Interleukin 1 receptor signaling regulates DUBA expression and facilitates Toll-like receptor 9–driven antiinflammatory cytokine production

Jose M. González-Navajas,[1,4] Jason Law,[1] Kim Phung Nguyen,[1] Meha Bhargava,[1] Mary Patricia Corr,[1] Nissi Varki,[2] Lars Eckmann,[1] Hal M. Hoffman,[3] Jongdae Lee,[1] and Eyal Raz[1]

[1]Department of Medicine, [2]Department of Pathology, and [3]Department of Pediatrics, University of California, San Diego, La Jolla, CA 92093

[4]Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Alicante 03010, Spain

The interleukin 1 receptor (IL-1R) and the Toll-like receptors (TLRs) are highly homologous innate immune receptors that provide the first line of defense against infection. We show that IL-1R type I (IL-1RI) is essential for TLR9-dependent activation of tumor necrosis factor receptor–associated factor 3 (TRAF3) and for production of the antiinflammatory cytokines IL-10 and type I interferon (IFN). Noncanonical K63-linked ubiquitination of TRAF3, which is essential for type I IFN and IL-10 production, was impaired in *Il1r1*$^{-/-}$ CD11c^{+} dendritic cells. In contrast, degradative ubiquitination of TRAF3 was not affected in the absence of IL-1R1 signaling. Deubiquitinating enzyme A (DUBA), which selectively cleaves K63-linked ubiquitin chains from TRAF3, was up-regulated in the absence of IL-1R1 signaling. DUBA short interference RNA augmented the TLR9-dependent type I IFN response. Mice deficient in IL-1RI signaling showed reduced expression of IL-10 and type I IFN and increased susceptibility to dextran sulphate sodium–induced colitis and failed to mount a protective type I IFN response after TLR9 ligand (CpG) administration. Our data identifies a new molecular pathway by which IL-1 signaling attenuates TLR9-mediated proinflammatory responses.

CORRESPONDENCE
Eyal Raz:
eraz@ucsd.edu

Abbreviations used: BMDC, BM-derived DC; DAI, disease activity index; DSS, dextran sulphate sodium; DUBA, deubiquitinating enzyme A; EMSA, electrophoretic mobility shift assay; IRF, IFN regulatory factor; MAPK, mitogen-activated protein kinase; TLR, Toll-like receptor; TRAF3, TNF receptor-associated factor 3.

The IL-1 receptor family includes 10 members, which contain IgG-like segments in the extracellular domain and a cytoplasmic toll/IL-1 receptor intracellular domain that is found in other Toll-like receptors (TLRs; Dinarello, 2009). The proinflammatory cytokines IL-1α and IL-1β bind the IL-1R type I (IL-1RI), leading to activation of NF-κB, the mitogen-activated protein kinase (MAPK), and certain IFN regulatory factors (IRFs; Fujita et al., 1989; Rivieccio et al., 2005). IL-1RI is constitutively expressed in most cell types (Dinarello, 1996), and it is the most studied member of the IL-1R family (Dinarello, 1996, 2009). Although the role of IL-1 in sterile inflammation, such as rheumatoid arthritis, gout, or autoinflammatory syndromes (Dinarello, 2009), has been extensively studied, its role in nonsterile inflammatory conditions, such as inflammatory bowel disease, has not been clearly defined (Bresnihan et al., 1998; Hoffman et al., 2004). Despite its role in inflammation, IL-1 signaling has been reported to protect mice from intestinal damage after *Citrobacter rodentium* infection (Lebeis et al., 2009) and from dextran sulphate sodium (DSS)–induced colitis (Kojouharoff et al., 1997; Lebeis et al., 2009). In contrast, administration of anti–IL-1β antibody improved DSS-induced colitis (Arai et al., 1998), and mice deficient in the NLRP3 inflammasome, a caspase-1–activating complex which regulates IL-1 and IL-18 maturation, are relatively resistant to intestinal inflammation induced in this model (Bauer et al., 2010). In this paper, we

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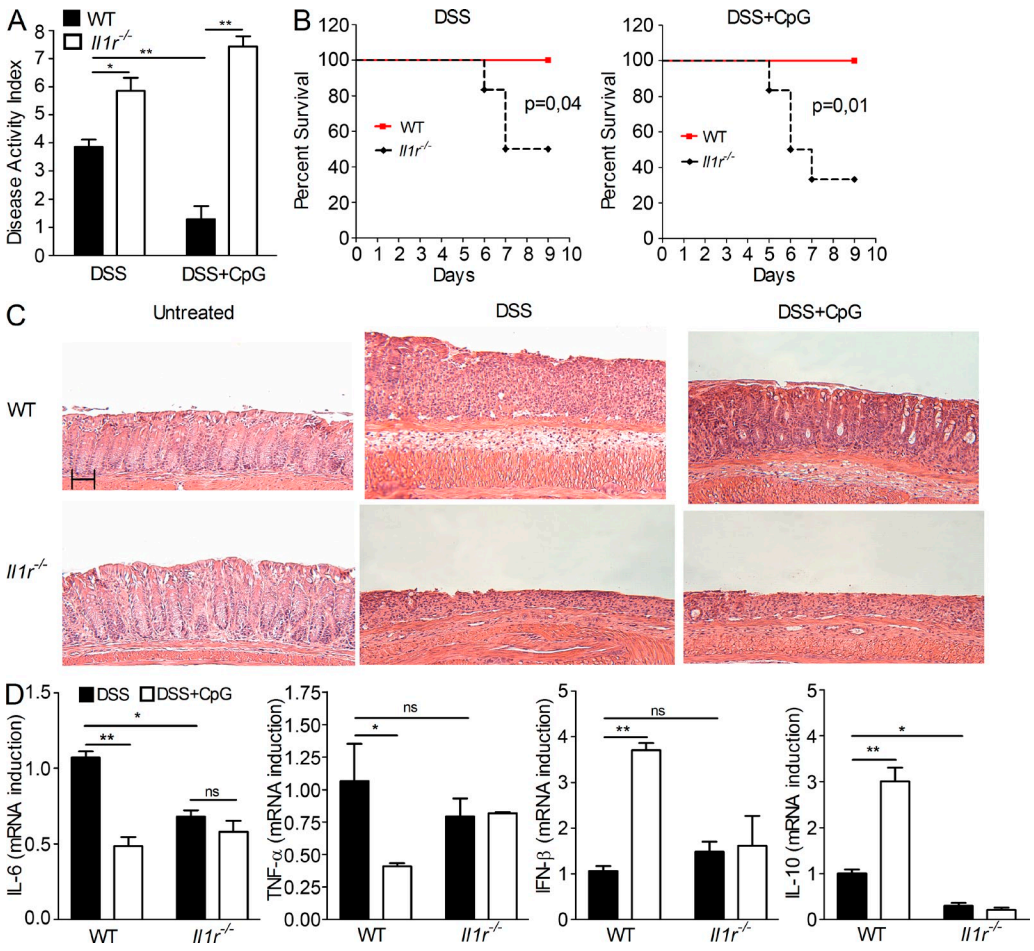

