

The NOD-Like Receptor NLRP12 Attenuates Colon Inflammation and Tumorigenesis

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SUMMARY

NLRP12 is a member of the intracellular Nod-like receptor (NLR) family that has been suggested to downregulate the production of inflammatory cytokines, but its physiological role in regulating inflammation has not been characterized. We analyzed mice deficient in *Nlrp12* to study its role in inflammatory diseases such as colitis and colorectal tumorigenesis. We show that *Nlrp12*-deficient mice are highly susceptible to colon inflammation and tumorigenesis, which is associated with increased production of inflammatory cytokines, chemokines, and tumorigenic factors. Enhanced colon inflammation and colorectal tumor development in *Nlrp12*-deficient mice are due to a failure to dampen NF- κ B and ERK activation in macrophages. These results reveal a critical role for NLRP12 in maintaining intestinal homeostasis and providing protection against colorectal tumorigenesis.

INTRODUCTION

Inflammation is generally considered to be a host protective response against infection and injury (Medzhitov, 2008). However, uncontrolled inflammation is a major risk factor for the development of cancer (Grivennikov et al., 2010). Colorectal cancer is the third most common form of cancer and the second leading cause of cancer-related death in developed countries (Eaden et al., 2001; Ekbom et al., 1990; Itzkowitz and Yio, 2004). Notably, patients with inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis are at increased risk for the development of colorectal cancer (Fiocchi, 1998). Although the precise molecular mechanism of IBD-related colorectal tumor formation is incompletely understood, it is widely viewed that cytokines, chemokines, matrix degrading enzymes, and growth factors produced during chronic inflam-

mation in IBD patients contribute to mutagenic transformation of colonic epithelial cells into neoplastic cells (Grivennikov et al., 2010).

Chronic inflammatory diseases of the gut are initiated by the aberrant interaction of the host immune system with commensal microflora (Goyette et al., 2007; Zaki et al., 2011). Innate immune receptors such as Toll-like receptors (TLR) at the surface of epithelial cells and immune cells initiate this inflammatory process by activating the downstream transcription factor NF- κ B, which is a central mediator of proinflammatory cytokine and chemokine production. However, a tight regulation of NF- κ B signaling is essential to maintaining a beneficial level of homeostatic interactions with the gut microflora. Therefore, deregulated NF- κ B signaling may represent a key mechanism contributing to gut inflammation, colitis, and colorectal tumorigenesis (Leu et al., 2003; van Vliet et al., 2005; Yu et al., 2009). Recent

Significance

Colorectal cancer is the third most common form of cancer and the second leading cause of cancer-related death in developed countries. Chronic inflammation shapes the tumorigenic micro-environment in the gut by inducing cytokines, chemokines, and other factors through NF- κ B, ERK, and STAT3 signaling. In this study, we showed that the NOD-like receptor family member NLRP12 plays a critical role in downregulating these tumor-inducing signaling pathways. Given the importance of anti-inflammatory signals in maintaining colonic homeostasis, these results reveal a regulatory mechanism controlling inflammation and tumorigenesis in the gut, and may help identify new therapeutic approaches to control inflammatory bowel diseases.

studies demonstrated a key role for molecules that negatively regulate NF- κ B activation in maintenance of gut homeostasis. For instance, enterocyte-specific deletion of the NF- κ B regulator A20 rendered mice hypersusceptible to colitis and colorectal tumorigenesis as a consequence of uncontrolled production of NF- κ B-dependent proinflammatory cytokines (Lee et al., 2000; Vereecke et al., 2010). Similarly, mice deficient in TIR8/SIGIRR, a molecule that negatively regulates Toll-like receptor (TLR)- and interleukin (IL)-1 receptor-mediated NF- κ B signaling, suffered from increased susceptibility to colitis and colorectal tumorigenesis (Garlanda et al., 2004; Xiao et al., 2010).

In addition to TLRs, the immune system makes use of a limited set of germ-line encoded pattern recognition receptors (PRRs) to induce the production of inflammatory cytokines in response to microbial components (Kawai and Akira, 2007). This includes C-type lectin receptors (CLRs), RIG-I-like receptors (RLRs), HIN-200 proteins and nucleotide binding, and oligomerization domain-like receptors (NLRs) (Inohara et al., 2005; Kanneganti et al., 2006, 2007). NLR proteins represent platform proteins that are characterized by the presence of a conserved nucleotide binding and oligomerization domain (referred to as NBD; NOD or NACHT domain) and are located in intracellular compartments (Kanneganti et al., 2007). NLRs are implicated in a multitude of innate immune signaling pathways ranging from the regulation of MAP kinase and NF- κ B signaling pathways by NOD1 and NOD2, over modulation of MHC class II genes by CIITA, to the assembly of caspase-1-activating protein complexes named “inflammasomes” by the NLR proteins NLRP1, NLRP3, and NLRC4 (Kanneganti et al., 2007). Unlike the above-mentioned NLRs, the *in vivo* role of the NLR protein NLRP12 is not clear. Notably, polymorphisms in the gene encoding NLRP12 have been linked with increased susceptibility to periodic fever syndromes and atopic dermatitis (Arthur et al., 2010; Borghini et al., 2011; J eru et al., 2008; Macaluso et al., 2007). NLRP12 was initially shown to regulate NF- κ B and caspase-1 activation (Wang et al., 2002). Moreover, NLRP12 was recently suggested to negatively regulate canonical and noncanonical NF- κ B signaling *in vitro* by targeting the kinases IRAK1 and NIK for proteasomal degradation (Arthur et al., 2007; Lich et al., 2007; Williams et al., 2005). However, *NLRP12* missense mutations in periodic fever syndrome patients were recently linked to increased caspase-1 activation rather than to inhibition of NF- κ B signaling (Borghini et al., 2011; J eru et al., 2010). Therefore, the physiological relevance of NLRP12-mediated regulation of NF- κ B pathways remains to be defined. In this study we focused on determining the physiological role of NLRP12 in regulating inflammation in a mouse model of colitis and colorectal tumorigenesis.

RESULTS

Generation of *Nlrp12*-Deficient Mice

Mouse NLRP12 shares with other NLRs a structural composition that exists of an amino-terminal Pyrin motif, followed by a central nucleotide-binding domain (NBD) and a C-terminal leucine rich repeat domain (see Figure S1A available online). The product is encoded on mouse chromosome 7 and contains 10 exons spanning a region of 28.3 kb. We initially investigated the expression pattern of murine *Nlrp12* transcripts in a variety of primary immune cell populations. Cells with the highest expression

levels of *Nlrp12* mRNA were neutrophils and T cells, followed by dendritic cells and macrophages, respectively (Figure 1A). Recently, it was reported that NLRP12 is expressed in the colon tissue (Lech et al., 2010). We further verified the expression of *Nlrp12* in different parts of the gastrointestinal tract and lymphoid organs. *Nlrp12* was found to be expressed in the small intestine, caecum, colon, spleen, liver, and mesenteric lymph nodes (MLN) (Figure 1B). To define the role of NLRP12 in regulating inflammatory responses in the gut, *Nlrp12*-deficient mice were generated by homologous recombination. To this aim, exon II encoding the Pyrin domain of *Nlrp12*—that is required for the recruitment of downstream effectors and functions of the protein—was replaced with a neomycin selection cassette in the targeting construct (Figures S1B and S1C). Positive embryonic stem (ES) cells were used to generate chimeric mice and *Nlrp12*^{-/-} mice were backcrossed to the C57BL/6 genetic background for 10 generations. *Nlrp12*^{-/-} mice were fertile and appeared healthy when housed in a specific pathogen-free environment.

Nlrp12-Deficient Mice Are Hypersusceptible to DSS-Induced Colitis

To define the role of NLRP12 in colitis-induced inflammation, *Nlrp12*^{-/-} mice were fed 3% DSS in drinking water for 5 days and susceptibility was monitored by measuring body weight, assessing stool consistency and rectal bleeding, and measuring colon length during both the acute (day 5) and recovery (days 9–20) stages of disease. Notably, disease progression and clinical scores of wild-type and *Nlrp12*-deficient mice were not statistically different during the acute phase of disease. However, wild-type mice started to recover once DSS was omitted from the drinking water, whereas *Nlrp12*-deficient mice suffered from continued body weight loss (Figure 1C), diarrhea, and rectal bleeding (Figure 1D). This inflammatory phenotype was further evidenced by the gross appearance of the colon. During the acute stage of colitis (at day 5), colons of wild-type and *Nlrp12*-deficient mice appeared similar (data not shown). At day 9, however, colons of *Nlrp12*-deficient mice were significantly shorter than those of wild-type mice (Figures 1E and 1F). To determine whether recovery in *Nlrp12*^{-/-} mice was simply delayed, or whether NLRP12-deficiency prevented healing responses at later time points as well, colon length, and weight of *Nlrp12*^{-/-} mice were analyzed at day 20 after DSS-induced colitis. Notably, colons of *Nlrp12*^{-/-} mice were significantly shorter and weighted more than those of wild-type mice (Figures S1D and S1E), suggesting that *Nlrp12*^{-/-} mice continued to suffer from colon inflammation 2 weeks after DSS administration was stopped. Consistent with an inflammatory phenotype, MLN and spleens of DSS-fed *Nlrp12*^{-/-} mice were found to be significantly heavier and enlarged at day 20 compared to those of wild-type mice (Figures S1F and S1G).

