The GTPase-activating protein GIT2 protects against colitis by negatively regulating Toll-like receptor signaling

Juncheng Wei[a,b,c,1], Chao Wei[a,b,c,1], Min Wang[b,c], Xiao Qiu[b,c,d], Yang Li[b], Yanzhi Yuan[b,c], Chaozhi Jin[b,c], Ling Leng[b,c], Jian Wang[b,c,2], Xiaoming Yang[b,c,2], and Fuchu He[b,c,2]

[a]Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, School of Basic Medicine, Peking Union Medical College, Beijing 100005, China; [b]Beijing Institute of Radiation Medicine, Beijing 100850, China; [c]State Key Laboratory of Proteomics, Beijing Proteome Research Center, National Engineering Research Center for Protein Drugs, Beijing 102206, China; and [d]Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing 100084, China

Edited by Richard A. Flavell, Yale School of Medicine and Howard Hughes Medical Institute, New Haven, CT, and approved May 8, 2014 (received for review May 14, 2013)

G protein-coupled receptor kinase-interactor 2 (GIT2) regulates thymocyte positive selection, neutrophil-direction sensing, and cell motility during immune responses by regulating the activity of the small GTPases ADP ribosylation factors (Arfs) and Ras-related C3 botulinum toxin substrate 1 (Rac1). Here, we show that *Git2*-deficient mice were more susceptible to dextran sodium sulfate (DSS)-induced colitis, *Escherichia coli*, or endotoxin-shock challenge, and a dramatic increase in proinflammatory cytokines was observed in *Git2* knockout mice and macrophages. GIT2 is a previously unidentified negative regulator of Toll-like receptor (TLR)-induced NF-κB signaling. The ubiquitination of TNF receptor associated factor 6 (TRAF6) is critical for the activation of NF-κB. GIT2 terminates TLR-induced NF-κB and MAPK signaling by recruiting the deubiquitinating enzyme Cylindromatosis to inhibit the ubiquitination of TRAF6. Finally, we show that the susceptibility of *Git2*-deficient mice to DSS-induced colitis depends on TLR signaling. Thus, we show that GIT2 is an essential terminator of TLR signaling and that loss of GIT2 leads to uncontrolled inflammation and severe organ damage.

GIT | IBD | TRAF6 | CYLD

Inflammatory bowel disease (IBD), which includes the two main forms Crohn's disease (CD) and ulcerative colitis (UC), is generally thought to develop from an abnormal immune response to the gut luminal microbiota in genetically predisposed individuals (1, 2). The innate immune system, which senses the commensal flora, is critical for intestinal homeostasis and tissue repair after injury (3). Toll-like receptor (TLR) signaling is responsible for the development of colitis (4).

Tight control of TLR signaling is crucial for the clearance of pathogens and for avoiding nonresolving inflammation (5, 6). Uncontrolled activation of the TLR-triggered signaling pathway promotes chronic inflammation and autoimmune diseases, such as IBD and obesity (7, 8). TLRs mediate host responses to microorganisms and induce inflammatory responses that are linked to the expression of proinflammatory cytokines and activation of the NF-κB pathway (7). Negative regulation of TLR signaling is crucial to maintain immune homeostasis. The tumor suppressor CYLD is a negative regulator of TLR signaling by deubiquitinating TRAF6 and NEMO (9–11). A deficiency in the catalytic domain of CYLD in mice causes elevated NF-κB activity, and those mice die shortly after birth (12).

GIT2 belongs to the group of ADP ribosylation factor (Arf)-directed GTPase activating proteins (GAPs). More than 24 proteins with Arf GAP domains have been identified in humans (13). To our knowledge, none of these proteins have been reported to directly regulate TLR signaling. GIT2 negatively regulates the activation of Arf GTPases through its N-terminal, Arf GTPase activating protein (GAP) domain (14) and of Rac GTPase through its interaction with PIX (p21-activated kinase-interacting exchange factor) (15). *Git2* deficiency leads to spontaneous splenomegaly, hypersusceptibility to infection, increased oxygen anion production by neutrophils, and impaired positive selection of CD4 single-positive thymocytes in thymus (16, 17). GIT2 is highly expressed in monocytes and macrophages, especially after stimulation by LPS (18, 19). Combining its known relationships with novel trans-eQTLs extends the connections of GIT2 to a host of inflammatory mediators (19). We identified that GIT2 interacted with the components of the NF-κB signaling pathway by a large-scale yeast two-hybrid screening (20). Cumulatively, these results show the important roles of GIT2 in innate and adaptive immunity. However, whether GIT2 directly regulates the intestinal immune response remains unknown, and the genetic evidence is lacking to support its physiological roles during intestinal immune responses by directly regulating the TLR signaling pathway.

Here, we show that knockout of *Git2* induced severe acute and chronic colitis after dextran sulfate sodium (DSS) treatment. *Git2*-deficient mice were more susceptible to infection by *Escherichia coli* and endotoxin shock. *Git2* deficiency resulted in a greater TLR-induced production of proinflammatory cytokines in vitro and in vivo. GIT2 inhibited the TLR-induced signaling pathway and was critical in inhibiting the activation of TRAF6. Moreover, GIT2 terminated the TLR signaling pathway by recruiting the deubiquitinating enzyme CYLD to the TRAF6 ubiquitin ligase. Thus, GIT2 is a critical terminator of TLR-induced inflammatory responses.

Significance

Inflammatory bowel disease is generally thought to develop from an abnormal immune response to the gut luminal microbiota in genetically predisposed individuals. Previous studies on G protein-coupled receptor kinase-interactor 2 (GIT2) have suggested that it regulates thymocyte positive selection, neutrophil-direction sensing, and cell motility during immune responses by regulating the activity of the small GTPases ADP ribosylation factors and Ras-related C3 botulinum toxin substrate 1. Here, we show that GIT2 protects against severe colitis in mice by inhibiting the ubiquitination of TNF receptor associated factor 6 and negatively regulating Toll-like receptor (TLR) signaling. GIT2 is essential for the resolution of TLR signaling, and the deficiency of *Git2* leads to uncontrolled inflammation and severe immune diseases.

Author contributions: J. Wei, C.W., X.Q., J. Wang, X.Y., and F.H. designed research; J. Wei, C.W., M.W., Y.L., and L.L. performed research; J. Wei, C.W., M.W., X.Q., Y.Y., C.J., J. Wang, X.Y., and F.H. analyzed data; and J. Wei, C.W., and J. Wang wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]J. Wei and C.W. contributed equally to this work.

[2]To whom correspondence may be addressed. E-mail: wangjian@bmi.ac.cn, xmyang2@bmi.ac.cn, or hefc@bmi.ac.cn.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1309218111/-/DCSupplemental.