Figure 1. *Il1r1*$^{-/-}$ mice are more susceptible to DSS-induced colitis than WT mice. (A) DAI score in WT and *Il1r1*$^{-/-}$ mice. Mice were given DSS (2%) in their drinking water for 7 d with or without pretreatment with CpG oligonucleotides (10 µg/mouse) 2 h before DSS administration. (B) Survival of WT and *Il1r1*$^{-/-}$ mice treated as described in A. (C) Hematoxylin and eosin staining of colon sections from untreated mice or WT and *Il1r1*$^{-/-}$ on day 7 of DSS treatment. Bar, 50 µm. (D) Quantitative PCR analysis of pro- and antiinflammatory mediators in colonic homogenates from WT and *Il1r1*$^{-/-}$ mice on day 7 of DSS treatment. (A–D) Data are representative of four different experiments ($n = 6$). Error bars represent mean ± SEM. ns, not significant. *, $P < 0.05$; **, $P < 0.01$.

describe a novel mechanism by which IL-1RI signaling modulates the TLR-dependent inflammatory response. We show that IL-1RI signaling down-regulates the expression of deubiquitinating enzyme A (DUBA) and consequently enhances the Lys63-linked ubiquitination of TNF receptor-associated factor 3 (TRAF3), which is necessary for the transcription of antiinflammatory cytokines.

RESULTS AND DISCUSSION

Genetic and pharmacologic targeting of IL-1RI exacerbates DSS-induced colitis

Mice exposed to orally delivered DSS develop acute colitis, displaying diarrhea, rectal bleeding, and weight loss. To better define how IL-1R contributes to colonic homeostasis, we exposed C57BL/6 (B6 and WT) and *Il1r1*$^{-/-}$ mice to DSS in the drinking water ad libitum. Surprisingly, *Il1r1*$^{-/-}$ mice were more susceptible to DSS colitis, as indicated by a higher disease activity index (DAI) score and an increased mortality compared with WT mice (Fig. 1, A and B). Furthermore, *Il1r1*$^{-/-}$ mice showed an impaired ability to recover from DSS-induced colitis and kept losing weight after DSS removal at day 7 (Fig. S1 A). In previous studies, administration of unmethylated CpG, a synthetic ligand for TLR9, was shown to attenuate DSS-induced colitis in mice, mainly via the induction of a type I IFN response (Rachmilewitz et al., 2002; Katakura et al., 2005). Accordingly, i.p. injection of CpG, before DSS administration, efficiently ameliorated the severity of colonic inflammation in WT mice (Fig. 1 A). In contrast, CpG administration resulted in a higher DAI score and further increased mortality in *Il1r1*$^{-/-}$ mice (Fig. 1, A and B). Histological analysis of the colonic tissues from the DSS-treated mice revealed that both WT and *Il1r1*$^{-/-}$ mice developed mucosal inflammation with epithelial ulcerations, crypt loss, depletion of goblet cells, and marked infiltration of mononuclear cells in the colonic lamina propria (Fig. 1 C). The extent of epithelial damage was more severe in *Il1r1*$^{-/-}$ mice in which DSS administration caused almost complete ablation of the colonic epithelium (Fig. 1 C).