To obtain further evidence of sustained inflammation in *Nlrp12*-deficient mice, colon tissue was analyzed histologically at days 5, 9, and 20 following DSS administration. Consistent with the clinical parameters discussed above, colons of *Nlrp12*-deficient mice contained markedly more infiltrating inflammatory cells, and displayed significantly more ulceration and hyperplasia during the recovery phase of the disease (at days 9 and 20), but not at early stages (Figures 1G and 1H). In agreement, colon tissue of *Nlrp12*-deficient mice contained significantly higher

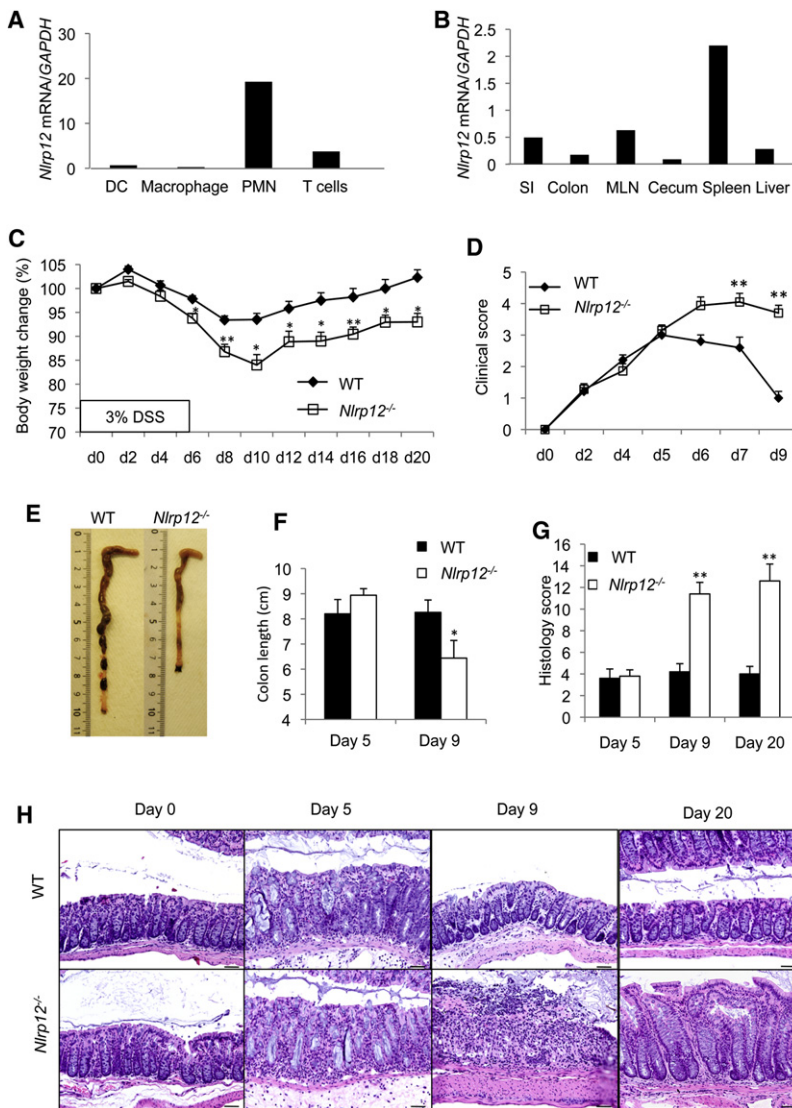


Figure 1. *Nlrp12*-Deficient Mice Are Hypersusceptible to DSS-Induced Colitis

(A) Real time PCR analysis for mRNA of *Nlrp12* in macrophages, dendritic cells (DC), PMN and T cells collected from wild-type mouse.

(B) Real time PCR analysis for mRNA of *Nlrp12* in liver, spleen, small intestine (SI), cecum, colon and MLN. (C, H) Wild-type and *Nlrp12*^{-/-} mice were fed a 3% DSS-solution in drinking water for 6 days followed by regular drinking water until the end of the study.

(C and D) Body weight (C) and clinical score (D; stool consistency plus rectal bleeding score) were scored daily. Data represent means \pm SEM; n = 20/group; *p < 0.05; **p < 0.01.

(E and F) Mice were sacrificed on day 5 and 9 to measure colon length. Histopathological changes in colon tissue collected at day 5, 9, and 20 were examined by H&E staining.

(G) Semiquantitative scoring of histopathology.

(H) Representative images of H&E staining of colon section at day 0, 5, 9, and 20 after DSS administration. Scale bar represents 50 μ m. Data represent means \pm SEM. *p < 0.05; **p < 0.01. See also Figure S1.

levels of proinflammatory cytokines than wild-type mice at day 9 (Figure S1H), but not at day 5 post-DSS administration (data not shown). Together, these results indicate that NLRP12 plays a critical role in resolving the inflammatory response following DSS-induced injury of the colonic epithelium.

NLRP12 Suppresses Colitis-Associated Tumorigenesis

The observation that *Nlrp12*-deficient mice suffered from sustained gut inflammation upon DSS-treatment, prompted us to investigate the role of *Nlrp12* in colitis-associated tumorigenesis. To this aim, we induced colon tumorigenesis by injecting a single dose of the DNA-methylating agent azoxymethane (AOM), which was followed by three cycles of 3% DSS-administration (Figure 2A). Changes in body weight were monitored daily throughout the study duration and colonic tumor burden was determined 12 weeks after AOM/DSS treatment. *Nlrp12*-deficient mice lost significantly more body weight relative to wild-type mice (Figure 2B) and showed increased rectal bleeding (Figure S2A). Consistently, *Nlrp12*-deficient mice had signifi-

cantly higher tumor burdens in the colon, although tumor size was not significantly different (Figures 2C–2E). Tumors in wild-type mice were mostly contained within the colorectal and distal areas of the colon, whereas tumors in *Nlrp12*-deficient mice were commonly found throughout the entire colonic tract (Figures S2B and S2C). Increased tumor burdens in *Nlrp12*-deficient mice were associated with more inflammation and hyperplasia (Figures 2F and 2G), and a higher incidence of dysplasia (Figure 2H). Histological examination of tumors and adenomatous polyps showed that all *Nlrp12*-deficient mice included in the study developed high-grade dysplasia, of which ~30% were classified as adenocarcinoma (Figures 2H and 2I). By contrast, only 20% of the wild-type cohort displayed high-grade dysplasia in the colon, and adenocarcinoma development was not evident in this group (Figure 2I). Collectively, these results indicate a critical role for NLRP12 in protection against colitis-associated tumorigenesis.