Results

Knockout of *Git2* Enhances Susceptibility to DSS-Induced Acute and Chronic Colitis. To determine the roles of GIT2 in pathogenesis of IBD, we examined the effect of *Git2* deficiency on DSS-induced acute colitis. All of the $Git2^{-/-}$ mice died after 9 d of DSS treatment whereas 90% of their $Git2^{+/+}$ littermates lived (Fig. 1*A*). The $Git2^{-/-}$ mice had remarkable weight loss compared with the wild-type mice (Fig. 1*B*). Additionally, $Git2^{-/-}$ mice showed several symptoms of severe colitis, including unformed or absent stool, blood in the rectum and shortened colonic length (Fig. 1*C*). The cytokines play important roles in the development of colitis (21). Our results showed that the levels of tumor necrosis factor α (TNFα), interleukin-6 (IL-6), and interleukin-17 (IL-17) in the sera of the $Git2^{-/-}$ mice with colitis were much higher than those from the $Git2^{+/+}$ mice (Fig. 1*D*). Additionally, histological analyses of the colons showed more severe symptoms, such as the disruption of mucosal structures and infiltration of massive inflammatory cells, in the $Git2^{-/-}$ mice that had been treated with DSS (Fig. 1*E*). Accordingly, the inflammation scores of the $Git2^{-/-}$ mice were higher throughout the course of colitis (Fig. 1*E*). We also observed greater production of nitric oxide synthase (NOS), a marker of inflammation, in the $Git2^{-/-}$ mice than in the wild-type mice after DSS induction (Fig. 1*F*).

We next evaluated the roles of GIT2 in the chronic progression of colitis. $Git2^{-/-}$ mice and their littermates were induced by administration of 2% (wt/vol) DSS on days 0–5, 20–25, and 40–45, and the mice were scarified and analyzed on day 54 (Fig. 2*A*). $Git2^{-/-}$ mice showed increased weight loss compared with controls after the initial and the third round of DSS (Fig. 2*B*). Consistent with the results in the acute model, DSS-treated $Git2^{-/-}$ mice showed significantly increased clinical features during disease progression (Fig. 2*C*), and colons from long-term DSS-induced $Git2^{-/-}$ mice were significantly shortened (Fig. 2*D*). After completion of the model (on day 55), substantially increased inflammation was shown through histopathological image analysis (Fig. 2*E*). These data show that GIT2 functions as a negative regulator in inflammation during recurring colitis.

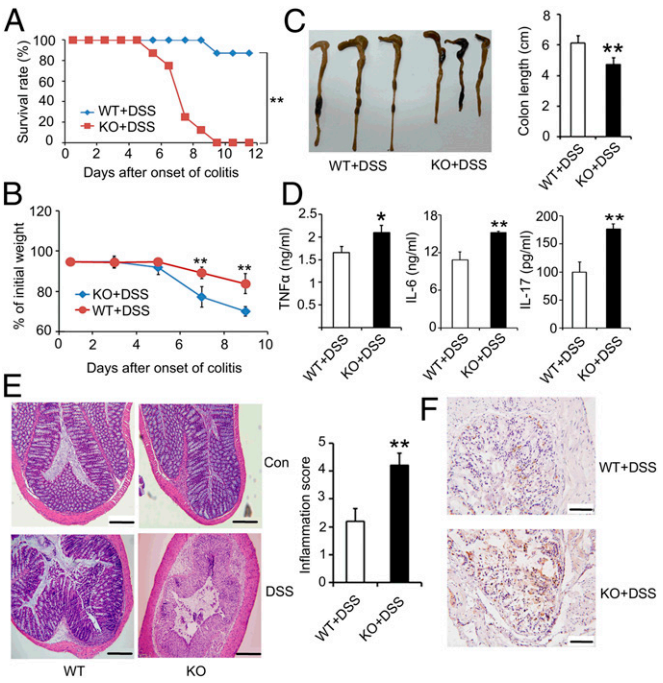

Fig. 1. Aggravated acute colitis is induced by DSS in $Git2^{-/-}$ mice. (*A*) $Git2^{+/+}$ ($n = 10$) and $Git2^{-/-}$ ($n = 9$) mice were fed with a 3.5% (wt/vol) DSS solution in drinking water for 5 d, and their survival was monitored until day 14 after treatment with DSS. (*B*) The mean changes in body weight of the $Git2^{+/+}$ and $Git2^{-/-}$ mice ($n = 7$ for each genotype) after being administered 2% (wt/vol) DSS were measured every other day until day 8. (*C*) $Git2^{+/+}$ and $Git2^{-/-}$ mice were treated as in *B*; colon lengths were measured. (*D*) The levels of IL-6, TNFα, and IL-17 in sera from $Git2^{+/+}$ or $Git2^{-/-}$ mice were quantified by ELISA after treatment as in *C*. (*E*) Histological changes in the colon tissue were examined by HE staining after treatment as in *C*. An inflammation score of colon tissue was performed as described in *SI Materials and Methods*. (*F*) The levels of NOS in colon tissue from the $Git2^{+/+}$ and $Git2^{-/-}$ ($n = 5$ for each genotype) mice were measured using immunohistochemistry after treatment as in *C*. (Original magnification: 100×.) (Scale bar: 200 μm.) The data are representative of three independent experiments (mean ± SEM, in *B, C, D* and *E*). $*P < 0.05$, $**P < 0.01$.

