The NLRP3 Inflammasome Protects against Loss of Epithelial Integrity and Mortality during Experimental Colitis

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DOI 10.1016/j.immuni.2010.03.003

SUMMARY

Decreased expression of the Nlrp3 protein is associated with susceptibility to Crohn's disease. However, the role of Nlrp3 in colitis has not been characterized. Nlrp3 interacts with the adaptor protein ASC to activate caspase-1 in inflammasomes, which are protein complexes responsible for the maturation and secretion of interleukin-1β (IL-1β) and IL-18. Here, we showed that mice deficient for Nlrp3 or ASC and caspase-1 were highly susceptible to dextran sodium sulfate (DSS)-induced colitis. Defective inflammasome activation led to loss of epithelial integrity, resulting in systemic dispersion of commensal bacteria, massive leukocyte infiltration, and increased chemokine production in the colon. This process was a consequence of a decrease in IL-18 in mice lacking components of the Nlrp3 inflammasome, resulting in higher mortality rates. Thus, the Nlrp3 inflammasome is critically involved in the maintenance of intestinal homeostasis and protection against colitis.

INTRODUCTION

Human inflammatory bowel disease (IBD), comprising ulcerative colitis and Crohn's disease, constitutes a major health problem in developed countries (Fiocchi, 1998). Ulcerative colitis exhibits a characteristic profile of chronic inflammation involving the distal colon and rectum and is generally recognized as an immune-mediated disorder resulting from abnormal interaction between colonic microflora and mucosal immune cells (Goyette et al., 2007). Excessive inflammatory and immune responses in the intestine are thought to be due to a breach in the epithelial barrier in the gut that segregates commensal microflora from the host's systemic organs (Strober et al., 2002). Indeed, deterioration of the mucus layer of the colon is prominent in patients with ulcerative colitis (Podolsky and Isselbacher, 1984; Rhodes, 1996). In addition, studies in rodents have linked tissue damage and disruption of the epithelial barrier in the gut to cytokine imbalances (Bouma and Strober, 2003). The production of these inflammatory mediators has been implicated in the pathogenesis of experimental colitis and IBD in humans (Podolsky, 2002).

The synthesis and secretion of proinflammatory cytokines is governed by germline-encoded receptors such as the toll-like receptor (TLR) and nucleotide-binding domain and leucine-rich repeat containing (NLR) protein family (Kanneganti et al., 2007; Kopp and Medzhitov, 2003). TLRs are membrane-bound receptors that detect pathogen-associated molecular patterns (PAMPs) in the extracellular milieu (Kawai and Akira, 2007). TLR activation results in the rapid transcriptional activation of effector genes, including cytokines and chemokines that drive recruitment and/or activation of immune cells at mucosal surfaces. This immune cell recruitment is believed to play an important role in protecting against bacterial dissemination but may also underlie the clinical manifestations associated with inflammation as well as tissue damage therein. For instance, mice lacking the flagellin receptor TLR5 developed spontaneous colitis (Vijay-Kumar et al., 2007). Although mice deficient for the lipopolysaccharide (LPS) receptor TLR4, the lipoprotein receptor TLR2 or the TLR signaling adaptor MyD88 do not display an overt intestinal phenotype, they develop exacerbated injury upon exposure to dextran sodium sulfate (DSS) (Araki et al., 2005; Fukata et al., 2005; Rakoff-Nahoum et al., 2004).

In addition to TLRs, several members of the cytosolic NLR family have been identified as key regulators of cytokine production (Kanneganti et al., 2007). Notably, the gene that encodes the NLR protein CARD15 (also known as NOD2) was associated with Crohn's disease (Hugot et al., 2001; Ogura et al., 2001). NOD2 was subsequently shown to mediate activation of the transcription factor NF-κB and MAP kinases (Girardin et al., 2003; Inohara et al., 2003). The NLR protein Nlrp3 (also referred to as Nalp3, CIAS1, or Cryopyrin) is involved in activation of the cysteine protease caspase-1 (Lamkanfi et al., 2007). Homotypic interactions between the pyrin domain in the N terminus of Nlrp3 and the bipartite adaptor protein ASC (encoded by *Pycard*) bridge the association of caspase-1 to Nlrp3 in a large protein complex referred to as the ''inflammasome'' (Martinon et al., 2002). Activated caspase-1 processes the cytosolic precursors of the related cytokines interleukin-1β (IL-1β) and IL-18, thus allowing secretion of the biologically active cytokines. Hence, mice lacking caspase-1 are defective in the maturation and secretion of IL-1β and IL-18 (Ghayur et al., 1997; Kuida et al., 1995; Li et al., 1995). IL-1β participates in the generation of systemic and local responses to infection, injury, and immunological challenges by generating fever, activating lymphocytes, and promoting leukocyte infiltration at sites of injury or infection (Dinarello, 1996). Although IL-18 lacks the pyrogenic activity of IL-1β, it is involved in the induction of several secondary proinflammatory cytokines, chemokines, cell adhesion molecules, and nitric oxide synthesis (Horwood et al., 1998; Olee et al., 1999).

Gain-of-function mutations within *NLRP3* have been associated with three autoinflammatory disorders characterized by skin rashes and prolonged episodes of fever in the absence of any apparent infection. These hereditary periodic-fever syndromes are Muckle-Wells syndrome (MWS), familial cold autoinflammatory syndrome (FACS), and neonatal-onset multisystem inflammatory disease (NOMID), and they are collectively referred to as the Cryopyrin-associated periodic syndromes (CAPS) (Agostini et al., 2004). Functional studies revealed that the disease-associated *NLRP3* mutations enhance caspase-1 activation and IL-1β secretion (Dowds et al., 2004). In addition, decreased *NLRP3* expression and IL-1β production was recently linked with increased susceptibility to Crohn's disease in humans (Villani et al., 2009). However, the role of the Nlrp3 inflammasome in colitis has not been characterized. To understand the role of the Nlrp3 inflammasome in colitis, we studied the response of *Nlrp3*$^{-/-}$, *Pycard*$^{-/-}$, and *Casp1*$^{-/-}$ mice to DSS-induced colitis. Our results indicated a major role for the Nlrp3 inflammasome in protection against DSS-induced colitis and revealed its protective function in intestinal homeostasis.

RESULTS

Nlrp3 Protects from Mortality and Morbidity after DSS and TNBS Administration

Oral administration of DSS is directly toxic to the colonic epithelium (Kitajima et al., 1999) and triggers inflammation by disrupting the compartmentalization of commensal bacteria in the gut (Rakoff-Nahoum et al., 2004). To study the contribution of Nlrp3 to the development of colitis, we first assessed the mortality rate of age- and sex-matched wild-type and *Nlrp3*$^{-/-}$ mice after oral administration of 4% DSS in drinking water. Only 20% of wild-type mice died during the DSS administration period, but a mortality rate higher than 80% was noted for the *Nlrp3*$^{-/-}$ cohort (Figure 1A). The experiment was repeated with a lower DSS concentration (3%) to study the phenotype of *Nlrp3*$^{-/-}$ mice under milder conditions. *Nlrp3*$^{-/-}$ mice suffered from more body weight loss from day 5 on (Figure 1B). Simultaneously, stool consistency scores of *Nlrp3*$^{-/-}$ mice became significantly worse compared to those of DSS-fed wild-type mice (Figure 1C). Differences in rectal bleeding were also apparent between the two groups, with *Nlrp3*$^{-/-}$ mice displaying significantly elevated scores relative to DSS-administered wild-type controls starting as early as day 2 (Figure 1D). The evaluation of colon length is the parameter with the lowest variability in the model of DSS-induced colitis (Okayasu et al., 1990). To further assess the severity of colitis, colon length was measured in DSS-fed wild-type and *Nlrp3*$^{-/-}$ mice. Colons of *Nlrp3*$^{-/-}$ mice were on average 20% shorter than those of wild-type mice treated with DSS (Figure 1E; Figure S1A available online).

