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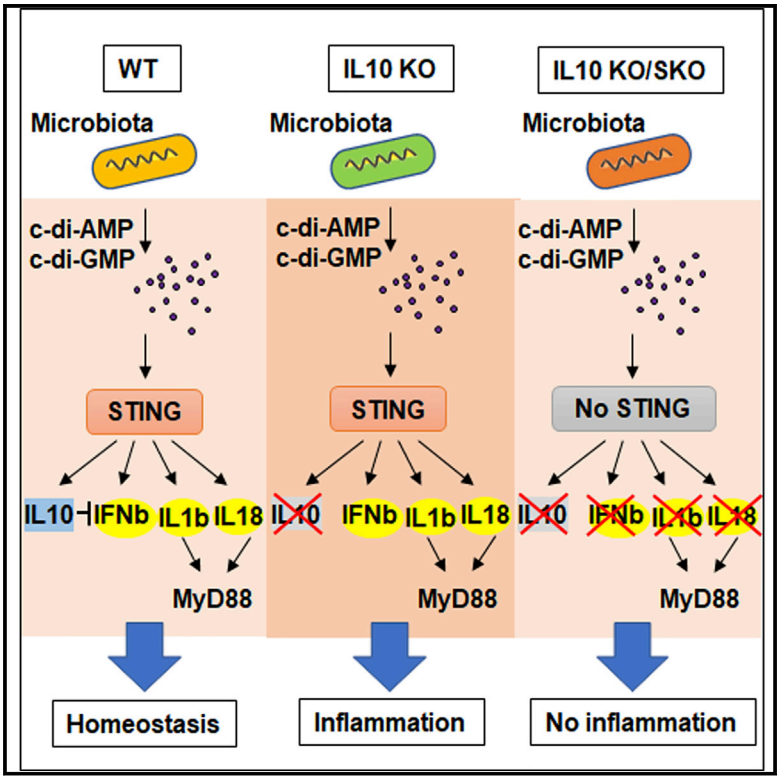
STING-Dependent Signaling Underlies IL-10 Controlled Inflammatory Colitis

Graphical Abstract



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In Brief

Ahn et al. find a key role for STING signaling in controlling gut homeostasis. Loss of STING reverses colitis observed in the absence of the anti-inflammatory cytokine IL-10 in mice. The interface of commensal bacteria with mononuclear phagocytes (MNPs) containing STING is responsible for stimulating pro- as well as anti-inflammatory cytokine expression.

Highlights

- Commensal bacteria stimulate STING signaling to control gut homeostasis
- Both pro- and anti-inflammatory (IL-10) cytokines expression is stimulated by STING
- STING-dependent pro-inflammatory cytokine activity is balanced by IL-10 production
- Monocyte lineages are primarily accountable for STING-mediated cytokine expression

Data and Software Availability

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STING-Dependent Signaling Underlies IL-10 Controlled Inflammatory Colitis

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SUMMARY

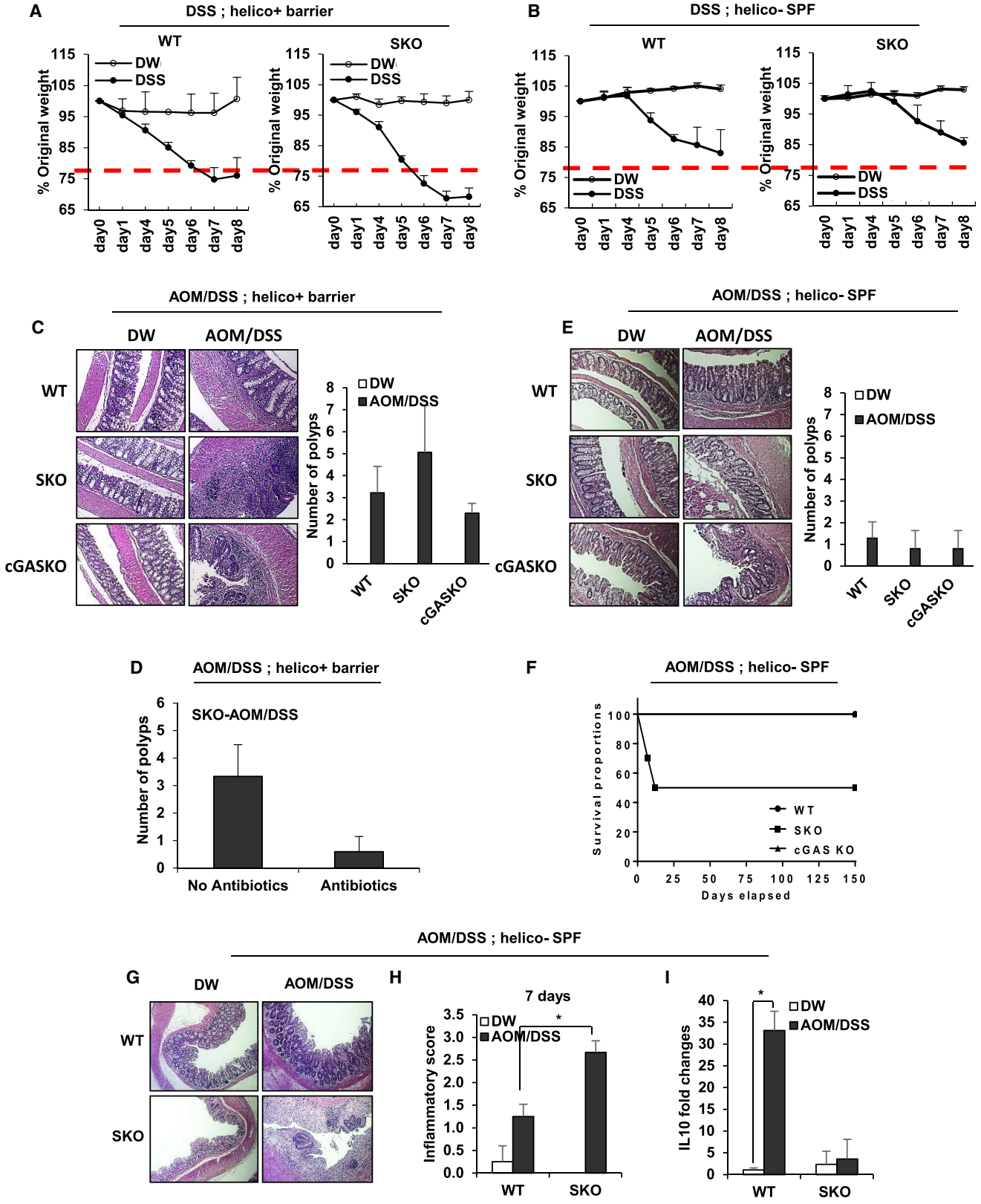
Intestinal immune homeostasis is preserved by commensal bacteria interacting with the host to generate a balanced array of cytokines that are essential for wound repair and for combatting infection. Inflammatory bowel disease (IBD), which can lead to colitis-associated cancer (CAC), is thought to involve chronic microbial irritation following a breach of the mucosal intestinal epithelium. However, the innate immune pathways responsible for regulating these inflammatory processes remain to be fully clarified. Here, we show that commensal bacteria influence STING signaling predominantly in mononuclear phagocytes to produce both pro-inflammatory cytokines as well as anti-inflammatory IL-10. Enterocolitis, manifested through loss of IL-10, was completely abrogated in the absence of STING. Intestinal inflammation was less severe in the absence of cGAS, possibly suggesting a role for cyclic dinucleotides (CDNs) indirectly regulating STING signaling. Our data shed insight into the causes of inflammation and provide a potential therapeutic target for prevention of IBD.

INTRODUCTION

The pro-inflammatory response, while essential for initiating wound repair and protection against pathogens, if uncontrolled, is known to drive a variety of maladies including rheumatoid arthritis, inflammatory bowel disease (IBD), and even cancer (de Souza and Fiocchi, 2016; Grivennikov et al., 2010; Nagata and Kawane, 2011; Saleh and Trinchieri, 2011; Trinchieri, 2012). Incidences of IBD, such as Crohn's disease and ulcerative colitis, are increasing, although the mechanistic causes remain to be clarified (de Souza and Fiocchi, 2016; Loftus, 2004; Sartor, 2006). Gut inflammatory responses are capably circumvented even though the gastrointestinal tract contains trillions of microbes (Belkaid and Hand, 2014). Indeed, most intestinal bacteria are considered commensal to the host, generating nutritional metabolites and even contributing toward facilitating the homeostasis of the immune system (Honda and Littman, 2016). However, damage to the intestinal mucous membrane, comprising the lamina propria and epithelial cells, enables microbes including non-commensal dysbiotic bacteria access to which cells respond by overproducing cytokines to generate an inflammatory state (Varol et al., 2010).