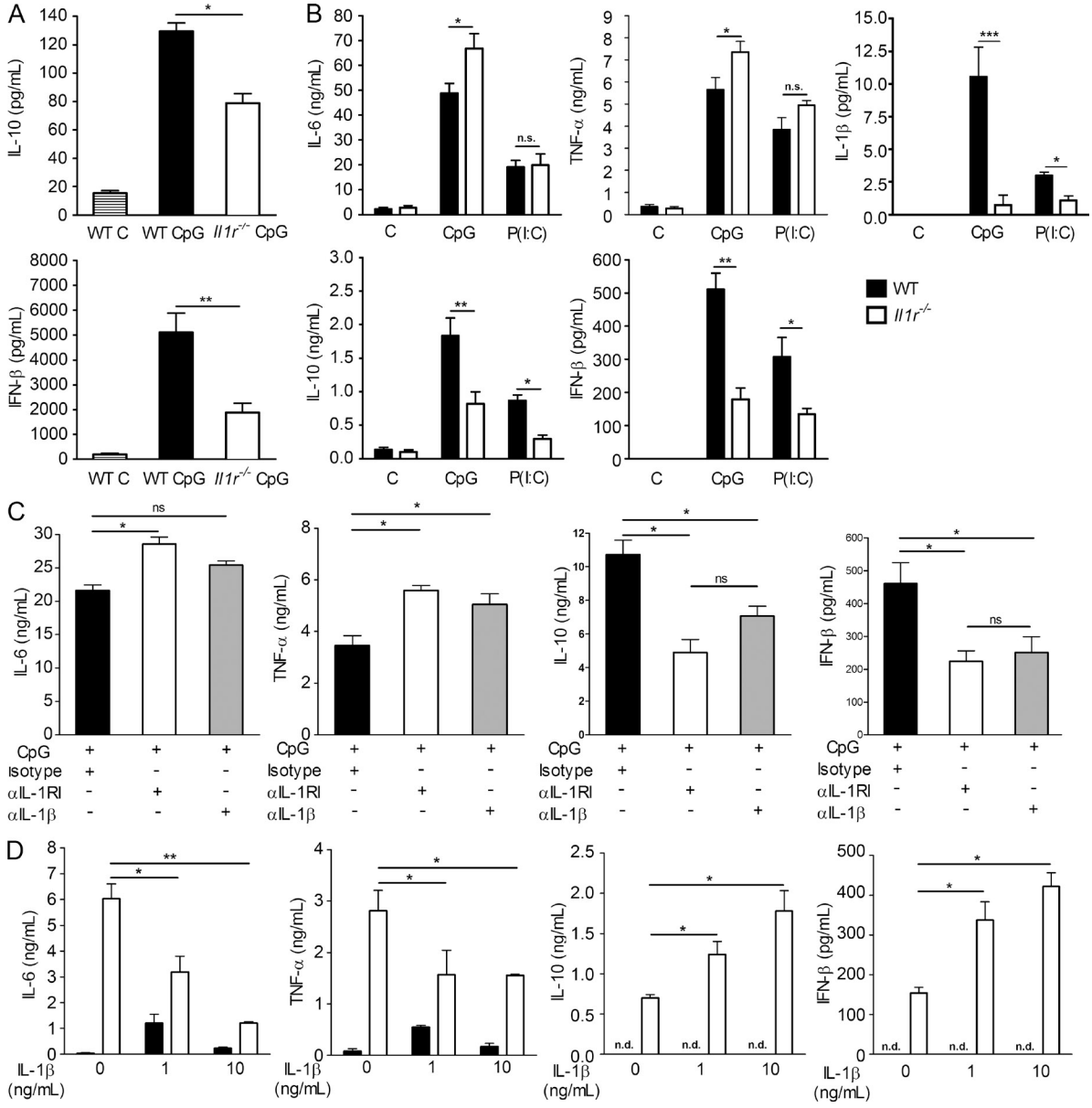

Figure 2. *Il1r1*$^{-/-}$ mice have impaired IL-10 and type I IFN response. (A) Cytokine levels in serum of WT and *Il1r1*$^{-/-}$ mice 2 h after i.v. administration of 50 µg CpG oligonucleotides or vehicle (C). Data are representative of two independent experiments ($n = 4$). (B) Cytokine levels in supernatants from WT and *Il1r1*$^{-/-}$ CD11c^{+} BMDCs 24 h after stimulation with 10 µg/ml CpG, 10 µg/ml Poly(I:C), or vehicle (C). (C) WT BMDCs were incubated with isotype control antibody, αIL-1RI blocking antibodies, or αIL-1β neutralizing antibody for 2 h. Cells were then stimulated with CpG for 24 h and cytokine levels were determined in the supernatants. (D) Effect of IL-1β prestimulation on cytokine production. WT BMDCs were cultured in the presence of 0, 1, or 10 ng/ml of recombinant IL-1β for 12 h. Cells were then washed and restimulated or not with 10 µg/ml CpG for 24 h, and the levels of cytokines were determined in the supernatants. (B–D) Data are representative of three different experiments. Error bars represent mean ± SEM. ns, not significant. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Importantly, although the administration of CpG highly reduced the DSS-induced damage in WT mice, it did not have any beneficial effect on colonic inflammation in *Il1r1*$^{-/-}$ mice (Fig. 1 C).

To determine potential causes for the differences in colitis severity and the differential response to CpG in WT versus *Il1r1*$^{-/-}$ mice, we measured the relative mRNA levels of pro- and antiinflammatory cytokines in colonic homogenates obtained from each group after 7 d of DSS, with or without CpG treatment. CpG administration decreased the mRNA levels of inflammatory cytokines, such as TNF and IL-6, in WT but not in *Il1r1*$^{-/-}$ mice (Fig. 1 D). More importantly, the administration of CpG resulted in increased mRNA levels of the antiinflammatory mediators IL-10 and IFN-β in the colonic tissues obtained from WT mice but not in those obtained from *Il1r1*$^{-/-}$ mice (Fig. 1 D).

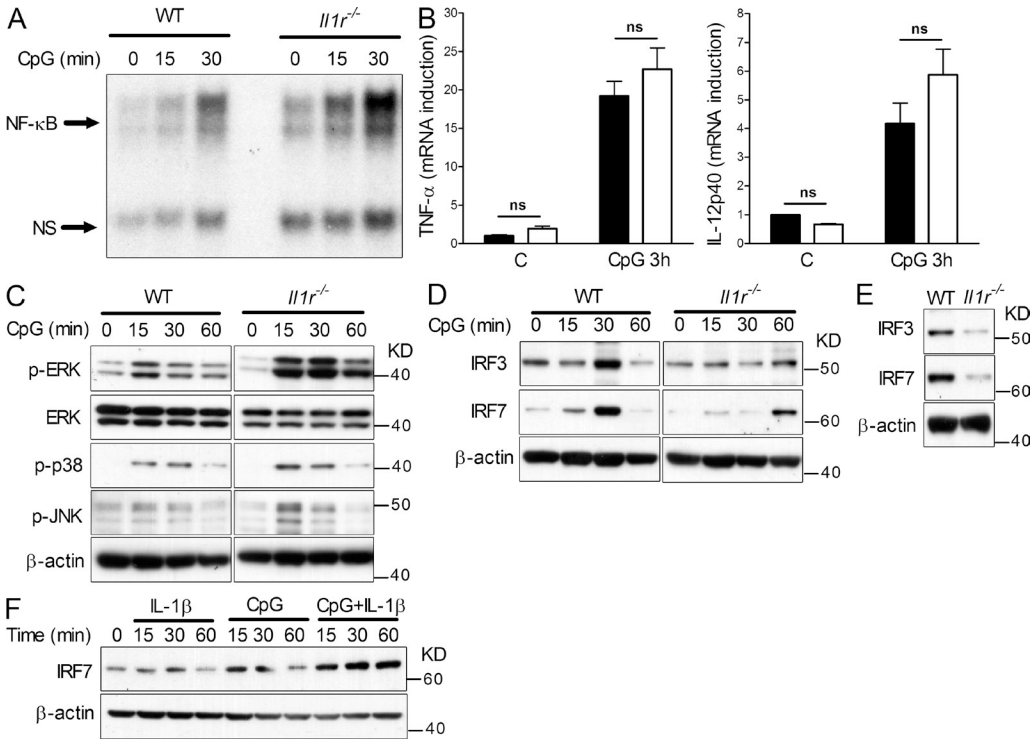

Figure 3. ***Il1r1*$^{-/-}$ BMDCs display impaired IRF3 and IRF7 responses.** (A) Electrophoretic mobility shift assay (EMSA) analysis of NF-κB activation in WT and *Il1r1*$^{-/-}$ BMDCs after CpG stimulation (10 µg/ml) for the indicated time periods. (B) BMDCs from WT and *Il1r*$^{-/-}$ mice were stimulated with 10 µg/ml CpG for 3 h, followed by RNA isolation and quantitative PCR analysis using GAPDH expression as internal reference. (C) Immunoblot analysis of MAPK activation in BMDCs from WT and *Il1r1*$^{-/-}$ stimulated with 10 µg/ml CpG oligonucleotides for the indicated time periods. (D) Immunoblot analysis of IRF3 and IRF7 in nuclear extracts of BMDCs from WT and *Il1r1*$^{-/-}$ mice stimulated as in C. (E) Baseline levels of IRF3 and IRF7 in nuclear extracts from freshly isolated unstimulated splenocytes from WT and *Il1r1*$^{-/-}$ mice. (F) WT BMDCs were left unstimulated (0) or were stimulated with 10 ng/ml of recombinant IL-1β, 10 µg/ml CpG, or both for the indicated time periods. Nuclear extracts were then isolated and IRF7 translocation was analyzed by immunoblotting. (A–F) Data are representative of three independent experiments. Error bars represent mean ± SEM. ns, not significant.

A recombinant form of IL-1R antagonist (IL-1Ra), also known as anakinra, is currently used in the treatment of rheumatoid arthritis and several human autoinflammatory diseases (Dinarello, 2009). To study the impact of IL-1Ra on the course of experimental colitis, we treated WT mice i.p. with either anakinra (1 mg/mouse, twice a day) or vehicle during the course of DSS administration. Blocking IL-1 activity with anakinra enhanced the severity of DSS-induced colitis in WT mice, as indicated by a higher DAI score and a more severe epithelial damage (Fig. S1, B and C). Anakinra treatment also resulted in increased levels of proinflammatory cytokines and reduced IFN-β production in supernatants from ex vivo–cultured colonic tissues (Fig. S1 D). Collectively, these results correlate with the phenotype of intestinal inflammation observed in *Il1r1*$^{-/-}$ mice and suggest that IL-1 signaling supports colonic homeostasis and attenuates colonic inflammation in this model.

