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Recognition of Commensal Microflora by Toll-Like Receptors Is Required for Intestinal Homeostasis

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Summary

Toll-like receptors (TLRs) play a crucial role in host defense against microbial infection. The microbial ligands recognized by TLRs are not unique to pathogens, however, and are produced by both pathogenic and commensal microorganisms. It is thought that an inflammatory response to commensal bacteria is avoided due to sequestration of microflora by surface epithelia. Here, we show that commensal bacteria are recognized by TLRs under normal steady-state conditions, and this interaction plays a crucial role in the maintenance of intestinal epithelial homeostasis. Furthermore, we find that activation of TLRs by commensal microflora is critical for the protection against gut injury and associated mortality. These findings reveal a novel function of TLRs—control of intestinal epithelial homeostasis and protection from injury—and provide a new perspective on the evolution of host-microbial interactions.

Introduction

All complex metazoans are colonized with a myriad of microbial organisms that comprise an indigenous microflora. While present at many of the interfaces with the external world, such as the oropharynx and skin of mammals, the overwhelming majority and diversity of the endogenous bacterial flora resides at the distal alimentary tract, most notably at the colon. It is here that the over 10^{13} resident bacteria confer many benefits to intestinal physiology comprising a truly mutualistic relationship (Hooper and Gordon, 2001). The metabolism of nutrients and organic substrates, the development of intestinal epithelium, vasculature, and lymphoid tissue, and the contribution to the phenomena of colonization resistance to pathogens are only a few of the ways in which the host benefits from the resident microflora present in the gut (Berg, 1996; Midvedt, 1999). However, the presence of commensal bacteria in the gut appears to be of crucial importance in the pathogenesis of human inflammatory bowel diseases (IBD), which include Crohn's disease and ulcerative colitis (Podolsky, 2002). These diseases are characterized by chronic inflammation of the intestine much of which is thought to be due to inappropriate activation of the immune system by commensal bacteria (Farrell and LaMont, 2002; Sartor, 2000). The nature and regulation of the host-commensal interactions in the gut is thus an area of intense scientific and clinical interest.

TLRs comprise a family of pattern-recognition receptors that detect conserved molecular products of microorganisms, such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA), recognized by TLR4 and TLR2, respectively (Takeda et al., 2003). TLRs function as sensors of microbial infection and are critical for the initiation of inflammatory and immune defense responses. Importantly the bacterial ligands recognized by TLRs are not unique to pathogens, but rather are shared by entire classes of bacteria, and are produced therefore by commensal microorganisms as well. It is not understood at present whether and how the host distinguishes between pathogenic and commensal bacteria. Moreover, the differences between the two groups of microorganisms are not always clear-cut. It is thought, however, that the sequestration of indigenous microflora by surface epithelia plays an important role in preventing TLR activation by commensals, whereas pathogenic bacteria are equipped with virulence factors that allow them to pass through epithelial barriers where they can be detected by TLRs expressed on macrophages and dendritic cells (Gewirtz et al., 2001; Sansonetti, 2002).

Given the importance of the microflora in intestinal inflammation, and the fact that TLRs play a major role in the recognition of bacterial products and induction of inflammation, we set out to test the in vivo effect of TLR ligation by commensal-derived products. Our results reveal an unexpected protective role of TLRs from epithelial injury, a crucial function of TLRs in intestinal epithelial homeostasis, and provide a new perspective on host-commensal symbiosis.

Results

TLR Signaling Protects from Mortality Caused by Intestinal Epithelial Injury

Current knowledge suggests that the disruption of the mucosal barrier upon injury to intestinal epithelial cells leads to the exposure of the multitude of TLR ligands produced by commensals to TLR-expressing cells, particularly macrophages, resident in the lamina propria of the intestine (Strober et al., 2002) resulting in a potent inflammatory response, intestinal inflammation, and corresponding injury. To test the effect of disrupted compartmentalization of commensals, we chose a model of intestinal injury and inflammation, the oral administration of dextran sulfate sodium (DSS), a sulfated polysaccharide known to be directly toxic to colonic epithelium (Kitajima et al., 1999).

To address the role of TLRs in intestinal inflammation, we used mice deficient in MyD88, an adaptor molecule essential for TLR-mediated induction of inflammatory cytokines (Takeda et al., 2003), as well as mice deficient in TLR2 and TLR4. Our hypothesis entering these studies was that MyD88$^{-/-}$ mice would not be able to mount a

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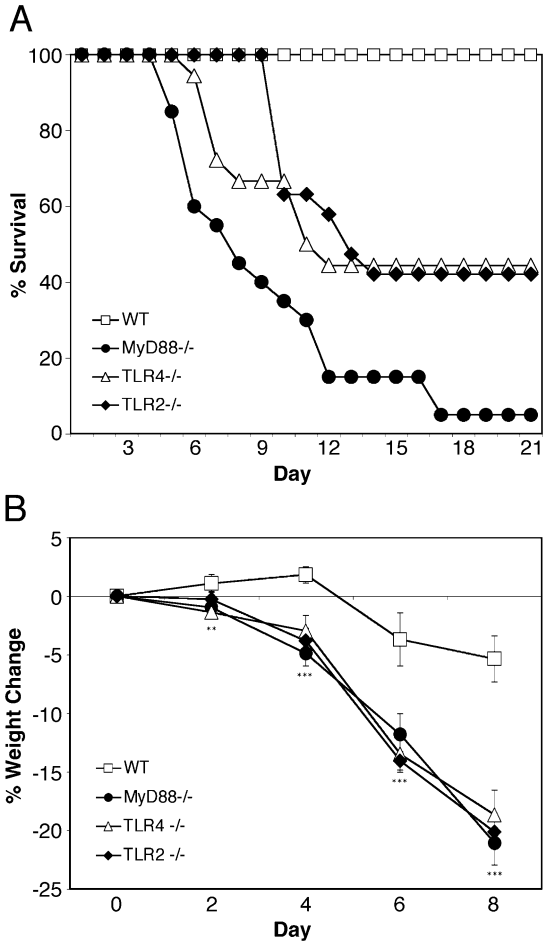

Figure 1. Increased Mortality and Morbidity in MyD88$^{-/-}$ Mice Following DSS Administration

wt control (N = 23); MyD88$^{-/-}$ (N = 20); TLR4$^{-/-}$ (N = 18); TLR2$^{-/-}$ (N = 25) mice were given 2% DSS in drinking water for seven days. Mice were changed to normal drinking water on day 8.

(A) Survival was monitored until day 21 after the start of DSS.

(B) Percent weight change of animals was determined by the following equation: % weight change = (weight at day X-day 0/weight at day 0) X-100. Error bars are ± SEM. ** = $p < 0.01$; *** = $p < 0.001$ (compared to wt) using the Student's test.

TLR-dependent inflammatory response to commensal bacteria, and therefore would manifest decreased intestinal pathology following DSS administration. To our surprise, MyD88$^{-/-}$ animals showed severe mortality and morbidity upon administration of DSS (2%; wt/vol) in drinking water for seven days (Figure 1A). This is in sharp contrast to wt mice, which had 100% survival and minimal morbidity at this low dose of DSS. In accordance with the observed differences in survival, MyD88$^{-/-}$ mice showed much more severe weight loss compared with wt controls (Figure 1B). Thus, MyD88-dependent signaling pathway appears to be critical for the protection against DSS-induced mortality and morbidity. MyD88 is a signaling adaptor used by all TLRs, as well as the IL-1 receptor (IL-1R) and IL-18R (Takeda et al., 2003). Previous DSS studies using animals deficient in interleukin-1β converting enzyme (ICE), which are unable to produce IL-1β or IL-18 (Siegmund et al., 2001) and using IL-18 antagonists (Sivakumar et al., 2002) have revealed an improvement of morbidity and disease phenotype compared to wt controls. Therefore the protective role of the MyD88 signaling pathway appears to be TLR specific, and hence related to TLR activation, presumably by the TLR ligands present in the colon. This conclusion was further supported by the compromised survival and severe weight loss observed in TLR2 and TLR4-deficient mice following DSS administration (Figure 1). These results indicated that while elimination of TLR4 or TLR2 increased the susceptibility to DSS-induced disease, the severe mortality seen in MyD88$^{-/-}$ animals was the result of defective signaling of multiple TLRs induced by various commensal-derived products.