Antibiotics are known to subdue the immune system signifying that mucosal commensal bacteria possibly contribute toward priming the immune system in the gut through a balanced production of a variety of innate immune regulated proteins, including a key anti-inflammatory cytokine interleukin-10 (IL-10) (Arthur et al., 2012, 2014; Honda and Littman, 2016; Uronis et al., 2009). Mice deficient in IL-10 can develop severe enterocolitis, resembling Crohn's disease unless they are treated with antibiotics (Hoentjen et al., 2003; Kühn et al., 1993; Madsen et al., 2000). The innate immune signaling pathways mainly responsible for pro-inflammatory cytokine production, normally suppressed by IL-10, remain to be clarified. However, loss of the Toll-like receptor (TLR) adaptor protein, myeloid-differentiation primary response protein (MyD88), in mononuclear phagocytes (MNPs) can eliminate inflammation in IL-10-deficient mice, suggesting a role for the TLR pathway or for pro-inflammatory cytokines that require MyD88 for signaling such as IL-1β or IL-18 (Hoshi et al., 2012; Rakoff-Nahoum et al., 2006; Salcedo et al., 2010). IL-10 production can also be initiated through TLR signaling or by type I interferon (IFN), which utilizes IFN-regulatory transcription factors (IRFs) and nuclear factor κB (NF-κB) to exert their effects (Ouyang et al., 2011). Following binding to the IL-10 receptor (IL-R1/2), IL-10 principally signals through STAT3 to prevent the pro-inflammatory effects of cytokines such as IL-12, IL-23, and IFN-λ (Chang et al., 2007; Hutchins et al., 2013; Manzanillo et al., 2015; Saraiva and O'Garra, 2010).

It has recently been shown that another key innate immune pathway, controlled by an endoplasmic reticulum (ER)-associated protein referred to as STING (stimulator of IFN genes), may also be involved in controlling inflammation (Ishikawa and Barber, 2008; Ishikawa et al., 2009). STING is activated by cyclic dinucleotides (CDNs) such as cyclic di-AMP or GMP (c-di-AMP, c-di-GMP) directly exuded by certain bacteria, or by cGAMP (cyclic GMP-AMP), which is generated by the cellular synthase cyclic GMP-AMP synthase (cGAS), following association with microbial or self-dsDNA species (Ablasser et al., 2013; Barber, 2014; Burdette et al., 2011). Here, we have evaluated the role of STING signaling in influencing intestinal inflammation and demonstrate that this innate immune pathway interacts with host commensal bacteria to play a key role in producing both pro- and anti-inflammatory cytokines that facilitate gut immune homeostasis.



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RESULTS

Commensal Bacteria-Host Interactions Influence STING Signaling

To evaluate the role of STING in influencing colitis, we orally treated mice containing (wild type [WT]) or lacking STING (STING knockout [SKO]) with dextran sodium sulfate (DSS), which can trigger intestinal inflammation (Ahn et al., 2015). Principally, the experiments were conducted with mice housed in a barrier facility, containing *Helicobacter* spp. (helico+ barrier), which is known to influence the outcome of colitis (Fox et al., 2011; Oliveira et al., 2004). We observed that mice lacking STING modestly lost weight in response to DSS treatment over an 8-day period, compared to similarly treated WT mice (day 5, $p = 0.005$; day 6, $p = 0.007$; day 7, $p = 0.027$) (Figure 1A). Correspondingly modest differences in histology were noted, which nevertheless indicated that mice lacking STING may play a role in early innate immune responses to colonic irritation, under these conditions (Figures S1A–S1C). However, similar treatment of mice housed in a *Helicobacter* spp.-negative environment (helico− specific pathogen-free [SPF]) indicated no significant differences in inflammatory responses to DSS treatment (Figures 1B and S1D). A similar observation, namely lack of notable inflammation in response to DSS treatment, was observed using mice deficient in the cGAS (Figures S1D–S1F). These data suggest that intestinal flora may play a role in influencing STING-dependent inflammatory responses. That STING signaling may be important in intestinal immunity has been demonstrated by observing that SKO mice, treated with azoxymethane/dextran sodium sulfate (AOM/DSS), which can induce colitis-associated cancer (CAC), develop increased polyp formations compared to control mice (Ahn et al., 2015). To further investigate the importance of STING signaling in intestinal immunity, we treated mice housed in a helico+ barrier room with AOM/DSS for approximately 4 months, and confirmed that SKO mice develop higher numbers of polyps compared to similarly treated WT mice (Figure 1C). To appraise the role of commensal bacteria in influencing this outcome, we correspondingly treated SKO mice with AOM/DSS in helico+ barrier housing conditions in the presence or absence of antibiotics. Our results demonstrated that antibiotic-treated SKO mice developed fewer polyps compared to untreated SKO mice, inferring a key role for bacteria in manipulating this event (Figure 1D). 16S ribosomal RNA sequence analysis of commensal microbial populations within the SKO mice housed under helico+ barrier housing conditions indicated, by principal coordinate analysis (PCoA) using Bray-Curtis dissimilarity at the operational taxonomic unit (OTU) level, significant bacterial differences to that of healthy WT mice (Figure S2). These included key differences in *Turicibacter* and *Odoribacter* species, implying that loss of STING signaling can influence commensal bacteria portfolios (Figure S2). Of interest was that cGAS-deficient mice (cGAS knockout [cGASKO]) did not appear to exhibit an increased amount of polyp formation compared to WT mice, suggesting that bacteria-produced CDNs rather than genomic DNA may play a role in triggering STING activity (Figure 1C). These data indicate that STING signaling is required to recognize AOM/DSS-induced DNA damage and initiate the wound repair processes, which if not instigated may enable microbial-influenced inflammatory events to facilitate CAC. This possibility was enforced by observing that AOM/DSS-treated SKO mice housed in a *Helicobacter* spp.-negative environment did not exhibit increased polyp formation (Figure 1E). Rather, we observed that SKO mice housed in *Helicobacter* spp.-free conditions had significantly higher inflammation and mortality rates compared to WT or cGASKO mice (Figures 1F–1H). This may be explained in part, by noting that SKO mice failed to generate anti-inflammatory IL-10 in response to AOM/DSS treatment (Figure 1I). Collectively, our observations suggest that STING may interact with commensal bacteria to generate anti-inflammatory cytokines such as IL-10 and play a role in maintaining gut immune homeostasis.

IL-10 Suppresses STING-Aggravated Inflammatory Colitis

That commensal bacteria can influence intestinal inflammation has been observed using other models of chronic colitis. For example, loss of IL-10, a major immunosuppressive cytokine, induces spontaneous colitis in mice because the effects of concomitant pro-inflammatory cytokine production including IFN-λ and IL-12 are not blocked (Davidson et al., 1998). Since antibiotics can eliminate the observed colitis and CAC in this model, commensal bacteria may constitutively stimulate innate immune signaling pathways resulting in the production of both pro-inflammatory cytokines as well as counterbalancing anti-inflammatory IL-10. However, the innate immune pathways that control host interactions with intestinal flora remain to be clarified. Given that antibiotics can also eliminate STING-dependent CAC and our observations of reduced levels of IL-10 in SKO mice

Figure 1. Commensal Bacteria-Host Interactions Influence Colonic Polyp Formation

(A and B) Body weight assessment of B6 background of wild-type (WT) and STING knockout (SKO) mice received 5% of dextran sodium sulfate (DSS) in drinking water for 7 days in helico+ barrier room (A) or helico− SPF room (B). The data are representative of at least two independent experiments.

(C) Representative photographs of H&E staining of colon tissues and the number of polyps in colon tissue of WT and SKO mice, either AOM/DSS treated or normal water treated in the helico+ barrier room.

(D) The number of polyps in AOM/DSS-treated SKO mice, either antibiotics treated or not. An antibiotic cocktail was administered in the drinking water of a separate cohort of mice for 1 month prior to AOM/DSS treatment.

(E) Representative photographs of H&E staining and the number of polyps in colon tissue of WT and SKO mice, either AOM/DSS treated or normal water treated in the helico− SPF room.