NLRP12 Dampens Inflammatory Responses after Colitis Induction

The splenomegaly of DSS-fed *Nlrp12*-deficient mice (Figure S1G) was also apparent in AOM/DSS-administered animals (Figures S3A and S3B). We therefore hypothesized that NLRP12 may protect from colitis-associated tumorigenesis by dampening immune cell activation and inflammatory responses in response to DSS-treatment. To characterize this possibility in additional detail, we carefully examined the histopathological changes that occur during early stages of tumor induction (at day 15 after AOM injection). In line with our hypothesis, histological analysis of colon sections revealed markedly more tissue damage, mucosal edema, inflammation, and hyperplasia in *Nlrp12*-deficient mice than in wild-type mice (Figures 3A and

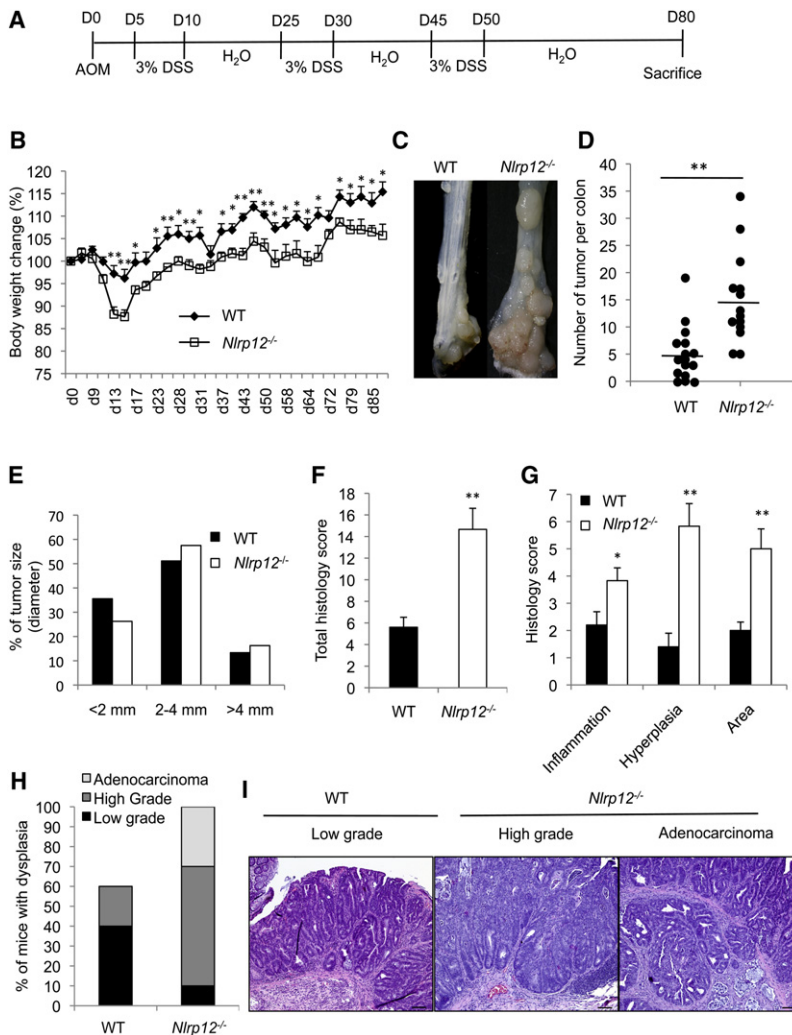


Figure 2. Increased Colitis-Associated Colorectal Tumorigenesis in *Nlrp12*-Deficient Mice

(A) WT (n = 16), *Nlrp12*^{-/-} (n = 14), were injected with AOM on day 0 (d0), and were then given a 3% DSS solution during three 5-day cycles as described in *Experimental Procedures*.
 (B) Body weight changes were monitored throughout the study.
 (C) Twelve weeks after AOM injection, mice were sacrificed to determine tumor development in the colon.
 (D and E) Number of tumors in the whole colon was counted (D), and diameter of the tumors (E) was measured at the end of the study. At the same time colons were processed for histopathological analysis.
 (F and G) H&E stained sections were scored for inflammation, ulceration, hyperplasia, and inflamed area.
 (H) Overall grading of dysplasia in each genotype.
 (I) Representative high magnification images showing dysplasia in colon tissue. Scale bar represents 100 μM. Data represent means ± SEM; *p < 0.05, **p < 0.01. See also *Figure S2*.

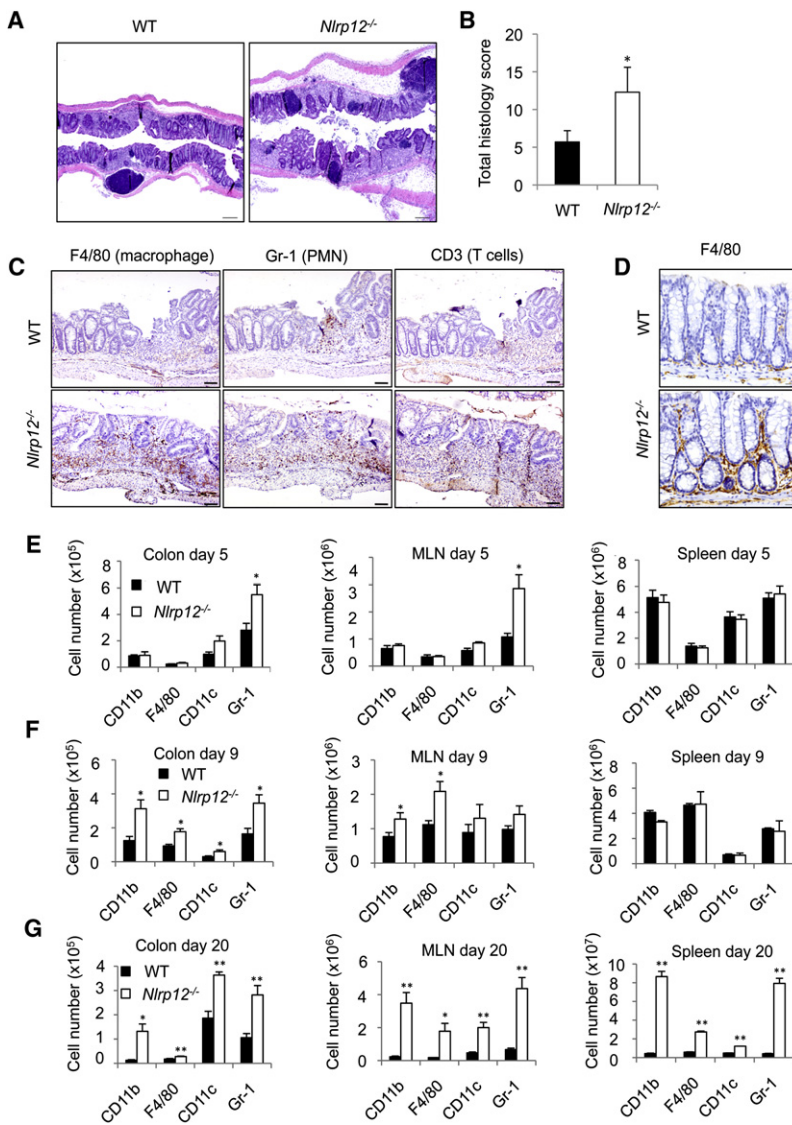
3B). Semiquantitative scores for colon inflammation, ulceration, and hyperplasia were all significantly higher in *Nlrp12*-deficient mice (*Figure S3C*). Moreover, *Nlrp12*-deficient colons showed increased infiltration of macrophages, PMNs, and T cells (*Figure 3C*). Hyperinfiltration of immune cells in *Nlrp12*^{-/-} mice was not confined to inflamed sections of the colon, but extended to the entire colon as evidenced by an increased F4/80-staining in relatively noninflamed parts of the *Nlrp12*^{-/-} colon (*Figure 3D*).

To further characterize the immune cell types associated with the induction of hyperinflammatory responses in the colon of *Nlrp12*^{-/-} mice, myeloid cells present in the colonic lamina propria were isolated at different stages of colitis and analyzed by flow cytometry. During early stages of colitis (at day 5), neutrophils were the most prevalent cell type found in the lamina propria of both wild-type and *Nlrp12*^{-/-} mice, but their number was nearly doubled in *Nlrp12*-deficient colons (*Figure 3E*). Notably, at later stages of colitis (days 9 and 20), cell counts of all analyzed myeloid cell types (CD11b⁺, F4/80⁺, CD11c⁺, Gr⁺) in *Nlrp12*^{-/-} mouse colons were significantly higher than in wild-type colons (*Figures 3F* and *3G*). At day 20, a similarly dramatic increase in infiltration of myeloid cell populations was evident in the mesen-

teric lymph node (MLN) and spleen of *Nlrp12*^{-/-} mice, although myeloid cell counts in these tissues were comparable to those of wild-type mice at earlier time points (*Figure 3G*; *Figure S3D*). Notably, number and frequency of myeloid cell populations in untreated control mice of wild-type and *Nlrp12*^{-/-} background were not different (data not shown). In addition to being more prevalent, a larger number of CD11b⁺ myeloid cells that were collected from the spleen and MLN of *Nlrp12*^{-/-} mice at day 20 produced IL-6 and TNF-α in response to LPS and following PMA plus ionomycin stimulation (*Figure 4A*). Moreover, the mean fluorescent intensity (MFI) for intracellular expression for IL-6 and TNF-α was significantly higher for *Nlrp12*-deficient CD11b⁺ cells than for wild-type cells, indicating that *Nlrp12*^{-/-} myeloid cells produced higher levels of these proinflammatory cytokines (*Figure 4B*). Thus, together these results indicate that NLRP12 plays a critical role in dampening the inflammatory response in myeloid cells and during DSS-induced colitis.