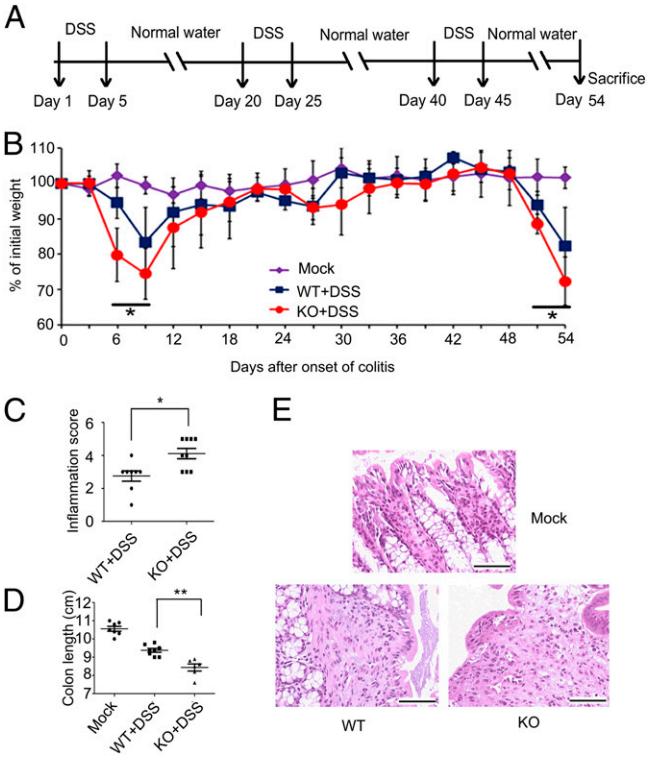

Fig. 2. Aggravated chronic colitis is induced by DSS in $Git2^{-/-}$ mice. (*A*) Schematic of the chronic colitis model. The mice were treat with three rounds of 2% (wt/vol) DSS for 5 d; the treatment was followed by 15 d of recovery. (*B*) The mean changes in body weight of the $Git2^{+/+}$ and $Git2^{-/-}$ mice were measured every 3 d until day 54. (*C*) The composite pathological scores were measured from $Git2^{+/+}$ and $Git2^{-/-}$ mice after treatment as in *A*. (*D*) The lengths of colons from $Git2^{+/+}$ and $Git2^{-/-}$ mice after treatment as in *A* were measured. (*E*) Histological changes in the colon tissue were examined by HE staining after treatment as in *A*. (Original magnification: 200×.) (Scale bar: 200 μm.) $*P < 0.05$, $**P < 0.01$.

DSS-Induced Colitis of the $Git2^{-/-}$ Mice Relied on Commensal Bacteria. $Git2^{-/-}$ mice did not spontaneously develop severe colitis that resembles the exposure to DSS. However, they indeed developed immunological abnormalities, such as the prominent inflammatory cell infiltration in the lamina propria of the colon, thickening of the muscular layer, and crypt damage in the inflamed areas (Fig. 3*A*). Subsequently, we examined whether hypersensitivity of the $Git2^{-/-}$ mice to DSS treatment depended on the commensal bacteria by administrating antibiotics. The disease severity was relieved in $Git2^{-/-}$ and $Git2^{+/+}$ mice during colitis that was induced by DSS after treatment with antibiotics, which is reflected in mucosal structures and inflammation scores (Fig. 3*B*), body weight (Fig. 3*C*), and colon length (Fig. 3*D*). These results indicated that the DSS-induced colitis of the $Git2^{-/-}$ mice relied on commensal bacteria.

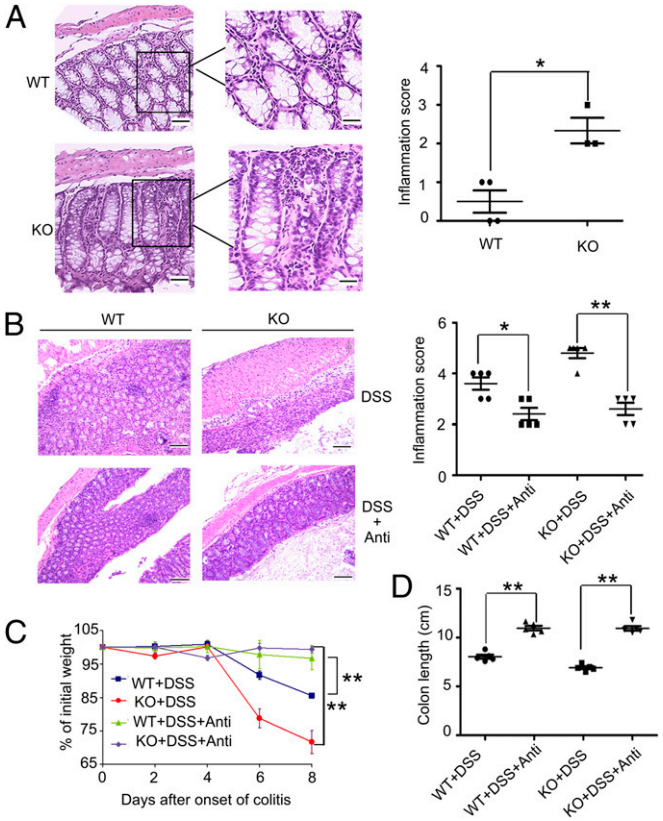

Fig. 3. DSS-induced colitis of the *Git2*$^{-/-}$ mice depends on commensal bacteria. (*A*) The colons of *Git2*$^{+/+}$ and *Git2*$^{-/-}$ mice (n = 3–4 per group) at 10 mo of age were dissected, and colitis was analyzed by HE staining. The inflammation scores were calculated according to the scoring system in *SI Materials and Methods*. (Original magnification: 200×.) (*B*) *Git2*$^{+/+}$ and *Git2*$^{-/-}$ mice were treated with a combination of antibiotics as described in *Materials and Methods* and fed 2% (wt/vol) DSS-solution in drinking water for 5 d, followed by regular drinking water for 3 d. The inflammation scores were calculated according to the scoring system in *SI Materials and Methods*. (Original magnification: 100×.) (Scale bar: 200 μm.) (*C* and *D*) *Git2*$^{+/+}$ and *Git2*$^{-/-}$ mice (n = 5 per group) were treated as in *B*, and weight loss and colon length were measured. *$P < 0.05$, **$P < 0.01$.

GIT2 Is Essential for Clearing Bacteria and Repressing the Inflammatory Response in *E. coli*-Induced Septic Peritonitis. To further confirm the roles of GIT2 in innate immunity, we infected mice with *E. coli* by i.p. injection. Within 40 h, 90% of the *Git2*$^{-/-}$ mice died due to a fatal shock by bacteria whereas 90% of the *Git2*$^{+/+}$ mice were still alive 32 h after infection with *E. coli* (Fig. 4*A*). The *Git2*$^{-/-}$ mice produced more proinflammatory cytokines, IL-6, and TNFα than the *Git2*$^{+/+}$ mice (Fig. 4*B*). The bacterial loading amounts of the livers, spleens, and lungs of the *Git2*$^{-/-}$ mice were significantly higher than those of the *Git2*$^{+/+}$ mice (Fig. 4*C*). It has been reported that proinflammatory cytokines promote the dissemination of *E. coli* (22–24). The *Git2*$^{-/-}$ mice also developed splenomegaly after bacterial infection (Fig. 4*D*). We also investigated whether the liver and the kidney of the mice were injured upon *E. coli* infection. The levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were higher in the sera of *Git2*$^{-/-}$ mice (Fig. 4*E*). Additionally, the levels of blood urea nitrogen (BUN), an indicator of kidney damage, were elevated in the sera of *Git2*$^{-/-}$ mice after bacterial infection (Fig. 4*E*). Thus, systematic failure and damage of multiple organs might cause the death of the *Git2*$^{-/-}$ mice upon bacterial infection. Histological analyses of the lungs from the *Git2* knockout mice showed thicker alveolar septa and dramatic pulmonary microvascular failure after infection with *E. coli*, as was evidenced by profound hemoconcentration in the pulmonary venules (Fig. 4*F*). These results suggest that *Git2* deficiency in mice leads to uncontrolled inflammatory innate responses and that *Git2*$^{-/-}$ mice are more susceptible to bacterial infection.