(F) Mortality rates of AOM/DSS-treated WT, SKO, and cGASKO mice in helico− SPF room.

(G and H) Representative photographs of H&E staining (G) and inflammation score (0, normal, to 3, most severe) (H) in either AOM/DSS treated or normal water treated in helico− SPF room for 7 days.

(I) qPCR analysis of IL-10 expression in colon from the mice same as (H). Error bars indicate SD. Statistical analysis was performed using Student's t test. $^{*}p \leq 0.05$.

treated with AOM/DSS (Figures 1D and 1I), we thus examined the importance of STING in the development of IL-10-controlled colitis and polyp formation. To achieve this, we generated IL-10$^{-/-}$STING$^{-/-}$ double-deficient mice (IL10KO/SKO). Our results indicated that, while IL-10$^{-/-}$ mice (IL10KO) developed severe colitis within 10 weeks, IL-10$^{-/-}$STING$^{-/-}$mice did not exhibit any significant intestinal inflammatory disease for over 19 weeks (Figure 2A). The pronounced thickening of the bowel wall and slightly shortened colon length characteristic for IL-10-deficient mice was also reduced in IL-10$^{-/-}$STING$^{-/-}$mice (Figures 2B–2D and S3A). The incidence of spontaneous polyp formation in IL-10-deficient mice was also completely eliminated in the absence of STING (Figures 2E and S3B). Gene expression profiles were measured on the various mice, using PCR and microarray analysis, which indicated that high levels of pro-inflammatory cytokine production including IL-1β, IL-22, and IL-12, as well as members of the Regenerating Family (Reg3b/g) typically detected in the colon of IL10KO mice, were similarly greatly repressed in the absence of STING signaling (Figures 2F and 2G). A similar effect was seen crossing cGAS-deficient mice with IL-10-deficient mice (IL10KO/cGASKO) (Figure S4A). However, we observed that 10% of the examined IL10KO/cGASKO mice developed polyps, perhaps again suggesting a direct role for CDNs on influencing STING signaling (Figures S4B and S4C). Our data thus indicate that STING signaling may play a significant role in the development of colitis in the absence of IL-10, plausibly by interacting with microbes to generate pro-inflammatory cytokines.

STING Signaling Does Not Require the Adaptor MyD88

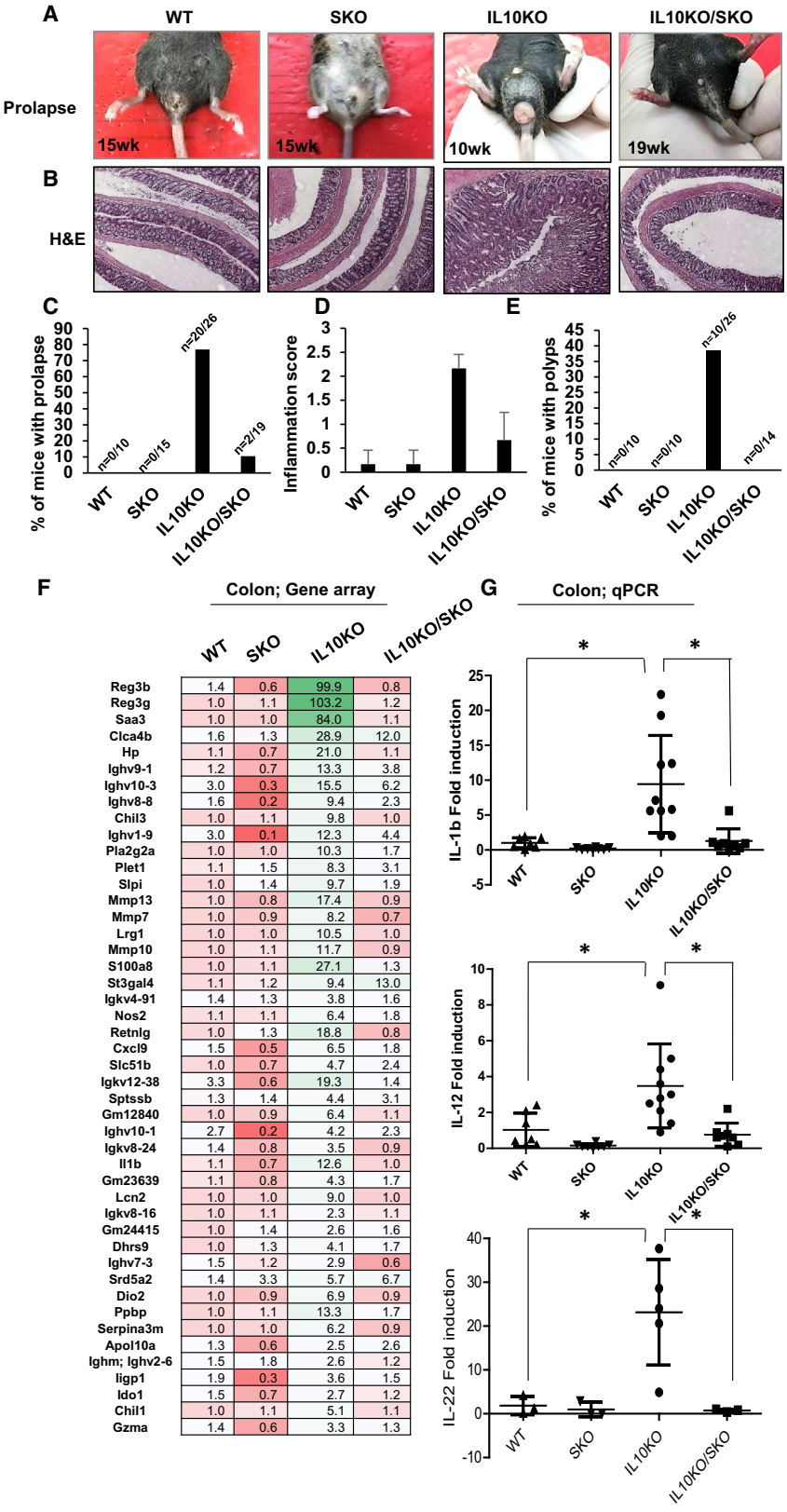
It is thus conceivable that STING signaling is stimulated following interaction with commensal bacteria. STING activation may invoke the generation of pro-inflammatory cytokines, which are normally suppressed by IL-10, to induce colitis. However, it is known that loss of MyD88 can also eliminate inflammatory colitis induced by IL-10 deficiency (Hoshi et al., 2012; Rakoff-Nahoum et al., 2006). Thus, it is acceptable that MyD88 may play a direct role in STING-dependent signaling or, alternatively, that STING-dependent pro-inflammatory cytokines may require downstream MyD88-dependent signaling to exert their effect. To determine this, we treated STING or MyD88-deficient murine embryonic fibroblasts (MEFs), bone marrow-derived macrophages (BMDMs) or dendritic cells (BMDCs) with exogenous CDNs or cytosolic dsDNA (IFN-stimulatory DNA [ISD]), which triggers STING signaling and type I IFN production. These data confirmed that STING but not MyD88 was required for CDNs or cytosolic dsDNA-dependent type I IFN production as determined by microarray analysis and PCR (Figures 3A–3D). We further confirmed that STING was not required for lipopolysaccharide (LPS)-driven innate immune signaling, which requires TLR4 and the adaptors MyD88 or TRIF to drive cytokine production such as IL-1b, at least in BMDMs or BMDCs (Figures 3E–3H). However, loss of STING was seen to somewhat abrogate IL1β production in response to LPS in MEFs, for reasons that presently remain unclear (Figures 3E and 3F). Of interest was that we observed that STING signaling could trigger STAT3 phosphorylation as well as STAT1 via cytosolic DNA stimulation. This was particularly noticeable in BMDCs compared to BMDMs (Figures 4A and S5). Cells retrieved from SKO mice were confirmed to retain sensitivity to LPS treatment, unlike those analyzed from MyD88-deficient mice (Figures 4B and S6). cGAS was seen to be required to activate STAT1 and STAT3 by ISD but not by exogenous CDNs, which acted on STING directly (Figure S5). Given these data, it is possible that commensal bacteria may stimulate STING signaling to induce cytokines that bind to receptors requiring MyD88 to exert their downstream pro-inflammatory effects. Thus, at least in part, loss of MyD88 may abrogate colitis manifested by IL-10 deficiency by preventing the action of STING-dependent pro-inflammatory cytokines.

