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C-Type Lectin SIGN-R1 Has a Role in Experimental Colitis and Responsiveness to Lipopolysaccharide

Sean P. Saunders,* Jillian L. Barlow,† Caitriona M. Walsh,* Agustin Bellsoi,† Philip Smith,* Andrew N. J. McKenzie,† and Padraic G. Fallon*

Pathogen recognition receptors (PRRs) function to maintain the balance between controlled responses to pathogens and uncontrolled innate immune activation leading to inflammation. In the context of commensal bacteria and the etiology of inflammatory bowel disease, although a role for the TLRs is known, there is a less defined function for C-type lectin receptors (CLRs). We demonstrate that mice deficient (−/−) in the CLR specific intracellular adhesion molecule-3 grabbing nonintegrin homolog-related 1 (SIGN-R1) (CD209b) have reduced susceptibility to experimental colitis, with a reduction in the disease severity, colon damage, and levels of the proinflammatory cytokines IL-1β, TNF-α, and IL-6. To determine whether SIGN-R1−/− mice had a systemic defect in innate activation, we examined the responsiveness of macrophages from SIGN-R1−/− mice to TLR ligands. SIGN-R1−/− peritoneal macrophages, but not bone marrow-derived macrophages, have a specific defect in IL-1β and IL-18 production, but not other cytokines, in response to the TLR4 ligand LPS. In vivo SIGN-R1−/− mice had significantly reduced susceptibility to LPS-induced shock. To address the synergistic relationship between SIGN-R1 and TLR4 in the context of experimental colitis, SIGN-R1/TLR4−/− mice were generated. SIGN-R1/TLR4−/− mice displayed reduced susceptibility to experimental colitis relative to severity of disease observed in wild-type or TLR4−/− mice. The in vivo use of a blocking mAb confirmed a functional role for SIGN-R1 in LPS-induced shock and experimental colitis. These data indicate a role for SIGN-R1 in the regulation of inflammation in a model of experimental colitis and illustrate that SIGN-R1 is a critical innate factor in response to LPS. The Journal of Immunology, 2010, 184: 2627–2637.

Inflammatory bowel disease (IBD) is a chronic disabling disorder of the gastrointestinal tract and includes the clinically subdivided conditions of Crohn’s disease (CD) and ulcerative colitis. The etiology of IBD is not fully determined. IBD arises from an inappropriate immune response to the resident intestinal microflora in genetically predisposed individuals (1). In humans, some 300–400 m² of the gastrointestinal mucosal surface area are in constant contact with potential pathogens, including ~10¹⁴ bacteria/g of gut tissue (2). At the interface between the intestinal epithelium and luminal contents, pathogen recognition receptors (PRRs) play a crucial role in the fine balance between maintaining immune tolerance to commensal bacteria, and uncontrolled innate immune activation leading to inflammation (3). The TLRs are the most extensively studied family of PRRs (4). TLRs recognize and bind pathogen associated molecular patterns (PAMPs) from various pathogens and thereby trigger activation of signaling pathways and induction of genes including proinflammatory cytokines (5). In the context of IBD, TLRs are expressed on intestinal epithelial cells, APCs and T cells within the lamina propria, functioning as both sentinels differentiating between commensal bacteria and pathogens in the lumen, and in recognition of the ingress of pathogen across the epithelium.

There is evidence that TLRs may cooperate with other classes of PRR in immunity (6). C-type lectin receptors (CLRs) represent another family of PRR that bind PAMP carbohydrate structures and contribute to innate and adaptive immunity (7). With respect to human infectious diseases, the most widely investigated CLR is dendritic cell (DC)-specific intracellular adhesion molecule-3 grabbing nonintegrin (SIGN) (CD209), which has been shown to recognize various pathogens, in particular certain mycobacteria, fungi, and viruses (8). Following binding of PAMPs by DC-SIGN, a signaling pathway is initiated that modulates TLR3-, 4- and 5–induced cytokine responses (9). Interestingly, a member of a family of DC-SIGN homologs described in mice, SIGN-related 1 (R1)/CD209b (10, 11), has also been shown to interact with the TLR signaling system, with SIGN-R1 associating with the TLR4-MD2 complex to enhance signal transduction in response to LPS in Gram-negative bacteria in vitro (12). Unlike DC-SIGN, SIGN-R1 is not expressed on DCs but is on splenic marginal zone, lymph node, and peritoneal macrophages (pMφs) (13–15) and can recognize glycans from different pathogens (16).

In view of the potential role for innate immunity and CLRs in regulation of intestinal inflammation, we evaluated the susceptibility of mice deficient in SIGN-R1 (17) to experimental colitis induced by treatment with dextran sodium sulfate (DSS). Because TLRs play an integral role in the pathogenesis of IBD, we sought to investigate the role of SIGN-R1 in response to TLR ligands. This study illustrates the potential for synergy between CLRs and TLRs in initiation and control of inflammatory responses.

Materials and Methods

**Mice**

SIGN-R1−/− mice (17) were backcrossed for more than eight generations to C57BL/6J strain. TLR2−/−, TLR4−/−, and MyD88−/− mice, on a congenic background.
PBS and mounted with Vectashield mounting medium (Vector Laboratories, was included in the last incubation with Abs. Finally, samples were washed in aFluor546 (Invitrogen, Carlsbad, CA). 7-Aminoactinomycin D (eBioscience) Tissue were then rinsed and incubated with streptavidin conjugated with Alex-egend San Diego, CA), and anti-mouse SIGN-R1-AlexaFluor647 (eBioscience). B220-Pacific Blue (eBioscience), anti-mouse CD4-biotin (clone RM4-5; Biol-conjugated Abs: anti-mouse CD11b-Pacific Blue (eBioscience), anti-mouse

in PBS for at least 30 min. After a brief rinse in PBS, sections were incubated with

in PBS for 15 min at room temperature and blocked with a 10% solution of FBS

first incubated in 4˚C acetone for 10 min and air-dried. The slides were then rinsed

with 1

g/ml; InvivoGen), MFG, 0.1 mg/ml; InvivoGen), and Taxol (25 µM; Sigma-Aldrich). Spermatozoids were harvested, and cytokine production was quantified using ELISA. ELISA reagents for detec-

tion of IL-6 and IL-12p40 were obtained from BD Biosciences (San Jose, CA), reagents for IL-1β, IL-10, and Tnf-α were from R&D Systems, and IL-18 was from MBL (Nagoya, Japan).