Enhanced Cytokine and Chemokine Production in *Nlrp12*-Deficient Mice Drives Hyperplasia and Tumorigenesis

Consistent with the enhanced infiltration and hyperactivation of myeloid cells in the absence of NLRP12, the production of proinflammatory cytokines such as IL-1β, IL-6, TNF-α, IL-17, and IL-15 were all found to be elevated in the colon of *Nlrp12*^{-/-} mice relative to the levels found in wild-type mice (*Figure 4C* and data not shown). Similarly, colons of *Nlrp12*-deficient mice contained higher levels of the chemokines G-CSF, eotaxin, KC, IP-10, MIP-1α, MIP-1β, and MIP2 (*Figure 4C* and data not shown). Given the association of higher tumor burdens with enhanced production of proinflammatory cytokines and chemokines in *Nlrp12*^{-/-} mice, we hypothesized that unlike most NLRs, NLRP12 may operate as a negative regulator of



inflammatory signaling pathways. Such mechanism may also explain its antitumor function because increased cytokine and chemokine production, along with tumorigenic growth factors, may create a microenvironment that promotes unwarranted cell proliferation and adenomatous polyp development in *Nlrp12*^{-/-} mice.

To understand the nature of the tumorigenic signals that are deregulated in *Nlrp12*^{-/-} mice, we first studied apoptosis induction in colon tissue of AOM/DSS-treated mice. However, mRNA and protein expression of the antiapoptotic protein Bcl-X_L as well as the number of TUNEL-positive cells in colon tissue of wild-type and *Nlrp12*^{-/-} mice were comparable (Figure S4). We next analyzed the expression of multiple cytokines and tumorigenic factors such as cyclooxygenase 2 (COX2) and inducible nitric oxide synthase (iNOS/NOS2) in colon tissue because these factors often drive tumorigenesis. The transcript level of proinflammatory cytokines such as IL-6 and TNF- α and the chemokine MIP2 were markedly more induced in *Nlrp12*^{-/-} mice relative to the levels in wild-type colon (Figure 5A). Unlike IL-6

Figure 3. *Nlrp12*-Deficiency Leads to Increased and Prolonged Inflammatory Responses in Colon Tissue

Wild-type and *Nlrp12*^{-/-} mice were injected with AOM and 5 days later mice were fed with 3% DSS in drinking water for 5 days. Distal colons were collected at day 15 after AOM injection and examined after H&E staining.

(A) Representative images of H&E stained sections of distal colon. Scale bar represents 200 μ M.

(B) Total histological scores of whole colon at the same time. Data represent means \pm SEM; n = 5/group; *p < 0.05.

(C) Colon tissue was immunostained for the macrophage marker F4/80, PMN marker Gr-1, and T cell marker CD3. Scale bar represents 100 μ M.

(D) F4/80 staining in noninflamed part of the colon at day 10 after DSS. Scale bar represents 200 μ M.

(E–G) In a separate experiment, mice were fed with 3% DSS for 5 days followed by normal drinking water until day 20. Colon, MLN, and spleen were collected at day 5, 9, and 20 after DSS. Colonic lamina propria cells, MLN cells, and splenocytes were analyzed flow cytometrically after staining for myeloid cell markers CD11b, F4/80, CD11c, and Gr-1. Total number of CD11b⁺, F4/80⁺, CD11c⁺, and Gr-1⁺ cells at day 5 (E), day 9 (F), and day 20 (G). Data represent means \pm SEM; n = 5/group; *p < 0.05; **p < 0.01. See also Figure S3.

and TNF- α , the levels of the tumor-suppressing cytokine IFN- γ and its effector IFN- γ -dependent NOS2 transcripts were not significantly changed (Figure 5A). In contrast, we measured 3-fold higher mRNA levels of the prostaglandin-synthesizing enzyme COX2, which has previously been linked to colon carcinogenesis (Buchanan and DuBois, 2006; Shiff and Rigas, 1999). Moreover, elevated COX2 and MIP2 expression was also evident in colonic tumors of *Nlrp12*-deficient mice (Figure 5B).

The expression of tumorigenic and proinflammatory genes is modulated by signal transduction pathways mediated by NF- κ B, MAPK, STAT, and AKT proteins. To understand whether these pathways and molecules were deregulated in the absence of *Nlrp12*, we examined activation of NF- κ B, MAPK, and STAT signaling pathways by western blotting. Indeed, significantly higher activation levels of NF- κ B, ERK, and STAT3 were observed in *Nlrp12*-deficient colon tissue at day 15 after AOM injection (day 10 after DSS) relative to the levels found in wild-type mice (Figures 5C and 5D). Hyperactivation of these inflammatory pathways is associated with an increased proliferation of epithelial cells in hyperplastic colon regions of AOM/DSS treated *Nlrp12*-deficient mice (Figures 5E and 5F). By contrast, untransformed colon tissue and unaffected colon regions of both *Nlrp12*^{-/-} and wild-type mice displayed similar proliferation levels as measured by BrdU staining (data not shown). Notably, hyperplastic colonic tissue in *Nlrp12*-deficient mice was surrounded by a massive infiltration of macrophages (Figure 5G), suggesting that myeloid cells may provide signals that promote tumorigenesis in the absence of NLRP12 signaling.

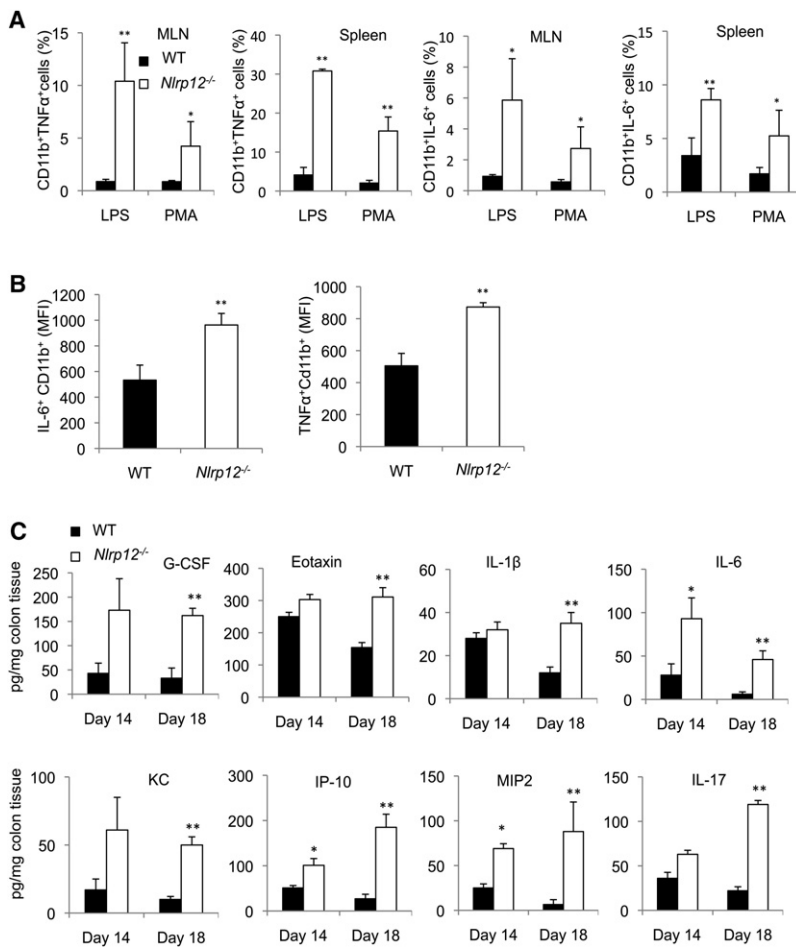


Figure 4. *Nlrp12*-Deficiency Leads to Enhanced Cytokine Production

WT and *Nlrp12*^{-/-} mice were fed with 3% DSS for 5 days followed by normal drinking water until day 20. MLN and spleen were collected at day 20 after DSS administration and cells were stimulated with either LPS (1 μg/ml) or PMA (25 ng/ml) plus ionomycin (500 ng/ml) for 5 hr. After stimulation cells were stained for myeloid cell marker CD11b, and intracellular cytokines IL-6 and TNFα.