STING Signaling Drives Pro-inflammatory, MyD88-Dependent Gene Induction

Key pro-inflammatory cytokines that bind to receptors requiring MyD88 to exert pro-inflammatory responses include IL-1β and IL-18 (Salcedo et al., 2010, 2013). The promoter region of both these cytokines are known to harbor NF-κB and STAT3 transcription factor binding sites, which, in turn, are stimulated directly or indirectly by STING signaling (Ahn et al., 2014, 2015; Barber, 2015). To confirm whether these cytokines can be induced in a STING-dependent manner, we treated BMDMs or BMDCs with CDNs or cytosolic DNA and first measured IL-1β induction by PCR. These data indicated that IL-1β production was indeed detectable in response to CDNs and dsDNA in a STING-dependent manner at least in these cell types (Figures 4C–4F). A similar study indicated that IL-18 production could also be augmented by STING signaling in BMDMs or BMDCs (Figures 4G–4J). Thus, the stimulation of STING signaling by CDNs/dsDNA can help increase the production of pro-inflammatory cytokines, IL-1β and IL-18, that require MyD88 to exert their influence in select cells. Therefore, the enhancement of colitis driven by loss of IL-10 may be partially explained by STING-dependent genes such as IL-1β and IL-18 exerting an inflammatory effect.

The Expression of Anti-inflammatory IL-10 Can Be Modulated by STING Signaling

The observation that loss of STING can rescue colitis manifested by loss of IL-10 implies not only that STING signaling may contribute toward the production of pro-inflammatory cytokines whose function is generally suppressed by IL-10 signaling but also that IL-10 expression itself may be regulated by STING as our preliminary data suggest (Figure 1I). IL-10 is expressed in a wide variety of immune-related cells including MNPs in response to a variety of stimuli including the LPS-triggered TLR4 pathway, requiring MyD88, as well as type I IFN (Saraiva and O'Garra, 2010). Indeed, the promoter region of IL-10 is known to harbor sites for STAT1, -3, NF-κB, and members of the IRF family. IL-10 binds to IL-10 receptors (IL-10R1/R2) and triggers the activation of STAT3 signaling to downregulate proteins involved in inflammation such as IL-12, IL-23 and IFN-γ. Upon analysis of BMDMs as well as BMDCs, we confirmed that IL-10 can also be induced by cytosolic DNA, in a STING-dependent manner (Figures 5A–5D). An additional member of the IL-10 family is IL-22, which is also inducible by NF-κB, STAT3-dependent signaling (Hutchins et al., 2013; Manzanillo et al., 2015). IL-22 is known to induce proinflammatory cytokines in the gut such



	WT	SKO	IL10KO	IL10KO/SKO
Reg3b	1.4	0.6	99.9	0.8
Reg3g	1.0	1.1	103.2	1.2
Saa3	1.0	1.0	84.0	1.1
Clca4b	1.6	1.3	28.9	12.0
Hp	1.1	0.7	21.0	1.1
Ighv9-1	1.2	0.7	13.3	3.8
Ighv10-3	3.0	0.3	15.5	6.2
Ighv8-8	1.6	0.2	9.4	2.3
Chil3	1.0	1.1	9.8	1.0
Ighv1-9	3.0	0.1	12.3	4.4
Pla2g2a	1.0	1.0	10.3	1.7
Plet1	1.1	1.5	8.3	3.1
Slpi	1.0	1.4	9.7	1.9
Mmp13	1.0	0.8	17.4	0.9
Mmp7	1.0	0.9	8.2	0.7
Lrg1	1.0	1.0	10.5	1.0
Mmp10	1.0	1.1	11.7	0.9
S100a8	1.0	1.1	27.1	1.3
St3gal4	1.1	1.2	9.4	13.0
Igkv4-91	1.4	1.3	3.8	1.6
Nos2	1.1	1.1	6.4	1.8
Retnlg	1.0	1.3	18.8	0.8
Cxcl9	1.5	0.5	6.5	1.8
Slc51b	1.0	0.7	4.7	2.4
Igkv12-38	3.3	0.6	19.3	1.4
Sptssb	1.3	1.4	4.4	3.1
Gm12840	1.0	0.9	6.4	1.1
Ighv10-1	2.7	0.2	4.2	2.3
Igkv8-24	1.4	0.8	3.5	0.9
Il1b	1.1	0.7	12.6	1.0
Gm23639	1.1	0.8	4.3	1.7
Lcn2	1.0	1.0	9.0	1.0
Igkv8-16	1.0	1.1	2.3	1.1
Gm24415	1.0	1.4	2.6	1.6
Dhrs9	1.0	1.3	4.1	1.7
Ighv7-3	1.5	1.2	2.9	0.6
Srd5a2	1.4	3.3	5.7	6.7
Dio2	1.0	0.9	6.9	0.9
Ppbp	1.0	1.1	13.3	1.7
Serpina3m	1.0	1.0	6.2	0.9
Apol10a	1.3	0.6	2.5	2.6
Ighm; Ighv2-6	1.5	1.8	2.6	1.2
Iigp1	1.9	0.3	3.6	1.5
Ido1	1.5	0.7	2.7	1.2
Chil1	1.0	1.1	5.1	1.1
Gzma	1.4	0.6	3.3	1.3

Figure 2. IL-10 Suppresses STING-Induced Inflammatory Colitis and CAC

(A–E) Representative photographs of rectal prolapse (A), representative photographs of H&E staining (B), percentage of mice with prolapse (C), inflammation score (D), and the number of polyps (E) in 10 ~19-week-old WT (n = 10), SKO (n = 15), IL10KO (n = 26), and IL10KO/SKO mice (n = 19).

(F) Gene array analysis of colon tissue from 8-week-old WT, SKO, IL10KO, and IL10KO/SKO mice (n = 5). Fold changes were estimated by WT mice, and the highest variable genes are shown. Pseudo-colors indicate transcript levels equal to below (red) or above (green).

(G) qPCR of IL-1β, IL-12, and IL-22 mRNA level in in each genotype of colon tissue. All data are the mean of at least seven mice. Error bars indicated SD. Statistical analysis was performed using Student's t test. *$p \leq 0.05$.