These clinical assessments were validated by histological examination of representative colon sections. In agreement with previous studies (Rakoff-Nahoum et al., 2004; Takagi et al., 2003), we observed marked histopathological changes in hematoxylin & eosin (H&E)-stained colons of DSS-treated wild-type mice characterized by crypt loss and infiltrating leukocytes (Figure 1F). However, only minimal evidence of necrosis and ulceration was evident in colons of wild-type mice. In contrast, colonic sections of DSS-fed *Nlrp3*$^{-/-}$ mice displayed severe transmural inflammation with focal areas of extensive ulceration and necrotic lesions. Inflammatory infiltrates filled the lamina propria and submucosa in areas where the mucosa was intact and often effaced the normal architecture of the tissue. Submucosal edema was often marked in areas of ulceration (Figure 1F). Semiquantitative scoring of these histological parameters confirmed that colitis severity in *Nlrp3*$^{-/-}$ mice was significantly higher than in wild-type mice (Figure 1G). Wild-type mice were attributed an overall histological score of 1.625 ± 0.27, whereas *Nlrp3*$^{-/-}$ mice were assigned a score of 3.78 ± 0.15 (Figure 1G). Consistent with the absence of disease in animals that were not fed DSS, no signs of inflammation or tissue damage were observed in colons of untreated wild-type and *Nlrp3*$^{-/-}$ mice (Figure S1B).

Intrarectal administration of 2,4,6-trinitrobenzenesulfonic acid (TNBS) represents an alternative model for the induction of acute colitis in mice through direct barrier destruction (Alex et al., 2009; Palmen et al., 1995). To assess whether Nlrp3 also exerts a protective role during acute TNBS-induced colitis, survival of wild-type and *Nlrp3*$^{-/-}$ mice was monitored for 5 days after intrarectal instillation of 150 mg/kg TNBS. As observed during acute DSS-induced colitis (Figure 1), *Nlrp3*$^{-/-}$ mice were significantly more susceptible to acute TNBS-induced mortality than wild-type mice (Figure S1C). In addition, macroscopic scoring of inflammation in colon confirmed that colitis severity in *Nlrp3*$^{-/-}$ mice was significantly higher than in wild-type mice (Figure S1D). Collectively, these results demonstrate that Nlrp3-dependent signaling is critical for protection against acute DSS- and TNBS-induced mortality and morbidity.

Nlrp3 Expression in Mucosal Epithelial Cells Is Critical for Protection against DSS-Induced Colitis

Nlrp3 is expressed in a wide range of immune cells as well as in epithelial cells (Kummer et al., 2007). To determine the cell populations that are critical for Nlrp3-dependent protection against DSS-induced colitis, we generated four groups of Nlrp3 bone marrow chimeras. In agreement with our previous results (Figure 1), *Nlrp3*$^{-/-}$ mice receiving *Nlrp3*$^{-/-}$ bone marrow presented with significantly worse symptoms of colitis relative to wild-type mice transplanted with wild-type bone marrow. Differences in clinical disease parameters between these groups such as body weight loss (Figure 2A), stool consistency (Figure 2B),

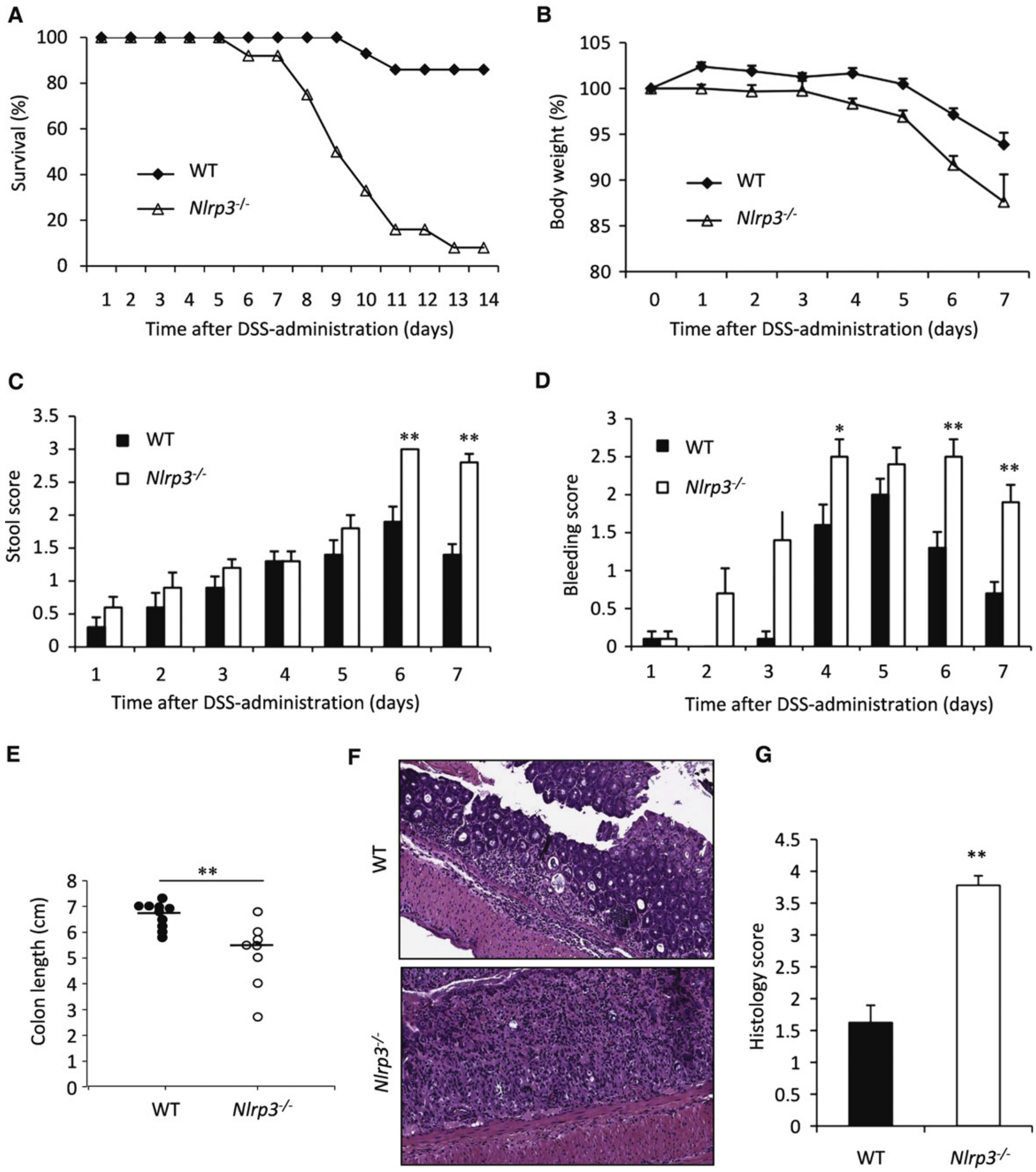

Figure 1. ***Nlrp3***$^{-/-}$ **Mice Are Hypersusceptible to DSS-Induced Colitis**

(A) Wild-type (n = 15) and *Nlrp3*$^{-/-}$ (n = 12) mice were fed a 4% DSS solution in drinking water for 5 days. Survival was monitored until day 14 after the start of DSS.

(B–G) Wild-type and *Nlrp3*$^{-/-}$ mice were treated with 3% DSS for 5 days, followed by regular drinking water for 2 days. (B) Body weight, (C) stool consistency, and (D) rectal bleeding score were scored daily.

(E) Mice were sacrificed on day 7 to measure colon length.

(F) At the same time, histopathological changes in colon tissue were examined by H&E staining.