Flow cytometry

Following peritoneal lavage, non-B/non-T cells were isolated from wild-type and SIGN-R1−/− mice by negative depletion using Dynabeads. Cells were used immediately or incubated for 24 h at 37˚C either with media alone or with 1 µg/ml LPS. Cells were stained with anti–CD11b-FITC (clone Mac-1; eBioscience, San Diego, CA), anti–SIGN-R1-AlexaFluor647 (clone 22D1; eBioscience), anti–TLR4-PE (clone MTS510; eBioscience) mAb and a hamster IgG isotype control (clone eBio229Arm; eBioscience).

Immunofluorescence on cryosections and confocal microscopy

Spleens or intestines were fixed in a solution of 1% paraformaldehyde in PBS at 4˚C for 3 h. Organs were then incubated for at least 2 h in a solution of sucrose 10%, 30%, and 70% in PBS (from low to high), respectively. The organs were then placed in optimal cutting temperature medium, frozen on dry ice, and stored at −70˚C until processed. Cryosections of 7 µm were cut at −20˚C (Leica Microsystems, Deerfield, IL), placed on poly-l-lysine–coated slides (VWR International, West Chester, PA), and air-dried overnight at room temperature. Samples were then stored at −20˚C or used for immunofluorescence. For this purpose, samples were first incubated in 4˚C acetone for 10 min and air-dried. The slides were then rinsed in PBS (three times) at room temperature and blocked with a 10% solution of PBS in PBS for at least 30 min. After a brief rinse in PBS, sections were incubated with conjugated Abs: anti-mouse CD11b-Pacific Blue (eBioscience), anti-mouse B220-Pacific Blue (eBioscience), anti-mouse CD4-biotin (clone RM4-5; Biologend San Diego, CA), and anti-mouse SIGN-R1-AlexaFluor647 (eBioscience). Tissue were then rinsed and incubated with streptavidin conjugated with Alexa-

af546 (Invitrogen, Carlsbad, CA). 7-Aminobenzoic acid D (eBioscience) was included in the last incubation with Abs. Finally, samples were washed in PBS and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Images were taken on a Carl Zeiss inverted microscope (LSM 710) and processed with ZEN 2008 (Carl Zeiss, Oberkothen, Germany).

DSS-induced colitis model

Colitis was induced by DSS treatment of mice (18, 19). Mice were treated with 2.5% DSS (35,000–50,000 kDa; MP Biomedical, Cleveland, OH) dissolved in deionized water for normal water. Body weight, food intake, occult blood in feces, and stool consistency/diarrhea were recorded daily for each mouse to determine the disease activity index (DAI), based on previous studies of DSS-induced colitis (18). The maximum DAI score was 12 based on assigning 1–4 scoring system for each parameter: score 0, no weight loss, normal stool, and no blood; score 1, 1–3% weight loss; score 2, 3–6% weight loss, loose stool (a loose stool was defined as the formation of a stool that readily becomes compacted upon handling), and blood visible in stool; score 3, 6–9% weight loss; and score 4, <9% weight loss, diarrhea, and gross bleeding. Blood in the feces of individual mice was detected using Hemdetect kits, as described previously (18). When mice were killed, the lengths of the colon were mea-

sured. For in vivo anti–SIGN-R1 mAb treatment, mice were injected i.p. with 50 µg anti–SIGN-R1 mAb (ER-TR9; BMA Biomedical) or control rat IgM (Serotec, Oxford, U.K.) each day from days 0 to 9. Mice were treated with 2.5% DSS and analyzed as described above.

Colon histology

An ~1-cm length of the distal colon was removed and fixed in 10% formaldehyde saline. The remaining colon was immediately snap frozen and stored at −80˚C. Tissue was embedded in paraffin, and for each mouse, three 5-µm sections were cut, at two levels separated by ~100 µm, and stained with H&E. All histology sections were examined in a blinded fashion by two observers independently. An arbitrary histological scoring system was used to quantify colon damage (18). A maximum combined score of 10 was determined from the severity of inflammatory cell infiltration (score 0, none; score 1, slightly dispersed cell infiltrate; score 2, moderately increased cell infiltrates forming occasional cell foci; and score 3, severely large areas of cell infiltrates causing loss of tissue architecture), extent of injury (score 0, none; score 1, mucosal; score 2, mucosal and submucosal; and score 3, transmural), and crypt damage (score 0, none; score 1, basal one-third damaged; score 2, basal two-thirds damaged; score 3, only surface epithelium intact; and score 4, loss of entire crypt and epithelium).

Colon cytokine and MPO analysis

Colons were homogenized using methods adapted from processing lung tissue (20). The protein content of colon extracts was determined. Levels of IL-1β, IL-6, IL-10, Tnf-α, and IL-12p40 were detected in colon extracts using ELISA (20). Myeloperoxidase (MPO) activity was detected using ODP as substrate and data interopolated from a MPO standard curve (Sigma-Aldrich). Levels of cytokines and MPO were expressed as picograms per milligram or units per milligram, respectively, relative to colonic protein.