In addition to TLRs, microbial products can be recognized by members of the NOD family of intracellular signaling proteins—NOD1 and NOD2 (Inohara and Nunez, 2003). In particular, mutations in NOD2 have been implicated in the predisposition to Crohn's disease, although the precise role of NOD2 in the pathoetiology or pathogenesis of this condition remains unknown (Hugot et al., 2001; Ogura et al., 2001). Unlike TLRs, NODs detect their microbial ligands in the cytosol. Both NOD1 and NOD2 signal activation of NF-κB and MAP kinases through the protein kinase RIP2 (Chin et al., 2002; Kobayashi et al., 2002). We found that, unlike MyD88-deficient mice, RIP2$^{-/-}$ mice are as resistant to DSS administration as wild-type mice (data not shown), suggesting that RIP2 dependent pathways do not play a major role in the susceptibility to colonic injury.

Severe Susceptibility to Colonic Injury in MyD88-Deficient Mice

We next analyzed the cause of death and morbidity of the MyD88$^{-/-}$ animals using several parameters. As opposed to wt mice, which remained active, mobile, and seemingly healthy throughout the duration of the experiment, MyD88$^{-/-}$ animals were observed to be moribund, with a hunched posture and defective grooming as early as day 5 post-DSS (data not shown). Analysis of colons at multiple time points after the administration of DSS revealed bleeding in the colons of MyD88$^{-/-}$ mice, which occurred with more rapid onset and with much greater extent and severity compared to control animals, observed as early as day 3 and being present throughout the colon by day 5 in the MyD88$^{-/-}$ group (Figures 2A and 2B). Concordant with this increased colonic bleeding, MyD88$^{-/-}$ animals became severely anemic with time as determined by the measurement of red blood cells and hematocrit concentration in circulating blood (Figures 2C and 2D). Thus, it appeared that MyD88$^{-/-}$ mice were dying of severe colonic bleeding and anemia upon administration of DSS, although the exact cause of death is difficult to determine with certainty. TLR2- and TLR4-deficient mice also exhibited colonic bleeding, thought not as severe as MyD88$^{-/-}$ animals (data not shown).

To investigate the possible mechanisms of the colonic bleeding in MyD88$^{-/-}$ animals, we conducted microscopic evaluation of the colons of experimental animals at multiple time points during the course of DSS administration. As seen from the comparison of representative photomicrographs taken on day 5 post-DSS, MyD88$^{-/-}$

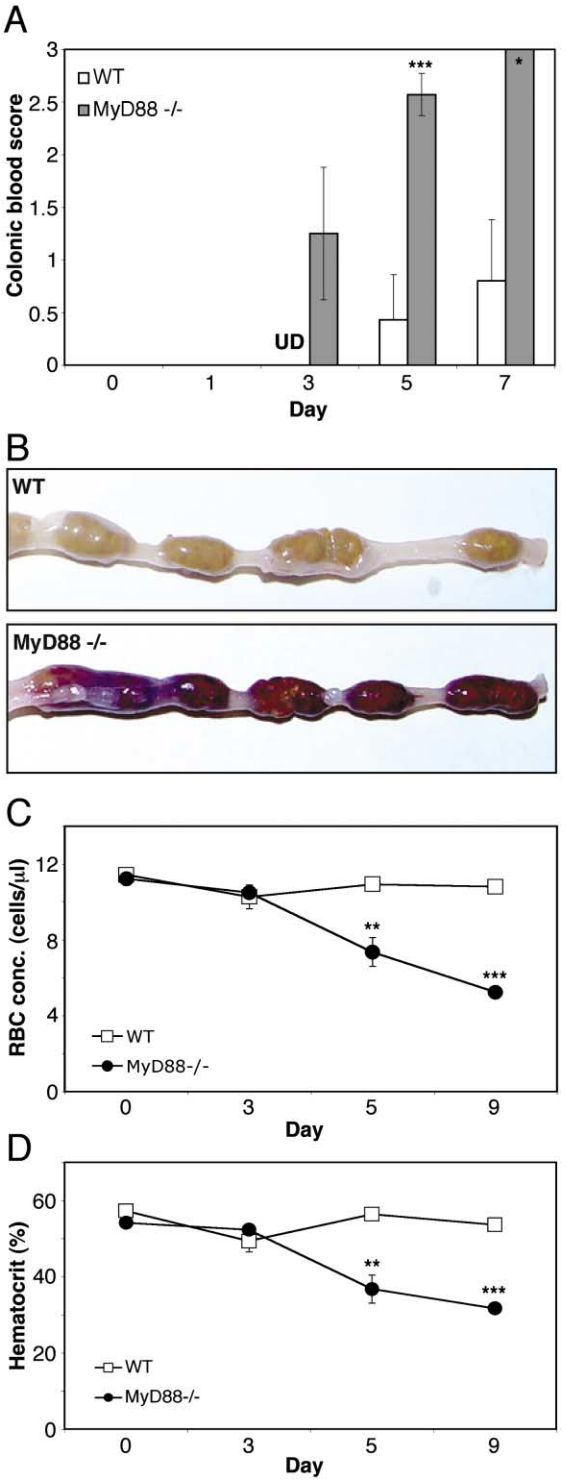

Figure 2. Severe Colonic Bleeding and Anemia in MyD88$^{-/-}$ Mice

(A) Kinetics of the severity of colonic bleeding. Colons of wt and MyD88$^{-/-}$ mice were removed at days 0, 1, 3, 5, and 7 postadministration of 2% DSS (N = 3–5 mice per time point). Scoring was as follows: 0 = lack of any gross blood visible throughout the entire colon; 1 = gross blood present in <1/3 of the colon; 2 = <2/3; 3 = >2/3 of the colon. UD = undetected.

(B) Photograph of a representative colon from wt and MyD88$^{-/-}$ mice at day 5 of DSS-treatment.

(C) RBC concentration and (D) Hematocrit values of peripheral blood taken at various time points during the administration of 2% DSS for seven days. Error bars represent ± SEM. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$ (compared to wt) using the Student's test.

colons show severe and extensive denudation of the surface epithelium (erosions) and mucodepletion of glands compared to wt control mice (Figure 3A). Histopathological scoring of colons revealed more severe ulceration and epithelial injury at days 3 and 5 in MyD88$^{-/-}$ mice compared to wt controls (Figures 3B and 3C).

Mortality of MyD88-Deficient Mice Is Not Due to Commensal Overgrowth

There were several possible reasons for the increased epithelial injury in colons of MyD88$^{-/-}$ mice, including damage due to uncontrolled overgrowth of commensal bacteria after disruption of the epithelial barrier, increased leukocytic infiltrate, or an inherent defect in epithelial resistance to injury and/or repair responses. The latter may be caused by a deficient induction of cytokines, cytoprotective, growth, and repair factors required for protection against injury. To address the first possibility, MyD88$^{-/-}$ animals were given a combination of broad-spectrum antibiotics in their drinking water for a range of 2–4 weeks prior to and during DSS administration in order to deplete the commensal flora and therefore prevent bacterial overgrowth. The sterility of the colons was confirmed by bacteriologic analysis of fecal contents (see below). Commensal depletion of MyD88$^{-/-}$ animals did not prevent the morbidity or mortality seen in untreated MyD88$^{-/-}$ animals as these two groups of animals died with similar kinetics and hemorrhagic colons (data not shown). In addition, the aerobic and anaerobic culture of spleens from MyD88$^{-/-}$ animals at various time points post-DSS administration revealed no bacteremia (data not shown), further suggesting that bacterial overgrowth was not the cause of pathology in DSS-treated MyD88$^{-/-}$ mice. Consistent with this conclusion, aerobic and anaerobic bacterial titers of colonic fecal cultures were comparable between untreated wt and MyD88$^{-/-}$ mice (data not shown).