GIT2 Protects Mice Against Endotoxin Challenge. We previously found that GIT2 associated with the components of the NF-κB signaling pathway, which suggested that GIT2 directly regulated immune response-related signaling pathways (20). Then, we challenged wild-type and *Git2* knockout mice with the endotoxin lipopolysaccharide (LPS), which is a TLR ligand. We found that 80% of *Git2*$^{-/-}$ mice died within 56 h of treatment whereas 50% of the *Git2*$^{+/+}$ mice were alive for 56 h, and 45% ultimately survived (Fig. S1*A*). After treatment with LPS, *Git2*$^{-/-}$ mice produced more IL-6 and TNFα than *Git2*$^{+/+}$ mice (Fig. S1*B*). The spleens from *Git2*$^{-/-}$ mice injected with LPS were larger than those from *Git2*$^{+/+}$ mice by gross examination (Fig. S1*C*). The lungs of *Git2*$^{-/-}$ mice were infiltrated with more inflammatory cells and exhibited a thicker alveolar septum than those of *Git2*$^{+/+}$ mice after LPS challenge (Fig. S1*D*). We also checked for expression of another inflammatory marker, cyclooxygenase-2 (COX-2), in the lungs of the mice (25). The number of COX-2-positive cells nearly doubled in the lungs of *Git2*$^{-/-}$ mice compared with those of *Git2*$^{+/+}$ mice after stimulation by LPS (Fig. S1*E*). These results demonstrate that GIT2 has an essential role in regulating the sensitivity of animals to LPS-induced lethal shock.

We further investigated the functions of GIT2 in regulating TLR signaling in bone marrow-derived macrophages (BMDMs). As expected, the transcription of TLR downstream genes (e.g., *Il1b*, *Il6*, *Nfkbib*, and *Tnf-α*) was significantly increased in *Git2* knockout BMDMs compared with *Git2*$^{+/+}$ BMDMs after treatment with LPS (Fig. S2*A*). Accordingly, the production of the proinflammatory cytokines TNFα and IL-6 was greater in *Git2*$^{-/-}$ BMDMs than in *Git2*$^{+/+}$ BMDMs that had been challenged with LPS and Pam_3CSK_4 (a TLR1 and TLR2 ligand) (Fig. S2*B*).

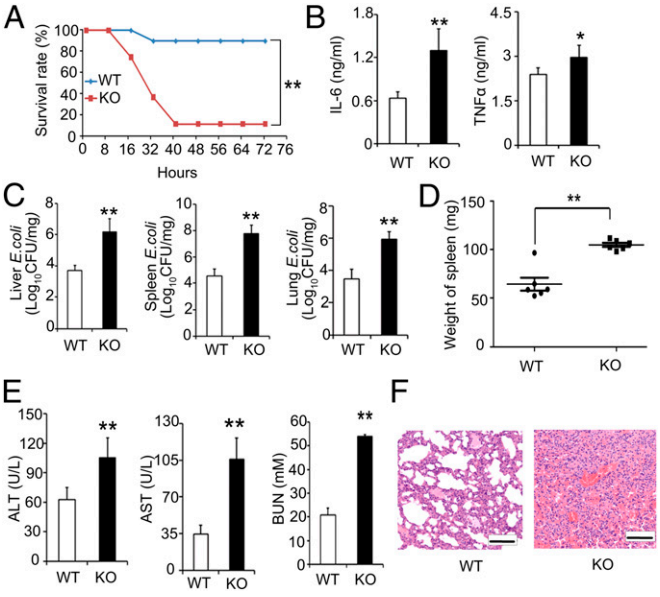

Fig. 4. *Git2*$^{-/-}$ mice are hypersensitive to *E. coli* infection. (*A*) Survival rate of *Git2*$^{-/-}$ (n = 10) and *Git2*$^{+/+}$ (n = 9) mice that were injected intraperitoneally with *E. coli* (1 × 10^7 CFUs). CFUs, colony-forming units. (*B*) ELISA of IL-6 and TNFα in the serum of *Git2*$^{+/+}$ and *Git2*$^{-/-}$ mice at 20 h after injection with *E. coli*. (*C*) Bacterial load in the liver, spleen, and lungs of *Git2*$^{+/+}$ and *Git2*$^{-/-}$ mice (n = 5 for each genotype) at 20 h after injection with *E. coli*. (*D*) Weight of the spleens from *Git2*$^{+/+}$ and *Git2*$^{-/-}$ mice (n = 6 for each genotype) after infection as in *A*. (*E*) The levels of ALT, AST, and BUN from the sera of *Git2*$^{+/+}$ and *Git2*$^{-/-}$ mice (n = 5 for each genotype) were measured after infection with *E. coli*. (*F*) HE staining of the lungs from *Git2*$^{+/+}$ and *Git2*$^{-/-}$ mice (n = 5 for each genotype) after infection as in *A*. (Original magnification: 100×.) (Scale bar: 200 μm.) *$P < 0.05$, **$P < 0.01$.

These data suggest that *Git2* deficiency enhances TLR-induced signaling in innate immune responses.

GIT2 Negatively Regulates TLR-Induced NF-κB Signaling. To validate the in vivo and in vitro results obtained with $Git2^{-/-}$ mice and cells, we examined the functional effects of GIT2 on NF-κB activation using a luciferase reporter gene assay. Overexpression of GIT2 significantly attenuated the NF-κB activation that was induced by inducer of TLRs [LPS, Poly (I:C), and ODN2006] and IL-1β/IL-18 in a dose-dependent manner in HEK293 cells (Fig. 5 *A* and *B* and Fig. S3). Moreover, we investigated the effect of GIT2 on NF-κB activation in HEK293 cells that was mediated by adaptor proteins or activators (i.e., MyD88, TRADD, RIP1, TRAF6, TRAF5, TRAF2, and cIAP1). We found that GIT2 inhibited the NF-κB activation that was mediated by these molecules, but the NF-κB activation that was induced by the NF-κB subunit p65 was unaffected by GIT2, which suggested that GIT2 inhibited NF-κB upstream of p65 (Fig. 5*C*). Conversely, knockdown of endogenous GIT2 using siRNA enhanced IL-1β–mediated NF-κB activation whereas a scrambled control siRNA didn't affect its activity (Fig. 5*D*). Knockdown of GIT2 also augmented TRAF6-mediated NF-κB activation (Fig. 5*E*). We further checked NF-κB and MAPK (mitogen-activated protein kinase) activation after treatment of BMDMs with LPS. The protein level of IκBα was reduced in $Git2^{-/-}$ BMDMs compared with those of $Git2^{+/+}$ BMDMs, which suggests an abnormal activation of NF-κB in the absence of GIT2 (Fig. 5*F*).