Il1r$^{-/-}$ mice display impaired production of IL-10 and type I IFN

Several mechanisms may be responsible for the increased susceptibility of *Il1r1*$^{-/-}$ mice to DSS-induced colitis. IL-1 promotes prostaglandin E2 (PGE_2) production via the induction of COX-2 (Mizel et al., 1981; Martin et al., 1994). PGE_2, in turn, was reported to inhibit mucosal inflammation in DSS-induced colitis in mice and rats (Kabashima et al., 2002; Nitta et al., 2002). Similarly, pretreatment with a low dose of IL-1 was also shown to suppress colonic inflammation in rabbits via the production of PGE_2 (Cominelli et al., 1990). Nevertheless, as type I IFN production by $CD11c^+$ DCs is partially responsible for CpG-dependent attenuation of colitis in WT mice in this model (Katakura et al., 2005; Abe et al., 2007), we reasoned that the impaired ability to produce type I IFN in response to TLR9 stimulation in *Il1r*$^{-/-}$ mice facilitated colonic inflammation. Indeed, the administration of recombinant mouse (rm) IFN-β ameliorated the severity of DSS-induced colitis (Fig. S2 A) and suppressed the inflammatory cytokine production in the colonic tissue of *Il1r1*$^{-/-}$ mice (Fig. S2 B).

Our data so far indicate a defect in IL-10 and type I IFN production in the colonic tissues of *Il1r*$^{-/-}$ mice. To explore whether these mice have a reflection of a systemic defect, we injected CpG i.v. and measured the serum levels of these cytokines. Indeed, we observed lower levels of these antiinflammatory cytokines in the serum of *Il1r*$^{-/-}$ as compared with WT mice (Fig. 2 A). Furthermore, BM-derived DCs (BMDCs) from *Il1r*$^{-/-}$ mice produced lower levels of IL-1β, IL-10,

and IFN-β in response to both TLR9 (CpG) and TLR3 [P(I:C)] stimulation as compared with those from WT mice (Fig. 2 B). In contrast, we observed increased levels of IL-6 and TNF in response to CpG but not to P(I:C) stimulation (Fig. 2 B). Furthermore, in vitro blockade of IL-1RI signaling in WT BMDCs, with either anti–IL-1RI blocking or anti–IL-1β neutralizing antibodies, resulted in reduced TLR9-dependent production of IL-10 and IFN-β levels and in augmented levels of TNF and IL-6 (Fig. 2 C). To further elucidate the antiinflammatory effects of IL-1RI signaling, we stimulated WT BMDCs with IL-1β. This stimulation led to a modest increase in the secretion of IL-6 and TNF without detectable levels of IL-10 and IFN-β (Fig. 2 D, solid bars). However, when IL-1β–pretreated cells were restimulated with CpG, we observed a significant reduction in the TLR9-dependent production of IL-6 and TNF and an increased production of IL-10 and IFN-β (Fig. 2 D, empty bars). Collectively, these in vitro results further support the antiinflammatory role of IL-1RI signaling observed in vivo (Fig. 1), especially in the context of TLR stimulation.

Signaling via IL-1RI is required for the activation of IRFs

Upon TLR stimulation, the production of pro- and anti-inflammatory cytokines is tightly regulated by the activation of different families of transcription factors, including NF-kB, MAPKs, and IRFs. In vitro activation of BMDCs from *Il1r1*$^{-/-}$ mice by CpG led to a modest increase in the activation of NF-kB and NF-kB target genes compared with WT BMDCs (Fig. 3, A and B). More significantly, this activation resulted in a marked increase in the phosphorylation levels of ERK and a mild increase in the activation of p38 and JNK MAPKs (Fig. 3 C). The increase in NF-kB and MAPK activation probably explain the augmented production of TNF and IL-6 by *Il1r1*$^{-/-}$ BMDCs in response to TLR9 stimulation. In contrast, the activation and nuclear translocation of IRF3 and IRF7, under the same conditions, were greatly reduced in *Il1r1*$^{-/-}$ BMDCs (Fig. 3 D). Importantly, freshly isolated splenocytes from *Il1r1*$^{-/-}$ mice also showed reduced nuclear translocation of IRF3 and IRF7 (Fig. 3 E). Furthermore, consistent with the stimulatory effect of IL-1β on the production of type I IFN (Fig. 2 D), IL-1β and CpG costimulation led to increased nuclear translocation of IRF7 when compared with CpG stimulation alone (Fig. 3 F). Collectively, these data suggest that IL-1RI signaling has a tonic stimulatory effect in the regulation of IRFs and consequently on the production of type I IFN.

IL-1RI signaling modulates the ubiquitination profile of TRAF3

TRAF3, an E3 ubiquitin ligase which interacts with both MyD88 and TRIF, is essential for the balanced production of type I IFN and proinflammatory cytokines upon TLR activation (Häcker et al., 2006; Oganesyan et al., 2006; Tseng et al., 2010). In particular, differences in the ubiquitination profile of TRAF3 have been reported to orchestrate these events. Although K48-linked ubiquitination leads to the degradation of TRAF3 and the activation of MAPKs and proinflammatory cytokines, K63-linked ubiquitination is essential for the activation of IRFs and subsequent type I IFN production (Tseng et al., 2010). Because *Il1r1*$^{-/-}$ BMDCs present an impaired activation of IRFs (Fig. 3) and production of IFN-β (Fig. 2), we investigated the fate of TRAF3 in these cells upon CpG stimulation. Although the baseline expression of TRAF3 was higher in WT cells, TRAF3 levels were reduced in both WT and *Il1r1*$^{-/-}$ BMDCs upon TLR9 stimulation (Fig. 4 A). The extent of TRAF3 degradation was very similar under each condition, as indicated by densitometric analysis of the bands, suggesting that IL-1RI deficiency does not affect the degradative K48-linked ubiquitination of TRAF3. Indeed, K48-linked ubiquitination was not affected in the absence of IL-1RI signaling (Fig. 4 B, top). In contrast, K63-linked ubiquitination was greatly reduced in *Il1r1*$^{-/-}$ as compared with WT BMDCs (Fig. 4 B, bottom). K63-linked ubiquitination in WT BMDCs was also shown in the absence of proteasomal inhibition, indicating that CpG induces K63 ubiquitination despite the K48-dependent degradation of TRAF3 (Fig. S3). Collectively, these results identified the