Mortality of MyD88-Deficient Mice Is Not Caused by Damage Due to Hyperinfiltrating Leukocytes

To address the second possibility (damage caused by infiltrating leukocytes) we compared the leukocytic infiltrate at various time points post-DSS administration in wt- and MyD88-deficient mice. Surprisingly, such analyses revealed no differences in overall infiltrating leukocytes (Figure 3D). Individual analysis of infiltrating leukocytes including lymphocytes, polymorphonuclear cells, and eosinophils revealed no differences between wt and MyD88$^{-/-}$ colons (Supplemental Figure S1 available at http://www.cell.com/cgi/content/full/118/2/229/DC1). Thus, differences in leukocytic infiltration were not responsible for the increased colonic epithelial injury in the MyD88$^{-/-}$ mice. This result is consistent with findings showing that DSS-mediated pathology is independent of T, B, and NK cells (Axelsson et al., 1996; Dieleman

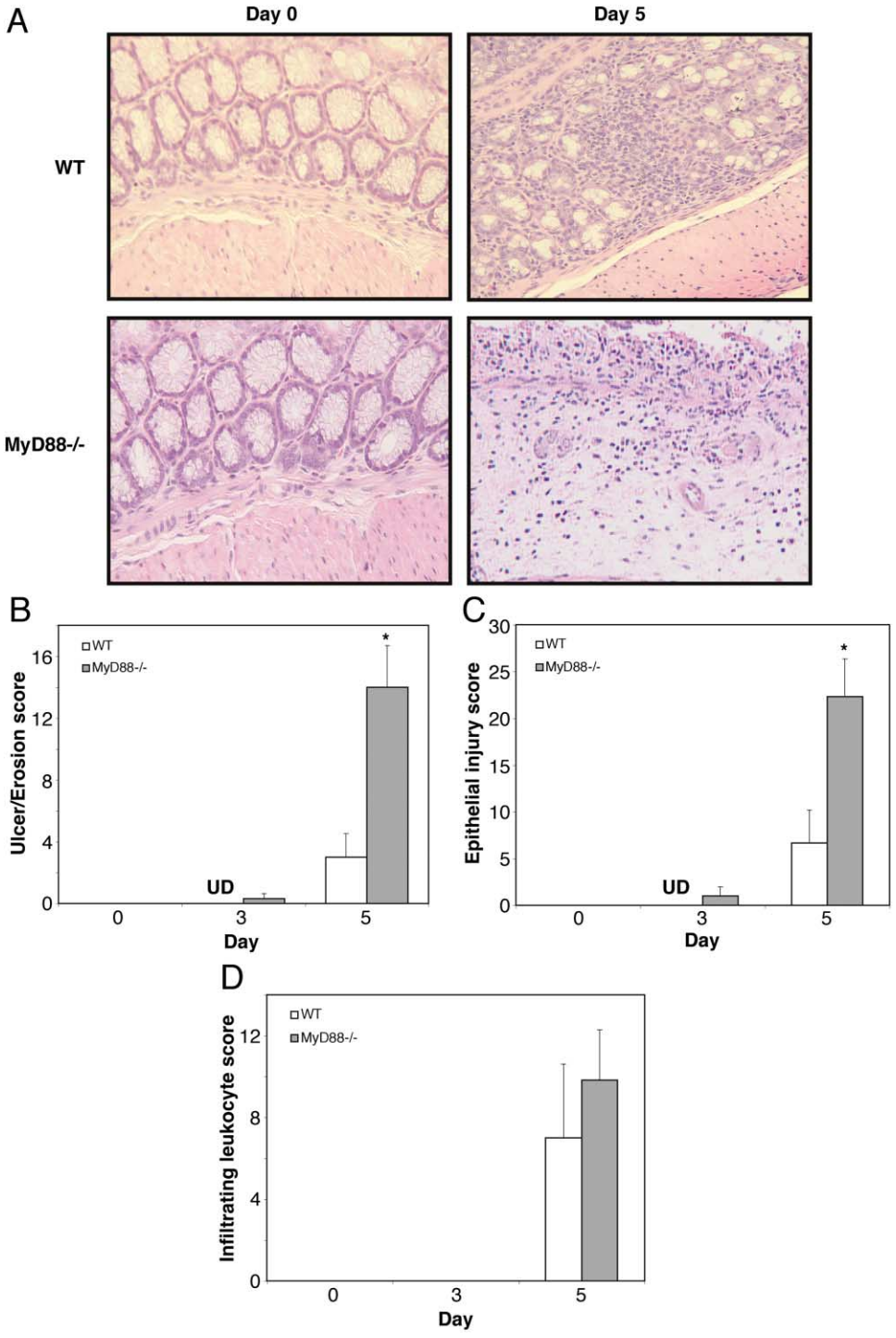

Figure 3. Colonic Epithelial Damage in MyD88$^{-/-}$ Mice Following DSS Administration

(A) Representative photomicrographs (magnification, ×200; hematoxylin and eosin staining) of colons from wt and MyD88$^{-/-}$ mice at days 0 and 5 of DSS administration. Histopathological scoring of (B) ulcer and erosions, (C) epithelial injury, and (D) infiltrating leukocytes of colons from wt and MyD88$^{-/-}$ mice at days 0, 3, and 5 of DSS administration. UD = undetected. See Experimental Procedures for a description of the scoring algorithm. Error bars represent ± SEM. * = $p < 0.05$ (compared to wt) using the Student's test.

et al., 1994). This conclusion is also supported by the fact that colonic bleeding in MyD88$^{-/-}$ mice was apparent as early as day 3 post-DSS (Figure 2A), whereas leukocytic infiltration was not evident until day 5 posttreatment (Figure 3D).

TLR Signaling Controls Homeostasis of Intestinal Epithelium

We next addressed the possibility that the increased intestinal damage in MyD88$^{-/-}$ mice was due to an inherent defect in the resistance of epithelial cells to direct

injury. Protection from intestinal injury such as chemical, mechanical, and radiation-induced is determined by many factors such as the balance of proliferation and differentiation along the crypt axis (Booth and Potten, 2001) as well as the production of mediators involved in protecting epithelial cells from initial injury (cytoprotective) and those involved in orchestrating repair response mechanisms such as restitution (Cho and Wang, 2002; Dignass, 2001).