LPS-induced shock model

SIGN-R1−/−, TLR4−/−, SIGN-R1/TLR4−/−, and wild-type mice had micro-

chip (Avid, Norco, CA) identity tags inserted s.c. to track individual mice, with the experimenter blind to the genotype of all the mice. The identity tags are preprogrammed with a unique set of digits that are generated and maintained within a battery-free and biologically inert, 2 × 13-mm biocompatible glass capsule. Microchip tags were implanted with a modified 3-cc Monoject syringe by s.c. injection. Mice from each group were randomized in boxes before treatment. Mice were injected i.p., with 10 or 20 mg/kg ultrapure LPS. A clinical score of disease severity for the assessment of endotoxin shock was applied: score 1, defined as piloerection fur and no behavior change; score 2, huddle reflex and responsive to stimuli; score 3, slower response to stimuli and docile; score 4, lack of curiosity, little or no response to stimuli, and immobile; and score 5, laboring breathing and moribund. Scores were assigned to each animal and averaged per group. Moribund mice with score 5 were humanely killed in compliance with animal health regulations. Sepsis scoring and mor-

tality were recorded for 96 h. Blood was removed 3 h after LPS challenge and serum isolated. As described above, ELISA was used to analyze serum levels of IL-1β, IL-18, IL-6, and Tnf-α. For in vivo anti–SIGN-R1 mAb treatment, mice were injected i.p., with 25 µg of anti–SIGN-R1 mAb (ER-TR9; BMA Biomedical, Augst, Switzerland) or control rat IgM (Serotec). LPS (10 mg/kg) was injected i.p. 30 min later, and mice were monitored as described above.

Statistical analysis

Statistical analysis was performed using GraphPad InStat. Results are presented as mean and SEM or SD where indicated. Differences, indicated as two-tailed p values, were considered significant when p < 0.05 as assessed by unpaired Student t test with Welch correction applied as necessary. The Kaplan–Meier method was used to evaluate survival in LPS-induced shock model.

Results

SIGN-R1−/− mice have a reduced susceptibility to DSS-induced colitis

To address the role of SIGN-R1 in colon inflammation, SIGN-R1−/− and wild-type mice were administered 2.5% DSS in the drinking water for 5 d and monitored for 10 d in total. Wild-type mice treated with DSS developed progressive weight loss from the fourth to eighth day, with ~20% reduction in body weight by day 10 (Fig. 1A). In wild-type mice, there was an increase in the Disease Activity Index (DAI), a combined scoring system for weight loss, diarrhea, and presence of blood in feces, from the third day of DSS exposure (Fig. 1B). In contrast, SIGN-R1−/− mice had less severe colitis, with no
more than a 5% loss in weight over the course of the treatment regimen (Fig. 1A). In addition, SIGN-R1 \(^{-/-}\) mice had a lower DAI, with a maximum score of 3 for SIGN-R1 \(^{-/-}\) mice relative to the DAI of 7–8 in wild-type animals. Although both groups of mice had significant (\(p, 0.05–0.0001\)) shortening of the colon following DSS treatment, the extent of shortening in length was significantly (\(p, 0.005\)) less in DSS-treated SIGN-R1 \(^{-/-}\) mice relative to wild-type–treated mice, which is consistent with the reduced severity of colitis (Fig. 1C,1D).

Furthermore, histology sections of the distal colon of DSS-treated mice showed that the SIGN-R1 \(^{-/-}\) mice had less DSS-induced inflammation and colon damage than comparably treated wild-type mice (Fig. 1E). Scoring of colon pathology confirmed the significantly reduced level of colon damage (\(p < 0.005\)) in DSS-treated SIGN-R1 \(^{-/-}\) mice relative to DSS-treated wild-type mice (Fig. 1F).

In homogenates of the colons, the levels of MPO activity, a marker for inflammation, was significantly (\(p < 0.05–0.0001\)) elevated in both SIGN-R1 \(^{-/-}\) and wild-type DSS-treated mice relative to corresponding untreated mice (Fig. 2A). However, MPO activity in DSS-treated SIGN-R1 \(^{-/-}\) mice was significantly lower (\(p < 0.0005\)) than in DSS-treated wild-type mice (Fig. 2A). Similarly, the levels of proinflammatory cytokines IL-1\(\beta\), IL-6, and TNF-\(\alpha\), which are associated with colon inflammation in the DSS colitis model, were elevated in all DSS-treated groups in comparison with cytokine detected in colons of untreated mice (Fig. 2B). However, consistent with the observed reduced pathology in SIGN-R1 \(^{-/-}\) mice, the levels of proinflammatory cytokines were significantly (\(p < 0.005–0.0001\)) lower in the colons of DSS-treated SIGN-R1 \(^{-/-}\) mice in comparison with DSS-treated wild-type mice (Fig. 2B). These data demonstrate that SIGN-R1 \(^{-/-}\) mice develop less severe colon inflammation relative to wild-type animals in the DSS model of colitis.

SIGN-R1 \(^{-/-}\) peritoneal macrophages have defective LPS-induced IL-1\(\beta\) and IL-18 production

In the DSS-induced colitis model, breakdown of the epithelial barrier leads to exposure to innate colon luminal PAMPs (21), leading to infiltration of macrophages into the lamina propria and, consequentially, increased proinflammatory cytokine expression in the colon (18, 22). To address whether SIGN-R1 \(^{-/-}\) mice develop ameliorated DSS-induced...
colitis because of a defect in Mϕ responsiveness to PAMP activation and proinflammatory cytokine production, we isolated Mϕs from the SIGN-R1−/− mice and exposed them to a panel of known TLR agonists in vitro. Flow cytometry confirmed expression of SIGN-R1 on pMϕs from wild-type animals (15) but not on pMϕs from SIGN-R1−/− mice (Fig. 3A). Strikingly, in response to ultrapure LPS, the secretion of IL-1β was significantly reduced in SIGN-R1−/− pMϕs, whereas levels of other proinflammatory cytokines IL-6, TNF-α, and IL-12p40, as well as the regulatory cytokine IL-10, produced in response to LPS was comparable to pMϕs from wild-type mice (Fig. 3B). In contrast, following