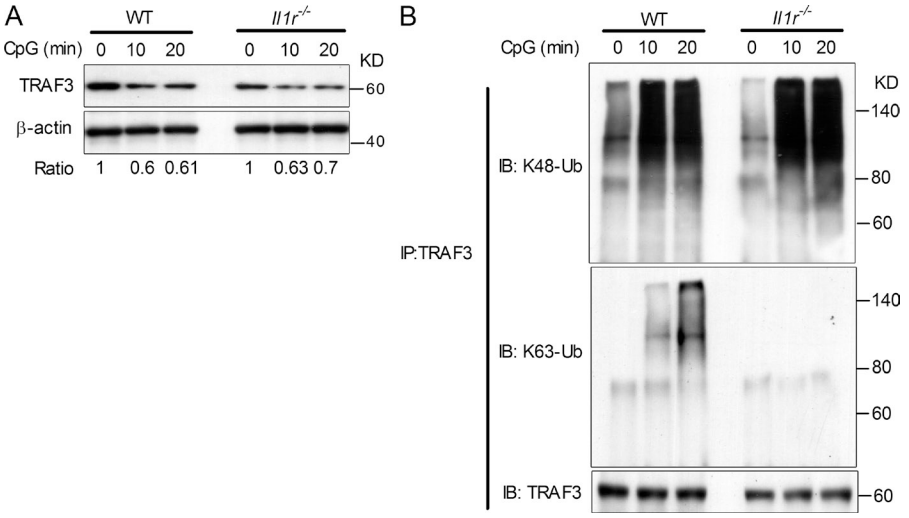

Figure 4. IL-1RI signaling modulates the ubiquitination profile of TRAF3. (A) Immunoblot analysis of TRAF3 in total cell lysates from WT and *Il1r1*$^{-/-}$ BMDCs stimulated with 10 µg/ml CpG for the indicated time periods. Ratio expresses the densitometry analysis of the TRAF3 bands normalized to the expression of β-actin. (B) TRAF3 ubiquitination assay. WT and *Il1r1*$^{-/-}$ BMDCs were treated with the proteasome inhibitor MG132 for 2 h and then stimulated with 10 µg/ml CpG for the indicated time periods. Overnight immunoprecipitation of TRAF3 was then followed by immunoblotting with anti–K48-linked ubiquitin (K48-Ub), anti–K63-linked ubiquitin (K63-Ub), or anti-TRAF3. (A and B) Data are representative of three different experiments.

role of IL-1RI signaling in the activation of TRAF3 and explain why *Il1r1*$^{-/-}$ BMDCs have lower production of IFN-β and IL-10 but increased or normal production of proinflammatory cytokines under the experimental conditions specified in this section.

DUBA inhibits type I IFN production in the absence of IL-1RI signaling

DUBs are proteases that cleave ubiquitin linkages. DUBA, a member of the ovarian tumor (OTU) domain–containing cysteine protease superfamily, was shown to suppress the type I IFN-dependent innate immune response by cleaving the K63 polyubiquitin chain on TRAF3 (Kayagaki et al., 2007). Cleavage resulted in the dissociation of TRAF3 from the downstream signaling complex and disruption of type I IFN production. To study the role of DUBA in the differential ubiquitination of TRAF3, we stimulated BMDCs from WT and *Il1r1*$^{-/-}$ mice with CpG and assayed its impact on DUBA protein levels. As shown in Fig. 5 A, CpG induces higher levels of DUBA protein in *Il1r1*$^{-/-}$ as compared with WT BMDCs. Importantly, steady-state levels of DUBA protein were increased in BMDCs (Fig. 5 A) as well as in freshly isolated splenocytes from *Il1r1*$^{-/-}$ mice (Fig. 5 B). It is of note that DUBA levels were not increased in splenocytes from *Tlr3*$^{-/-}$ or *Tlr9*$^{-/-}$ mice (Fig. S4), indicating that the dysregulation of DUBA expression is not a common characteristic in all TLR-deficient cells. Consistent with the protein data, *Duba* mRNA levels were increased in freshly isolated splenocytes and mesenteric LN-derived cells from *Il1r1*$^{-/-}$ mice (Fig. 5 C), suggesting that IL-1RI signaling negatively regulates DUBA transcription. Finally, we determined whether DUBA is responsible for the reduced production of IL-10 and IFN-β in *Il1r1*$^{-/-}$ BMDCs. DUBA knockdown by siRNA transfection in *Il1r1*$^{-/-}$ BMDCs (Fig. 5 D) resulted in significantly increased IL-10 and IFN-β production and decreased secretion of IL-6, but not TNF, in CpG-stimulated *Il1r1*$^{-/-}$ BMDCs (Fig. 5 E).

Our findings suggest that IL-1RI signaling positively regulates TLR-dependent type I IFN production. In the absence of IL-1 signaling, DUBA expression levels are increased and, therefore, the cleavage of K63-linked polyubiquitin chains of TRAF3, upon TLR stimulation, is facilitated. This effect results in reduced activation of TRAF3 and, consequently, diminished type I IFN and IL-10 responses.

IL-1 is an endogenous cytokine that utilizes TLR signaling pathways, suggesting its close relation to innate defense networks against microbial threats. By regulating DUBA levels, IL-1RI signaling equilibrates the pro- and antiinflammatory cytokine production in response to exogenous microbial TLR stimulation. Our results have uncovered new antiinflammatory and protective properties of this well-known and pleiotropic proinflammatory cytokine. They also reveal a novel mechanism by which IL-1RI signaling restrains TLR-mediated inflammatory responses.

The therapeutic potential of blocking IL-1RI signaling has been recognized for >20 yr, resulting in the generation of numerous compounds. The development of IL-1RA as a therapy for sepsis, an overwhelming inflammatory response to infection, was unsuccessful. However, patients suffering from sterile inflammatory diseases, such as rheumatoid arthritis and