To address the first possibility, that a homeostatic imbalance of intestinal epithelium in the absence of TLR signaling may be responsible for the observed increased susceptibility to colonic injury, we examined the baseline proliferative state of colonic crypts of wt and MyD88$^{-/-}$ mice. Proliferating cells were labeled by intraperitoneal (i.p.) injection of BrDU 24 hr prior to harvesting colons. Analysis of BrDU-positive intestinal epithelial cells revealed an increased number of proliferating cells in MyD88$^{-/-}$ mice compared to wt controls (Figure 4A, top two images). Importantly, the proliferating cells were clearly present in the middle and upper regions of the crypt in the MyD88$^{-/-}$ mice. These areas of the crypt are remote from the stem cell area and are normally fully differentiated and nonproliferating. To exclude a possibility that BrDU-positive cells recently migrated from the stem cell area, we performed a 2 hr BrDU labeling and again found proliferating cells at the stem cell area as well as in the middle and upper regions of the crypts in MyD88$^{-/-}$ mice (Figure 4A, bottom two images) in addition to a significant increase in the number of proliferating cells at baseline (Figure 4E; day 0). Both the expanded proliferative zone and increase in number of proliferating cells in MyD88-deficient mice suggests dysregulated proliferation and differentiation of intestinal epithelium in the absence of TLR signals. Consistent with the higher number of proliferating cells per crypt, the average total number of cells per crypt was higher in MyD88$^{-/-}$ mice compared to wt controls (Figure 4B), and markers of cycling cells, such as cyclin D1 (Figure 4C) and c-*myc* (data not shown) were upregulated in these cells.

Much of the evidence for the relationship between intestinal epithelial cell cycling and susceptibility to injury comes from studies of radiation-induced injury (Neta and Okunieff, 1996; Booth and Potten, 2001). The defect observed in intestinal epithelial homeostasis in the absence of TLR signaling should render these animals particularly susceptible to radiation-induced injury. Accordingly, MyD88$^{-/-}$ mice showed severe mortality upon γ irradiation compared to wt controls (Supplemental Figure S2 available on *Cell* website). Compensatory proliferation of intestinal epithelial cells is part of the normal repair response to injury and is a reflection of the ability of cells that have survived the injurious insult to proliferate and repopulate the crypt. Crypts with increased numbers of epithelial cells in cell cycle have been shown to be more susceptible to radiation-induced injury as determined by their inability to undergo compensatory proliferation and repopulate the crypt (Booth and Potten, 2001). Therefore, we analyzed the susceptibility to radiation-induced injury in wt and MyD88$^{-/-}$ mice by comparing the compensatory proliferation and crypt repopulation in the colons of animals 3.5 days postirradiation. Consistent with the abnormally high level of epithelial proliferation at baseline, MyD88$^{-/-}$ mice sustained more severe radiation-induced epithelial damage (Figure 4D). These mice showed pronounced defects in crypt repopulation and compensatory proliferation compared to wt mice, evidenced by the decreased number of BrDU positive cells present in the colons 3.5 days postirradiation (Figures 4D and 4E), and the shortened villus length compared to wt mice (Figure 4D).

Although proliferative status is a major determinant of radiation sensitivity, compromised expression of cytoprotective and repair factors by MyD88$^{-/-}$ epithelium (see below) has likely also contributed to increased susceptibility to radiation-induced injury.

TLR-Mediated Recognition of Commensals in the Colon Regulates Production of Tissue Protective Factors

In addition to homeostatic imbalance, increased susceptibility to intestinal injury may be due to defective production of cytoprotective and reparative factors. IL-6, TNF, and KC-1, in addition to their role in inflammation and host defense, play direct roles in protecting various cell types, including neurons (Wang et al., 2002), hepatocytes (Bohan et al., 2003), vascular endothelium (Waxman et al., 2003), renal (Ueland et al., 2003) and lung epithelium (Ward et al., 2000), and keratinocytes (Lin et al., 2003) from injury. In the intestine, in vivo and in vitro studies have shown that these factors play beneficial roles in the response to injury through a direct effect on intestinal epithelium and the initiation of repair responses such as restitution (Yoo, 2002). In particular, IL-6 has been shown to be crucial in protecting the intestinal epithelium from injury (Tebbutt et al., 2002) possibly by regulating intestinal trefoil factor, an indispensable mediator of colonic epithelial repair (Mashimo et al., 1996). It has been unknown, however, whether commensals and TLRs are responsible for the induction of these cytokines for cytoprotection under normal conditions or during intestinal epithelial injury. To address this possibility, we measured the amounts of TNF, IL-6, and KC-1 produced by the colons of mice both at the steady state before administration of DSS and after intestinal injury by DSS. We found that colons of wt mice produced all three factors prior to DSS administration (Figure 5A), and that production of IL-6 and KC-1 was significantly upregulated at various time points after the administration of DSS (Figure 5B). In contrast, colons of MyD88$^{-/-}$ mice produced very low levels of these factors at the uninjured state and were not able to induce these factors after DSS administration despite severe intestinal injury (Figures 5A and 5B). This result demonstrates the important point that commensal bacterial products stimulate TLRs under normal conditions with intact epithelium, as the production of cytokines was dependent on the presence of commensals and a functional TLR-signaling pathway. Indeed removal of commensal flora by antibiotic treatment in vivo (Figure 6; see below) completely eliminated the MyD88-dependent production of cytokines by the colon (Figure 5A). The effect of commensal depletion also demonstrates that the secretion of cytokines was caused by commensal bacteria present in vivo, rather than by tissue manip-

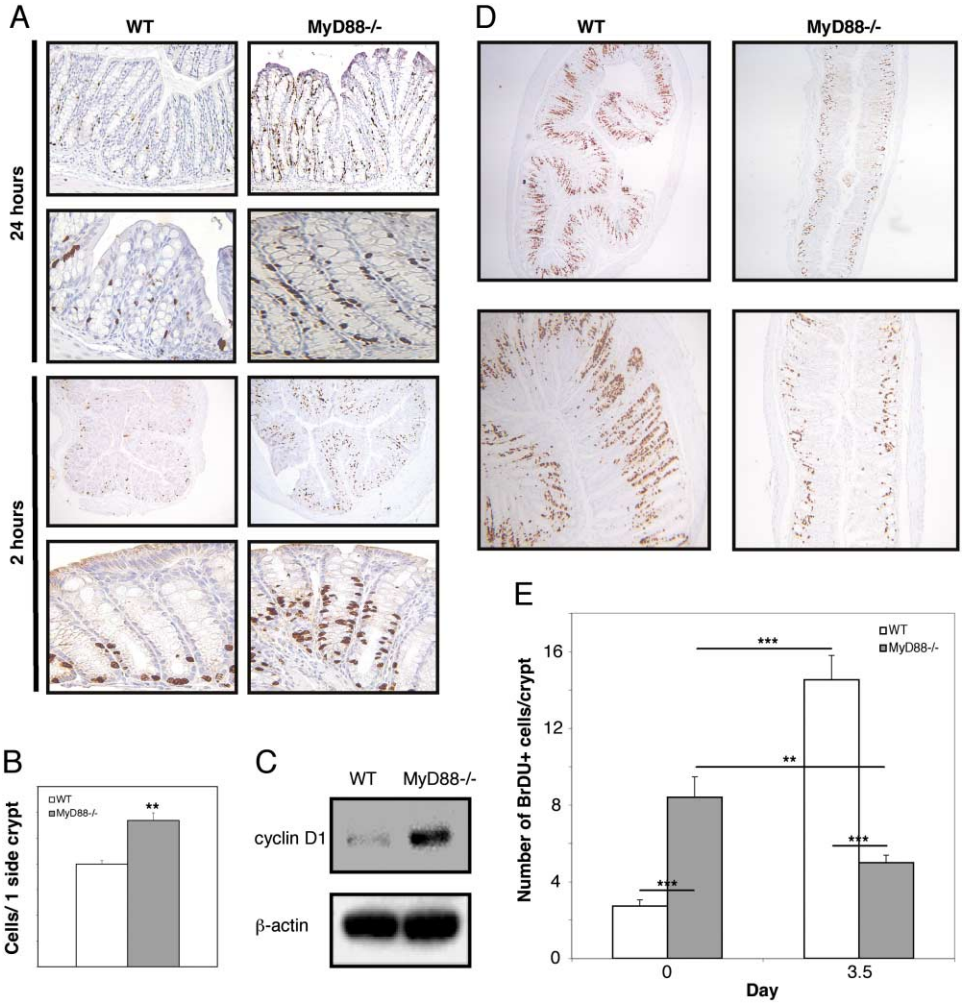

Figure 4. Defects in Steady-State Intestinal Epithelial Homeostasis in the Absence of TLR Signaling

(A) Photomicrographs of immunohistochemical staining for BrDU from sections of colons of wt and MyD88$^{-/-}$ mice injected with 1 mg/ml BrDU and sacrificed 24 hr (upper images) and 2 hr (lower images) later. Sections were counterstained with hematoxylin. Magnifications: For 24 hr: upper, ×200; lower, ×400; For 2 hr: upper, ×100; lower, ×400.