(A) Frequency of CD11b⁺TNFα⁺ and CD11b⁺IL-6⁺ cells. (B) Mean fluorescent intensity (MFI) of IL-6⁺CD11b⁺ and TNFα⁺CD11b⁺ cells in LPS stimulated MLN and spleen. Data represent means ± SEM; n = 5/group; *p < 0.05, **p < 0.01. In a separate experiment, wild-type and *Nlrp12*^{-/-} mice were injected with AOM and 5 days later mice were fed with 3% DSS in drinking water for 5 days.

(C) Whole colons were collected at day 14 and day 18 after AOM injection and homogenates were used to determine cytokines by multiplex ELISA. Data represent means ± SEM; n = 7–8/group; *p < 0.05, **p < 0.01.

cient immune cells that was collected at day 15 after AOM injection (day 10 after DSS) showed increased NF-κB and ERK activation (Figures 6D and 6E). Together, these results suggest that *Nlrp12*-deficient myeloid cells, particularly macrophages, fail to silence NF-κB and ERK signaling pathways, which ultimately results in elevated cytokine levels, inflammatory responses, and colon tumorigenesis during colitis.

NLRP12 Negatively Regulates NF-κB and ERK Signaling in Macrophages

To gather further evidence for deregulated NF-κB and ERK signaling in *Nlrp12*-deficient

macrophages, bone marrow-derived macrophages from wild-type and *Nlrp12*-deficient mice were stimulated with LPS, and activation of NF-κB, MAPK, and STAT3 was analyzed by western blotting at different time points. In agreement with the notion that NF-κB activation is enhanced in *Nlrp12*-deficient cells, both IκBα degradation and phosphorylation were found to be increased over a range of time points covering 10 min and up to 2 hr after LPS exposure (Figures 7A and 7B). Furthermore, enhanced phosphorylation of ERK was apparent in LPS-stimulated *Nlrp12*-deficient macrophages (Figure 7A). In contrast, activation levels of STAT3 and the MAP kinases p38 and JNK were comparable to those of wild-type macrophages (Figure 7A and data not shown), confirming the specificity of these results. Enhanced NF-κB and ERK activation in *Nlrp12*-deficient macrophages was not due to deregulated inflammasome activity, because caspase-1 activation in response to the inflammasome activators LPS plus ATP, and upon infection with *Salmonella* or *Listeria* was not affected (Figure S5A).

We next addressed the question whether the increased activation of NF-κB and ERK in *Nlrp12*-deficient macrophages is confined to the TLR4 ligand LPS, or a general response seen with other TLR ligands as well. Wild-type and *Nlrp12*^{-/-} macrophages stimulated with the TLR2 ligand Pam3-CSK4 and the

NLRP12 Signaling in the Hematopoietic Compartment Is Critical for Protection against Colitis and Colitis-Associated Tumorigenesis

To determine the cell populations that contribute to NLRP12-mediated protection against tumorigenesis, we generated 4 groups of *Nlrp12* bone marrow chimeras (Figure 6A). Eight weeks after bone marrow reconstitution, mice were subjected to tumor induction using AOM plus DSS. NLRP12 deficiency in either compartment led to increased body weight loss compared to wild-type mice (Figure 6A), suggesting that NLRP12 may contribute to protection against colitis in both immune and non-immune cells. However, the body weight of *Nlrp12*-deficient mice with wild-type hematopoietic cells later recovered to level similar to those of wild-type mice, whereas mice lacking NLRP12 in the hematopoietic compartment failed to do so (Figure 6A). This suggests that NLRP12 signaling in immune cells is critical to controlling colitis, whereas its role in epithelial cells may be redundant. Consistently, the groups lacking *Nlrp12* in the hematopoietic compartment had significantly higher tumor burdens and shortened colons (Figures 6B and 6C). On the other hand, no significant differences in tumor burdens and colon length were observed between wild-type and *Nlrp12*-deficient mice having wild-type bone marrow cells (Figures 6B and 6C). In agreement, colon tissue of chimera groups with *Nlrp12*-defi-

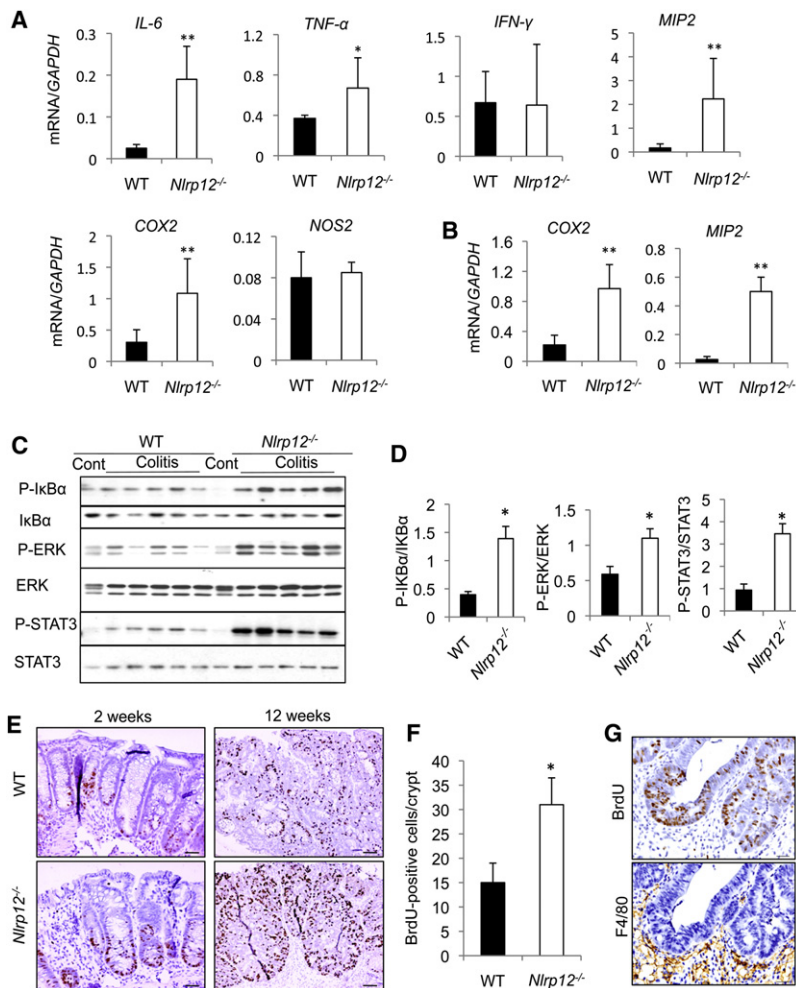


Figure 5. Enhanced Proinflammatory Cytokine Levels in Colon Tissue of *Nlrp12*-Deficient Mice Augments Production of Tumor-Inducing Factors

Wild-type and *Nlrp12*^{-/-} mice were injected with AOM and 5 days later mice were fed with 3% DSS in drinking water for 5 days.

(A) Distal colons collected at day 15 after AOM injection were used to isolate RNA for expression analysis of *IL-6*, *TNF- α* , *IFN- γ* , *MIP2*, *COX2*, and *NOS2* by real time PCR. Data represent means \pm SEM; n = 5–7/group; *p < 0.05, **p < 0.01.

(B) Real time PCR analysis for *COX2* and *MIP2* in distal colon tumors collected at 12 weeks after AOM/DSS treatment. Data represent means \pm SEM; n = 5–7/group; **p < 0.01.

(C) Whole colon tissue homogenates collected at 15 days after AOM injection (10 days after DSS) were examined for activation of NF- κ B, p38, ERK, and STAT3 by western blot analysis. Each lane corresponds individual mouse.

(D) Densitometric analysis of p-I κ B α , p-ERK, and p-STAT3 relative to their total protein. Data represent means \pm SD; n = 5/group; *p < 0.05. In a separate experiment, mice were injected intraperitoneally with BrdU either at day 15 or 12 weeks after AOM injection. Colon sections were stained for BrdU-positive cells and macrophages.

(E) Representative images of BrdU staining at 15 days and 12 weeks after tumor induction. Scale bar represents 50 μ M.

(F) Number of BrdU-positive cells at day 10 after DSS treatment (day 15 after AOM). Data represent means \pm SEM; n = 4 mice/group; 3 different section per mouse; 50 field per section; *p < 0.05.

(G) Serial sections of *Nlrp12*^{-/-} mouse colon at day 10 (day 15 after AOM) were stained for macrophage marker F4/80 (lower panel) and BrdU-positive cells (upper panel). Scale bar represents 20 μ M. See also Figure S4.