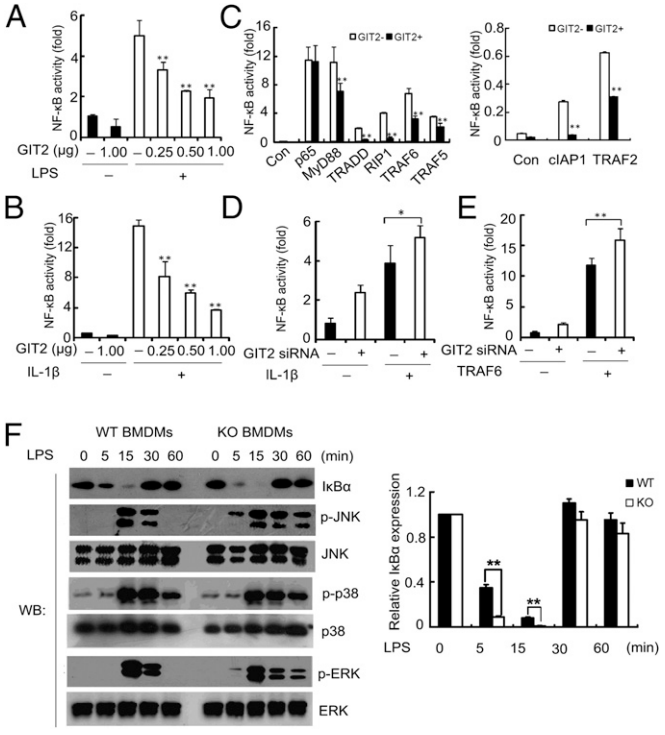

Fig. 5. GIT2 inhibits the activation of the TLR-induced NF-κB and MAPK pathways. (*A*) Luciferase reporter gene assay of NF-κB activation after LPS stimulation of HEK293 cells that expressed TLR4, MD2 (myeloid differentiation protein 2), and increasing amounts of GIT2. (*B*) NF-κB activation was measured after IL-1β stimulation in HEK293 cells transfected with increasing amounts of GIT2. (*C*) NF-κB activation was measured after transfection of HEK293 cells with the indicated NF-κB activators and GIT2. (*D and E*) NF-κB activation was examined in HEK293 cells after treatment with IL-1β or TRAF6 and knockdown of GIT2 by siRNA. (*F*) Immunoblot analysis of IκBα, JNK, p-JNK, p38, p-p38, ERK, and p-ERK in lysates of $Git2^{+/+}$ and $Git2^{-/-}$ BMDMs after treatment with LPS for 0–60 min. Densitometry analysis of IκBα level was performed by Image Lab software (Bio-Rad). Con, control. $^{*}P < 0.05$, $^{**}P < 0.01$.

Phosphorylated JNK and ERK were sustained up to 60 min in $Git2^{-/-}$ BMDMs after stimulation with LPS whereas p-JNK and p-ERK levels in $Git2^{+/+}$ BMDMs were decreased to baseline levels within 60 min (Fig. 5*F*). To determine whether $Git2^{-/-}$ mice have constitutively activated NF-κB compared with their WT littermates, $Git2^{-/-}$ mice and their littermates were killed, and the splenocytes were isolated and stimulated immediately with LPS for indicated times. The expression of IκBα and p-IκBα was analyzed by Western blot. The results indicated that the NF-κB pathway was constitutively activated in $Git2^{-/-}$ mice (Fig. S4). All of these results show that GIT2 negatively regulates TLR-induced NF-κB and MAPK signaling.

GIT2 Blocks the Ubiquitination of TRAF6 by Recruiting CYLD. GIT2 has been shown to interact with TRAF1 by yeast two-hybrid screening (20). TRAF6, another member of the TRAF family, is an essential adaptor for TLR/IL-1R–induced NF-κB activation. We speculated that GIT2 might directly associate with TRAF6. Therefore, the interaction between TRAF6 and GIT2 was validated in HEK293 cells by coimmunoprecipitation assays (Fig. 6*A*), and the association between GIT2 and TRAF6 was enhanced after treatment with IL-1β (Fig. 6*B*). The ubiquitination of TRAF6 through Lys63 (K63)-linked polyubiquitin chains is essential for the activation of TLR downstream signaling (26). We found that the ubiquitination of TRAF6 was repressed by GIT2 in a dose-dependent manner in HEK293 cells (Fig. 6*C*, *Left*). In contrast, ubiquitination of TRAF3 was unaffected by GIT2 (Fig. S5). To confirm the constitutive activation of TRAF6 under baseline noninflammatory conditions, $Git2^{-/-}$ mice and their WT littermates were killed, and ubiquitination levels of TRAF6 from the splenocytes were analyzed by coimmunoprecipitation assay. The results indicated that TRAF6 was indeed constitutively activated in the *Git2*-deficient cells (Fig. S6). We also found that GIT2 specifically inhibited the ubiquitination of TRAF6 when cotransfected with the K48R ubiquitin mutant but not with the K63R ubiquitin mutant (Fig. 6*C*, *Right*). This finding suggests that GIT2 blocks the formation of K63-linked polyubiquitin chains on TRAF6. Oligomerization of TRAF6 is a key step for its ubiquitination and activity (27). However, we noticed that oligomerization of TRAF6 was not influenced by GIT2 (Fig. S7). CYLD targets TRAF6 for deubiquitination to terminate TLR-triggered activation of NF-κB (28). We found that GIT2 interacted with CYLD in HEK293 cells (Fig. 6*D*) and that GIT2 augmented the association of TRAF6 with CYLD (Fig. 6*E*). The endogenous binding of TRAF6, GIT2, and CYLD was enhanced after treatment of BMDMs with LPS (Fig. 6*F*). Furthermore, GIT2 augmented the deubiquitination of TRAF6 by CYLD (Fig. 6*G*) and knockdown *cyld* with specific shRNA blocked the deubiquitination of GIT2 to TRAF6 (Fig. 6*H*). Collectively, we show that GIT2 blocks the ubiquitination of TRAF6 by recruiting CYLD.