(B) Average number of cells per one side of colonic crypt of wt and MyD88$^{-/-}$ mice.

(C) Protein lysates isolated from colonic epithelium of wt and MyD88$^{-/-}$ were analyzed by Western blot for cyclin D1 and β-actin.

(D) Photomicrographs of 2 hr BrdU staining of wt and MyD88$^{-/-}$ colons 3.5 days after 10 Gy whole body irradiation; upper ×40, lower ×100.

(E) Average number of BrDU+ cells per crypt at 2 hr postinjection of BrDU at baseline (day 0) and 3.5 days after 10 Gy whole body irradiation. Error bars represent ± SEM. ** = $p < 0.01$, (compared to wt) using the Student's test.

ulation in vitro. Although IL-6 and TNF are known to protect against intestinal injury, here we used these cytokines as markers of protective responses, rather than their sole mediators. Indeed, a magnitude of other cytoprotective and repair factors is likely to be involved in TLR-mediated protection from the injury (data not shown).

TLR Signaling in the Colon Controls Expression of Cytoprotective Heat-Shock Proteins

In addition to growth factors and cytokines, members of the heat-shock protein (hsp) families, hsp25 and hsp72, are well established to play a cytoprotective role in many cell types including intestinal epithelial cells (Malago et al., 2002). Recently it has been shown that these hsps can be upregulated in vitro in intestinal epithelial cell lines by bacterial products (Kojima et al., 2003). We therefore tested whether the steady-state expression of hsp25 and hsp72 is compromised in the colonic epithelium of MyD88$^{-/-}$ mice in vivo. We found that expression of both hsp25 and hsp72 was severely diminished in the colonic epithelium of MyD88$^{-/-}$ mice (Figure 5C), suggesting that these cytoprotective proteins are constitutively induced by commensal products through TLRs at the steady state. Importantly, intraepithelial and lamina propria lymphocytes, which are known to express these hsps (Kojima et al., 2003), did not contribute to the differences in hsp expression, as numbers of different subpopulations of these cells (αβ, γδ TCR, CD3, CD4, CD8 T cells, and B cells) were comparable between wt and MyD88$^{-/-}$ intestines (data not shown). Expression of the hsps can be induced either directly

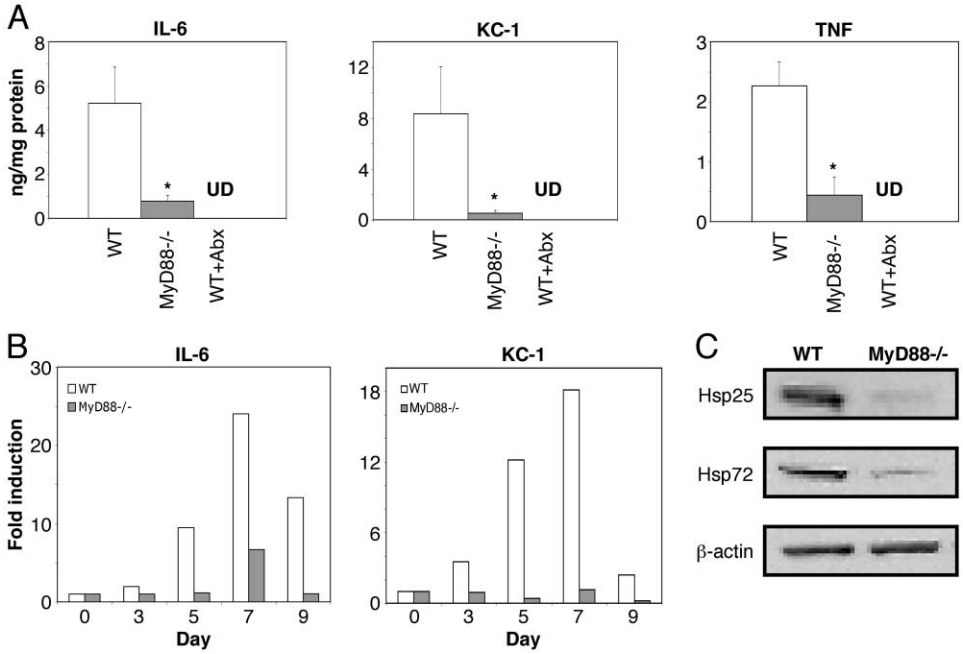

Figure 5. MyD88 Dependent Induction of Cytokines in the Colon by Commensals

(A) Baseline endogenous production of IL-6, KC-1, and TNF by the colons of uninjured wt, MyD88$^{-/-}$, and wt mice depleted of commensals by four week administration of broad-spectrum antibiotics (wt + Abx); UD = undetected.

(B) Induction of IL-6 and KC-1 in wt and MyD88$^{-/-}$ colons at 3, 5, 7, and 9 days after the initiation of DSS. Fold induction was determined by dividing the concentration of factor at each time point by the value at day 0. Data is representative of 2–3 experiments per time point. Factors are derived from spontaneous release into supernatant after 24 hr whole organ culture of colons in serum-free media. Cytokines in the supernatant were measured by ELISA and were normalized for the amount of cytokine per mg of total protein in supernatant.

(C) Protein lysates isolated from colonic epithelium of wt and MyD88$^{-/-}$ mice were analyzed by Western blot for Hsp25, Hsp72, and β-actin. Error bars represent ± SEM. * = $p < 0.05$ (compared to wt) using the Student's test.

by TLR signaling in the epithelial cells, or indirectly, through the cytokines induced by TLRs. In either case, TLR signal-dependent expression of hsps 25 and 72 explains, in part, the protection from epithelial injury by TLR signaling.

Commensal Microflora Is Required for the Protection from Intestinal Epithelial Injury

Taken together these results indicated that TLR signaling via the MyD88-dependent pathway conferred protection from the mortality, morbidity, colonic bleeding, and intestinal epithelial damage caused by the administration of the injurious agent DSS. These results also suggested that commensal bacteria may be responsible for triggering TLRs and conferring protection from direct epithelial damage. To determine the role of commensal microflora in protection against colon injury, wt mice were depleted of all detectable commensals by a four-week oral administration of vancomycin, neomycin, metronidazole, and ampicillin (V/N/M/A) (Figure 6A). The mice that received this combination of antibiotics showed severe mortality and morbidity when given DSS (Figure 6B). Consistent with the hypothesis that commensal bacteria were responsible for the induction of protective factors via TLRs, colons of commensal-depleted animals showed no detectable levels of IL-6, TNF, or KC-1 before and after administration of DSS (Figure 5A and data not shown). In contrast to mice completely devoid of detectable commensals in the gut, animals in which only certain classes of commensal bacteria were depleted through administration of selective antibiotics (Figure 6A) showed 100% survival (Figure 6B), with minimal morbidity and colonic bleeding similar to wt animals not given antibiotics (data not shown). This result suggested that (1) it is not any particular group of commensal bacteria that provides protection, and (2) commensals that are not depleted by antibiotics can still activate TLRs and induce protective and repair responses.