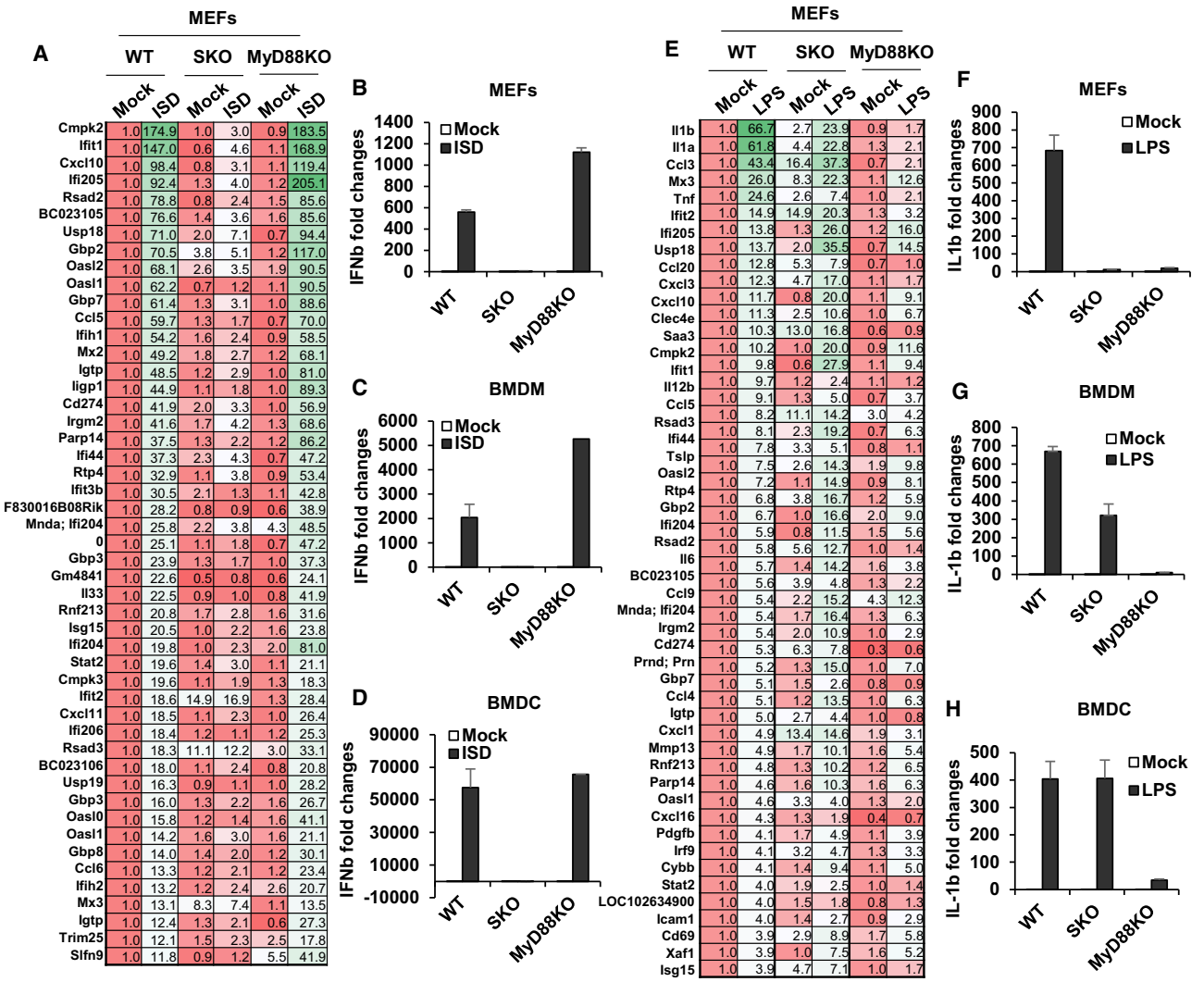


Figure 3. STING Signaling Does Not Require the Adaptor MyD88

(A and E) Gene array analysis from total RNA purified in WT, SKO, and MyD88KO mouse embryonic fibroblasts (MEFs) treated with 3 μg/mL ISD (A) or 1 μg/mL LPS (E) for 6 hr. Fold changes were estimated by WT mice and the highest variable genes are shown. Pseudo-colors indicate transcript levels equal to below (red) and above (green).

(B–D) qPCR analysis of IFNβ mRNA level in MEFs (B), bone marrow-derived macrophages (BMDMs) (C), and BMDCs (D) treated the same as in (A).

(F–H) qPCR analysis of IL-1β mRNA level in MEFs (F), BMDMs (G), and BMDCs (H) treated the same as in (E).

Error bars indicate SD.

as the S100 family, IL-6, and IL-8 (Andoh et al., 2005; Kolls et al., 2008; Zenewicz et al., 2008). Examination of the colon retrieved from IL-10-deficient mice indicated the presence of significant levels of IL-22 was eliminated in the absence of STING, as described (Figure 2G). Thus, STING signaling can influence both the production of pro-inflammatory cytokines such as IL-1 and IL18 and directly or indirectly IL-22, *in vitro* and *in vivo* to influence the outcome of colitis.

Monocyte Lineages Are Predominantly Responsible for STING-Mediated Pro-inflammatory Responses

Our data indicate that STING signaling is required to initiate wound-healing processes in response to AOM/DSS injury. Loss of STING facilitates polyp formation perhaps as a result of dysbiotic microbial infiltration of the lamina propria, which initiates STING-independent inflammatory responses. Furthermore, loss of STING may also reduce anti-inflammatory IL-10 production. To start to delineate the cells including MNPs responsible for STING's role in maintaining gut immune homeostasis, mice with STING floxed allele(s) were crossed with different cre-recombinase expressing mouse lines (LysM-SKO, which deleted STING from macrophages and neutrophils and CD11c-SKO, which deleted STING from dendritic cells) (Bouabe and Okkenhaug, 2013; Caton et al., 2007; Clausen et al., 1999). To examine the role of STING signaling in the macrophage and dendritic cell subsets, the LysM-SKO and CD11c-SKO mice

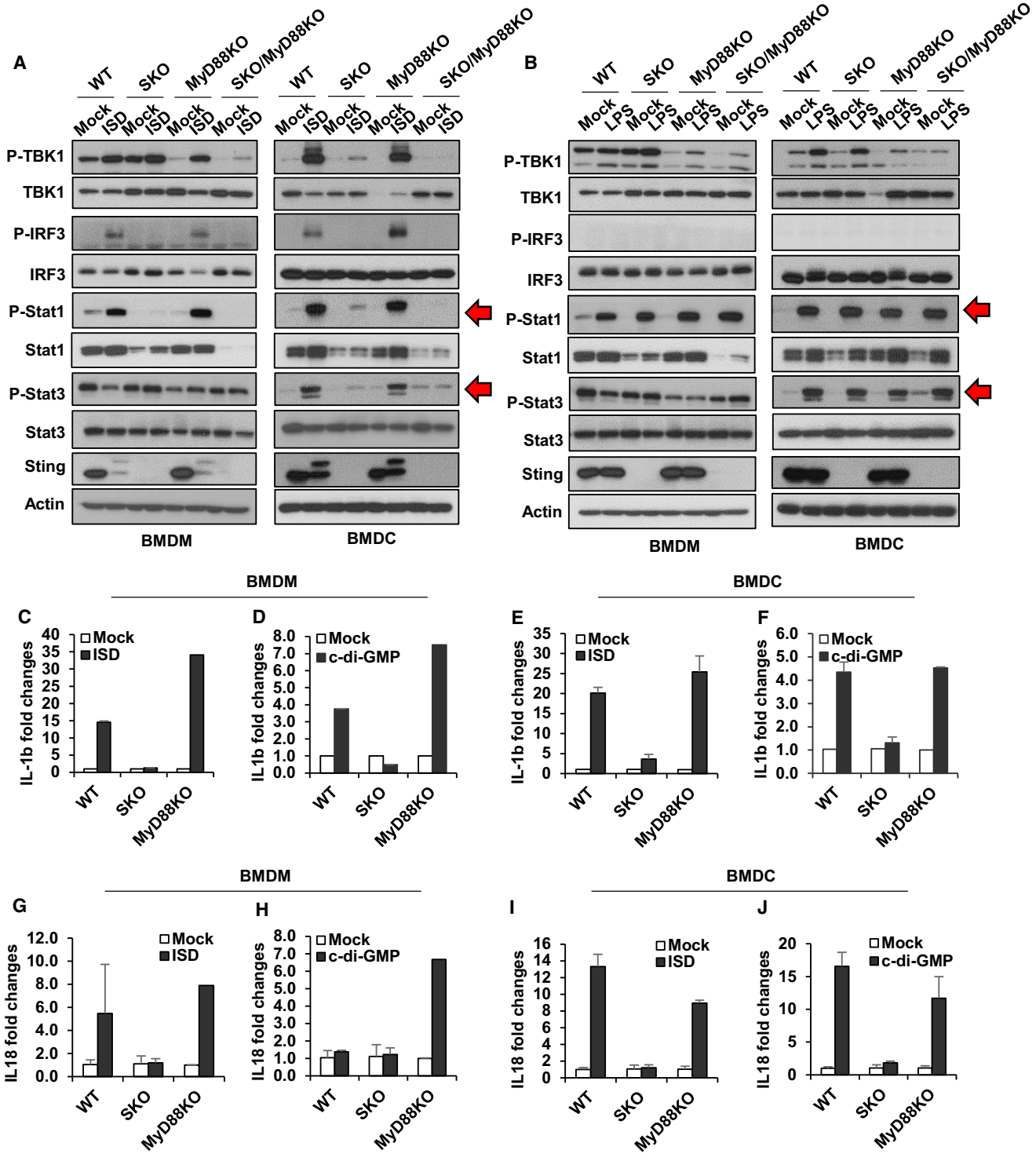


Figure 4. STING Signaling Drives Pro-inflammatory, MyD88-Dependent Gene Induction

(A and B) Immunoblot analysis to determine the levels of pTBK1, pIRF3, pStat1, pStat3, and STING in BMDMs or BMDCs treated with 3 μg/mL ISD (A) or 3 μg/mL LPS (B) for 6 hr.

(C–F) qPCR analysis of IL-1β mRNA level in BMDMs (C and D) or BMDCs (E and F) treated with 3 μg/mL ISD (C and E) or 3 μg/mL c-di-GMP (E and F) for 6 hr.

(G–J) qPCR analysis of IL-18 mRNA level in BMDMs (G and H) or BMDCs (I and J) treated with 3 μg/mL ISD (G and I) or 3 μg/mL c-di-GMP (H and J) for 6 hr. Data are representative of at least two independent experiments. Error bars indicated SD.