**FIGURE 3.** Impaired IL-1β and IL-18 production by pMϕs from SIGN-R1−/− mice in response to LPS. A, Flow cytometry detection of SIGN-R1 on CD11b+ pMϕs in wild-type but not SIGN-R1−/− mice. B, Production of IL-1β, IL-6, TNF-α, IL-12, and IL-10 by pMϕs from wild-type and SIGN-R1−/− mice cultured with media or stimulated with Pam3CSK4 (300 ng/ml), PGN (10 μg/ml), poly(I:C) (50 μg/ml), ultrapure LPS (10 ng/ml), or CpG (5 μg/ml). *p < 0.05 lower IL-1β produced in LPS-treated SIGN-R1−/− pMϕs relative to cells from wild-type mice. C, Cytokine production by BMMϕs treated as described for pMϕs. D, IL-1β production by pMϕs treated with ultrapure LPS (uLPS; 0.1, 1, and 10 ng/ml), crude standard LPS (sLPS; 10 ng/ml), or Taxol (25 μM). E, IL-18 production by pMϕs treated with ultrapure LPS (10 ng/ml). Data shown are mean ± SEM of triplicate wells and are representative of two to three separate experiments.
stimulation with other TLR agonists assayed [TLR1/TLR2 ligand, Pam3CSK4; TLR2 ligand, bacterial PGN; TLR3 ligand, synthetic dsRNA poly(IC); and TLR9 ligand, CpG-DNA]; levels of cytokine secretion were comparable in pMδs from wild-type and SIGN-R1/−/− mice (Fig. 3B), indicating that the defect in IL-1β secretion in pMδs from SIGN-R1/−/− mice is exclusive to signaling through TLR4. SIGN-R1 expression has not been observed on BMMδs (14, 15), and we confirmed the lack of SIGN-R1 expression by flow cytometry (data not shown). To confirm that the IL-1β defect in pMδs was indeed because of SIGN-R1 deficiency, we generated BMMδs from SIGN-R1/−/− and wild-type mice and stimulated them with a panel of TLR agonists. Secretion levels of all cytokines analyzed were comparable in BMMδs from both mouse genotypes (Fig. 3C), confirming that LPS-induced IL-1β production by pMδs is specifically regulated by SIGN-R1.

In contrast to the use of ultrapure LPS, devoid of contamination that can stimulate with crude LPS or the LPS-mimetic Taxol, which is known to signal through TLR4 (24), IL-1β production from pMδs from SIGN-R1/−/− mice was comparable to cells from wild-type mice (Fig. 3D), whereas there was a dose-dependent defect in IL-1β specific to ultrapure LPS (Fig. 3D). The specific defect in IL-1β secretion by pMδs from SIGN-R1/−/− mice raised the possible involvement of the inflammasome pathway, with IL-18 release, which is regulated by the inflammasome (25) similar to IL-1β, and diminished in pMδs from SIGN-R1/−/− mice (Fig. 3E). These data infer that the absence of SIGN-R1 expression on pMδs, there is a specific impaired production of IL-1β and IL-18 in response to the TLR4 ligand LPS.

SIGN-R1/−/− mice have reduced susceptibility to LPS-induced shock

The in vivo data showing a defect in IL-1β secretion by LPS-stimulated SIGN-R1/−/− pMδs (Fig. 3B, 3D) and in vitro findings by others showing an association between SIGN-R1 and the TLR4-MD2 complex in response to LPS (12) prompted us to elucidate whether SIGN-R1/−/− mice had altered responses in vivo to LPS-induced shock. We first confirmed earlier studies (12) that LPS treatment upregulates SIGN-R1 expression on pMδs (Fig. 4A, 4B). Furthermore, as pMδs from SIGN-R1/−/− mice had comparable surface expression of TLR4 as wild-type mice (Fig. 4C), the reduced responsiveness to LPS was not because of absence of TLR4 on SIGN-R1/−/− pMδs. SIGN-R1/−/− and wild-type mice were injected with 10 mg or 20 mg/kg LPS, doses that cause ~50 or ~90% mortality in wild-type mice, respectively. SIGN-R1/−/− mice developed reduced clinical signs of shock after LPS challenge, as determined by the bactericidal activity of circulating phagocytes (data not shown), with significantly higher survival (p < 0.05) of SIGN-R1/−/− mice relative to wild-type animals at both LPS doses (Fig. 4D). Consistent with reduced susceptibility to LPS-induced shock, the levels of early proinflammatory cytokines IL-1β, IL-18, IL-6, and TNF-α in serum 3 h after challenge were significantly lower (p < 0.05–0.0001) in SIGN-R1/−/− mice relative to wild-type animals (Fig. 4E).

TLR4/−/− and SIGN-R1/TLR4/−/− mice are unresponsive to LPS

To further address the interplay between SIGN-R1 and TLR4 in recognition of LPS, SIGN-R1/−/− mice were crossed to TLR4−/− mice, generating SIGN-R1/TLR4/−/− mice, and pMδs responsiveness to PAMP activation and the production of proinflammatory cytokines were analyzed. Flow cytometry confirmed lack of TLR4 expression on pMδs from TLR4−/− and SIGN-R1/TLR4−/− mice, but its presence on wild-type and SIGN-R1/−/− cells (data not shown). In response to the TLR4 ligand LPS, the secretion of IL-1β, IL-6, and TNF-α was not detected in TLR4−/− or SIGN-R1/TLR4−/− pMδs. Following stimulation with other TLR agonists assayed [Pam3CSK4, PGN, poly(I:C), and CpG-DNA], levels of cytokine secretion were comparable in pMδs from wild-type, SIGN-R1/−/−, TLR4−/−, and SIGN-R1/TLR4−/− mice (Fig. 5A). As shown above (Fig. 3B, 3D), the secretion of IL-1β following LPS treatment was reduced in SIGN-R1−/− pMδs (Fig. 5A).