Recognition of Commensal-Derived Ligands by TLRs Is Required for the Protection from Colonic Injury

Collectively, these data suggested that commensal bacterial products engage TLRs and thus confer protection against DSS-induced intestinal epithelial injury. However, it remained possible that the protective effect of commensal microflora was due to the beneficial effect of a particular species of commensals, through specific metabolic activity. To prove that the protection was due to recognition of commensal products by TLRs (rather than due to the metabolic activity of commensals themselves), intestinal microflora-depleted animals were given either purified LPS or LTA in drinking water for one week prior to and during the administration of DSS. LPS and LTA would mimic the ability of gram-negative and gram-positive commensals, respectively, to trigger TLRs, but not metabolic or any other bioactivity of microflora. Remarkably, administration of either LPS (TLR4

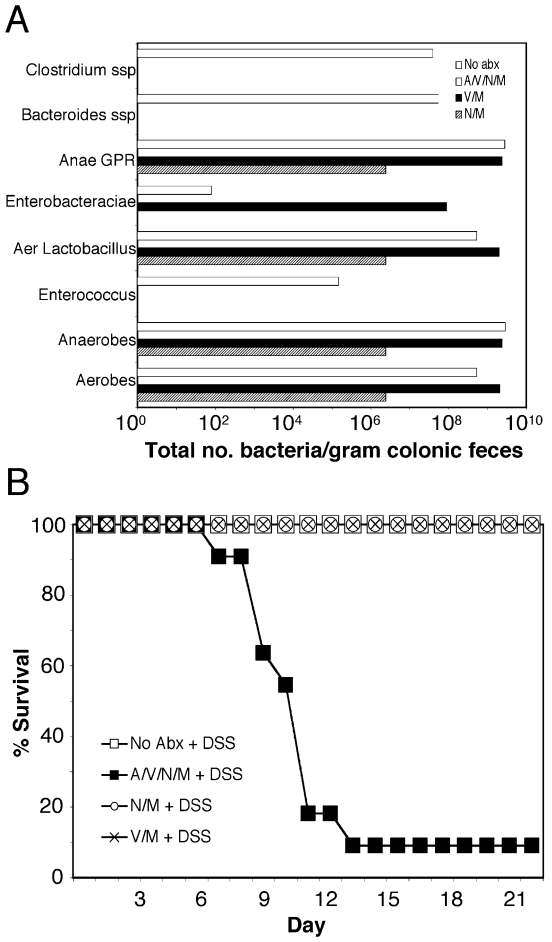

Figure 6. Depletion of Colonic Microflora by Broad-Spectrum Antibiotics

(A) Animals were given ampicillin (A; 1 g/L), vancomycin (V; 500 mg/L), neomycin sulfate (N; 1 g/L), and/or metronidazole (M; 1 g/L) in drinking water for four weeks prior to beginning DSS treatment. Three depletion protocols were used: A/V/N/M, V/M, and N/M. After four weeks on antibiotics, colonic fecal matter was cultured aerobically and anaerobically, and commensal bacteria were identified and quantified using biochemical analysis, morphologic appearance, and Gram staining.

(B) Survival of animals treated with the above combinations of antibiotics for four weeks upon administration of 2% DSS in drinking water for seven days.

ligand) or LTA (TLR2 ligand) via the oral route completely protected animals from the DSS-induced mortality, morbidity, and severe colonic bleeding seen in mice with colons depleted of commensal microflora (Figures 7A–7C). Analysis of colonic epithelium for cytoprotective heat-shock proteins showed a loss of hsp 25 and 72 expression when commensals were depleted by antibiotics (Figure 7D). This expression was upregulated upon oral administration of LPS to these commensal-depleted mice (Figure 7D), correlating with the rescue from colonic injury upon DSS administration seen in these mice. Hsps are presumably not the only factors responsible for LPS-mediated protection in this model, as other LPS induced protective mechanisms are likely to be involved.

Titration of LPS in drinking water showed that the protective effect required LPS dose between 10 μg/μl (100% survival) and 10 ng/μl (30% survival) (Supplemental Figure 3 available on *Cell* website). Finally, the specificity and TLR-dependence of LPS-mediated rescue was confirmed using TLR4$^{-/-}$ and TLR2$^{-/-}$ mice. As expected, oral LPS did not rescue any of the commensal depleted TLR4-deficient mice from DSS-induced mortality, while most of the commensal-depleted TLR2-deficient mice were rescued by oral LPS (Figure 7E). Collectively, these results demonstrate that it is indeed the recognition of commensal bacterial products by TLRs that is responsible for the protection from mortality caused by intestinal epithelial damage.

Discussion

The results presented here reveal a new role of commensal microflora and the innate immune system in mammalian physiology. In addition to the well-appreciated beneficial effects of commensals due to their metabolic activity, interaction of commensal bacterial products with host microbial pattern recognition receptors plays a critical role in resistance to epithelial injury and presumably in other aspects of epithelial homeostasis. Thus, unexpectedly, recognition of commensal bacterial products by the receptors that are critical for defense against pathogens represents a critical component of the symbiosis between the host and indigenous microflora. It remains to be seen whether this interaction may be responsible for some of the other known beneficial effects of commensals on host biology.

Our findings also reveal a new, nonimmune function of TLRs, maintenance of epithelial homeostasis, and protection from direct epithelial injury. This is consistent with recent findings that TLRs may directly induce the expression of several factors (in addition to heat-shock proteins, IL-6, TNF, and KC-1) which are involved in cytoprotection, tissue repair, and angiogenesis, such as COX-2 (Rhee and Hwang, 2000), KGF-1 (Putnins et al., 2002), KGF-2 (Sanale et al., 2002), HGF (Sugiyama et al., 1996; Yoshioka et al., 2001), TGF-β1 (van Tol et al., 1999), VEGF (Li et al., 2001; Zheng et al., 2002), and angiogenin-4 (Hooper et al., 2003). Many of these factors have been shown to be crucial in protecting the gut from injury (Dignass, 2001; Podolsky, 1999).

Our results suggest that this TLR-mediated protection may work through two possible mechanisms that are not mutually exclusive. The first is the steady-state induction of protective factors, via the constitutive detection of lumenally derived TLR ligands on commensals by TLRs expressed on colonic epithelium. Indeed, expression of at least some TLRs, most notably TLR4, has been described in both human and mouse intestinal epithelium (Cario et al., 2000, 2002; Ortega-Cava et al., 2003). Interestingly in this regard, a recent study showed that the in vivo ablation of NF-κB activation in colonic epithelium resulted in increased susceptibility to ischemic injury (Chen et al., 2003). Also consistent with this possibility is the increased proliferation of colonic epithelial cells observed in MyD88$^{-/-}$ mice (Figure 4). This by itself would make them more susceptible to damage (Neta and Okunieff, 1996; Booth and Potten, 2001). Both inflammatory cytokines and NF-κB activation are known