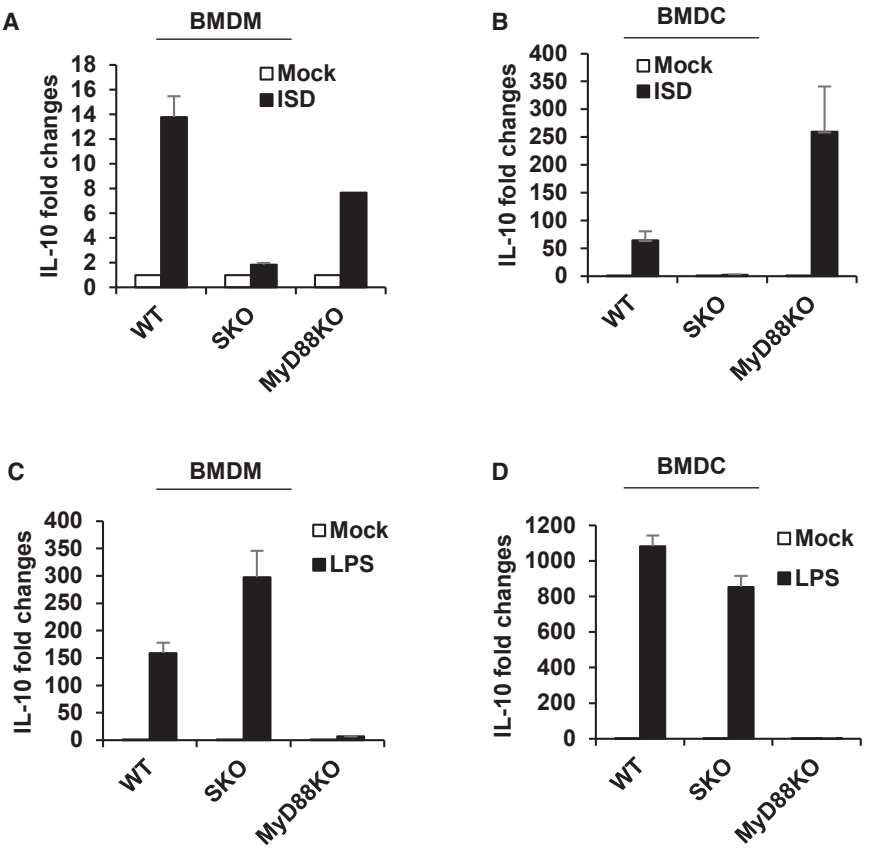


Figure 5. IL-10 Can Be Modulated by STING Signaling

(A–D) qPCR analysis of IL-10 mRNA level in BMDMs (A and C) or BMDCs (B and D) treated with 3 μg/mL ISD (A and B) or 1 μg/mL LPS (C and D) for 6 hr. Data are representative of at least two independent experiments. Error bars indicated SD.

were treated with AOM/DSS as described. Body weight was monitored, and effects on the colon including polyp formation were observed. This analysis indicated that WT, SKO, LysM-SKO, and CD11c-SKO mice all exhibited modest weight reduction as a result of treatment (Figures 6A–6D). However, the LysM-SKO and CD11c-SKO mice were noted to lose slightly less weight (Figures 6C and 6D). In addition, both LysM-SKO and CD11C-SKO mice exhibited less inflammation and polyp formation in response to AOM/DSS treatment and compared to SKO mice (LysM-SKO, $p = 0.0005$; CD11C-SKO, $p = 0.0019$) (Figures 6E and 6F). Our data would suggest that, *in vivo*, STING signaling in MNPs representing both macrophages and DCs' lineages may play an important role in recognizing microbes, and/or DNA-damaging events, to generate cytokines that can pre-dominantly aggravate inflammatory colitis. However, STING signaling in other cell types may also contribute toward maintaining gut homeostasis since SKO mice, which do not express STING in any tissue, exhibit different responses to carcinogenic events *in vivo* compared to WT and LysM-SKO/CD11c-SKO mice (Figures 6E and 6F) (Ahn et al., 2015). Our data underscore the importance of STING-innate immune signaling in interacting with commensal bacteria and controlling inflammatory colitis.

DISCUSSION

Our data demonstrate a key role for STING signaling in interacting with commensal bacteria and influencing gut immune homeostasis. STING-deficient mice (SKO) were found to harbor altered portfolios of commensal bacteria compared to similarly housed wild-type mice, possibly due to loss of an important innate immune pathway enabling the dominance of select species for reasons that presently remain unclear. Significantly, loss of STING signaling was found to reverse the severe form of colitis that ensues in mice in the absence of the key anti-inflammatory cytokine IL-10. Thus, it is possible that CDNs produced from intracellular bacteria, as well as microbial DNA, may constitutively stimulate STING-dependent signaling. Plausibly, under normal conditions, intracellular microbes may obtain access to scavenging intestinal epithelial cells and/or MNPs and activate STING signaling, or conversely that exogenous CDNs produced from extracellular microbes may gain access to such immune cells that harbor STING. It is also possible that self-DNA may trigger the production of CDNs in immune cells, although the observation that treatment of STING-deficient mice with antibiotics eliminated the incidence of polyp formation would argue against this. While it is known that different housing conditions that contain varying bacteria species, as well as the background of the mice themselves, can affect the outcome of inflammatory stimuli, our data would nevertheless suggest an important role for STING signaling in interacting with the microbiome (De Robertis et al., 2011; Laukens et al., 2016; Mähler et al., 1998).

The production of IL-10 requires NF-κB or IRF transcription factor activation, which can be triggered by TLR stimulation or by IFN (Chang et al., 2007; Ouyang et al., 2011). Since STING signaling utilizes these same pathways, it is perhaps unsurprising that STING can also stimulate the production of IL-10. Our data indicate that, through interaction with commensal bacteria, STING signaling may play a major role in maintaining an appropriate amount of immunosuppressive IL-10. Without STING-dependent IL-10 production, levels of STING-dependent pro-inflammatory cytokines may increase and trigger an inflammatory state. These aggravating cytokines may be predominantly generated by MNPs, as our data suggest, since LysM-SKO/CD11c-SKO exhibited less inflammation in response to carcinogens. The MNP population implicated here include both P1 ($CD11c^+CD11b^-$, $CD103^+CX3CR1^-DEC205^+F4/80^-$) and P2 ($CD11c^+CD11b^+$, $CD11c^+CD103^-CX3CR1^+$ macrophage

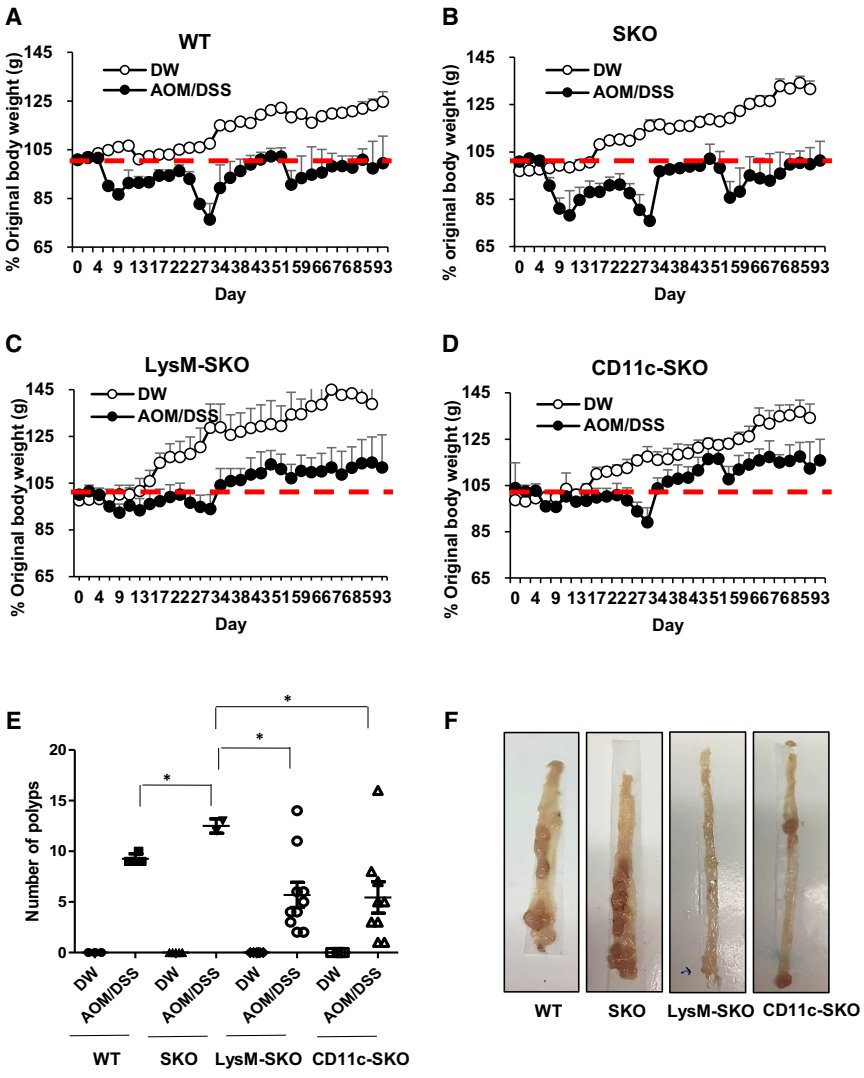


Figure 6. Monocytes Are Predominantly Responsible for STING-Mediated Pro-inflammatory Responses