Following injection of wild-type, SIGN-R1−/−, TLR4−/−, and SIGN-R1/TLR4−/− mice with 20 mg/kg LPS, a dose that causes ~90% mortality in wild-type mice, there were significant (p < 0.001) differences in survival between groups (Fig. 4D). Although SIGN-R1−/− were partially protected from LPS-induced death, the TLR4−/− and SIGN-R1/TLR4−/− mice developed no mortalities (Fig. 5B) and no overt clinical signs of shock after LPS challenge (data not shown). Additionally, there were no detectable levels of proinflammatory cytokines IL-1β, IL-6, and TNF-α in serum of TLR4−/− and SIGN-R1/TLR4−/− mice 3 h after LPS challenge (Fig. 5C). In contrast, as shown above (Fig. 4E), SIGN-R1−/− mice had lower levels of proinflammatory cytokines IL-1β, IL-6, and TNF-α in the serum relative to wild-type animals (Fig. 5C).

Role for SIGN-R1 and TLR4 in susceptibility to colitis

SIGN-R1/−/−, TLR4/−/−, and SIGN-R1/TLR4/−/− mice were evaluated for susceptibility to colon inflammation after exposure to DSS-induced colitis. In view of the role of the epithelium in the DSS colitis model (26) and expression of TLR4 on intestinal epithelial cells (27), we confirmed by confocal microscopy (Fig. 6A) and Western immunoblotting (data not shown) that SIGN-R1 is not expressed on intestinal epithelial cells but is present on marginal zone Mδs in the spleen (Fig. 6A). As shown above, the pathological and immunological responses following DSS treatment of SIGN-R1−/− were attenuated relative to wild-type animals (Fig. 6). Following DSS treatment, TLR4−/− mice develop a dual spectrum of disease different from wild-type animals (28, 29). Although TLR4−/− mice developed overt colitis, the pathology was milder relative to wild-type animals with nonsignificant reduction in weight loss and less severe colon inflammation (Fig. 6B, 6F). Additionally, TLR4−/− mice differed from wild-type mice with respect to the DSS-induced damage to the colon, with significantly elevated DAI scores for the first 5 d of DSS exposure in TLR4−/− mice compared with wild-type mice (Fig. 6B). This was primarily due to marked rectal bleeding in TLR4−/− mice, which was not detected in other DSS-treated groups (Fig. 6D). In contrast to TLR4−/− mice, DSS treatment of SIGN-R1/TLR4−/− led to a reduced susceptibility to disease that was comparable to SIGN-R1−/− mice for all parameters addressed (Fig. 6).

All DSS-treated groups had a decrease in colon length compared with the untreated control groups (Fig. 6C, 6E). However, the length of colons of DSS-treated SIGN-R1−/− and SIGN-R1/TLR4−/− mice was significantly (p < 0.01–0.005) longer than DSS-treated wild-type and TLR4−/− groups (Fig. 6E). There was no difference in change in colon length between SIGN-R1−/− and SIGN-R1/TLR4−/− mice (Fig. 6C, 6E). Furthermore, quantification of colon pathology confirmed significantly decreased inflammation in SIGN-R1−/− and SIGN-R1/TLR4−/− mice compared with the wild-type and TLR4−/− mice, with reduced pathology in TLR4−/− mice (Fig. 6F). Levels of colon MPO support histological findings, with a significant decrease in MPO activity in SIGN-R1−/− and SIGN-R1/TLR4−/− mice compared with the wild-type and TLR4−/− groups (Fig. 7A). Again, no difference was observed between SIGN-R1−/− and SIGN-R1/ TLR4−/− mice. Levels of proinflammatory cytokines IL-1β, IL-6, and TNF-α in colon homogenates were significantly lower in SIGN-R1−/− and SIGN-R1/TLR4−/− mice compared with the wild-type and TLR4−/− groups (Fig. 7B). Therefore, SIGN-R1 deficiency protects mice from DSS-induced colitis independently of TLR4.

Blocking SIGN-R1 in vivo attenuates severity of LPS-induced shock and colitis

To validate data from SIGN-R1−/− mice showing an in vivo role for SIGN-R1 in response to LPS and in a DSS-induced model of colitis, we used an anti-SIGN-R1 mAb (ER-TR9), which blocks...
binding to SIGN-R1 in vivo (14, 30). In the LPS-induced shock model, C57BL/6J mice were injected with anti–SIGN-R1 mAb or a control mAb, 30 min before LPS challenge. Mice treated with anti–SIGN-R1 mAb developed transient overt clinical disease relative to mice injected with the control mAb, with no mortalities (p, 0.05) in anti–SIGN-R1 mAb-treated mice compared with control animals (Fig. 8A). Therefore, blocking SIGN-R1 in mice reduces the magnitude of LPS-induced proinflammatory cytokine shock.

As we have also shown reduced severity of disease in SIGN-R1−/− mice in the DSS model of colitis (Figs. 1, 5), C57BL/6J mice were injected with anti–SIGN-R1 mAb or a control mAb daily for 9 d during DSS treatment. Treatment of mice with anti–SIGN-R1 mAb attenuated the pathological and immunological responses relative to control mAb DSS-treated animals (Fig. 8B–D). Anti–SIGN-R1 mAb-treated mice had ~5% loss in weight over the course of the treatment regimen (Fig. 8A) and lower DAI, with a maximum score of 2 relative to the DAI of 6–7 in control mAb DSS-treated animals (Fig. 8C). Mice treated with blocking SIGN-R1 mAb had significantly (p < 0.05) longer colon lengths relative to control mAb DSS-treated animals (Fig. 8D). Furthermore, scoring of histology sections of the colon confirmed reduced colon damage in anti–SIGN-R1 mAb-treated mice (data not shown). The use of a mAb that blocks SIGN-R1 validates in vivo data from SIGN-R1−/− mice.