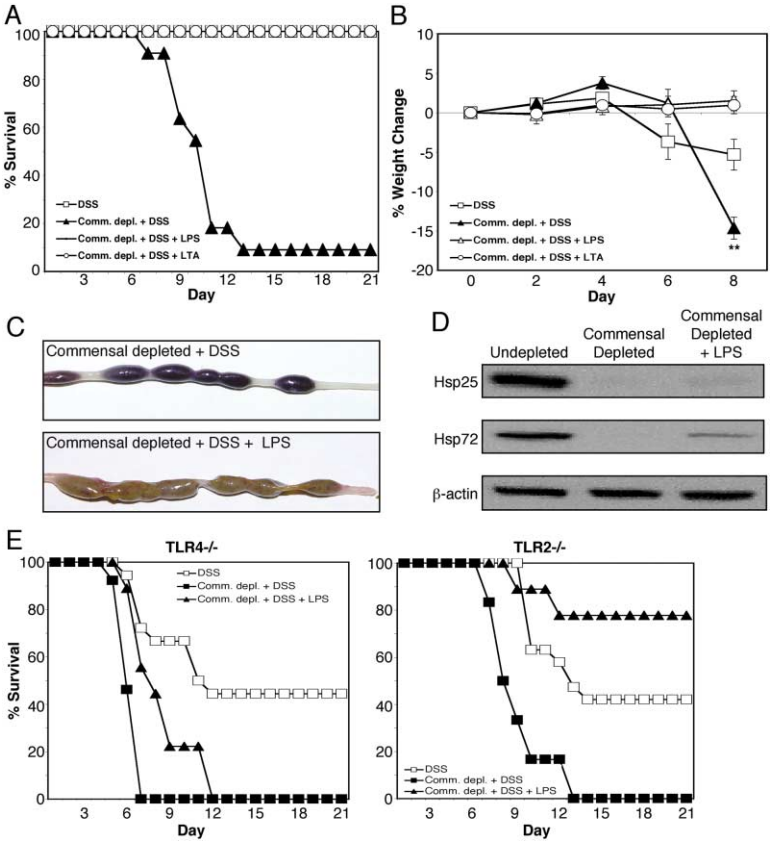

Figure 7. Protection from Gut Injury Is Dependent on Recognition of Commensal-Derived Ligands by TLRs

(A) Survival and (B) percent weight change of wt animals depleted of commensals by a four week regimen of A/V/N/M (Comm. depl. + DSS), commensal-depleted animals reconstituted with either 50 μg/μl of purified *E. coli* 026:B6 LPS (Comm. depl. + DSS + LPS) or 12.5 μg/μl *S. aureus* LTA (Comm. depl. + DSS + LTA), and undepleted mice (DSS) after seven day administration of 2% DSS. LPS and LTA were administered in drinking water for the week prior to and during DSS exposure.

(C) Photograph of representative colons from commensal-depleted wt mice with and without oral reconstitution of LPS at day five of DSS-treatment.

(D) Protein lysates isolated from colonic epithelium of wt animals, without antibiotics (undepleted) commensal depleted, and commensal depleted and reconstituted with oral LPS, were analyzed by Western blot for Hsp25, Hsp72, and β-actin.

(E) Survival of TLR4$^{-/-}$ and TLR2$^{-/-}$ depleted of commensals by a four week regimen of A/V/N/M (Comm. depl. + DSS), commensal-depleted animals reconstituted with 50 μg/μl of purified *E. coli* 026:B6 LPS (Comm. depl. + DSS + LPS), and undepleted mice (DSS) after seven day administration of 2% DSS. Error bars represent ±SEM. ** = $p < 0.01$ (Commensal depleted + DSS + LPS and + LTA compared to Commensal depleted + DSS) using the Student's test.

to have antiproliferative effects on epithelial cells (Basile et al., 2003; Booth and Potten, 2001; Neta and Okunieff, 1996; Seitz et al., 1998, 2000). Moreover, NF-κB participates in regulating epithelial cell turnover in the colon as p50-deficient colons have been shown to have extensive proliferative zones and elongated crypts (Inan et al., 2000), similar to what we find in MyD88$^{-/-}$ mice. Therefore, the increased epithelial proliferation in MyD88$^{-/-}$ colons is likely to be due, at least in part, to the disruption of TLR-induced NF-κB activation and cytokine production.

The second possibility is that commensal-derived TLR ligands may induce the production of protective factors upon epithelial damage. It has long been appreciated that mesenchymal-epithelial crosstalk is crucial to the orchestration of responses to tissue injury (Clark, 2003). For example, the desequestration of luminal repair factors from basolateral receptors is a mechanism used to detect injury and to induce a repair response in both vascular and respiratory systems. Upon disruption of the vascular endothelium, von Willebrand's factor is exposed to type IV collagen present in the basement membrane allowing for the initiation of hemostasis (de Groot, 2002). In the upper respiratory tract, epithelial damage desequesters heregulin, normally present on the apical side of epithelial cells, allowing its access to basolaterally located receptors and triggering them to initiate repair responses (Vermeer et al., 2003). In a similar manner, the detection of commensal-derived ligands by TLRs expressed on cells resident below the basement membrane, such as fibroblasts and macrophages, may represent a signal that disruption of the epithelial barrier has occurred. The two mechanisms are not mutually exclusive, since TLR activation on epithelial cells and myeloid cells can conceivably induce distinct tissue protection and repair responses. Indeed our results showing (1) abnormalities in intestinal epithelial homeostasis at the steady state and a baseline defect in the production of factors such as heat-shock proteins and cytokines and (2) defects in the induced production of these factors following colonic injury, suggest that both epithelial detection of commensals and mesenchymal detection following damage are involved in commensal-TLR interaction required for protection. It would be interesting to determine the possible qualitative differences and relative contributions of these two mechanisms of commensal-TLR interaction.

Inflammatory mediators have long been appreciated for their role in host defense and wound healing (Nathan, 2002). Excessive uncontrolled inflammation, however, results in a variety of pathological conditions, including, at the extreme, septic shock. Evolution of the inflammatory response is thus a result of a trade-off between its beneficial and detrimental effects. Similarly, recognition of commensal bacteria by TLRs plays an important beneficial role in the control of intestinal epithelial homeostasis and protection from direct injury, while dysregulated interaction between commensals and TLRs may promote chronic inflammation and tissue damage, such as that seen in IBD. Our findings emphasize the exis-

tence of a crucial balance between the protective effect of TLR activation by commensals, and the detrimental effect of this interaction when it becomes dysregulated. The importance of this balance is particularly relevant for some areas of medical practice as the common clinical regimens associated with intestinal tissue damage (such as radiation, chemotherapy, and colonic surgery) are universally accompanied by treatment with antibiotics to prevent opportunistic infections. Our findings suggest that complete depletion of microflora may have its own detrimental effects on tissue repair and regeneration and that highly controlled stimulation of TLRs by their ligands (natural or synthetic) may play a beneficial role in certain situations associated with tissue damage.

Recognition of molecular patterns common to entire classes of microorganisms allows a small number of germline-encoded receptors, such as TLRs, to detect a multitude of potential microbial pathogens. However, the direct consequence of pattern recognition strategy is the lack of discrimination by the pattern recognition receptors between pathogenic and commensal microorganisms. It is thought that "undesired" innate immune response to commensals is normally prevented due to the sequestration of microflora by mucosal epithelial barriers, such as intestinal epithelium. This work demonstrates that the ability of TLRs to recognize commensal bacterial products is not simply an unavoidable cost of pattern recognition of infection. Rather, it has its own beneficial and crucial role in mammalian physiology. We therefore propose that mammalian TLRs have at least two distinct functions—protection from infection and control of tissue homeostasis (at least in the case of surface epithelia). Both functions depend on the recognition of microorganisms—pathogens and commensals, respectively. This dual function may explain why some of the TLR-induced gene products, such as inflammatory cytokines and chemokines, are involved in both host defense and tissue repair responses. It would be interesting to determine which of the two TLR functions evolved first.

Experimental Procedures

Mice

MyD88$^{-/-}$, TLR4$^{-/-}$, TLR2$^{-/-}$, and wt littermates mice were bred and maintained under specific pathogen-free conditions at the animal facility of Yale University School of Medicine. These strains are maintained as F2 generations from 129/SvJ X C57BL/6.

Induction of DSS Colitis

Mice received 2% (wt/vol) DSS (40,000 kDa; ICN Biochemicals), ad libitum, in their drinking water for seven days, then switched to regular drinking water. The amount of DSS water drank per animal was recorded and no differences in intake between strains were observed. For survival studies, mice were followed for 21 days post start of DSS-treatment. Mice were weighed every other day for the determination of percent weight change. This was calculated as: % weight change = (weight at day X-day 0/weight at day 0) X 100. Animals were monitored clinically for rectal bleeding, diarrhea, and general signs of morbidity, including hunched posture and failure to groom.