(G) Semiquantitative scoring of histopathology was performed as described in Experimental Procedures. Data represent means ± SE of a representative experiment. *p < 0.05; **p < 0.01.

and colonic bleeding (Figure 2C) all reached statistical significance by day 7 after DSS administration. Incidence and severity of colitis in *Nlrp3*$^{-/-}$ mice receiving wild-type bone marrow was comparable to that of *Nlrp3*$^{-/-}$ mice transplanted with *Nlrp3*$^{-/-}$ bone marrow (Figures 2A–2C), suggesting that Nlrp3 expression in nonhematopoietic cells is more important for protection against colitis than Nlrp3 expression in leukocytes. Indeed, wild-type mice transplanted with *Nlrp3*$^{-/-}$ bone marrow were less sensitive to DSS-induced colitis and presented with body weight changes, diarrhea, and bleeding scores that were comparable to those of wild-type mice (Figures 2A–2C). The marked improvement in the clinical manifestation of colitis in the latter

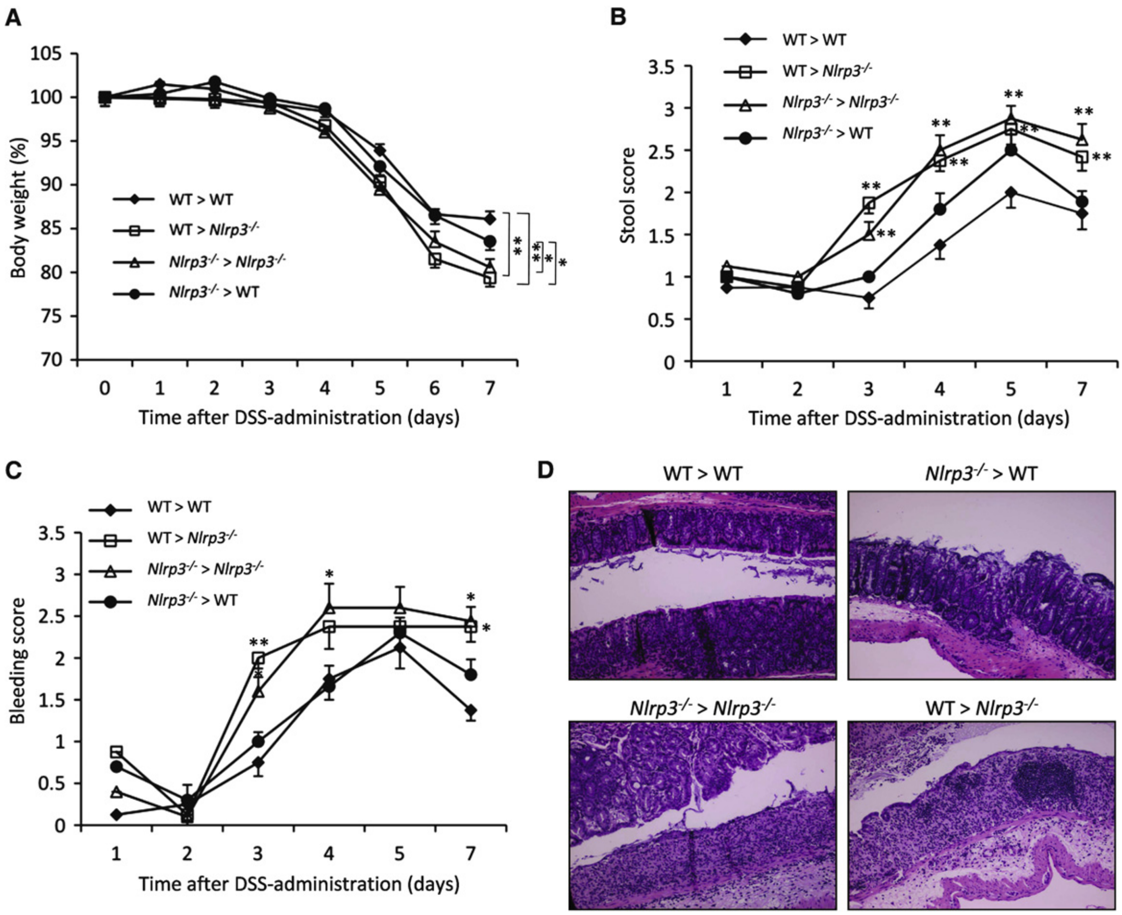

Figure 2. Nlrp3 Signaling in Nonhematopoetic Cells Is Critical for Protection against DSS-Induced Injury

Mice (n = 8–10/group) were treated with 3% DSS for 5 days, followed by regular drinking water for 2 days. (A) Body weight, (B) stool consistency, and (C) rectal bleeding were scored daily. (D) Mice were sacrificed on day 7 to examine histopathological changes in colon tissue by H&E staining.

groups was confirmed by less signs of severe histopathology in H&E-stained sections of the lamina propria of wild-type mice that received wild-type or *Nlrp3*$^{-/-}$ bone marrow (Figure 2D). In contrast, *Nlrp3*$^{-/-}$ mice presented with extensive crypt destruction and edema regardless of the Nlrp3 status of the transplanted bone marrow (Figure 2D, bottom). In agreement, colon homogenates of DSS-fed *Nlrp3*$^{-/-}$ recipients contained higher amounts of inflammatory cytokines and chemokines relative to wild-type recipients (Figure S2). Overall, these results suggest that Nlrp3 expression in local cells of the colonic mucosa is critical for protection against DSS-induced colitis.

Inflammasome Signaling Downstream of Nlrp3 Confers Protection against DSS-Induced Colitis

Nlrp3 recruits ASC and caspase-1 into a large protein complex termed the "inflammasome" (Kanneganti et al., 2007; Lamkanfi and Dixit, 2009). To determine whether Nlrp3 inflammasome activation is implicated in protection against colitis, we assessed the response of mice lacking the downstream inflammasome components ASC and caspase-1. Similar to *Nlrp3*$^{-/-}$ mice (Figure 1A), *Pycard*$^{-/-}$ and *Casp1*$^{-/-}$ mice were highly susceptible to DSS-induced colitis, with nearly all *Pycard*$^{-/-}$ and *Casp1*$^{-/-}$ mice dying within 2 weeks after administration of 4% DSS (Figure 3A). As seen with *Nlrp3*$^{-/-}$ mice, *Pycard*$^{-/-}$ and *Casp1*$^{-/-}$ mice displayed significantly more body weight loss (Figure 3B), higher stool consistency scores (Figure 3C), and rectal bleeding (Figure 3D) when fed on a milder regimen of 3% DSS. Moreover, the colon length of *Pycard*$^{-/-}$ and *Casp1*$^{-/-}$ mice was significantly reduced (Figure 3E; Figure S3A). Finally and as observed for *Nlrp3*$^{-/-}$ mice (Figure 1F), H&E-stained colon sections of DSS-fed *Pycard*$^{-/-}$ and *Casp1*$^{-/-}$ mice displayed severe transmural inflammation with focal areas of extensive ulceration and necrotic lesions (Figures 3F and 3G). The role of the Nlrp3 inflammasome in protection against DSS-induced colitis is not limited to the acute phase of disease as shown by the fact that *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$ mice also suffered from increased body weight loss, diarrhea, and reduced colon length during chronic disease (Figures S3B–S3F). These results demonstrate that Nlrp3 inflammasome activation is critical for protection against DSS-induced colitis.

IL-18 Maturation by the Nlrp3 Inflammasome Confers Protection against DSS-Induced Colitis

The Nlrp3 inflammasome is responsible for the maturation and secretion of the related cytokines IL-1β and IL-18 (Kanneganti et al., 2006; Mariathasan et al., 2006; Sutterwala et al., 2006).