Discussion

IBD pathogenesis arises after a compromise in the integrity of the intestinal barrier leading to dysregulation in the innate immune response to commensal bacteria (26). In this study, we have shown that mice deficient in the macrophage-expressed C-type lectin SIGN-R1 have

**FIGURE 4.** A role for SIGN-R1 in susceptibility to LPS-induced shock. A, Representative flow cytometry images showing upregulation of SIGN-R1 on CD11b+ pMφs from wild-type mice following LPS treatment in vitro. SIGN-R1−/− cells were also treated with LPS. B, Histogram of SIGN-R1 expression on CD11b+ pMφs from wild-type mice. C, Histogram of TLR4 expression on CD11b+ pMφs from wild-type and SIGN-R1−/− mice. Staining of TLR4−/− pMφs was included as a control. D, Kaplan-Meier survival curves of wild-type and SIGN-R1−/− mice after i.p. injection with ultrapure LPS (10 or 20 mg/kg). E, Serum levels of IL-1β, IL-18, IL-6, and TNF-α 3 h after i.p. challenge with 10 mg/kg LPS. Data are mean ± SE (six to nine mice per group) and are representative of two separate experiments. Statistical differences between serum cytokines were determined by Student t tests.
FIGURE 5. TLR4−/− and SIGN-R1/TLR4−/− mice are unresponsiveness to LPS. A. Production of IL-1β, IL-6, and TNF-α by pMφs from wild-type, SIGN-R1−/−, TLR4−/−, and SIGN-R1/TLR4−/− mice cultured as in Fig. 3. *p < 0.05 lower IL-1β produced in LPS-treated SIGN-R1−/− pMφs relative to cells from wild-type mice. Data shown are mean ± SEM of triplicate wells and are representative of two to three separate experiments. B. Kaplan-Meier survival curves of wild-type, SIGN-R1−/−, TLR4−/−, and SIGN-R1/TLR4−/− mice after i.p. injection with 20 mg/kg ultrapure LPS. C. Serum levels of IL-1β, IL-6, and TNF-α 3 h after i.p. challenge with 20 mg/kg LPS. Data are mean ± SE (six to nine mice per group) and are representative of two separate experiments. Statistical differences between serum cytokines was determined by Student t tests.

Reduced susceptibility to experimental colitis. We also show in an experimental model of LPS-induced endotoxin shock that SIGN-R1−/− mice have reduced early systemic production of proinflammatory cytokines and are partially protected from LPS-induced mortality. Our data suggest that amelioration in disease in SIGN-R1−/− mice arises from an innate defect in recognition of LPS. This was demonstrated ex vivo with pMφs from SIGN-R1−/− mice having attenuated LPS-induced production of IL-1β and IL-18 but responding normally to other TLR ligands tested. These data suggest a role for the C-type lectin SIGN-R1 in recognition of LPS and regulation of inflammation in a model of experimental colitis. Furthermore, the use of an anti–SIGN-R1 mAb that blocks in vivo binding to SIGN-R1 (14, 30) ameliorated LPS-induced shock and the development of acute DSS-induced colitis, thus validating functional data from SIGN-R1−/− mice.

IBD may arise following a loss of barrier integrity and consequential activation of the innate immune system by exposure to the indigenous intestinal flora, evoking inflammation as a result of cell infiltration of the colon and resulting proinflammatory cytokine release (1). In the colitis model used in this study, DSS, a sulfated polymer, induces intestinal injury by a direct toxic effect on epithelial cells of the basal crypt, with a loss of integrity in the mucosal barrier allowing infiltration of intestinal bacteria (21). Subsequently, colon inflammation develops with an infiltration of macrophages, neutrophils, and eosinophils into the colon and increased production of the inflammatory cytokines IL-1β, TNF-α, and IL-6, which contribute to the inflammation and damage to the colon (21, 31). In this study, the severity of all DSS-induced pathology was significantly reduced in SIGN-R1−/− mice relative to wild-type animals, suggesting that recognition of bacterial surface carbohydrates by SIGN-R1 may contribute to the development of intestinal inflammation. Although SIGN-R1 is a murine homolog of human DC-SIGN, these data do not infer a role for DC-SIGN in IBD. However, in CD patients DC-SIGN–positive DCs have been reported in the colon and mesenteric lymph nodes (32–34). DCs are now thought to have a significant role in the pathogenesis of IBD (35). The CD209 gene encoding for DC-SIGN is located on the genetic region 19p13, a gene locus, which has been associated with linkage to IBD (36), but a CD209 functional polymorphism (rs4804803) was not associated with susceptibility to IBD (37). Nevertheless, the data presented in this study would suggest further investigation is warranted in to the potential for DC-SIGN to have a role in IBD.

In the context of the role of PRRs in DSS-induced colitis, the TLR family has been investigated using TLR2−/− and TLR4−/− and MyD88−/− mice (29, 38). Previous studies have shown that TLR4−/− mice develop a more severe clinical phenotype in response to DSS-induced colitis with colonic bleeding and increased weight loss relative to wild-type mice (29, 39, 40). In our hands, upon DSS treatment, TLR4−/− mice also developed more severe rectal bleeding relative to wild-type mice following DSS treatment, despite having an overall reduction in colon pathology. TLR4−/− mice develop two aspects of altered disease: with both a reduction in inflammation in the colon and paradoxically impaired mucosal repair leading to epithelial damage. A decrease in colon inflammatory infiltration has been reported in TLR4−/− mice with a marked reduction in neutrophils (29, 40), but these studies do not show the same amelioration of colon pathology in TLR4−/− as was observed in our data. Such differences between studies on TLR4−/− mice may be explained by the two recent reports (41, 42) highlighting the role of intestinal microbiota in modulation of the immune response and the variations in intestinal microbiota component populations between mice of the same strain but bred in different facilities. Disparities in the resident microbiota between animal facilities will have a significant impact on the repertoire and magnitude of the intestinal flora, evoking inflammation as a result of cell infiltration of the colon, with activation of the innate immune system by exposure to the indigenous flora, evoking inflammation as a result of cell infiltration of the colon, with activation of the innate immune system by exposure to the indigenous flora, evoking inflammation as a result of cell infiltration of the colon, with activation of the innate immune system by exposure to the indigenous flora, evoking inflammation as a result of cell infiltration of the colon, with activation of the innate immune system by exposure to the indigenous flora, evoking inflammation as a result of cell infiltration of the colon, with activation of the innate immune system by exposure to the indigenous flora, evoking inflammation as a result of cell infiltration of the colon, with activation of the innate 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TLR4, in the context of the proinflammatory cascade as induced in this experimental model of colitis.