The Association of TRAF6 and CYLD with GIT2 Is Essential to Inhibit the Activation of NF-κB. GIT2 has an ARF GTPase-activating protein (ARF-GAP) domain, three ankyrin (ANK) repeats, a Spa2-homology domain (SHD), and two paxillin-binding sites (PBSs) (Fig. S8*A*). The N1 mutant of GIT2, which contains only the ARF-GAP domain, lost the ability to bind to TRAF6 (Fig. S8*B*). However, the mutants (M, N2, N3, and C2) of GIT2 were able to associate with TRAF6 (Fig. S8*B*), which suggested that the PBS domain mediated the binding of TRAF6 to GIT2. Interestingly, the additional TRAF6-binding domain of GIT2, which contains two regions (PBS1 and PBS2), can also bind to paxillin (29). We then tested the ability of the GIT2-deletion mutants for the ability to bind CYLD and found that the N1, N2, and C2 mutants of GIT2 could not bind to CYLD (Fig. S8*C*). In contrast, the N3, C1, and M mutants of GIT2 interacted with CYLD (Fig. S8*C*). These data suggest that the SHD domain mediates the interaction between GIT2 and CYLD. Next, we investigated the effects of the GIT2 mutants on NF-κB activation. The M fragment of GIT2, which contains the PBS1 and SHD that mediate its interaction with TRAF6 and CYLD,

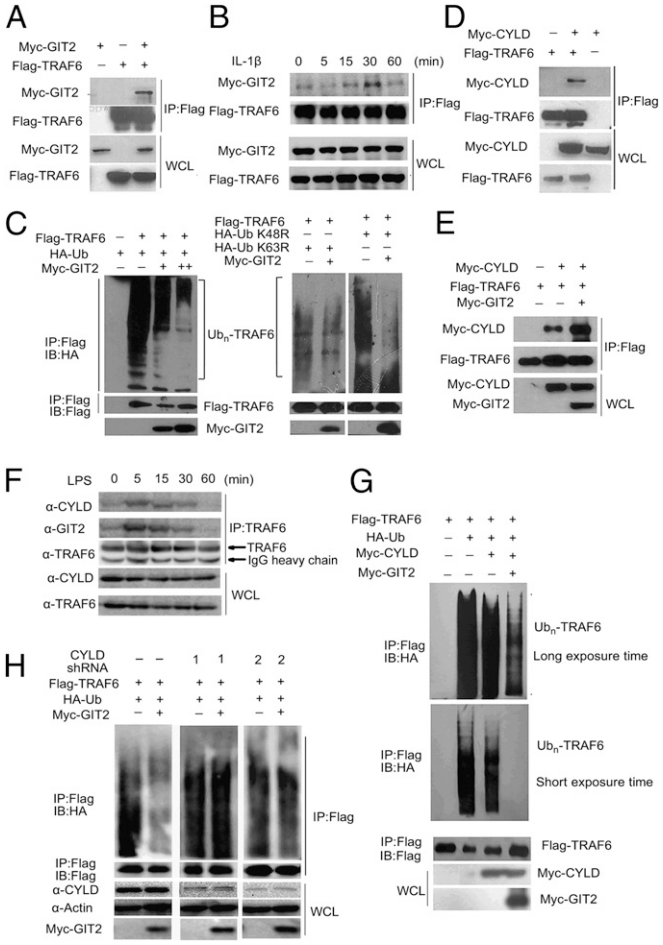

Fig. 6. GIT2 inhibits the ubiquitination of TRAF6 by recruiting CYLD. (*A and B*) Immunoblot analysis of anti-Flag immunoprecipitates of lysates from HEK293 cells that were cotransfected with Myc-GIT2 and Flag-TRAF6. WCL, whole cell lysates; IP, immunoprecipitates. (*C*) Immunoprecipitation and immunoblot analysis of HEK293 cells that were cotransfected with various combinations of Flag-TRAF6, Myc-GIT2, HA-tagged wild-type ubiquitin (HA-Ub), Ub(K48R), and Ub(K63R). (*D*) Immunoprecipitation and immunoblot analysis of the interactions in HEK293 cells transiently expressing Flag-TRAF6 and Myc-CYLD. (*E*) HEK293 cells were transfected with Flag-TRAF6, Myc-CYLD, and Myc-GIT2 as shown. Lysates from those cells were immunoprecipitated with anti-Flag antibody and immunoblotted with anti-Myc and anti-Flag antibodies. (*F*) Endogenous interaction of GIT2, CYLD, and TRAF6 in BMDMs after LPS stimulation at indicated times. (*G*) HEK293 cells were transfected with Flag-TRAF6, Myc-CYLD, Myc-GIT2, and HA-Ub as shown, and cell lysates were immunoprecipitated with anti-Flag antibody and immunoblotted with indicated antibody. (*H*) Immunoprecipitation and immunoblot analysis of HEK293 cells that were cotransfected with various combinations of Flag-TRAF6, Myc-GIT2, HA-Ub, and shRNA of CYLD.

inhibited TRAF6 and IL-1β–induced NF-κB activation (Fig. S8 *D* and *E*). However, the C2 mutant of GIT2, which did not associate with CYLD, lost the ability to repress NF-κB activity (Fig. S8 *D* and *E*). Unexpectedly, the N1 mutant of GIT2 inhibited NF-κB activation, which suggests that GIT2 regulates NF-κB activity through other mechanisms.

Susceptibility of *Git2*-Deficient Mice to DSS-Induced Colitis Depends on TLR Signaling. TLR and inflammasome signaling both play critical roles in regulating colitis (30). We determined whether the susceptibility of *Git2*-deficient mice to DSS-induced acute colitis depended on TLR signaling. The severe colitis symptoms of $Git2^{-/-}$ mice, including shorter colon length (Fig. 7*A*) and remarkable weight loss (Fig. 7*B*), were rescued after being treated with Polymyxin B (PMB), which specifically neutralized the effect of LPS. Moreover, we stimulated BMDMs with agonists of NLRP3 (CPPD and MSU), and the levels of IL-1β or IL-18 were comparable in wild-type and $Git2^{-/-}$ BMDMs (Fig. S9). Next, we determined the effects of IL-1β or IL-18 signaling in regulating DSS-induced colitis. Wild-type and $Git2^{-/-}$ mice were treated with IL-1β neutralizing antibody or IL-18 neutralizing antibody. The colon lengths of $Git2^{-/-}$ mice treated with IL-1β neutralizing antibody or IL-18 neutralizing antibody were similar to $Git2^{-/-}$ mice treated with PBS (Fig. 7*C*). These results indicated that TLR signaling, but not IL-1β or IL-18 signaling, plays a central role in regulating DSS-induced colitis of $Git2^{-/-}$ mice. Furthermore, *Git2*, *Myd88*, or *Git2*/*Myd88* knockout mice were fed with DSS to induce colitis. The susceptibility of *Git2*-deficient mice to DSS-induced acute colitis was rescued by *Myd88* knockout (Fig. 7*D*).

TLR signaling plays critical roles in intestinal epithelial cells and leukocytes of the lamina propria during IBD (31, 32). To determine the cell populations that are critical for *Git2*-dependent protection against colitis, we generated radiation-induced reciprocal bone marrow chimeras from WT and *Git2* KO mice. The mice lacking *Git2* either on hematopoietic or non-hematopoietic cells had higher death rates and more weight loss than the wild-type mice transplanted with wild-type bone marrow (Fig. 7 *E* and *F*). These results indicated that both of the hematopoietic cells and local cells of the colonic mucosa are important to the *Git2*-dependent protection against DSS-induced colitis.