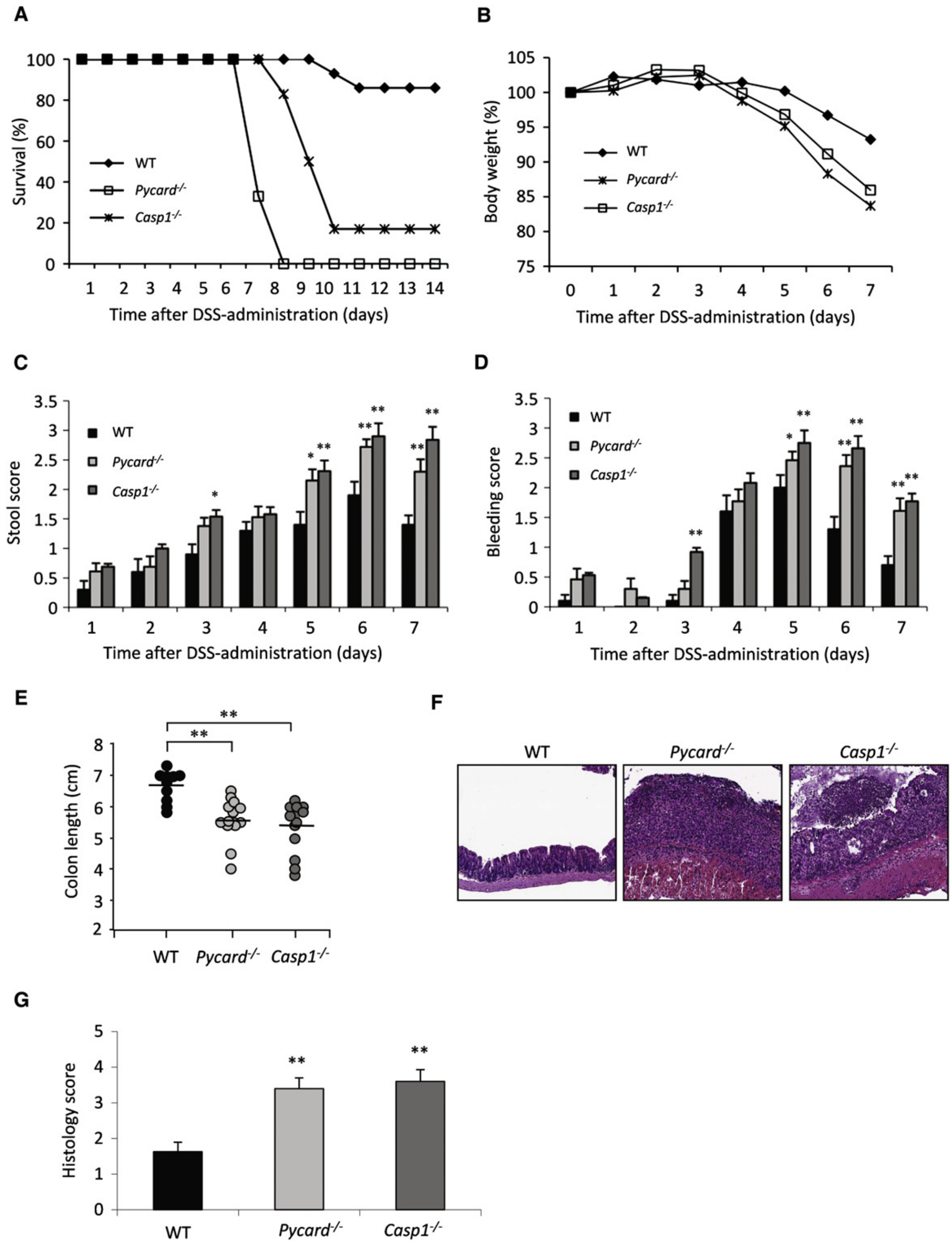

Figure 3. Essential Role for the Nlrp3 Inflammasome Components ASC and Caspase-1 in Protection against DSS-Induced Colitis

(A) Wild-type, *Pycard*$^{-/-}$, and *Casp1*$^{-/-}$ mice (n = 7–10) were fed a 4% DSS solution in drinking water for 5 days. Survival was monitored until day 14 after the start of DSS.

(B–G) Wild-type, *Pycard*$^{-/-}$, and *Casp1*$^{-/-}$ mice (n = 10–14) were fed a 3% DSS solution in drinking water for 5 days, followed by regular drinking water for 2 days.

(B) Body weight, (C) stool consistency, and (D) rectal bleeding were scored daily.

(E) Mice were sacrificed on day 7 to measure colon length.

(F) Histopathological changes in colon tissue were examined by H&E staining.

(G) Semiquantitative scoring of histopathology was performed as described in Experimental Procedures.

Data represent means ± SE of a representative experiment. $^*p < 0.05$; $^{**}p < 0.01$.

Notably, IL-18 has previously been associated with protection against DSS-induced colitis (Takagi et al., 2003). We therefore determined the amounts of IL-1β and IL-18 in serum of DSS-treated animals. IL-1β amounts in serum of wild-type, *Pycard*$^{-/-}$, and *Casp1*$^{-/-}$ mice barely rose above those of untreated animals at the three time points analyzed (days 1, 3, and 7; data not shown). Similarly, IL-1β amounts produced by colonic tissue from DSS-fed wild-type mice remained below 200 pg/ml, although caspase-1-deficient cells secreted even less IL-1β (Figure S4A). Unlike IL-1β, IL-18 was highly induced in the serum of DSS-treated wild-type mice, but not in *Pycard*$^{-/-}$ and *Casp1*$^{-/-}$ mice (Figure 4A). Local IL-18 production in the colon was also induced in response to DSS treatment as evidenced by the markedly increased IL-18 immunoreactivity (Figure 4B). In agreement with an important role for IL-18 downstream of the Nlrp3 inflammasome, colons of *caspase-1*$^{-/-}$ mice contained significantly less mature IL-18 relative to DSS-fed wild-type mice (Figure 4C). The results of the bone marrow chimera studies (Figure 2) suggested that cells of the colonic mucosa represent a critical site of Nlrp3 inflammasome activation during DSS-induced colitis. To provide additional support for the colonic mucosa as an important site for Nlrp3 inflammasome activation, we determined the amounts of mature IL-18 produced by isolated colonic epithelial cells. As in total colon extracts (Figure 4C), colonic epithelial cells isolated from DSS-fed *Casp1*$^{-/-}$ mice produced markedly less mature IL-18 than those of wild-type mice (Figure 4D). Isolated epithelial cells from colonic epithelia stained positive for the epithelial cell marker cytokeratin-18 (Figure S4B). Finally, we tested the role of IL-18 in protection against DSS-induced colitis. To this end, DSS-fed *Casp1*$^{-/-}$ mice received a daily injection of saline or 0.5 μg recombinant IL-18 for 4 consecutive days. In agreement with an important role for IL-18 downstream of the Nlrp3 inflammasome, *Casp1*$^{-/-}$ mice treated with recombinant IL-18 lost significantly less body weight when compared to those receiving PBS (Figure 4E). Thus, Nlrp3 inflammasome signaling through IL-18 confers protection against DSS-induced colitis.

The Nlrp3 Inflammasome Is Required for Preservation of Epithelial Integrity after DSS Administration

IL-18 has been linked to repair and restitution of ulcerated epithelium (Reuter and Pizarro, 2004), and colitis was previously shown to be more severe under conditions in which epithelial cell integrity is compromised (Rakoff-Nahoum et al., 2004). We therefore investigated the role of the Nlrp3 inflammasome in maintaining epithelial integrity in the gut. The intestinal barrier permeability in *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$ mice appeared normal prior to DSS treatment (Figure 5A). However, the Nlrp3 inflammasome is important for regulation of gastrointestinal permeability after DSS-induced injury because significantly more FITC-dextran was recovered in serum of DSS-treated *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$ mice (Figure 5A).

The decreased barrier function in the absence of Nlrp3 inflammasome signaling could be explained by increased apoptosis of epithelial cells and/or decreased cell proliferation. We first characterized the extent of apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. The number of TUNEL-positive cells in colonic tissue of DSS-treated *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$ mice was comparable to that of wild-type mice (data not shown), indicating that the absence of Nlrp3 inflammasome signaling does not affect apoptosis. 5′-bromo-2′-deoxy-uridine (BrdU) staining was subsequently used to determine the role of the Nlrp3 inflammasome in epithelial cell proliferation. The epithelial crypts of DSS-treated *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$ mice presented with significantly less BrdU-positive cells (Figures 5B and 5C). Untreated wild-type, *Nlrp3*$^{-/-}$, and *Casp1*$^{-/-}$ mice all showed comparable amounts of BrdU staining in colonic crypts, suggesting that the Nlrp3 inflammasome is specifically required for epithelial cell proliferation after DSS-induced injury. Therefore, activation of the Nlrp3 inflammasome induces a compensatory proliferative response of epithelial cells in order to preserve the integrity of the epithelial layer during DSS-induced colitis.