As TLR4 signals through MyD88, in common with TLR2, we attempted to address the implication of SIGN-R1 cooperating with other innate mediators by generating mice with a combined deficiency in SIGN-R1 and MyD88 or TLR2 and SIGN-R1. SIGN-R1/MyD88 and SIGN-R1/TLR2 mice were produced, and we noted no additional in vivo or in vitro effects than previously reported with the respective individual gene-deficient mice (data not shown). Male and female SIGN-R1+/− MyD88−/− crosses were established, but despite genotyping >200 pups, although MyD88−/− mice were produced, we were unable to generate MyD88−/− and SIGN-R1 heterozygous or homozygous offspring. Previously, it has been reported that MyD88 homozygous mice are nonviable when crossed to mice that express a pathogenic mutant of superoxide dismutase 1, but MyD88−/− mice were successfully generated expressing a lower pathogenic superoxide dismutase 1 mutant (43). Therefore, the failure to generate dual MyD88−/− and SIGN-R1 heterozygous or homozygous mice suggests a critical role for this gene combination in development and survival, although we have no further data to support this.

FIGURE 6. SIGN-R1/TLR4−/− mice have reduced severity of DSS-induced colitis. A, Confocal microscopy immunofluorescence of intestine cryosections stained for SIGN-R1 (blue), CD11b (red), and 7-aminoactinomycin D (green); spleen cryosections stained for SIGN-R1 (blue), CD4 (orange), and B220 (red). Original magnification ×200. Wild-type, SIGN-R1−/−, TLR4−/−, and SIGN-R1/TLR4−/− mice were exposed to DSS as described in Fig. 1. B, Daily changes in weight and DAI. C, Representative images of gross appearance of the colon from untreated wild-type mouse or DSS-treated mice groups showing relative shortening in colon length and marked hemorrhagic colon in TLR4−/− mice. D, Detection of blood (blue) in feces of five of five TLR4−/− mice but not all other groups of mice after 5 d treatment with DSS. E, Colon lengths of untreated and DSS-treated mice. F, Representative histological images of colon from DSS-treated mice (H&E stained; original magnification ×200). G, Histology scores of colon cross-sections. Data are mean ± SE (four to nine mice per group) and are representative of three separate experiments. Statistical differences between groups were determined by Student t tests.
More severe colitis was recently observed in mice deficient in macrophage galactose-type C-type lectin-1 (MGL1/CD301) following DSS treatment (44). This contrasts with our data where mice deficient in SIGN-R1 had reduced disease in the same model. As MGL1 and SIGN-R1 differ in cellular expression, and also in their ligand recognition, it highlights the diversity of roles for different CLRs in pathogen recognition and inflammatory responses. The exacerbated colitis in MGL1−/− mice was attributed to the absence of an IL-10–producing macrophage population in deficient mice (44). Intriguingly, regulation of IL-10 production via CLRs has also been previously reported for Dectin-1 and also DC-SIGN (45–48).

With respect to DC-SIGN, engagement of ligands from a range of different pathogens activated a pathway that modulated signaling by various TLRs and altered IL-10 gene transcription through a TLR-dependent mechanism (9). In SIGN-R1−/− mice, we have seen no defect in IL-10 production in this study or indeed in previous work on helminth-infected SIGN-R1−/− mice (49). Others have reported reduced IL-10 production in SIGN-R1−/− mice (48), but this may reflect differences in the pathogen ligand interacting with SIGN-R1.

Previous in vitro work has demonstrated that SIGN-R1 associates with the TLR4-MD2 complex on the plasma membrane and SIGN-R1 binds *E. coli* LPS, enhancing both TLR4 oligomerization and downstream signaling via IκB-α degradation (12). It was shown that anti-SIGN-R1 Ab treatment blocks cytokine production from pMøs (12). Although we see a specific defect in IL-1β and IL-18 production in pMøs from C57BL/6j SIGN-R1−/− strain mice, they reported a pan defect in all cytokines measured using anti-SIGN-R1 mAb-treated pMøs from C3H/HeN mice. Additionally, the previous use of whole *E. coli* (12) compared with the here-in use of ultrapure LPS, devoid of contaminants, also negates the direct comparison between both data sets, and may account for the discrepancy. LPS has been shown to increase expression of SIGN-R1 on mouse pMøs (12), as shown in this study (Fig. 4B). Our data show that pMøs from SIGN-R1−/− mice have diminished responses to TLR4 activation via LPS, but responded normally to other TLR ligands tested. As TLR4 expression is unaltered on SIGN-R1−/− mice (49), this may be TLR4 dependent in murine Kupffer cells (50), which is consistent with our data for SIGN-R1/TLR4−/− mice following LPS injection. This may be due to comparing the response of defined pMø cells ex vivo with the complex multicellular system in the in vivo environment.