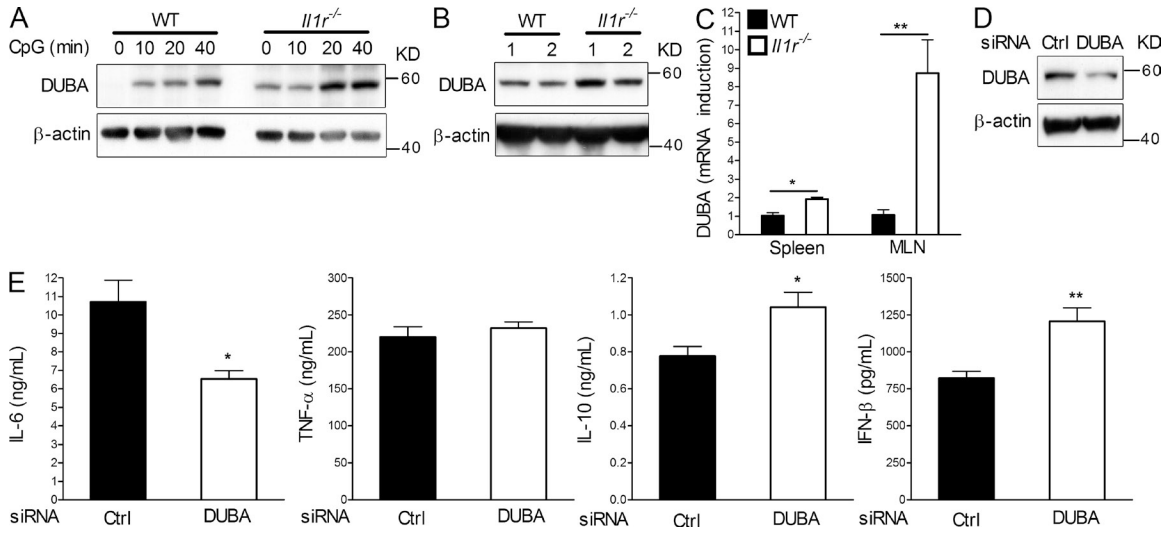

Figure 5. DUBA inhibits type I IFN production by *Il1r1*$^{-/-}$ BMDCs. (A) Immunoblot analysis of DUBA in total cell lysates from WT and *Il1r1*$^{-/-}$ BMDCs stimulated with 10 µg/ml CpG for the indicated time periods. (B) Immunoblot analysis of DUBA expression in total cell lysates from freshly isolated splenocytes from WT and *Il1r1*$^{-/-}$ mice. (C) Quantitative PCR analysis of *Duba* mRNA expression in freshly isolated splenocytes and mesenteric LN-derived cells from WT and *Il1r1*$^{-/-}$ mice. (D) Immunoblot analysis of DUBA expression in total cell lysates from *Il1r*$^{-/-}$ BMDCs transfected with 0.5 µM of either control (Ctrl) or DUBA siRNAs. (E) Pro- and antiinflammatory cytokine production in *Il1r1*$^{-/-}$ BMDCs after DUBA knockdown. *Il1r1*$^{-/-}$ BMDCs were transfected as described in D. 24 h after transfection, cells were stimulated with 10 µg/ml CpG oligonucleotides for an additional 24 h and cytokine production was determined by ELISA. (A–E) Data are representative of three different experiments. Error bars represent mean ± SEM. *, P < 0.05; **, P < 0.01.

autoinflammatory syndromes, systemic onset juvenile idiopathic arthritis, and gout were shown to benefit from blocking IL-1RI signaling (Bresnihan et al., 1998; Emsley et al., 2005; Pascual et al., 2005; Dinarello, 2009). Our findings may help to explain these clinical observations and suggest that IL-1R blockade can be harmful in certain infections or in nonsterile inflammatory conditions.

MATERIALS AND METHODS

Mice. 6–10-wk-old mice were used for all the experimental procedures. Specific pathogen-free (SPF) C57BL/6 (B6 and WT) mice were purchased from Harlan Sprague Dawley Inc. *Il1r1*$^{-/-}$ mice on the B6 background (The Jackson Laboratory) were bred in our animal facility under SPF conditions. *Tlr3*$^{-/-}$ and *Tlr9*$^{-/-}$ mice on the B6 background were provided by S. Akira (Osaka University, Osaka, Japan). All experimental procedures were approved by the University of California, San Diego Institutional Animal Care and Use Committee.

Reagents. 1018 CpG-ODN (5′-TGACTGTGAACGTTCGAGATGA-3′) was purchased from TriLink Biotechnologies (Katakura et al., 2005; Abe et al., 2007). Synthetic analogue of dsRNA (Poly(I:C)) was purchased from InvivoGen. All TLR ligands were LPS free. DSS was purchased from MP Biomedicals. rmIFN-β was purchased from Millipore. rmIL-1β was purchased from BD. Anakinra was obtained from Amgen.

Antibodies. Anti–mouse IL-1β (clone B122) and anti–mouse IL-1α (clone ALF-161) antibodies were purchased from eBioscience, anti–mouse CD121a (IL-1 receptor, type I; clone 35F5) antibody from BD, anti-IRF3, anti-IRF7, and anti-OTUD5 (DUBA) antibodies from Abcam, anti-ERK, anti–p-ERK, anti-p-p38, and anti–p-JNK antibodies from Cell Signaling Technologies, anti-TRAF3 antibody from Santa Cruz Biotechnology, Inc., anti-ubiquitin Lys63-specific (clone Apu3) and anti-ubiquitin Lys48-specific (clone Apu2) antibodies from Millipore, and anti–β-actin antibody from Sigma-Aldrich.

DSS-induced colitis. WT (B6) and *Il1r1*$^{-/-}$ mice were given 2% DSS (wt/vol) dissolved in sterile water ad libitum for 7 d. Groups of mice were treated with 10 μg CpG-ODN per mouse i.p., 2 h before DSS administration. The DAI score, combining weight loss and bleeding, was determined as previously described (Rachmilewitz et al., 2004; Katakura et al., 2005; Abe et al., 2007).

Histological evaluation. Preparation, H&E staining, and histological evaluation of colonic tissues was performed as described in our previous publications (Katakura et al., 2005; González-Navajas et al., 2010).

Anakinra treatment. WT mice (B6) were exposed to 2% DSS in the drinking water for 7 d as previously described (Rachmilewitz et al., 2004; Katakura et al., 2005; Abe et al., 2007). Starting 1 d before DSS exposure, mice were treated with two daily injections s.c. of anakinra (1 mg/mouse) or saline solution (vehicle). Mice were monitored daily for body weight loss and signs of intestinal inflammation.

rmIFN-β treatment. *Il1r1*$^{-/-}$ mice were treated with daily injections of rmIFN-β (1,000 U/mouse) or vehicle i.p. during DSS exposure as described in DSS-induced colitis. Mice were monitored daily for body weight loss and signs of intestinal inflammation.

Isolation of RNA and quantitative RT-PCR. The isolation of RNA and quantitative RT-PCR were performed as described in our previous publications (Katakura et al., 2005; González-Navajas et al., 2010). In brief, isolation of RNA was performed using RNeasy Mini kit (QIAGEN). After isolation, RNA was treated with DNase I (Invitrogen) to digest contaminating DNA. Synthesis of cDNA by reverse transcription was performed using Superscript III First-Strand system (Invitrogen). Quantitative real-time PCR was performed on an AB7300 (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems). GAPDH expression was used as internal reference. RT-PCR primers for specific target genes (Table S1) were designed based on their reported sequences and synthesized by IDT Technologies.

Measurement of cytokine levels in colonic tissue. Colonic explants were obtained and cultured as previously described (Abe et al., 2007; González-Navajas et al., 2010). After 24–36 h of culture, cytokine levels from the supernatants were measured using sandwich ELISAs for IL-1β, IL-6, TNF, and IL-10 (eBioscience).

IFN-β ELISA. Analysis of IFN-β levels was performed as previously described (Weinstein et al., 2000). In brief, 96-well plates were coated overnight with 1 μg/ml of rat anti–mouse IFN-β monoclonal antibody (Abcam). The plates were then blocked for 2 h before the addition of culture supernatants or recombinant IFN-β standard (Millipore). After incubation (overnight at 4°C or 2 h at 37°C), plates were washed and 50 U/ml of rabbit anti–mouse IFN-β (PBL) was added per well. The plates were incubated for 60 min at room temperature, washed, and then 3 μg/ml anti–rabbit IgG-HRP was added per well. The bound peroxidase was finally detected by the addition of TMB substrate (Sigma-Aldrich).

In vitro stimulation of BMDCs. BMDCs from WT and *Il1r1*$^{-/-}$ mice were prepared as previously described (Lutz et al., 1999; Datta et al., 2003). CD11c^{+} cells were then isolated by positive selection using MACS Microbeads, according to the manufacturer's protocol (Miltenyi Biotec). For cytokine production, BMDCs were incubated with 10 μg/ml CpG-ODN or 20 μg/ml Poly(I:C) and the culture supernatants were collected after 24 h.

For neutralization studies, WT BMDCs were incubated with 2 μg/ml anti-IL1RI or 5 μg/ml anti–IL-1β antibodies for 2 h. Cells were then stimulated with 10 μg/ml CpG-ODN for 24 h.