For kinetics studies, animals were sacrificed at various time points post the start of DSS treatment including days 0, 1, 3, 5, 7, and 9.

Radiation-Induced Injury

Mice were exposed to 10 Gy of γ radiation at a rate of 1.8 Gy/min in a 137Cs irradiator. For survival experiments, mice were reconstituted with 3×10^6 bone marrow cells one day postradiation and placed on prophylactic antibiotics to control for mortality due to radiation-induced bone marrow depletion.

Scoring of Colonic Bleeding

Colonic bleeding was determined by a gross colonic blood scoring system as previously described (Siegmund et al., 2001). Colons were analyzed immediately after excision. Scoring was as follows: 0 = lack of any gross blood visible throughout the entire colon; 1 = gross blood present in <1/3 of the colon; 2 = <2/3; 3 = >2/3 of the colon.

Histological Scoring

Colons were excised and cut into three equal segments to be named proximal, middle, and distal colon. Tissue was fixed with 10% neutral formalin, paraffin embedded, sectioned at 3-6 μm, and stained with hematoxylin and eosin. Sections were analyzed in a blinded manner by a trained gastroentero-pathologist. Inflammatory infiltrate was scored using two different types of criteria, extent and severity of injury and character of infiltrate, where infiltrating leukocyte extent/severity score equals area of involved plus score of severity per each layer of the intestine: mucosal, submucosal, muscularis propria, and adventitia. Inflammatory character equals severity of infiltrate plus area involved for each of the following: lymphocytes, neutrophils, plasma cells, and eosinophils. Epithelial injury score equals % area of section plus mucodepletion of glands plus intraepithelial lymphocytes plus ulcer/erosion. Ulcer/erosion score equals ulcer/erosion plus area involved. Histopathological changes were scored on a scale of 0–3 (where 0 = none; 1 = mild; 2 = moderate; 3 = severe) for each parameter. Area involved was scored as follows: 0 = no involvement; 1 = <25% of section; 2 = <50%; 3 = <75%; 4 = <100%. A score was determined for each part of the colon: proximal, middle, and distal. Total scores are the sum of the scores of each individual segment.

Analysis of Red Blood Cells in Peripheral Blood

In order to determine the anemic status of experimental animals, mice were anaesthetized using metaphane and eyebled using heparin-coated capillary tubes (Fisher Scientific). Blood was transferred to microtainer tubes with K2-EDTA (Becton-Dickinson) and inverted multiple times. Red blood cell (RBC) concentration and hematocrit (percentage of whole blood in RBC) were determined by standard hematological analysis in the clinical hematology lab in the Department of Laboratory Medicine of Yale-New Haven Hospital.

Colon Organ Culture

A modification of the protocol of Siegmund et al. (2001) was used. Briefly, 1 cm segments of all three parts of the colon were washed in cold PBS supplemented with penicillin and streptomycin (Gibco). These segments were cultured in 24-well flat bottom culture plates (Falcon) in serum-free RPMI 1640 medium (Gibco) supplemented with penicillin and streptomycin. After 24 hr, supernatant fluid was collected and stored at -20°C until analyzed.

Cytokine Measurement by Enzyme-Linked Immunosorbant Assay (ELISA)

Paired antibodies (α-mouse purified and biotinylated) and recombinant standards for TNF, IL-6, (BD Bioscience Pharmingen), and KC-1 (R&D Systems) were used to quantify factors present in supernatants of whole colon cultures. Levels in whole colon culture supernatant were standardized to the amount of total protein in supernatant by quantification by BCA analysis (Pierce) and presented as ng of cytokine per mg of protein in supernatant.

Depletion of Gut Commensal Microflora

Animals were provided ampicillin (A; 1 g/L; Sigma), vancomycin (V; 500 mg/L; Abbott Labs), neomycin sulfate (N; 1 g/L; Pharmacia/Upjohn), and metronidazole (M; 1 g/L; Sidmack Labs) in drinking water for four weeks prior to beginning DSS treatment and during the course of DSS administration based on a variation of the commensal depletion protocol of Fagarasan et al. (2002). A duration of four weeks of antibiotic treatment was chosen based on both empiric bacteriologic analysis of commensal growth in feces and also to

ensure that detritus of commensal bacteria which includes TLR ligands was absent from colons for one week prior to the administration of DSS. Three combinations of antibiotics were administered; for complete depletion of commensal as verified by bacteriologic analysis of colonic feces, a combination all four antibiotics was used (A/V/N/M). For selective depletions of certain classes of commensals, vancomycin and metronidazole (V/M) and neomycin sulfate and metronidazole (N/M) were used.

Bacterial Culture

For the determination of colonic microflora, fecal matter was removed from colons using sterile technique, placed in 15 ml conical tubes with thyoglycolate, weighed, and vortexed until homogenous. Contents were diluted and plated on universal and differential media for the growth of anaerobes and aerobes. Colonies were counted after incubation at 37°C for 48 hr (aerobes) and 72 hr (anaerobes). Anaerobic cultures were grown in an anaerobic chamber in the clinical microbiology lab in the Department of Laboratory Medicine of Yale-New Haven Hospital. After counting, colonies were picked and identified by biochemical analysis, morphologic appearance, and Gram staining.

To determine bacteremia, spleens were excised under aseptic conditions, placed in thioglycolate, and made into suspension using sterile frosted glass slides. Different dilutions of these suspensions were plated, cultured aerobically and anaerobically, and analyzed as described above for fecal contents.

In Situ Intestinal Migration and Proliferation

Cells in S phase were labeled by i.p. administration of 1 mg/ml of 5′-bromo-2′-deoxyuridine (BrDU) in PBS. Intestines were excised at 2 or 24 hr postinjection and the same segment of colon (4 cm from distal end) was fixed in 10% neutral formalin buffer and embedded in paraffin. Immunohistochemistry was performed using a BrDU staining kit from BD Biosciences. Tissues were counterstained with hematoxylin. The number of cells per crypt column was quantified by counting the number of cells in intact, well-oriented crypts in which adjacent nuclei and lumen were visible.

Isolation of Protein from Colonic Epithelial Cell

Epithelial cells from the large intestine of mice were isolated using the protocol of Saam and Gordon (1999). Protein lysates of colonic epithelial cells were made with a cocktail of protease inhibitors, quantified by BCA, and stored at −70°C.

Western Blot

Colonic protein lysates were resolved on Bis-Tris polyacrylamide gels and transferred to Immobilon paper. Blots were probed with anticyclin D1, c-*myc* (Santa Cruz), Hsp25, Hsp72 (Stressgen), and β-actin (Sigma), followed by the appropriate species specific horseradish peroxidase 2° antibody (Sigma) and developed using the ECL detection system (Amersham).

Reconstitution of Commensal-Depleted Animals with TLR Ligands

wt animals were depleted of commensals using the 4-week, A/V/N/M regimen. At week 3, drinking water was supplemented with 50 μg/μl, 10 μg/μl, or 10 ng/μl of purified *E. coli* 026:B6 LPS (Sigma) or 12.5 μg/μl of *S. aureus* LTA (Invivogen) and was continued in drinking water for the duration of DSS administration. The highest concentration of LPS (50 μg/μl) was selected to assure bioavailability of LPS at the intestinal lumen based on the oral LPS administration protocol of Tamai et al. (2002).

Statistical Analysis

Statistical analysis was performed using the paired Student's t test. P values < 0.05 were considered significant. Error bars represent ± SEM.

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