Increased Intestinal Barrier Permeability Results in Commensal Overgrowth and Bacteremia

It is well established that commensal microflora in the lumen of the colon play an essential role during intestinal inflammation (Rembacken et al., 1999; Sutherland et al., 1991; Turunen et al., 1998). In addition, a functional Nlrp3 inflammasome may be required to mount a proper immune response to prevent commensal overgrowth. We therefore asked whether the profound disruption of the epithelial barrier in the colon of DSS-fed *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$ mice caused commensal overgrowth and bacteremia. To this end, mice were administered 3% DSS for 7 days and the number of colony-forming units (CFUs) in different tissues was determined at day 9. Significantly more bacteria were counted in the stool, liver, colon, and mesenteric lymph nodes (MLN) of *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$ mice relative to DSS-fed wild-type mice (Figure 6A). Increased bacteremia in *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$ mice was due to DSS treatment as shown by the fact that untreated mice showed similar bacterial counts in the stool and colon (Figure S5A) and their systemic organs were devoid of bacteria (data not shown). Systemic dissemination of bacteria and bacterial components triggers an exuberant cytokine and chemokine inflammatory response. To gain additional evidence of bacteremia, we measured a variety of cytokines and chemokines in serum of DSS-fed *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$ mice. In agreement with the increased bacterial dissemination in *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$ mice, the amounts of the chemokines eotaxin, G-CSF, KC, and MCP-1 were all significantly higher in serum of *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$ mice relative to DSS-fed wild-type mice (Figures 6B–6E). In addition, serum concentrations of the proinflammatory cytokines IL-6 and TNF-α were also dramatically higher in *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$ mice when compared to wild-type mice (Figures 6F and 6G).

We also assessed local cytokine and chemokine production in colon tissue and found these to be consistent with those in serum. The amounts of KC, eotaxin, G-CSF, MCP-1, and IL-6 were all higher in colons of *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$ mice relative to those of DSS-fed wild-type mice (Figure S5B). To characterize the immune cells responsible for the increased production of chemokines and cytokines in the colon, we examined the expression of cell surface markers on mononuclear cells that infiltrated the lamina propria and submucosa. Significantly increased numbers of neutrophils and macrophages (F4/80$^+$ cells) were observed in the colon of DSS-fed *Nlrp3*$^{-/-}$ and

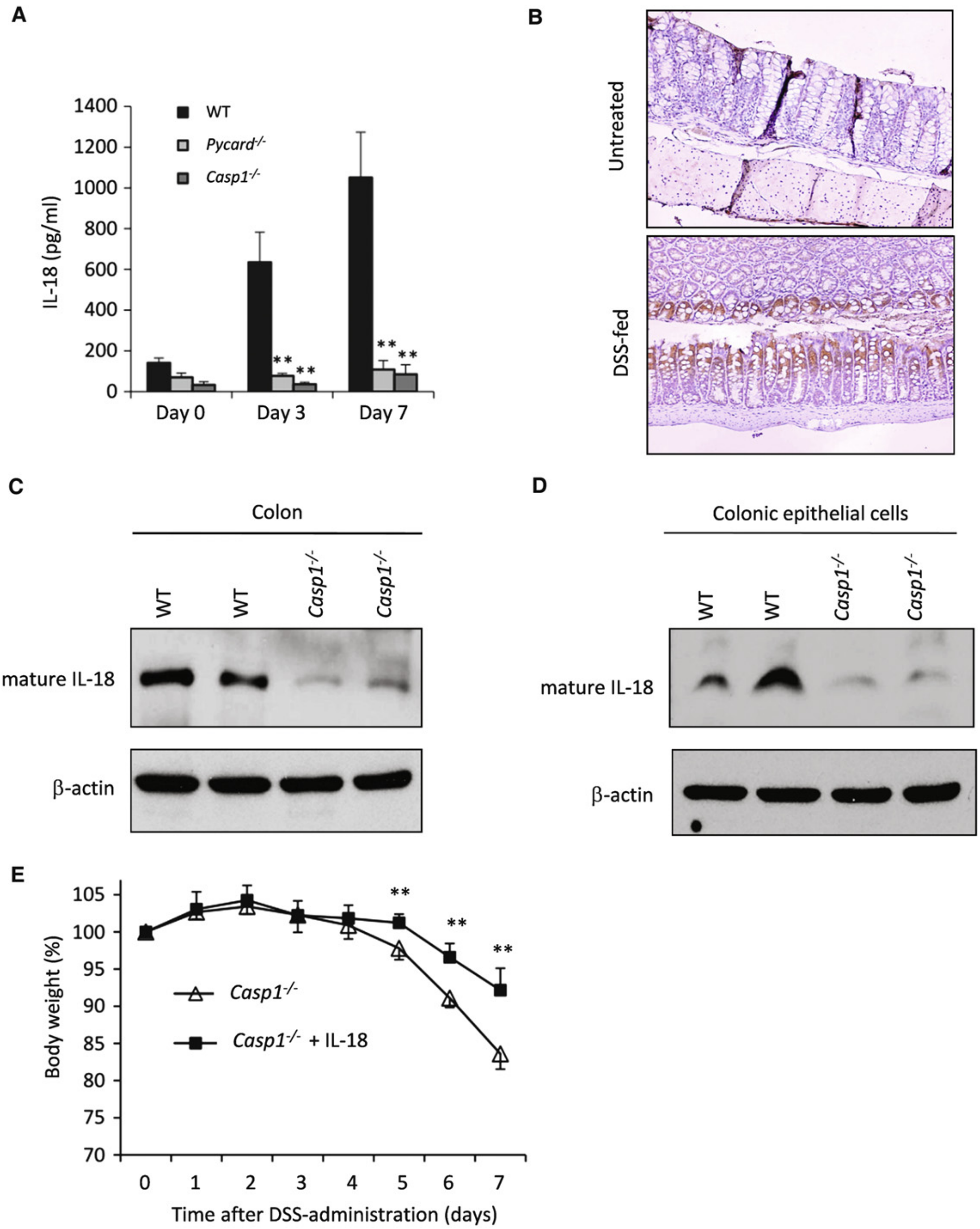

Figure 4. IL-18 Production by the Nlrp3 Inflammasome Is Required for Protection against DSS-Induced Colitis

(A) Wild-type, *Pycard*$^{-/-}$, and *Casp1*$^{-/-}$ mice were fed a 3% DSS solution in drinking water for 5 days, followed by regular drinking water for 2 days. Serum IL-18 concentrations on days 0 (n = 5/group), 3 (n = 5/group), and 7 (n = 10/group) was determined by multiplex assay.

(B–D) At day 7, colons were collected (B) and sections were stained for IL-18 (C, D), and colonic epithelial cells were isolated to determine the concentrations of mature IL-18 by immunoblotting. Blots were reprobed for β-actin.

(E) *Casp1*$^{-/-}$ mice (n = 5/group) were fed a 3% DSS solution in drinking water for 5 days, followed by regular drinking water for 2 days. One cohort simultaneously received a daily injection of 0.5 μg recombinant IL-18, whereas the control group was injected with saline. Body weight change was monitored daily for 7 days. Data represent means ± SE. **$p < 0.01$.

Casp1$^{-/-}$ mice (Figure S5C, left). In contrast, CD3 (T cell) and CD45R (B cell) staining were not significantly different in wild-type and inflammasome-deficient mice (Figure S5C, right).