For pretreatment studies, WT BMDCs were cultured in the presence of 1 or 10 ng/ml rmIL-1β for 12 h. After collection of the supernatants, cells were washed and restimulated with CpG-ODN for 24 h.

For immunoblot analysis, BMDCs were stimulated with 10 μg/ml CpG-ODN for different periods of time. Cells were then collected and total cell lysates were obtained using RIPA buffer (Sigma-Aldrich). For some experiments, nuclear and cytosolic protein fractions were separated as previously described (Lee et al., 2000, 2006).

EMSA. Translocation of activated NF-κB into the nucleus was measured by EMSA using consensus NF-κB oligonucleotides (Santa Cruz Biotechnology, Inc.) as previously described (Lee et al., 2000, 2006).

Immunoprecipitation and ubiquitination assays. WT and *Il1r1*$^{-/-}$ BMDCs were prepared as described in In vitro stimulation of BMDCs and cultured with 10 μM of the proteasome inhibitor MG132 for 2 h. Cells were then stimulated with 10 μg/ml CpG-ODN for different periods of time and total cell lysates were prepared in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% (vol/vol) Triton X-100, 1% (vol/vol) deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 mM *N*-ethylmaleimide. Proteins (200 μg per sample) were boiled at 95°C in the presence of 0.1% SDS to remove noncovalently associated proteins. Samples were then immunoprecipitated overnight with constant mixing at 4°C with 2 μg/ml TRAF3 antibody (Santa Cruz Biotechnology, Inc.) and protein A–agarose beads (10 μl per 100 μl of total lysate). After extensive washing with lysis buffer, the immunocomplexes and any noncovalently bound proteins were dissociated by boiling in 4X LDS sample buffer (Invitrogen) and subjected to SDS page, followed by immunoblotting with rabbit anti-ubiquitin Lys63- (clone Apu3) or anti-ubiquitin Lys48 (clone Apu2)-specific antibodies (Miltenyi Biotec) and a light chain–specific anti–rabbit IgG-HRP as secondary antibody (Jackson ImmunoResearch Laboratories).

Gene silencing by siRNA. Negative control siRNA and OTUD5 (DUBA) siRNA were purchased from Santa Cruz Biotechnology, Inc. DUBA siRNA sequences, consisting of a pool of three target-specific 19–25-nt siRNA duplexes, are specified in Table S2. Transfection of siRNA was performed by electroporation using a mouse DC nucleofection kit and a Nucleofector II device (Lonza) as previously described (González-Navajas et al., 2010).

Statistical analysis. Values are displayed as mean ± SD. Statistical differences between groups were analyzed using the nonparametric Mann-Whitney *U* test for quantitative data. For the comparison of survival curves, the nonparametric log-rank test was performed. All the p-values are two-tailed, and p-values <0.05 were considered significant. All calculations were performed using Prism 4.0 software (GraphPad Software, Inc.).

Online supplemental material. Fig. S1 shows that anakinra treatment exacerbates intestinal inflammation after DSS injury. Fig S2 shows that treatment with rmIFN-β ameliorates colitis in *Il1r1*$^{-/-}$ mice. Fig. S3 shows that CpG triggers the K63-linked ubiquitination of TRAF3 in the absence of proteasomal inhibition. Fig. S4 shows that DUBA is not overexpressed in Tlr32/2 or Tlr92/2 mice. Table S1 shows oligonucleotides used for quantitative PCR analysis. Table S2 shows siRNA duplexes used for DUBA silencing experiments. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20101326/DC1.

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J.M. González-Navajas designed and performed experiments, analyzed data and wrote the paper; J. Law, K.P. Nguyen, and M. Bhargava performed experiments; M.P. Corr screened the mouse lines and edited the manuscript; N. Varki and L. Eckmann conducted the histological evaluation; H.M. Hoffman and J. Lee edited the manuscript and provided conceptual advice; and E. Raz designed the study, analyzed data, and wrote the paper.

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REFERENCES

Abe, K., K.P. Nguyen, S.D. Fine, J.H. Mo, C. Shen, S. Shenouda, M. Corr, S. Jung, J. Lee, L. Eckmann, and E. Raz. 2007. Conventional dendritic cells regulate the outcome of colonic inflammation independently of T cells. *Proc. Natl. Acad. Sci. USA*. 104:17022–17027. doi:10.1073/pnas.0708469104

Arai, Y., H. Takanashi, H. Kitagawa, and I. Okayasu. 1998. Involvement of interleukin-1 in the development of ulcerative colitis induced by dextran sulfate sodium in mice. *Cytokine*. 10:890–896. doi:10.1006/cyto.1998.0355

Bauer, C., P. Duewell, C. Mayer, H.A. Lehr, K.A. Fitzgerald, M. Dauer, J. Tschopp, S. Endres, E. Latz, and M. Schnurr. 2010. Colitis induced in mice with dextran sulfate sodium (DSS) is mediated by the NLRP3 inflammasome. *Gut*. 59:1192–1199. doi:10.1136/gut.2009.197822

Bresnihan, B., J.M. Alvaro-Gracia, M. Cobby, M. Doherty, Z. Domljan, P. Emery, G. Nuki, K. Pavelka, R. Rau, B. Rozman, et al. 1998. Treatment of rheumatoid arthritis with recombinant human interleukin-1 receptor antagonist. *Arthritis Rheum*. 41:2196–2204. doi:10.1002/1529-0131(199812)41:12<2196::AID-ART15>3.0.CO;2-2

Cominelli, F., C.C. Nast, R. Llerena, C.A. Dinarello, and R.D. Zipser. 1990. Interleukin 1 suppresses inflammation in rabbit colitis. Mediation by endogenous prostaglandins. *J. Clin. Invest*. 85:582–586. doi:10.1172/JCI114476

Datta, S.K., V. Redecke, K.R. Prilliman, K. Takabayashi, M. Corr, T. Tallant, J. DiDonato, R. Dziarski, S. Akira, S.P. Schoenberger, and E. Raz. 2003. A subset of Toll-like receptor ligands induces cross-presentation by bone marrow-derived dendritic cells. *J. Immunol*. 170:4102–4110.

Dinarello, C.A. 1996. Biologic basis for interleukin-1 in disease. *Blood*. 87:2095–2147.