TLR3 ligand poly(I:C) showed that ligation of both receptors induced enhanced NF- κ B and ERK activation in *Nlrp12*-deficient macrophages (Figures 7C–7F), indicating a role for NLRP12 in negatively regulating NF- κ B and ERK signaling downstream of multiple TLRs. In agreement with the in vivo data presented earlier, expression of *IL-6*, *KC*, *TNF- α* , *COX2* and *MIP2* were all significantly increased in LPS-stimulated *Nlrp12*-deficient macrophages at the mRNA (Figure 7G) and protein levels (Figure S5B).

Previous studies suggested NLRP12 to suppress canonical NF- κ B activation by preventing IRAK1 phosphorylation, and to downregulate noncanonical NF- κ B signaling by inducing proteasomal degradation of NIK (Lich et al., 2007; Williams et al., 2005). To determine whether the canonical and/or noncanonical NF- κ B signaling was altered in *Nlrp12*-deficient macrophages upon LPS stimulation, we analyzed the phosphorylation status of the noncanonical effector P100, and that of P105 for canonical NF- κ B signaling. Interestingly, the levels of phosphorylated P105 were markedly induced in *Nlrp12*-deficient macrophages after LPS-stimulation (Figure 8A). In contrast, the levels of phosphorylated P100 appeared comparable in LPS-stimulated wild-type and *Nlrp12*^{-/-} macrophages (Figure 8A). To understand whether NLRP12 suppresses canonical NF- κ B pathway during

LPS stimulation, we performed western blot analysis of phospho-P105 and phospho-P100 in cytosolic lysate and RelA (p65) and RelB in nuclear lysate of macrophages. As shown in Figure 8B, phosphorylated P105 levels were markedly higher in *Nlrp12*^{-/-} macrophages than in wild-type cells. Moreover, increased amounts of RelA were detected in the nucleus of *Nlrp12*-deficient macrophages that had been stimulated with LPS (Figure 8C). Finally, we measured the DNA-binding activity of p65 in nuclear isolates of LPS-stimulated macrophages. A significantly higher p65 DNA-binding activity was measured in nuclear lysates of LPS-stimulated *Nlrp12*-deficient macrophages (Figure 8D). Although our results do not exclude the possibility of NLRP12 regulating noncanonical NF- κ B signaling in response to TNF- α receptor family ligands such as CD40L (Lich et al., 2007), it confirms that NLRP12 potently downregulates TLR-induced activation of canonical NF- κ B signaling, as previously suggested (Jéru et al., 2010). In conclusion, NLRP12 dampens inflammation and colon tumorigenesis by attenuating activation of NF- κ B and ERK signaling in myeloid cells. In the absence of NLRP12, upregulated production of inflammatory cytokines and tumorigenic factors drives the transformation of epithelial cells and supports colitis-associated tumorigenesis.

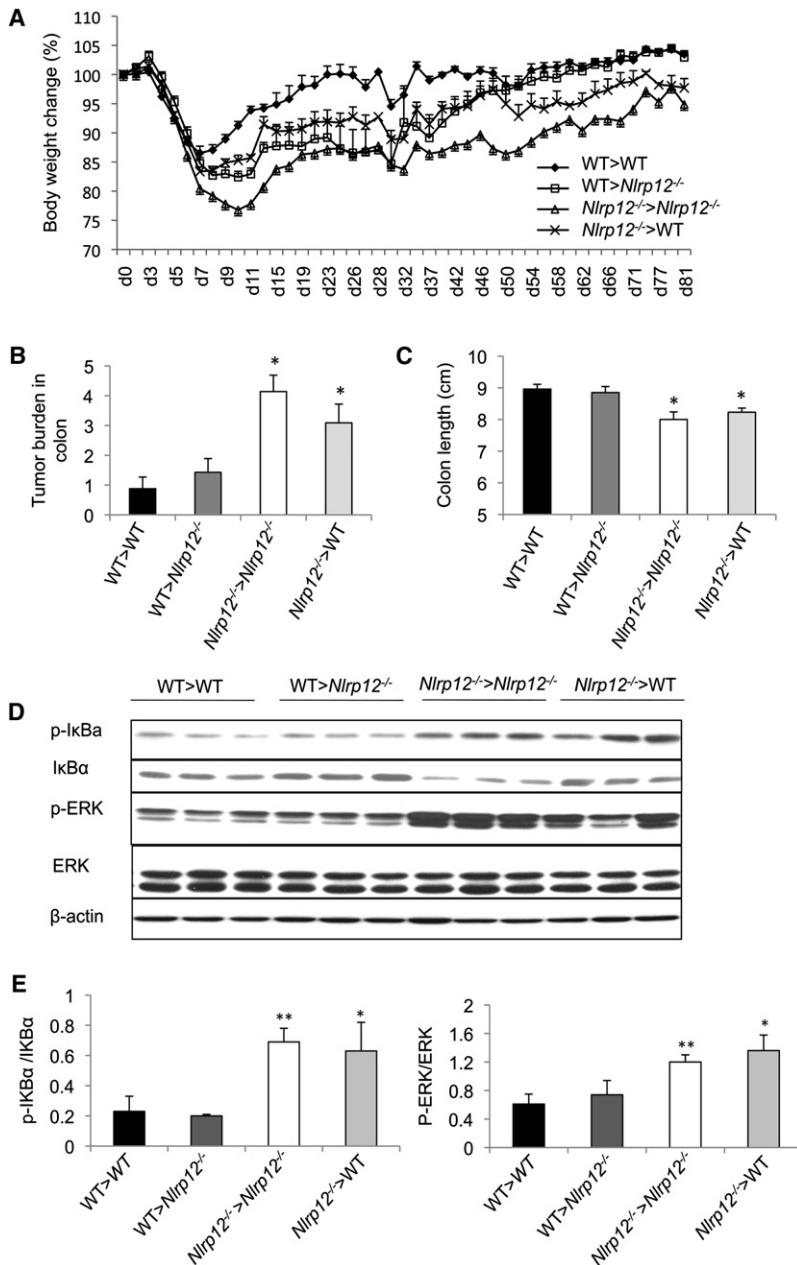


Figure 6. NLRP12 Signaling in Hematopoietic Cells Is Critical for Protection Against Colon Tumorigenesis

Bone marrow chimera mice for *Nlrp12* were generated as described in [Experimental Procedures](#). Eight weeks after, mice (8–10/group) were induced tumorigenesis using AOM/DSS regimen as described in [Experimental Procedures](#).

(A) Body weight was monitored every alternate day.

(B and C) Tumor burden in whole colon and length of colon were measured at 12 weeks after tumor induction. Data represent means \pm SEM; * $p < 0.05$.

(D) In a separate study, colon tissue collected at 15 days after AOM injection were processed for western blot analysis for $\text{I}\kappa\text{B}\alpha$, p- $\text{I}\kappa\text{B}\alpha$, ERK, and p-ERK.

(E) Densitometric analysis of p- $\text{I}\kappa\text{B}\alpha$ and p-ERK relative to their total protein. Data represent means \pm SD; $n = 3/\text{group}$; * $p < 0.05$; ** $p < 0.01$.

layer (Medzhitov, 2007). However, a tight regulation of inflammatory signaling pathways is critical to maintain immune responses at homeostatic levels. Excessive inflammation is destructive by itself and activates cells of the adaptive immune system, which may ultimately result in the development of autoimmunity (Liew et al., 2005). Therefore, the mechanisms controlling inhibition of NF- κ B and other inflammatory signaling pathways are equally important as those driving inflammation. Indeed, deletion of negative regulators of NF- κ B such as A20, TIR8, and DUBA was previously shown to lead to increased susceptibility to DSS-induced colitis (Garlanda et al., 2004; González-Navajas et al., 2010; Lee et al., 2000; Vereecke et al., 2010; Xiao et al., 2010). Similarly, we showed here that *Nlrp12* deficiency leads to increased susceptibility to colitis and colitis-associated tumorigenesis in mice by a failure to dampen inflammatory signaling pathways.