Discussion

IBDs constitute a significant health burden impacting the quality of life of 2.2 million Europeans and 1.4 million Americans, and its peak onset is in persons 15–30 y of age (1). Genetic studies strongly suggest the influence of genetic factors in the etiology of

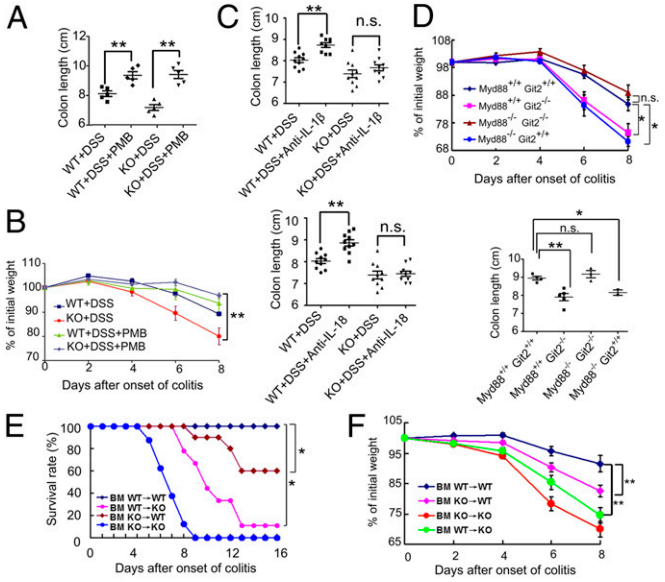

Fig. 7. Susceptibility of *Git2*-deficient mice to DSS-induced colitis depends on TLR signaling. (*A and B*) $Git2^{+/+}$ and $Git2^{-/-}$ mice were treated with PMB and fed 2% (wt/vol) DSS-solution in drinking water for 5 d, followed by regular drinking water for 3 d. The weight loss and colon length of mice were measured. (*C*) Colon length of $Git2^{+/+}$ and $Git2^{-/-}$ mice was measured after treatments with DSS and IL-1β or IL-18 neutralizing antibody on day 8. (*D*) $Git2^{-/-}$, $Myd88^{-/-}$, $Git2^{-/-}$ $Myd88^{-/-}$ double knockout mice, and wild-type mice were fed 2% (wt/vol) DSS, and weight loss was measured for 8 d and colon length of the mice was measured on day 10. (*E and F*) Bone-marrow cells isolated from wild-type or $Git2^{-/-}$ mice were injected into lethal-irradiated wild-type or $Git2^{-/-}$ recipients. Eight Weeks after bone-marrow transplant, the survival rate was monitored until day 16, and weight loss was measured every other day for 8 d. $^*P < 0.05$, $^{**}P < 0.01$.

IBD (3). Here, we show that GIT2 is an essential regulator of the severity of acute and chronic DSS-induced colitis. *Git2*-deficient mice treated with DSS demonstrated more severe symptoms, including shorter colons and higher inflammation scores. Furthermore, the $Git2^{-/-}$ mice and their WT counterparts showed no significant difference in colon length or inflammatory response after treatment with antibiotics and DSS, which indicated that the DSS-induced colitis of the $Git2^{-/-}$ mice relied on commensal bacteria. To determine the role of GIT2 in immune responses, we directly stimulated the $Git2^{-/-}$ and WT mice with *E. coli* or LPS. *Git2*-deficient mice were more susceptible to these challenges and produced more proinflammatory cytokines.

It has been shown that *Git2*-deficient mice demonstrate an immunodeficiency with splenomegaly and high susceptibility to infection (16). Whether GIT2 directly regulates intestinal immunity and the exact mechanism by which GIT2 negatively regulates TLR signaling remain unknown. In this study, we showed that GIT2 negatively regulated TLR-triggered immune responses in vivo and in vitro. GIT2 blocked TLR signaling by deubiquitinating TRAF6 and enhancing the binding of CYLD to TRAF6. The ubiquitin-editing enzyme CYLD is a critical negative regulator of inflammation and of the activation of the transcription factor NF-κB (28). CYLD negatively regulates TLR- and TNF-family signaling by inhibiting TRAF6 ubiquitination and activating downstream signaling events (10). However, CYLD did not remove polyUb chains that were conjugated to TRAF6 in vitro (11). We demonstrated that GIT2 at least partially repressed TLR signaling by recruiting CYLD to deubiquitinate TRAF6. Polyubiquitination of TRAF6 may facilitate the interaction between GIT2 and CYLD, which terminates its activity. In conclusion, our results show that GIT2 negatively regulates the activation of TLR signaling to resolve the immune response and that the loss of GIT2 leads to susceptibility to infectious diseases and colitis. We provide evidences that GIT2 negatively regulates inflammation by direct regulating of TLR signaling. The GPCR pathway has important functions in autoimmunity and inflammation (33), and GIT2 may target two critical pathways that control inflammation. Future studies on the roles of GIT2 in inflammation will aid in the development of disease therapeutics.

Materials and Methods

Mice. $Git2^{-/-}$ mice were obtained from the RIKEN BioResource Center (Japan), and $Myd88^{-/-}$ mice were a kind gift from Shizuo Akira (Hyogo College of Medicine, Hyogo, Japan) (34). All mice were bred in a specific pathogen-free facility, and all animal experiments were approved by the Animal Ethics Committee of the Academy of Military Medical Science. *Myd88* and *Git2* double-knockout mice were generated by crossing.

DSS-Induced Colitis. Colitis was induced in $Git2^{+/+}$ and $Git2^{-/-}$ mice by administering 2% (wt/vol) DSS (36-50 kDa; MP Biomedical) that was dissolved in drinking water for five consecutive days as described previously, followed by regular water (35). Various assays were performed as described in *SI Materials and Methods*.

Bacterial Infection. *E. coli* (ATCC25922, American Type Culture Collection) were inoculated into tryptic soy broth and incubated in a shaking incubator for 10 h at 37 °C. The mice were intraperitoneally injected with 1×10^7 CFUs of *E. coli*. Subsequently, serum and organs were collected and analyzed.

Inhibition of Commensal Microflora. Mice were treated with selective antibiotics for 4 wk as follows: metronidazole (1 g/L; Sigma), neomycin (1 g/L; Amresco), vancomycin (500 mg/L; Amresco), and ampicillin (1 g/L; Amresco). After 4 wk, colitis was induced by DSS.

