IBD. Moreover, a recent study reported that different modes of DC activation is the central processor and a key effector in colonic homeostasis³¹. Therefore, modulating DCs and TLR signaling pathways represent potential methods for controlling intestinal inflammation.

Continuous activation of the immune system by ubiquitous pathogens would overwhelm the host. Therefore, the negative regulation of TLR signaling and function is necessary. It is also possible that the aforementioned disease states result from either

overactivation of TLRs or dysregulation of endogenous TLR-signaling inhibition^{32,33}. One of the negative regulators of TLR4 signaling is IRAK-M, which is an inhibitor of IRAK-1/IRAK-4 signaling specifically expressed in monocyte lineages²⁴. As shown previously^{23,34}, IRAK-M expression is associated with LPS tolerance in DCs. Although it should be noted that other factors, such as A20 or NF- κ B p50, also influence LPS tolerance^{35,36}, IRAK-M appears to have a substantial role in the cascade. Further experiments such as the use of IRAK-M^{-/-} mice in the experimental colitis model are needed in order to confirm whether IRAK-M is involved in colonic homeostasis, and whether it is an effective target for modulating the immune system in IBD.

There has been a rapid increase in the prevalence of IBD in industrialized countries³⁷. The hygiene hypothesis proposes that the present clean surroundings in less exposure to bacteria are involved in development of this disease³⁷. Similarly to IBD, epidemiological data in humans support a differential dose model, with endotoxin exposure correlated with both increased and decreased incidences and severities of lung diseases, such as bronchial asthma³⁸. Furthermore, LPS tolerance is thought to be involved in the development of these diseases³⁹. In our studies, we observed that LPS tolerance is able to protect mice from acute intestinal inflammation. Further experiments using chronic colitis models that also examine commensal bacteria, such as colitis in IL-10^{-/-} mice, are needed, as aberrant LPS tolerance and less DC modulation may be causative factors in IBD.

In conclusion, LPS tolerance has a protective effect against experimental colitis and IRAK-M is one of the most likely candidates to be involved in the observed phenomenon. Our data also suggest that IBD results from a defect in LPS tolerance or negative regulators of TLR signaling, and that modulating this signaling pathway may provide a novel treatment approach for this disease.

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COMPETING INTEREST STATEMENT

The authors declare that they have no competing financial interests.

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