In this study we propose that NLRP12 activity in macrophages plays a major role in attenuating colon inflammation and tumorigenesis in mice. Our interpretation is based on several evidences. At first, we demonstrated that

DISCUSSION

Our studies demonstrate that NLRP12 plays an essential role in the suppression of proinflammatory cytokines and chemokines by controlling the activation of NF- κ B and ERK pathways in response to microbial components and in colitis and colorectal tumorigenesis. Colorectal tumorigenesis is a leading cause of cancer-related death. IBD is a predisposing factor of colorectal cancer (Itzkowitz and Yio, 2004). Chronic colitis develops due to hyperactivation of immune cells upon permeabilization of the colonic epithelial barrier in genetically susceptible hosts (Hill and Artis, 2010). Inflammatory processes initiated upon detection of commensal flora by NLRs and other PRRs primarily aim to control the infection and to restore the damage to the epithelial

NLRP12 activity in the mouse myeloid compartment is critical in protection against colitis-associated colon tumorigenesis. Second, *Nlrp12*-deficient macrophages are hyperresponsive to TLR ligands, showing increased activation of NF- κ B and ERK. Increased macrophage density in tumor tissue is strongly linked with poor prognosis of human cancer (Chen et al., 2005). A growing body of evidence suggests that activated macrophages in human colorectal tumors produce tumor promoting cytokines such as IL-6 and TNF- α , chemokines KC and MIP2, enzymes matrix metalloproteinases, COX2 and NOS2, and growth factors (Qian and Pollard, 2010). Notably, NLRP12 is expressed in both human and mouse monocytic cells (Lich et al., 2007; Williams et al., 2005). It was shown that human monocytic cells having mutation in NLRP12 are hyperinflammatory in nature and linked

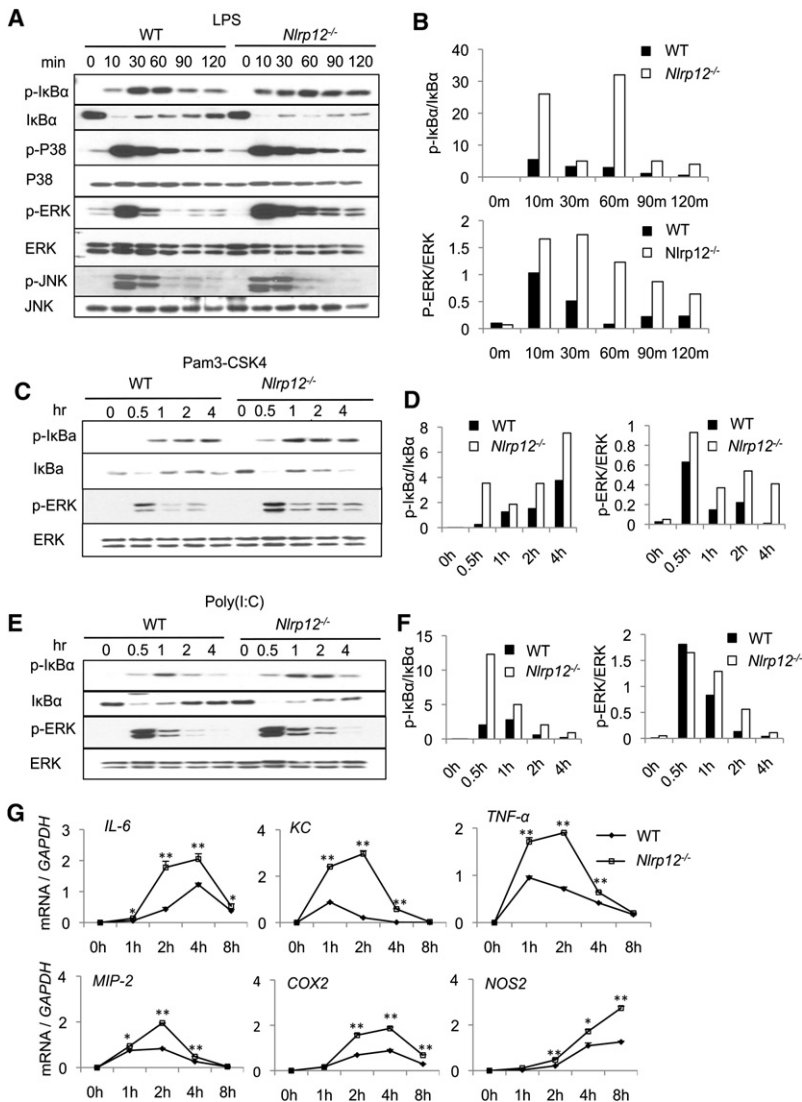


Figure 7. NLRP12 Negatively Regulate NF-κB and ERK Activation in Macrophages

(A–F) Bone marrow-derived macrophages were cultured as described in Experimental Procedures and were stimulated with (A) LPS (1 μg/mL), (C) Pam3-CSK4 (5 μg/mL) (E) poly(I:C) (1 μg/mL). Cell lysates collected at indicated time points were analyzed for p-IκBα, IκBα, p-P38, p-ERK, ERK, p-JNK, and JNK by western blotting. Densitometric analysis of band intensity of p-IκBα and p-ERK relative to IκBα and ERK of LPS- (B), Pam3CSK4- (D), and Poly(I:C)-treated (F) cells.

(G) LPS-stimulated wild-type and *Nlrp12*-deficient macrophages were collected at different time points and mRNA was isolated for real-time quantitative PCR analysis of *IL-6*, *KC*, *TNF-α*, *MIP2*, *COX2*, and *NOS2* synthesis. Data represent means ± SD of triplicate wells; **p* < 0.05; ***p* < 0.01. See also Figure S5.

as COX2, by activating the oncogenic transcription factor cMyc (Wilkins and Sansom, 2008). STAT3 regulates the proinflammatory cytokines IL-17 and IL-23, the antiapoptotic protein Bcl-xL and several growth factors (Yu et al., 2009). Therefore, higher activation of NF-κB, ERK and STAT3 in *Nlrp12*-deficient colon tissue during the recovery stage of the disease strongly supports our phenotypic observation as well as explains the mechanism of increased tumor incidence in *Nlrp12*-deficient mice.

In summary, our study provides evidence for an anti-inflammatory and antitumorigenic role for NLRP12 in vivo by negatively regulating NF-κB and ERK signaling. Considering the importance of anti-inflammatory signals in maintaining colonic homeostasis, our study on the anti-inflammatory function of NLRP12 in colitis and colon tumorigenesis bears enormous importance. This study demonstrates a regulatory mechanism of intestinal inflammation and tumorigenesis by PRRs and paves the way to

to inflammatory disease periodic fever syndrome (Jéru et al., 2008). Mutations in other NLR genes such as *NOD2* and *NLRP3* were previously shown to be associated with autoinflammatory diseases and IBD (Hugot et al., 2001; Maeda et al., 2005; Miceli-Richard et al., 2001; Ogura et al., 2001; Schoultz et al., 2009; Villani et al., 2009; Zaki et al., 2010). Therefore, hyperinflammatory nature of *Nlrp12*-deficient macrophages as seen in this study of mouse model of colorectal tumorigenesis suggests a critical role of NLRP12 in the protection of human colon inflammation and colorectal cancer.

Several tumorigenic factors produced by tumor-associated macrophages are regulated by the signaling pathways NF-κB, STAT3 and ERK. The critical role of NF-κB in both mouse models and human colorectal tumorigenesis is well-known (Gretten et al., 2004; Luo et al., 2004; Terzic et al., 2010). Recent studies in a mouse model of colorectal tumorigenesis demonstrated that both ERK and STAT3 activation are an integral part of tumor development (Bollrath et al., 2009; Kujawski et al., 2008; Lee et al., 2010). ERK regulates several tumorigenic factors such

further understanding the role of NLR proteins in gastrointestinal disorders. This may help identify new therapeutic approaches to control this increasingly important health problem.

EXPERIMENTAL PROCEDURES

Mice

Nlrp12 knock-out mice were generated by homologous recombination in ES cells by replacement of exon II of the *Nlrp12* gene encoding the N-terminal Pyrin domain with an IRES-β-gal-neomycin-resistance cassette via a targeting vector (Figure S1B). A positive ES clone was used to generate chimeric mice. 129/C57BL/6 chimeric mice were crossed with C57BL/6 females to generate heterozygous mice. *Nlrp12*^{-/-} mice were further backcrossed to C57BL/6 background for 10 generations. Male mice 8–10 weeks of age maintained in a pathogen-free facility were used in this study. Animal studies were conducted under protocols approved by St. Jude Children's Research Hospital Committee on Use and Care of Animals.

