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

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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.
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).

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.
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 $\text{Il1r1}^{-/-}$ mice (Fig. 1 D). More importantly, the administration of CpG resulted in increased mRNA levels of the antiinflammatory mediators IL-10 and IFN-$\beta$ in the colonic tissues obtained from WT mice but not in those obtained from $\text{Il1r1}^{-/-}$ mice (Fig. 1 D).

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 $\text{Il1r1}^{-/-}$ mice (Fig. 1 C).

To determine potential causes for the differences in colitis severity and the differential response to CpG in WT versus $\text{Il1r1}^{-/-}$ 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 $\text{Il1r1}^{-/-}$ mice (Fig. 1 D). More importantly, the administration of CpG resulted in increased mRNA levels of the antiinflammatory mediators IL-10 and IFN-$\beta$ in the colonic tissues obtained from WT mice but not in those obtained from $\text{Il1r1}^{-/-}$ mice (Fig. 1 D).
PGE2, 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 PGE2 (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 Il11r−/− 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 Il1r1−/− 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 Il1r1−/− as compared with WT mice (Fig. 2 A). Furthermore, BM-derived DCs (BMDCs) from Il11r−/− mice produced lower levels of 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.
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 secretion of IL-6 and TNF (Fig. 2 D, empty bars). Collectively, these 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 antiinflammatory 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-/- BMDCs 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.](image-url)
role of IL-1RI signaling in the activation of TRAF3 and explain why II1r1−/− 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 II1r1−/− 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 II1r1−/− 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 II1r1−/− 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 II1r1−/− 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 II1r1−/− BMDCs. DUBA knockdown by siRNA transfection in II1r1−/− 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 II1r1−/− 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...
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. Il1r1−/− 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΄-TGACTGTAACGTTCGAGATGA-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 3F5) antibody from BD, anti–IFR3, anti–IFR7, 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–TRAFA3 antibody from Santa Cruz Biotechnology, Inc., anti–ubiquitin Lys63-specific (clone Aplu3) and anti–ubiquitin Lys48-specific (clone Apla2) 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 analysis 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.

rmILN-β treatment. Il1r1−/− mice were treated with daily injections of rmILN-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 (PBL) and then incubated for 2 h at 4°C or 2 h at 37°C. Plates were then 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 Aplu3) or anti-ubiquitin Lys48– (clone Apla2)–specific antibodies (Miltenyi Biotec) and a light chain–specific anti–rabbit IgG-HRP as secondary antibody (Jackson Immune Research Laboratories).
Gene silencing by siRNA. Negative control siRNA and OTUD5 (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-α accelerates intestinal inflammation after DSS injury. Fig S3 shows that CpG triggers the K63-linked ubiquitination of TRAF3 in the absence of protective inhibition. Fig. S4 shows that DUBA is not overexpressed in terms with rmIFN-α and Figure S5 shows that DUBA is not overexpressed in ameliorates colitis in mice treated with rmIFN-α. Table S2 shows siRNA duplexes used for DUBA proteasomal inhibition. Fig. S4 shows that DUBA is not overexpressed in ameliorates colitis in mice treated with rmIFN-α and Figure S5 shows that DUBA is not overexpressed in terms with rmIFN-α.

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.).

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