(A–D) Body weight assessment in WT (A), SKO (B), LysM-SKO (C), and CD11c-SKO (D) mice treated with AOM/DSS or normal water as control. LysM-SKO for AOM/DSS (n = 10), LysM-SKO for DW control (n = 6), CD11c-SKO for AOM/DSS (n = 9), CD11c-SKO for DW control (n = 5), WT for AOM/DSS (n = 4), WT for DW control (n = 3), SKO for AOM/DSS (n = 4), and SKO mice for DW control (n = 5).

(E and F) Number of polys (E) and representative photographs of polyps in colon (F). All data are the mean of at least three mice. Error bars indicated SD. Statistical analysis was performed using Student's t test. *$p \leq 0.05$.

[MP2]) subsets as determined using CD11c-Cre and LysM-Cre mice, respectively. However, the cytokine profile triggered in response to STING activation may vary depending on the cell type. In the complete absence of STING, SKO mice were more prone to inflammation and polyp formation in response to DSS and AOM/DSS treatment, respectively. We postulate that DSS-induced inflammation may enable microbes access to STING-containing immune cells such as $CD11c^{+}CD11b^{+}$ (P2). STING signaling may be important for facilitating rapid wound healing and antimicrobial processes, which if not enabled allow microbes access to the lamina propria where they can aggravate STING-independent inflammatory responses, such as through the TLR pathway (Salcedo et al., 2010). AOM-instigated DNA damage events also likely trigger intrinsic STING-dependent cytokine production, and loss of STING may enable DNA-damaged cells to escape the immune system and proliferate (Ahn et al., 2015). Loss of extrinsic STING signaling in antigen-presenting cells (APCs) may also affect anti-tumor T cell responses, and additionally enable pre-cancerous cells to escape (Woo et al., 2014). It is presently unclear why cGAS-deficient (cGASKO) mice exhibited less pronounced inflammatory responses or polyp formation following DSS or AOM/DSS treatment, compared to SKO mice. However, in these transient early-immediate circumstances, it may be that CDNs generated by bacteria exert a more important effect upon STING signaling than self or microbial cytosolic dsDNA species (Danilchanka and Mekalanos, 2013).

That the elimination of STING signaling prevents colitis-associated with IL-10 deficiency suggests that STING signaling may be responsible for the generation of liable pro-inflammatory cytokines in this model (Figure 7). STING-dependent signaling certainly seems capable of influencing the production of pro-inflammatory cytokines IL-1β, IL-18, and possibly IL-22 at least indirectly, which after binding to appropriate receptors require the adaptor MyD88 to stimulate additional pro-inflammatory production (Ahn et al., 2015; Salcedo et al., 2013). In this light, it is well known that loss of MyD88 can also eliminate colitis associated with loss of IL-10 (Hoshi et al., 2012; Rakoff-Nahoum et al., 2006). This would suggest that MyD88 plays a role in STING signaling or facilitates the downstream effects of STING-triggered pro-inflammatory cytokines such as IL-1β and IL-18. Our data would favor the latter model since Myd88 did not affect cytosolic dsDNA-dependent signaling. Similarly, STING was not directly required for LPS signaling in BMDMs or BMDCs, which predominantly required MyD88 or TRIF. However, it is also likely that the interaction of commensal bacteria with members of the TLRs may also play a key role in IL-10 production and that both the STING and the TLR pathways synergistically function to maintain immune homeostasis of the gut (Hoshi et al., 2012; Uronis et al., 2009). Since loss of TLR4 does not eliminate IL-10-mediated suppression, this may suggest that at least this TLR may not be a major

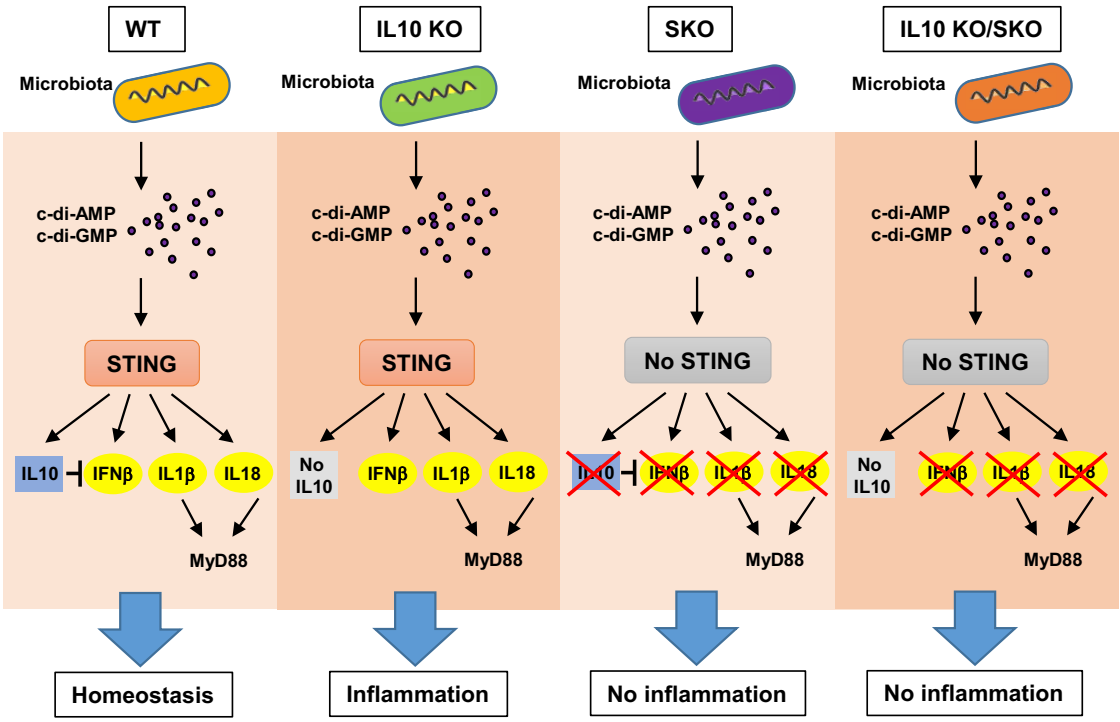


Figure 7. A Schematic Illustration of STING Signaling Responsible for the Generation of Pro-inflammatory Cytokines to Prevent Colitis Associated with IL-10 Deficiency

cause of pro-inflammatory cytokines that drive colitis (González-Navajas et al, 2010). Collectively, our data indicate that STING may play a key role in maintaining intestinal immune homeostasis and be a key producer of anti-inflammatory IL-10 as well as pro-inflammatory cytokines and type I IFN. Thus, deregulation of the STING pathway, which is being more commonly associated with a variety of autoinflammatory disease such as Aicardi-Goutieres syndrome (AGS) and STING-associated vasculopathy within onset in infancy (SAVI), may also play a role in IBD. Therefore, the development of therapeutics that may target the STING pathway may have benefit in the treatment of such malaise.