Induction of DSS-Induced Colitis

Acute colitis was induced with 3% (w/v) DSS (molecular mass 36–40 kDa; MP Biologicals) dissolved in sterile, distilled water ad lib for the experimental

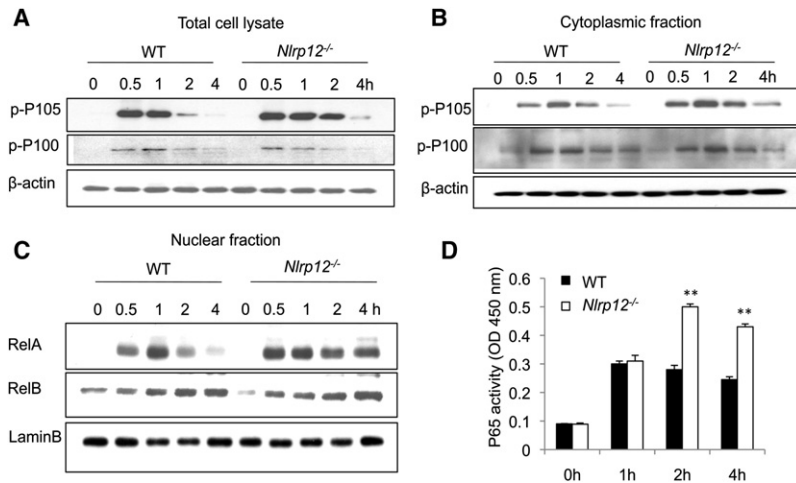


Figure 8. NLRP12 Is a Negative Regulator of Canonical NF- κ B Signaling Pathway in Response to LPS

(A and B) Bone marrow-derived macrophages from wild-type and *Nlrp12*^{-/-} mice were stimulated with LPS (1 μ g/mL). Total cell lysate (A) and cytoplasmic fraction (B) were analyzed for phosphorylation of P100 and P105 by western blotting.

(C) Western blot analysis of NF- κ B complex component RelA and RelB in nuclear fraction.

(D) 5 μ g nuclear protein was assayed for p65 DNA binding activity using commercial ELISA kit as described in [Experimental Procedures](#). Absorbance at 450 nm represents corresponding DNA binding activity of P65 (RelA) in the nuclear extract. Data represent means \pm SD of triplicate wells in ELISA assay of a single sample; **p < 0.01.

days 1–5 followed by normal drinking water until the end of the experiment. The DSS solutions were made fresh on day 3.

Clinical Scoring of Colitis

Scoring for stool consistency and occult blood was done as previously described (Zaki et al., 2010). Briefly, stool scores were determined as follows: 0 = well-formed pellets, 1 = semiformal stools that did not adhere to the anus, 2 = semiformal stools that adhered to the anus, 3 = liquid stools that adhered to the anus. Bleeding scores were determined as follows: 0 = no blood by using hemocult (Beckman Coulter), 1 = positive hemocult, 2 = blood traces in stool visible, 3 = gross rectal bleeding. Stool consistency scores and bleeding scores were added and presented as clinical score.

Induction of Colorectal Cancer

Mice were injected intraperitoneally with 10 mg/kg AOM (Sigma). After 5 days, 3% DSS was given in drinking water over 5 days followed by regular drinking water for 2 weeks. This cycle was repeated twice and mice were sacrificed 4 weeks after the last DSS cycle.

Histopathological Analysis

Formalin-preserved colon sections were processed and embedded in paraffin by standard techniques. Longitudinal sections of 5 μ m thick were stained with hematoxylin and eosin (H&E) and examined by a pathologist blinded to the experimental groups. Colitis scores were assigned based on the extent and severity of inflammation, ulceration, and hyperplasia of the mucosa. Severity scores for inflammation were as follows: 0 = normal (within normal limits); 1 = mild (small, focal, or widely separated, limited to lamina propria); 2 = moderate (multifocal or locally extensive, extending to submucosa); 3 = severe (transmural inflammation with ulcers covering >20 crypts). Scores for ulceration were as follows: 0 = normal (no ulcers); 1 = mild (1–2 ulcers involving up to a total of 20 crypts); 2 = moderate (1–4 ulcers involving a total of 20–40 crypts); 3 = severe (>4 ulcers or over 40 crypts). Mucosal hyperplasia scores were assigned as follows: 0 = normal (within normal limits); 1 = mild (crypts 2–3 times normal thickness, normal epithelium); 2 = moderate (crypts 2–3 times normal thickness, hyperchromatic epithelium, reduced goblet cells, scattered arborization); 3 = severe (crypts >4 times normal thickness, marked hyperchromasia, few to no goblet cells, high mitotic index, frequent arborization). Scoring for extent of lesions: 0 = normal (0% involvement); 1 = mild (up to 30% involvement); 2 = moderate (30%–70% involvement); 3 = severe (over 70% involvement). For immunohistochemistry, formalin-fixed paraffin-embedded tissues were cut into 4 μ m sections and slides were stained with antibodies against the macrophage marker F4/80, neutrophil marker Gr-1, and T cell marker CD3.

In Situ Intestinal Proliferation Assay

The number of proliferating cells in intestinal epithelium was determined using the immunoperoxidase staining protocol with the thymidine analog

5'-bromo-2'-deoxyuridine (BrdU) as described earlier (Zaki et al., 2010). In brief, 1 mg/ml BrdU in PBS was injected intraperitoneally. Three hours later, colon tissue was collected, fixed in 10% neutral buffered formalin, and embedded in paraffin. Immunohistochemistry was performed using an in situ BrdU staining kit (BD Bioscience). Tissues were counterstained with hematoxylin.

In Vitro Signaling Assays

Bone marrow cells were cultured in L-cell-conditioned IMDM medium supplemented with 10% FBS, 1% nonessential amino acid, and 1% penicillin-streptomycin for 5 days to differentiate into macrophages. Bone marrow derived macrophages were seeded in 12-well cell culture plates, cultured overnight, and stimulated with ultrapure *Escherichia coli*-derived LPS (Invivogen), Pam3CSK4 (Invivogen), or Poly (I:C) (Invivogen). For analysis of caspase-1 activation, macrophages were cultured with LPS for 3 hr and then with 5 mM ATP (Sigma) for 30 min or infected with *Salmonella typhimurium* or *Listeria monocytogenes* for 4 hr.

Bone Marrow Chimeras

Bone marrow transfer was used to create *Nlrp12*^{-/-} chimera mice wherein the genetic deficiency of *Nlrp12* was confined to either circulating cells (*Nlrp12*^{-/-} > WT chimera) or nonhematopoietic tissue (WT > *Nlrp12*^{-/-}). Briefly bone marrows were collected from femur and tibia of congenic WT (expressing CD45.1 leukocyte antigen) or *Nlrp12*^{-/-} (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 \times 10⁹/ml. One hundred microliters 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 > *Nlrp12*^{-/-} (WT cells expressing CD45.1 into *Nlrp12*^{-/-} expressing CD45.2); *Nlrp12*^{-/-} > *Nlrp12*^{-/-} (*Nlrp12*^{-/-} cells expressing CD45.2 cells into *Nlrp12*^{-/-} expressing CD45.2); and *Nlrp12*^{-/-} > WT (*Nlrp12*^{-/-} 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 efficiency was verified after 6 weeks by staining for CD45.1 and CD45.2 in blood cells using FITC-conjugated anti-CD45.1 and PE-conjugated anti-CD45.2. All chimera mice used in this study had ~95% reconstitution.

Isolation of Lamina Propria Cells

Colons were dissected, washed with ice-cold PBS supplemented with antibiotics (penicillin plus streptomycin), and cut into small pieces. Colons pieces were then incubated with RPMI medium supplemented with 3% FBS, 0.5 mM DTT, 5mM EDTA, and antibiotics at 37°C for 30 min with gentle shaking. After removing epithelial layer, the remaining colon segments were incubated at 37°C with RPMI medium containing 0.5% CollagenaseD (Roche) and 0.05% DNase (Roche) for 30 min with gentle shaking. The supernatant was passed through 70 μ m cell strainer to isolate lamina propria cells.

NF- κ B Activity Assay

Nuclear p65 DNA binding activity was determined by ELISA-based NF- κ B activity assay (Cayman, Cat #10007889) according to manufacturer instructions. Briefly, 10 μ l (5 μ g) nuclear extract was incubated in 96-well ELISA plate precoated with a specific dsDNA sequence containing NF- κ B response element. A primary antibody for NF- κ B (p65) was then added that was detected by a HRP-conjugated secondary antibody. Finally P65 activity was measured as a colorimetric readout at 450 nm.

Statistical Analysis

Data are represented as mean \pm standard deviation or standard error of the mean. Statistical significance was determined by Student's t test; $p < 0.05$ was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at [doi:10.1016/j.ccr.2011.10.022](https://doi.org/10.1016/j.ccr.2011.10.022).

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