Other Material and Methods. Other material and methods are described in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Dr. Hisataka Sabe (Osaka Bioscience Institute) and the RIKEN BioResource Center for providing the $Git2^{-/-}$ mice, and Dr. Li Tang (Beijing Institute of Radiation Medicine) and Dr. Hong Tang (Institute of Biophysics, Chinese Academy of Sciences) for providing some of the $Myd88^{-/-}$ mice. We are grateful for the critical comments of the manuscript by Drs. Xuemin Zhang (National Center of Biomedical Analysis), Xuetao Cao (Second Military Medical University) and Genhong Cheng (University of California Los Angeles). This work was supported by National High-Tech R&D Program of China Grant 2012AA020201, Special Funds for Major State Basic Research of China Grants 2011CB910600 and 2012CB910200, National International Cooperation Project Grant 2011DFB30370, Beijing Municipal Natural Science Foundation Grant 5122014, and Beijing NOVA Program Grant 2011014.

1. Abraham C, Cho JH (2009) Inflammatory bowel disease. *N Engl J Med* 361(21):2066–2078.
2. Danese S, Fiocchi C (2011) Ulcerative colitis. *N Engl J Med* 365(18):1713–1725.
3. Saleh M, Elson CO (2011) Experimental inflammatory bowel disease: Insights into the host-microbiota dialog. *Immunity* 34(3):293–302.
4. Rakoff-Nahoum S, Hao L, Medzhitov R (2006) Role of toll-like receptors in spontaneous commensal-dependent colitis. *Immunity* 25(2):319–329.
5. Murray PJ, Smale ST (2012) Restraint of inflammatory signaling by interdependent strata of negative regulatory pathways. *Nat Immunol* 13(10):916–924.
6. Li HY, et al. (2008) Deactivation of the kinase IKK by CUEDC2 through recruitment of the phosphatase PP1. *Nat Immunol* 9(5):533–541.
7. Marshak-Rothstein A (2006) Toll-like receptors in systemic autoimmune disease. *Nat Rev Immunol* 6(11):823–835.
8. Cook DN, Pisetsky DS, Schwartz DA (2004) Toll-like receptors in the pathogenesis of human disease. *Nat Immunol* 5(10):975–979.
9. Trompouki E, et al. (2003) CYLD is a deubiquitinating enzyme that negatively regulates NF-kappaB activation by TNFR family members. *Nature* 424(6950):793–796.
10. Kovalenko A, et al. (2003) The tumour suppressor CYLD negatively regulates NF-kappaB signalling by deubiquitination. *Nature* 424(6950):801–805.
11. Xia ZP, et al. (2009) Direct activation of protein kinases by unanchored polyubiquitin chains. *Nature* 461(7260):114–119.
12. Trompouki E, et al. (2009) Truncation of the catalytic domain of the cylindromatosis tumor suppressor impairs lung maturation. *Neoplasia* 11(5):469–476.
13. Inoue H, Randazzo PA (2007) Arf GAPs and their interacting proteins. *Traffic* 8(11): 1465–1475.
14. Premont RT, Claing A, Vitale N, Perry SJ, Lefkowitz RJ (2000) The GIT family of ADP-ribosylation factor GTPase-activating proteins: Functional diversity of GIT2 through alternative splicing. *J Biol Chem* 275(29):22373–22380.
15. Ku GM, Yablonski D, Manser E, Lim L, Weiss A (2001) A PAK1-PIX-PKL complex is activated by the T-cell receptor independent of Nck, Slp-76 and LAT. *EMBO J* 20(3):457–465.
16. Mazaki Y, et al. (2006) Neutrophil direction sensing and superoxide production linked by the GTPase-activating protein GIT2. *Nat Immunol* 7(7):724–731.
17. Phee H, et al. (2010) Regulation of thymocyte positive selection and motility by GIT2. *Nat Immunol* 11(6):503–511.
18. Su AI, et al. (2004) A gene atlas of the mouse and human protein-encoding transcriptomes. *Proc Natl Acad Sci USA* 101(16):6062–6067.
19. Orozco LD, et al. (2012) Unraveling inflammatory responses using systems genetics and gene-environment interactions in macrophages. *Cell* 151(3):658–670.
20. Wang J, et al. (2011) Toward an understanding of the protein interaction network of the human liver. *Mol Syst Biol* 7:536.
21. Knutson CG, et al. (2013) Chemical and cytokine features of innate immunity characterize serum and tissue profiles in inflammatory bowel disease. *Proc Natl Acad Sci USA* 110(26):E2332–E2341.
22. Haziot A, et al. (1996) Resistance to endotoxin shock and reduced dissemination of gram-negative bacteria in CD14-deficient mice. *Immunity* 4(4):407–414.
23. Han C, et al. (2010) Integrin CD11b negatively regulates TLR-triggered inflammatory responses by activating Syk and promoting degradation of MyD88 and TRIF via Cbl-b. *Nat Immunol* 11(8):734–742.
24. Xu S, et al. (2012) Constitutive MHC class I molecules negatively regulate TLR-triggered inflammatory responses via the Fps-SHP-2 pathway. *Nat Immunol* 13(6):551–559.
25. Seibert K, Masferrer JL (1994) Role of inducible cyclooxygenase (COX-2) in inflammation. *Receptor* 4(1):17–23.
26. Lomaga MA, et al. (1999) TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. *Genes Dev* 13(8):1015–1024.
27. Wang Y, et al. (2006) Association of beta-arrestin and TRAF6 negatively regulates Toll-like receptor-interleukin 1 receptor signaling. *Nat Immunol* 7(2):139–147.
28. Massoumi R (2010) Ubiquitin chain cleavage: CYLD at work. *Trends Biochem Sci* 35(7): 392–399.
29. Turner CE, et al. (1999) Paxillin LD4 motif binds PAK and PIX through a novel 95-kD ankyrin repeat, ARF-GAP protein: A role in cytoskeletal remodeling. *J Cell Biol* 145(4):851–863.
30. Lin KM, et al. (2014) IRAK-1 bypasses priming and directly links TLRs to rapid NLRP3 inflammasome activation. *Proc Natl Acad Sci USA* 111(2):775–780.
31. Asquith MJ, Boulard O, Powrie F, Maloy KJ (2010) Pathogenic and protective roles of MyD88 in leukocytes and epithelial cells in mouse models of inflammatory bowel disease. *Gastroenterology* 139(2):519–529, 529.e1–529.e2.
32. Brandl K, et al. (2010) MyD88 signaling in nonhematopoietic cells protects mice against induced colitis by regulating specific EGF receptor ligands. *Proc Natl Acad Sci USA* 107(46):19967–19972.
33. Zlotnik A, Yoshie O (2012) The chemokine superfamily revisited. *Immunity* 36(5): 705–716.
34. Adachi O, et al. (1998) Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* 9(1):143–150.
35. Liu Z, et al. (2011) The kinase LRRK2 is a regulator of the transcription factor NFAT that modulates the severity of inflammatory bowel disease. *Nat Immunol* 12(11): 1063–1070.