These results suggest that the increased DSS-induced morbidity and lethality in the absence of Nlrp3 inflammasome signaling may be caused by commensal overgrowth and

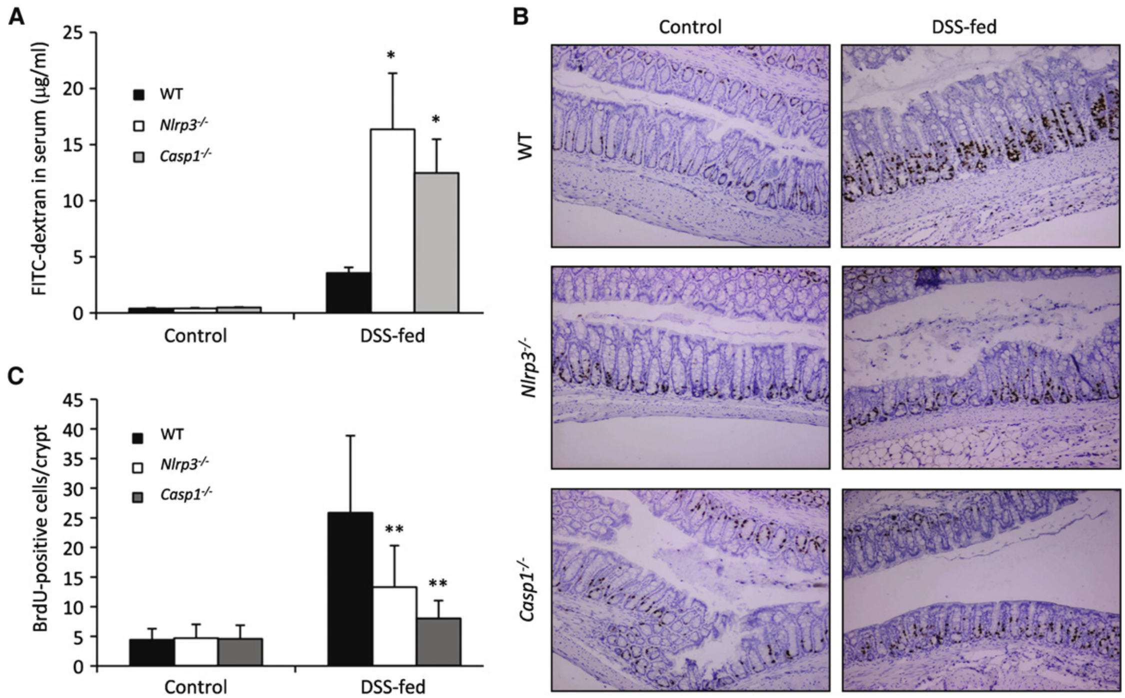

Figure 5. The Nlrp3 Inflammasome Is Required for Protection against Epithelial Barrier Permeabilization and Epithelial Cell Proliferation during DSS-Induced Colitis

(A) Wild-type, *Nlrp3*$^{-/-}$, and *Casp1*$^{-/-}$ mice (n = 5/group) were fed a 3% DSS solution in drinking water for 5 days, followed by regular drinking water for 2 days. Control and DSS-fed mice were subsequently fed FITC-dextran, and FITC-dextran amounts in serum were determined 3 hr later. Data represent means ± SE; *$p < 0.05$.
(B) Control (left) and DSS-fed (right) mice were then injected intraperitoneally with BrdU before colon sections were prepared to visualize BrdU-positive cells.
(C) Quantification of BrdU-positive cells per crypt in colons of untreated and DSS-fed wild-type, *Nlrp3*$^{-/-}$, and *Casp1*$^{-/-}$ mice. 100 crypts/mouse colon of three mice/genotype were analyzed. Data represent mean ± SE; **$p < 0.001$.

bacteremia after the breach of the intestinal barrier. An exaggerated immune response to these commensal bacteria may further exacerbate disease severity. To address the role of commensal bacteria in the increased colitis severity in inflammasome-deficient mice, we examined whether clinical parameters of DSS-induced colitis could be ameliorated with antibiotics. *Nlrp3*$^{-/-}$ mice were administered a 3% DSS solution alongside a combination of the selective antibiotics metronidazole, neomycin, and vancomycin from day 2 on. Disease severity was compared to *Nlrp3*$^{-/-}$ mice that were fed a 3% DSS solution without antibiotics. A dramatic improvement in the clinical scores of the antibiotic-treated arm was observed over *Nlrp3*$^{-/-}$ mice that did not receive antibiotics (Figures S5D–S5F). For instance, body weight loss in the antibiotics-treated arm was around 6%, whereas the group that was refused antibiotics presented with a loss of more than 20%. Prominent improvements in other clinical features including stool consistency and rectal bleeding were also noted for antibiotics-treated *Nlrp3*$^{-/-}$ mice. These marked improvements prompted us to examine the affect of antibiotics treatment on mortality after administration of a 4% DSS solution. As before, ~80% of placebo (PBS)-treated *Nlrp3*$^{-/-}$ mice had died 2 weeks after DSS administration. In contrast, all *Nlrp3*$^{-/-}$ mice that were coadministered antibiotics remained alive by the end of the experiment (data not shown). These results indicate that overgrowth of colonic microflora contributed significantly to the increased DSS-induced morbidity and lethality of *Nlrp3*$^{-/-}$ mice.

DISCUSSION

We show here that *Nlrp3*$^{-/-}$ mice were significantly more susceptible to DSS-induced colitis. Similar to *Nlrp3*$^{-/-}$ mice, *Pycard*$^{-/-}$ and *Casp1*$^{-/-}$ mice were more sensitive to colitis-associated body weight loss, diarrhea, rectal bleeding, and mortality during both the acute and chronic phase of disease, indicating a key role for the Nlrp3 inflammasome in protection against DSS-induced colitis. The role of the Nlrp3 inflammasome in protection against colitis is not limited to the DSS-induced model because *Nlrp3*$^{-/-}$ mice also suffered from increased body weight loss, diarrhea, and reduced colon length in the acute TNBS-induced colitis model. Oral administration of DSS and TNBS is directly toxic to the gut and causes crypt destruction, mucosal erosion, and ulceration. Epithelial damage induces a localized repair response characterized by increased division of stem cells at the base of crypts to replace damaged enterocytes (Radtke and Clevers, 2005). IL-18 production by the Nlrp3 inflammasome in colonic epithelial cells was identified as a crucial mediator of repair of the mucosal barrier and protection against DSS-induced colitis. Indeed, IL-18 has previously been associated to repair and restitution of ulcerated epithelium (Reuter and Pizarro, 2004). Mature IL-18 generated by the Nlrp3 inflammasome may subsequently bind to the IL-18R expressed on intestinal epithelial cells and local immune cells in the gut to exert its functions. Notably, the TLR4-MyD88 signaling axis has also been implicated in maintenance of