Dinarello, C.A. 2009. Immunological and inflammatory functions of the interleukin-1 family. *Annu. Rev. Immunol*. 27:519–550. doi:10.1146/annurev.immunol.021908.132612

Emsley, H.C., C.J. Smith, R.F. Georgiou, A. Vail, S.J. Hopkins, N.J. Rothwell, and P.J. Tyrrell; Acute Stroke Investigators. 2005. A randomised phase II study of interleukin-1 receptor antagonist in acute stroke patients. *J. Neurol. Neurosurg. Psychiatry*. 76:1366–1372. doi:10.1136/jnnp.2004.054882

Fujita, T., L.F. Reis, N. Watanabe, Y. Kimura, T. Taniguchi, and J. Vilcek. 1989. Induction of the transcription factor IRF-1 and interferon-beta mRNAs by cytokines and activators of second-messenger pathways. *Proc. Natl. Acad. Sci. USA*. 86:9936–9940. doi:10.1073/pnas.86.24.9936

González-Navajas, J.M., S. Fine, J. Law, S.K. Datta, K.P. Nguyen, M. Yu, M. Corr, K. Katakura, L. Eckman, J. Lee, and E. Raz. 2010. TLR4 signaling in effector CD4+ T cells regulates TCR activation and experimental colitis in mice. *J. Clin. Invest*. 120:570–581. doi:10.1172/JCI40055

Häcker, H., V. Redecke, B. Blagoev, I. Kratchmarova, L.C. Hsu, G.G. Wang, M.P. Kamps, E. Raz, H. Wagner, G. Häcker, et al. 2006. Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. *Nature*. 439:204–207. doi:10.1038/nature04369

Hoffman, H.M., S. Rosengren, D.L. Boyle, J.Y. Cho, J. Nayar, J.L. Mueller, J.P. Anderson, A.A. Wanderer, and G.S. Firestein. 2004. Prevention of cold-associated acute inflammation in familial cold autoinflammatory syndrome by interleukin-1 receptor antagonist. *Lancet*. 364:1779–1785. doi:10.1016/S0140-6736(04)17401-1

Kabashima, K., T. Saji, T. Murata, M. Nagamachi, T. Matsuoka, E. Segi, K. Tsuboi, Y. Sugimoto, T. Kobayashi, Y. Miyachi, et al. 2002. The prostaglandin receptor EP4 suppresses colitis, mucosal damage and CD4 cell activation in the gut. *J. Clin. Invest*. 109:883–893.

Katakura, K., J. Lee, D. Rachmilewitz, G. Li, L. Eckmann, and E. Raz. 2005. Toll-like receptor 9-induced type I IFN protects mice from experimental colitis. *J. Clin. Invest*. 115:695–702.

Kayagaki, N., Q. Phung, S. Chan, R. Chaudhari, C. Quan, K.M. O'Rourke, M. Eby, E. Pietras, G. Cheng, J.F. Bazan, et al. 2007. DUBA: a deubiquitinase that regulates type I interferon production. *Science*. 318:1628–1632. doi:10.1126/science.1145918

Kojouharoff, G., W. Hans, F. Obermeier, D.N. Männel, T. Andus, J. Schölmerich, V. Gross, and W. Falk. 1997. Neutralization of tumour necrosis factor (TNF) but not of IL-1 reduces inflammation in chronic dextran sulphate sodium-induced colitis in mice. *Clin. Exp. Immunol*. 107:353–358. doi:10.1111/j.1365-2249.1997.291-ce1184.x

Lebeis, S.L., K.R. Powell, D. Merlin, M.A. Sherman, and D. Kalman. 2009. Interleukin-1 receptor signaling protects mice from lethal intestinal damage caused by the attaching and effacing pathogen Citrobacter rodentium. *Infect. Immun*. 77:604–614. doi:10.1128/IAI.00907-08

Lee, J., L. Mira-Arbibe, and R.J. Ulevitch. 2000. TAK1 regulates multiple protein kinase cascades activated by bacterial lipopolysaccharide. *J. Leukoc. Biol*. 68:909–915.

Lee, J., J.H. Mo, K. Katakura, I. Alkalay, A.N. Rucker, Y.T. Liu, H.K. Lee, C. Shen, G. Cojocaru, S. Shenouda, et al. 2006. Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells. *Nat. Cell Biol*. 8:1327–1336. doi:10.1038/ncb1500

Lutz, M.B., N. Kukutsch, A.L. Ogilvie, S. Rössner, F. Koch, N. Romani, and G. Schuler. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods*. 223:77–92. doi:10.1016/S0022-1759(98)00204-X

Martin, M., D. Neumann, T. Hoff, K. Resch, D.L. DeWitt, and M. Goppelt-Struebe. 1994. Interleukin-1-induced cyclooxygenase 2 expression is suppressed by cyclosporin A in rat mesangial cells. *Kidney Int*. 45:150–158. doi:10.1038/ki.1994.18

Mizel, S.B., J.M. Dayer, S.M. Krane, and S.E. Mergenhagen. 1981. Stimulation of rheumatoid synovial cell collagenase and prostaglandin production by partially purified lymphocyte-activating factor (interleukin 1). *Proc. Natl. Acad. Sci. USA*. 78:2474–2477. doi:10.1073/pnas.78.4.2474

Nitta, M., I. Hirata, K. Toshina, M. Murano, K. Maemura, N. Hamamoto, S. Sasaki, H. Yamauchi, and K. Katsu. 2002. Expression of the EP4 prostaglandin E2 receptor subtype with rat dextran sodium sulphate

colitis: colitis suppression by a selective agonist, ONO-AE1-329. *Scand. J. Immunol.* 56:66–75. doi:10.1046/j.1365-3083.2002.01096.x

Oganesyan, G., S.K. Saha, B. Guo, J.Q. He, A. Shahangian, B. Zarnegar, A. Perry, and G. Cheng. 2006. Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response. *Nature*. 439:208–211. doi:10.1038/nature04374

Pascual, V., F. Allantaz, E. Arce, M. Punaro, and J. Banchereau. 2005. Role of interleukin-1 (IL-1) in the pathogenesis of systemic onset juvenile idiopathic arthritis and clinical response to IL-1 blockade. *J. Exp. Med.* 201:1479–1486. doi:10.1084/jem.20050473

Rachmilewitz, D., F. Karmeli, K. Takabayashi, T. Hayashi, L. Leider-Trejo, J. Lee, L.M. Leoni, and E. Raz. 2002. Immunostimulatory DNA ameliorates experimental and spontaneous murine colitis. *Gastroenterology*. 122:1428–1441. doi:10.1053/gast.2002.32994

Rachmilewitz, D., K. Katakura, F. Karmeli, T. Hayashi, C. Reinus, B. Rudensky, S. Akira, K. Takeda, J. Lee, K. Takabayashi, and E. Raz. 2004. Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. *Gastroenterology*. 126:520–528. doi:10.1053/j.gastro.2003.11.019

Rivieccio, M.A., G.R. John, X. Song, H.S. Suh, Y. Zhao, S.C. Lee, and C.F. Brosnan. 2005. The cytokine IL-1beta activates IFN response factor 3 in human fetal astrocytes in culture. *J. Immunol.* 174:3719–3726.

Tseng, P.H., A. Matsuzawa, W. Zhang, T. Mino, D.A. Vignali, and M. Karin. 2010. Different modes of ubiquitination of the adaptor TRAF3 selectively activate the expression of type I interferons and proinflammatory cytokines. *Nat. Immunol.* 11:70–75. doi:10.1038/ni.1819

Weinstein, S.L., A.J. Finn, S.H. Davé, F. Meng, C.A. Lowell, J.S. Sanghera, and A.L. DeFranco. 2000. Phosphatidylinositol 3-kinase and mTOR mediate lipopolysaccharide-stimulated nitric oxide production in macrophages via interferon-beta. *J. Leukoc. Biol.* 67: 405–414.