Confirming the pivotal role played by TLR4 in LPS recognition, SIGN-R1/TLR4−/− mice are protected from LPS-induced shock and colitis. A, Clinical scores and Kaplan-Meier survival curves of wild-type mice treated with anti–SIGN-R1 (ER-TR9) mAb or a control mAb prior to LPS (10 mg/kg) treatment. Wild-type mice treated with anti–SIGN-R1 (ER-TR9) mAb, control mAb, or untreated, prior to exposure to 2.5% DSS in drinking water for 5 d, followed by 5 d on normal water, were assessed for daily weight changes (B), DAI (C), and lengths of colons (D) measured at autopsy. Data are mean ± SE (four to eight mice per group) and are representative of three separate experiments. Statistical differences between groups were determined by Student *t* tests.
response to LPS (data not shown). BMMØs, which do not express SIGN-R1 (15), have an intact capacity to respond to LPS, suggesting that the observed defect in SIGN-R1-deficient pMØ cytokine responsiveness is crucially dependent on the absence of the SIGN-R1 molecule. Thus, in wild-type mice, SIGN-R1 is expressed on pMØs and has a functional role in LPS recognition. In contrast, BMMØs respond to stimuli and function independent of SIGN-R1, because it is not expressed. This indicates that different MØ populations may have evolved different mechanisms of signal transduction, some which may require a synergistic cooperation with different classes of PRR and some which may not.

The observed specific defect in both LPS-induced IL-1β and IL-18 secretion, but not seen in levels of other cytokines assayed, is indicative of a dysregulation in a specific activation signaling pathway interacting in the cascade from TLR4-SIGN-R1 interaction with LPS. The reduced secretion of IL-1β and IL-18 by LPS-activated SIGN-R1−/− pMØs, with no effect on the production of TNF-α and IL-6, suggests that the defect is specific to inflammasome-mediated caspase-1 activation (24). This leads to an attenuation of the innate immune response and IL-1β– and IL-18–dependent recruitment of inflammatory mediators (51). Indeed, mice deficient in components of the inflammasome pathway such as apoptosis-associated speck-like protein containing a caspase recruiting domain (apoptosis-associated speck-like protein containing a caspase recruiting domain [ASC]) or caspase-1 have been shown to be refractory to LPS-induced toxicity (52, 53), similar to the data on SIGN-R1−/− mice in this study. Interestingly, in the case of the ASC−/− mice, Yamamoto et al. (53) show that upon LPS stimulation, macrophages fail to process procaspase-1, resulting in a failure to process pro–IL-1β and pro–IL-18, with secreted levels of other proinflammatory cytokines, TNF-α and IL-6, not affected. Thus, the overlaps in the response of SIGN-R1−/− and ASC−/− mice, with the defect specific to IL-1β and IL-18 activation, and not other cytokines, are indicative of a similar defect associated with the inflammasome. LPS primes macrophages through TLR4 signaling leading to the synthesis and cellular accumulation of pro–IL-1β in an NF-κB–dependent manner (24). We postulate that SIGN-R1 may be a secondary activation signal in response to LPS, acting in synergy with TLR4 molecules; whether this is a result of co-localization on the plasma membrane or via intracellular cross-talk mechanisms remains undetermined. It is possible that SIGN-R1 upstream activation is involved in inducing inflammasome complex assembly, multimerization of the adaptor molecule ASC, and recruitment and cleavage of procaspase-1, thereby mediating release of IL-1β and IL-18 (54). Indeed, in a recent study, Ferwerda et al. (55) show synergy in IL-1β production in response to muramyl dipeptide stimulation between Nod2 and TLR4 proteins, which were meditated in a caspase-1–dependent manner, illustrating amplification of the inflammatory stimulus by multiple innate receptors.

The data strongly suggest SIGN-R1 involvement with inflammasome-mediated caspase-1 activation, yet undoubtedly, the mechanism of the signal transduction cascade via SIGN-R1 and TLR4, and the potential for interplay with the inflammasome, needs to be formally dissected. But what is evident is that SIGN-R1 is an additional innate factor critical to the response to LPS and the activation of a TLR4-initiated inflammatory response. The data illustrate a novel role for a CLR acting in synergy with a TLR in recognition of pathogen, thereby fully activating the innate immune response. In the context of colitis, as we verify an absence of SIGN-R1 expression on enterocytes, which express TLR4 (27), it is unlikely that SIGN-R1 interacts with TLR4 in the intestinal epithelium. It is interesting at this point to reconsider the ex vivo defect in IL-1β and IL-18 secretion by SIGN-R1−/− pMØs, in the context of SIGN-R1−/− mice being refractory to the DSS-induced colitis. A previous study using a mouse deficient in IL-1β–converting enzyme, which is critical for the processing of functional IL-1β and IL-18, showed that these mice were protected from DSS-induced colitis (54). Furthermore, use of an IL-18–blocking Ab also ameliorated the deleterious effects of DSS-induced colitis (56). Such studies are illustrative of the importance of inflammasome activation, as well as release of IL-18 and IL-1β, in the initiation of DSS-induced colitis and consistent for SIGN-R1 functioning in this pathway.

Increasingly, attention is focusing on using TLR agonists and antagonists, or the targeting of TLR downstream signaling, as a possible future therapeutic approach to ameliorate inflammation (57). As SIGN-R1−/− mice have less severe DSS-induced colitis and also reduced susceptibility to LPS-induced mortality, this illustrates the potential of targeting the CLR to disrupt signaling in innate immune responses that lead to inflammatory diseases. The use herein of a mAb that blocks SIGN-R1 in vivo has validated the strategy of targeting CLR as approach to suppressing LPS-TLR4 responses in endotoxin shock or gastrointestinal diseases. The relatively limited cellular distribution of CLR may lead to the development of future therapeutics, which may have a more focused efficacy than current therapies.

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Disclosures

The authors have no financial interests of interest.

References


Corrections


The last name of the fourth author was misspelled. The correct name should be Agustin Bellosi.

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