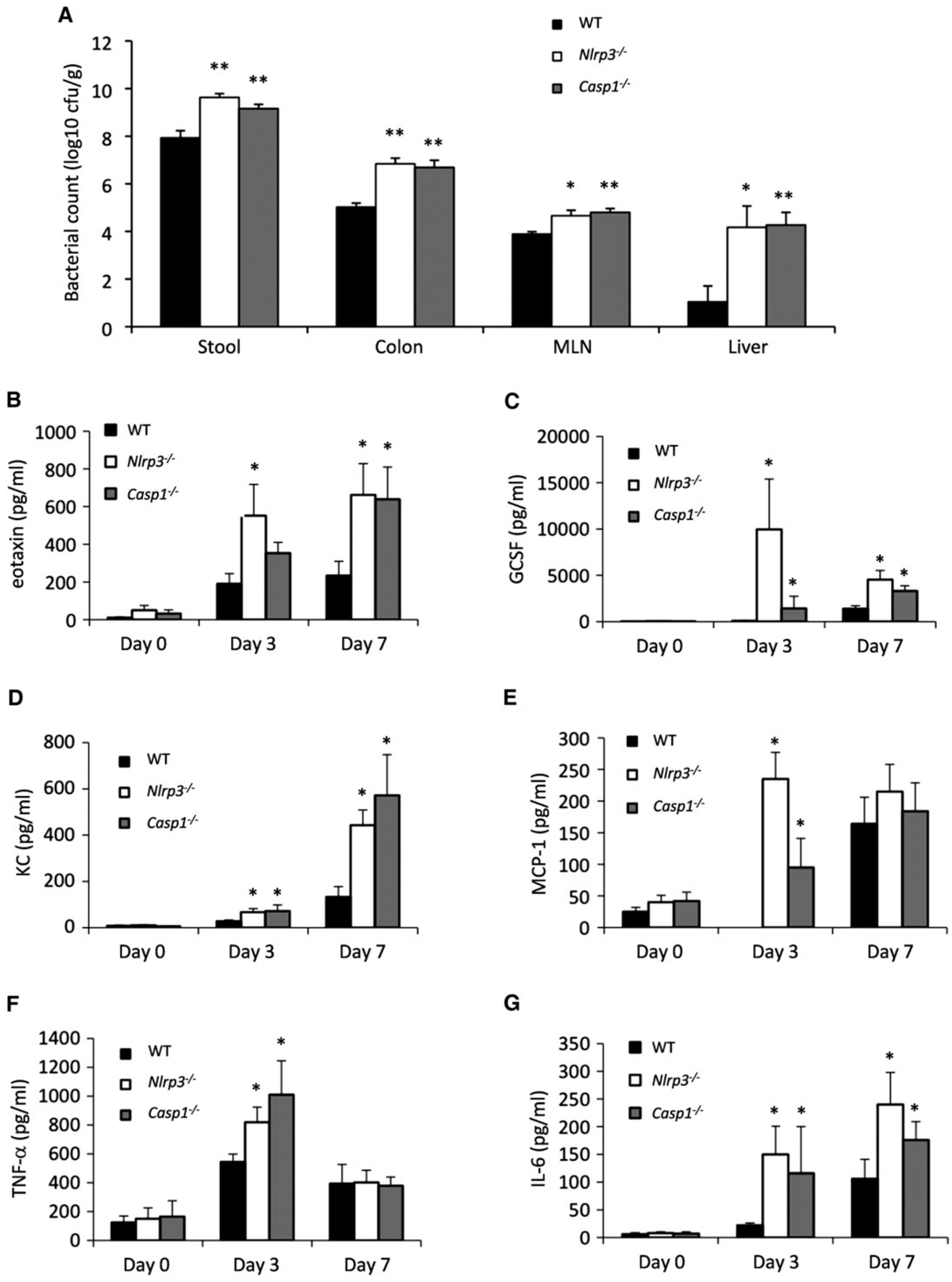

Figure 6. Increased Systemic Dissemination of Commensal Microflora and Cytokine Production in *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$ Mice during DSS-Induced Colitis

(A) Wild-type, *Nlrp3*$^{-/-}$, and *Casp1*$^{-/-}$ mice (n = 8/group) were fed a 3% DSS solution in drinking water for 5 days, followed by regular drinking water for 2 days. Bacterial counts in stool, colon, MLN, and liver of DSS-fed wild-type, *Nlrp3*$^{-/-}$, and *Casp1*$^{-/-}$ mice were determined at day 9.

(B–G) Serum amounts of (B) eotaxin, (C) GCSF, (D) KC, (E) MCP-1, (F) IL-6, and (G) TNF-α were measured at days 3 and 7 by multiplex assay (n = 5 mice/group). Data represent means ± SE. *$p < 0.05$, **$p < 0.01$.

epithelial cell homeostasis in the gut and protection against DSS-induced colitis (Fukata et al., 2005; Rakoff-Nahoum et al., 2004). This suggests that MyD88 contributes to epithelial cell homeostasis in the gut both at the level of TLR4 signaling and downstream of the IL-18R. In addition to IL-18, the cytokines IL-11 and IL-22 have been identified as important regulators of gastrointestinal mucosal biology (Keith et al., 1994; Zenewicz et al., 2008). It remains to be determined whether these cytokines operate in a hierarchical cascade or interact in a network of parallel pathways to confer protection against destruction of the mucosal barrier.

Earlier studies with *Casp1*$^{-/-}$ mice and the caspase-1 inhibitor pralnacasan suggested a detrimental rather than a protective role for caspase-1 in DSS-induced colitis (Bauer et al., 2007; Loher et al., 2004; Siegmund et al., 2001b). However, our observation that *Casp1*$^{-/-}$ mice are more susceptible to DSS-induced colitis is in agreement with a growing body of evidence suggesting a protective role for Nlrp3 inflammasome-mediated IL-18 production during colitis. First, mice lacking the other inflammasome components Nlrp3 and ASC were also more susceptible to DSS-induced colitis. Second, both *Il18*$^{-/-}$ and *Il18r1*$^{-/-}$ mice were shown to display increased susceptibility to DSS-induced colitis, which was associated with greater lethality and more severe histopathological changes (Takagi et al., 2003). Third, *Il1r*$^{-/-}$ mice also showed increased intestinal damage and histopathology during DSS-induced colitis (Lebeis et al., 2009). Finally, several previous studies reported the development of more severe DSS-induced colitis in mice lacking the adaptor protein MyD88, which is required for the production of the caspase-1 substrates IL-1β and IL-18, as well as for signaling downstream of their respective receptors (Araki et al., 2005; Fukata et al., 2005; Rakoff-Nahoum et al., 2004). Noteworthy, the results from the gene-deleted mouse models described above are sometimes in conflict with reports using (bio)chemical approaches for neutralization of caspase-1 and IL-18. For instance, experiments in IL-18-deficient mice suggested a beneficial role for IL-18 during DSS-induced colitis (Takagi et al., 2003), whereas IL-18 neutralization with recombinant IL-18 binding protein (Sivakumar et al., 2002) and IL-18 antibodies suggested a detrimental role for IL-18 (Siegmund et al., 2001a). In addition to differences in experimental design, characteristics inherent to (bio)chemical neutralization and gene-deleted mouse models may have contributed to the different outcomes. On the one hand, chemical and biochemical inhibitors are most suited for therapeutic intervention in patients, although they are unlikely to achieve complete neutralization of the desired target and may suffer from pleiotropic effects that could interfere with disease outcome. On the other hand, gene-targeted deletion in mice is a surer approach for complete removal of the protein under study. However, the possibility that gene deletion may trigger mild developmental defects that go unnoticed but nevertheless may influence the disease phenotype cannot be completely excluded. Thus, (bio)chemical neutralization and gene-targeted deletion approaches each have particular advantages and both should be considered to further our knowledge on the mechanisms underlying human disease.

EXPERIMENTAL PROCEDURES

Mice

Nlrp3$^{-/-}$, *Pycard*$^{-/-}$, and *Casp1*$^{-/-}$ mice backcrossed to C57BL/6 background for at least 10 generations have been described before (Lamkanfi et al., 2008; Thomas et al., 2009). Mice were housed in a pathogen-free facility and the animal studies were conducted under protocols approved by St. Jude Children's Research Hospital Committee on Use and Care of Animals. All mice were male 8–10 weeks old and maintained in an SPF facility. All experiments were conducted under protocols approved by the St. Jude Children's research Hospital Committee on Use and Care of Animals.

Induction of DSS-Induced Colitis

For survival studies, acute colitis was induced with 4% (w/v) DSS (molecular mass 36–40 kDa; MP Biologicals) dissolved in sterile, distilled water ad libitum for the experimental days 1–5 followed by normal drinking water until the end of the experiment (day 14). The DSS solutions were made fresh on day 3. For all other experimental read-outs, DSS-induced colitis was induced by feeding mice 3% (w/v) DSS during 5 days, followed by normal drinking water until the end of the experiment on day 7. For bacterial count determination, mice continued to receive a 3% DSS solution until day 7 and bacterial numbers were determined on day 9.

Determination of Clinical Scores

Body weight, stool consistency, and the presence of occult blood were determined daily up to day 7. The baseline clinical score was determined on day 1. Scoring for stool consistency and occult blood was done as described previously (Wirtz et al., 2007). In brief, stool scores were determined as follows: 0, well-formed pellets; 1, semiformed stools that did not adhere to the anus; 2, semiformed stools that adhered to the anus; 3, liquid stools that adhered to the anus. Bleeding scores were determined as follows: 0, no blood as tested with hemoccult (Beckman Coulter); 1, positive hemoccult; 2, blood traces in stool visible; 3, gross rectal bleeding.