EXPERIMENTAL PROCEDURES

Mice

SKO mice (Sting$^{-/-}$) were generated in our laboratory (Ishikawa and Barber, 2008). We have generated B6 background WT and SKO from original clone of B6/129 background SKO mice. Our original SKO mice in 129/B6 mixed background were backcrossed onto a WT B16 background more than seven times until the hetero mice (STING$^{+/-}$) were >97% B16 in our conventional mice room (Helico spp +). To generate *Helicobacter*-free STING KO mice, we utilized an *in vitro* fertilization method and the oocytes were transferred to *Helicobacter*-free recipient females. We bred the hetero mice (STING$^{+/-}$) to generate WT and SKO mice in a specific pathogen-free room (Helico spp −). Both Helico+ and Helico− rooms were inspected by the same Rodent Health Surveillance authorities quarterly by Division of Veterinary Resources at University of Miami. All use autoclaved caging, supplies, water, and irradiated food. cGASKO mice were kindly provided by Dr. Herbert W. Virgin IV (Washington University School of Medicine). MyD88 knockout mice (MyD88KO) and IL-10 knockout mice (IL10KO) were purchased from The Jackson Laboratory. To generate SKO mice on IL10KO background, B6/129 background SKO mice were crossed to B6/129 background IL10KO mice. The IL10KO mice and IL10KO/SKO mice were bred in a Helico+ room. We used Helico+ WT and SKO mice as controls. To generate the conditioning SKO mice, we developed animals with the STING gene floxed. In brief, the exons 1–5 were flanked with loxp sites in C57/BL6-derived embryonic stem (ES) cells in order to render STING susceptible to Cre-mediated recombination. The floxed STING mice were crossed to mice expressing Cre under a cell-specific promoter (LysM-Cre, CD11C-Cre) to generate LysM-SKO and CD11c-SKO mice. Mice care and study were conducted under approval from the Institutional Animal Care and Use Committee (IACUC) of the University of Miami. Mouse genotypes from tail biopsies were determined by real-time PCR with specific probes designed for each gene by commercial vendor (Transnetyx).

Acute DSS Colitis

WT and SKO mice, 6–8 weeks of age, were divided into experimental and control groups. Mice in the experimental group received 5% DSS (MP 160110; molecular weight [MW], 36,000–5,000) for 7 days. The following day, the mice were sacrificed, and colon was removed to proceed with histology. Distilled water was administered to the control group mice. The mice were monitored every day to evaluate disease activity index.

AOM/DSS Induced Colitis-Associated Tumor Induction

WT, SKO, and cGASKO mice were injected intraperitoneally with AOM (MP 180139; Sigma-Aldrich; A5486) at a dose of 10 mg/kg. DSS at 3% was administered in the drinking water for 7 days every 3 weeks. DSS cycle was repeated four times. At 125 days, the mice were sacrificed, and the colon was resected and flushed with PBS to count polyps. Colon were fixed in formalin for histology and frozen for RNA expression analysis. For antibiotic treatment, mice were treated with an antibiotic cocktail of ampicillin (1 g/L), neomycin (1/L), metronidazole (1 g/L), and vancomycin (500 mg/L) in their drinking water for 4 weeks prior to AOM/DSS administration.

V4 16S rRNA Gene Sequencing

Stool was collected from WT and SKO mice and the commensal microbiota composition was evaluated by V4 hypervariable region of the 16S rRNA gene sequencing by Second Genome (The Microbiome Company). Second Genome performed nucleic isolation with the MoBio PowerMag Microbiome kit (Carlsbad, CA) according to the manufacturer's guidelines and optimized for high-throughput processing. All samples were quantified via the Qubit Quant-iT dsDNA High Sensitivity Kit (Invitrogen, Life Technologies, Grand Island, NY). To enrich the sample for bacterial 16S V4 rDNA region, DNA was amplified utilizing fusion primers designed against the surrounding conserved regions, which are tailed with sequences to incorporate Illumina (San Diego, CA) adapters and indexing barcodes. Each sample was PCR amplified with two differently barcoded V4 fusion primers. Samples that met the post-PCR quantification minimum were advanced for pooling and sequencing. For each sample, amplified products were concentrated using a solid-phase reversible immobilization method for the purification of PCR products and quantified by qPCR. A pool containing 16S V4 enriched, amplified, and barcoded samples were loaded into a MiSeq reagent cartridge, and then onto the instrument along with the flow cell. After cluster formation on the MiSeq instrument, the amplicons were sequenced for 250 cycles with custom primers designed for paired-end sequencing. Samples are processed in a good laboratory practices (GLP)-compliant service laboratory running quality management systems for sample and data tracking. The laboratory implements detailed standard operating procedures (SOPs), equipment and process validation, training, audits, and document control measures. Quality control (QC) and quality assurance (QA) metrics are maintained for all sample handling, processing, and storage procedures.

Primary Cell Culture

MEFs were obtained from embryonic day 15 (E15) embryos by a standard procedure as described (Ishikawa and Barber, 2008). BMDCs were isolated from hind-limb femurs of 8- to 10-week-old mice. Briefly, the marrow cells were flushed from the bones with DMEM (Invitrogen), 10% heat-inactivated fetal calf serum (FCS) (Invitrogen) with a 23G needle and incubated at 37°C for 4 hr. After harvesting floating cells, approximately 2×10^7 cells were seeded in 10-cm dish with complete DMEM including 10 ng/mL recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) (GM-CSF; R&D Systems) or 10 ng/ml of recombinant mouse M-CSF (M-CSF; R&D Systems) for dendritic cells (BMDCs) or macrophages (BMDMs).

Histopathology

Mice were sacrificed, and the colon tissues were fixed in 10% formalin for 48 hr. All processes for paraffin block and H&E staining were performed at the Pathology Research Resources Histology Laboratory in University of Miami.

Gene Array Analysis

Total RNA was isolated from cells or tissues with RNeasy Mini kit (74104; QIAGEN, Valencia, CA). RNA quality was analyzed by Bionalyzer RNA 6000 Nano (Agilent Technologies, Santa Clara CA). Gene array analysis was examined by Illumina Sentrix BeadChip Array (Mouse WG6, version 2) (Affymetrix, Santa Clara, CA) at the Oncogenomics Core Facility, University of Miami. Raw intensity values from Illumina array are uploaded on GeneSpring software from Agilent. Values are *Quantile* normalized and $\log_2$ transformed to the median of all samples. Significantly differential expressed genes are computed using the Student's t test and selected using threshold of p value ≤ 0.05. Hierarchical clustering and visualization of selected differentially expressed genes were performed on GeneSpring using *Pearson Correlation* distance method and linkage was computed using the *Ward* method. Gene expression profiles were processed, and statistical analysis was performed at the Sylvester Comprehensive Cancer Center Bioinformatics Core Facility at University of Miami.

Real-Time qPCR

Total RNA were reverse-transcribed using M-MLV Reverse Transcriptase (Promega). Real-time PCR was performed using TaqMan Gene Expression Assay (Applied Biosystems) for innate immune genes and inflammatory cytokines (IFNb, Mm00439552; IL-1b, Mm01336189; IL-18, Mm00434225; IL-10, Mm01288386; Life Technologies).

Immunoblot Analysis

Equal amounts of proteins were resolved on SDS-polyacrylamide gels and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). After blocking with 5% blocking reagent, membranes were incubated with various primary antibodies (and appropriate secondary antibodies). The image was resolved using an enhanced chemiluminescence system (ECL) (Thermo Scientific) and detected by autoradiography. Antibodies included rabbit polyclonal antibody against STING, which was developed in our laboratory as described previously in Ishikawa and Barber, 2008; other antibodies were obtained from the following sources: β-actin (Sigma-Aldrich; A2228), p-IRF3 (Cell Signaling; CST4947), IRF3 (Cell Signaling; CST4302), p-TBK1 (Cell Signaling; CST15483), TBK1 (Abcam; ab12116), p-p65 (Cell Signaling; CST3033), p65 (Cell Signaling; CST), p-Stat3 (Cell Signaling; CST9145), Stat3 (Cell Signaling; CST4904), p-Stat1 (Cell Signaling; CST9167), and Stat1 (Cell Signaling; CST14994).

Statistical Analysis

All statistical analysis was performed by Student's t test unless specified. The data were considered to be significantly different when $p < 0.05$.

DATA AND SOFTWARE AVAILABILITY

The accession numbers for the microarray data reported in this paper are GEO: GSE107809, GSE107810, and GSE107811.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.11.101.

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AUTHOR CONTRIBUTIONS

J.A. designed and carried out most of the experiments. S.S. performed animal experiments. S.C.O. advised on experimental design. G.N.B. designed the experiments and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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