Histopathology and Immunohistochemistry

After day 7, the entire colon was excised to measure the length of the colon and the weight of cecum. Colons were washed, fixed in 10% buffered formaldehyde, and embedded in paraffin. Tissue sections were stained with hematoxylin & eosin (H&E). Histology was scored by a pathologist in a blinded fashion as a combination of inflammatory cell infiltration (score 0–3) and tissue damage (score 0–3). The presence of occasional inflammatory cells in the lamina propria was scored as 0, increased numbers of inflammatory cells in the lamina propria was assigned score 1, confluence of inflammatory cells extending into the submucosa was scored as 2, and transmural extension of the infiltrate was scored as 3. For tissue damage, no mucosal damage was scored as 0, lymphoepithelial lesions were scored as 1, surface mucosal erosion or focal ulceration was scored as 2, and extensive mucosal damage and extension into deeper structures of the bowel wall was scored as 3. The combined histological score ranged from 0 (no changes) to 6 (extensive infiltration and tissue damage).

For immunohistochemistry, formalin-fixed paraffin-embedded tissues were cut into 4 μm section and slides were stained for neutrophil, macrophage, T cell, and B cell via the immunoperoxidase method with neutrophil, F4/80, CD3, and CD45R/B220 antibodies, respectively. IL-18 immunostaining was performed with a rat anti-mouse IL-18 antibody (MBL).

Recombinant IL-18

Recombinant IL-18 (MBL International) was injected intraperitoneally at a concentration of 0.5 μg per mouse in 100 μl phosphate-buffered saline (PBS) on days 0, 1, 2, 3, and 4.

Cytokine Measurements

Serum was collected from blood drawn by cardiac puncture at the indicated time points. To measure the cytokine amounts in colon tissue, a part of colon was homogenized mechanically in PBS containing 1% NP-40 and complete protease inhibitor cocktail (Roche). Mouse cytokines and chemokines in serum and colon homogenate were measured with Luminex (Bio-Rad) and ELISA (R&D Systems) assays.

Immunoblotting

Tissue homogenates were lysed in lysis buffer solution (150 mM NaCl, 10 mM Tris [pH 7.4], 5 mM EDTA, 1 mM EGTA, 0.1% Nonidet P-40) supplemented with a protease inhibitor cocktail tablet (Roche). Samples were clarified, denatured with SDS buffer, and boiled for 5 min. Proteins were separated by SDS-PAGE and transferred on to nitrocellulose membranes. The membranes were immunoblotted with primary antibodies and proteins detected with appropriate secondary anti-rat antibody conjugated to horseradish peroxidase followed by enhanced chemiluminescence. IL-18 antibodies were from MBL.

Isolation of Colonic Epithelial Cells
Colonic epithelial cells were isolated as described before (Greten et al., 2004). In brief, colons were dissected, washed with PBS, and cut into small pieces. Colon segments were incubated in HBSS supplemented with 5 mM EDTA and 0.5 mM DTT for 30 min at 37°C with gentle shaking. Cells in the supernatants were filtered through a 70 μm cell strainer and washed twice. Enrichment for colonic epithelial cells was determined as the percentage of cells staining positive for the epithelial cell-specific marker cytokeratin-18. 85%–90% of isolated cells stained positive for cytokeratin-18.

Bacterial Culture
Samples of stool, colon, and liver tissue were collected in 5 ml of a 3% thioglycolate solution and homogenized. Different dilutions of the obtained suspensions were plated on blood agar and BHI agar and incubated at 37°C for 48 hr. Bacterial counts were determined by colony-forming assay.

Depletion of Commensal Bacteria
To inhibit overgrowth of commensal bacteria during DSS administration, mice were treated with selective antibiotics: metronidazole (1g/L; Sigma) for killing anaerobic bacteria, neomycin (1g/L; Sigma) for killing gram-negative bacteria, and vancomycin (50 mg/Kg/day; Sigma) for inhibition of gram-positive staphylococci and streptococci. Antibiotics treatment was started at day 2 after DSS administration and continued until day 9. Metronidazole and neomycin was added in drinking water, and vancomycin was given by oral gavage once daily.

Bone Marrow Chimeras
Bone marrow transfer was used to create *Nlrp3*$^{-/-}$ chimera mice wherein the genetic deficiency of *Nlrp3* was confined to either circulating cells (*Nlrp3*$^{-/-}$ > WT chimera) or nonhematopoietic tissue (WT > *Nlrp3*$^{-/-}$). In brief, bone marrows were collected from femur and tibia of congenic WT (expressing CD45.1 leukocyte antigen) or *Nlrp3*$^{-/-}$ (expressing CD45.2 leukocyte antigen) donor mice by flushing with HBSS. After several washing steps, cells were resuspended in PBS at a concentration of 1 × 10^8/ml. 100 μl of this cell suspension was injected retro-orbitally in irradiated donor mice. Four chimera groups were generated WT > WT (WT cells expressing CD45.1 into WT expressing CD45.2); WT > *Nlrp3*$^{-/-}$ (WT cells expressing CD45.1 into *Nlrp3*$^{-/-}$ expressing CD45.2); *Nlrp3*$^{-/-}$ > *Nlrp3*$^{-/-}$ (*Nlrp3* expressing CD45.2 cells into *Nlrp3*$^{-/-}$ expressing CD45.2); and *Nlrp3*$^{-/-}$ > WT (*Nlrp3*$^{-/-}$ cells expressing CD45.2 into WT expressing CD45.1). The use of CD45.1-expressing congenic mice facilitated verification of proper reconstitution in the chimera mice. Bone marrow reconstitution was verified after 5 weeks by staining for CD45.1 and CD45.2 in blood cells with FITC-conjugated anti-CD45.1 and PE-conjugated anti-CD45.2. 7 weeks after bone marrow transfer, mice were fed with 3% DSS for 5 days. Body weight change, stool consistency, and rectal bleeding were monitored daily. At day 7, mice were sacrificed to collect colon tissue for H&E staining.

In Vivo Intestinal Permeability Measurement
In vivo assay to assess epithelial barrier permeability was performed with an FITC-labeled Dextran method as described (Furuta et al., 2001). In brief, food and water were withdrawn and mice were gavaged with permeability tracer FITC-dextran (M*w* 4000; Sigma-Aldrich) at a concentration 60 mg/100 g body weight. Blood was collected by heart puncture and FITC-dextran amount in serum was measured with a fluorescence spectrophotometer setup with emission and excitation wavelengths of, respectively, 490 nm and 520 nm. FITC-dextran concentration was determined from standard curves generated by serial dilution of FITC-dextran.

In Situ Intestinal Proliferation Assay
The number of proliferating cells in intestinal epithelium was detected by immunoperoxidase staining for thymidine analougue 5′-bromo-2′deoxyuridine (BrdU) as described (Rakoff-Nahoum et al., 2004). In brief, 1 mg/ml BrdU in PBS was injected intraperitoneally. 2 hr later, colon tissue was collected and 4 cm of distal colon was fixed in 10% neutral buffered formalin and embedded in paraffin. Immunohistochemistry was performed with an in situ BrdU staining kit (BD Bioscience). Tissues were counterstained with hematoxylin. The number of BrdU-positive cells per intact and well-oriented crypt was determined.

Statistical Analysis
Data are represented as mean ± SEM. Differences in group survival and bacteremia were analyzed with the Kaplan-Meier test with Prism5 (GraphPad Software). In all other cases, statistical significance was determined by Student's t test. $p < 0.05$ was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at doi:10.1016/j.immuni.2010.03.003.

ACKNOWLEDGMENTS

We thank A. Coyle, E. Grant, J. Bertin (Millennium Pharmaceuticals), G. Nuñez (University of Michigan), and R. Flavell (Yale) for generous supply of mutant mice. M.L. is supported by the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen. This work was supported by National Institutes of Health Grant AR056296, a Cancer Center Support Grant (CCSG 2 P30 CA 21765), and the American Lebanese Syrian Associated Charities (ALSAC) to T.-D.K.

Received: May 31, 2009
Revised: January 11, 2010
Accepted: March 1, 2010
Published online: March 18, 2010

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