UNIVERSITY OF CALIFORNIA, SAN DIEGO

Anti-inflammatory Cytokine Production: Regulation of DUBA Expression Proves as a New Link Between Interleukin 1 Receptor Signaling and Toll-like Receptor 9

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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The Thesis of Meha Bhargava is approved and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

Anti-inflammatory Cytokine Production: Regulation of DUBA expression Proves as a New Link Between Interleukin 1 Receptor Signaling and Toll-like Receptor 9

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Meha Bhargava

Master of Science in Biology

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Professor Eyal Raz, Chair

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 anti-inflammatory 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 $Il1r1^{-/-}$ $CD11\ c^{+}$ 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 response.

INTRODUCTION

The primary function of the intestinal tract is the digestion and absorption of nutrients. The intestinal lumen is lined with a specialized simple epithelium, which performs the primary functions of digestion and absorption, and also importantly forms a barrier against luminal pathogens. The intestinal epithelium is the most vigorously self-renewing tissue of adult mammals with single layer of epithelial cells that is renewed every 4-5 days. The lumen of the large intestine, as well as the stomach and small intestine, in mice and other mammals harbor an extensive bacterial flora. These commensal microbes have been shown to stimulate the production of many gut plasma cells, most of which produce IgA, throughout mouse development.

An intact intestinal microbiota is necessary not only for effective colonization resistance, but also for effective communication with the cytokine network. In ulcerative colitis (UC), a type of inflammatory bowel disease (IBD), a break in the intestinal epithelial integrity causes normally commensal bacteria to produce an inflammatory response in the host. The intestinal mucosa thus becomes infiltrated with T lymphocytes responsive to microbial antigens (Bienenstock, book). It has been suggested that the immune responses stimulated by enteric antigens bowel inflammation seen in colitis mice originates from aberrant cytokine production.

Activated innate immune cells mediate a variety of protective and proinflammatory mucosal immune responses, with key roles in clearance of microbial pathogens, recruitment of effector cells into the inflammatory focus,

tissue destruction and remodeling, and antigen presentation. These activities apply to both acute and chronic inflammatory processes at mucosal surfaces, including IBDs. Inflammatory cytokines, most notably, interleukin-1 (IL-1), tumor necrosis factor (TNF), and IL-6 are secreted by a variety of activated innate immune cells of hematologic, mesenchymal, epithelial, and endothelial origin (Bienenstock, book).

IL-1 occurs in two forms, IL-1α and IL-1β, which share similar receptor-binding properties and thus have overlapping functions. IL-1α and IL-1β levels are low in the normal intestine but are dramatically increased during active inflammation (Cominelli *et al.*, 1992). Lamina propria macrophages, particularly newly immigrated cells with monocyte features, account for the majority of IL-1 production (McAlindon *et al.,* 1998). IL-1 is a highly inflammatory cytokine and the margin between clinical benefit and unacceptable toxicity in humans is exceedingly narrow (Dinarello, 1996). The ability of IL-1 Receptor antagonist (IL-1Ra) to prevent the clinical features and haematological and biochemical changes in patients with certain autoinflammatory syndromes indicates a central role for IL-1 in these disorders (Hoffman, 1994).

Although the role of IL-1 in sterile inflammation, such as rheumatoid arthritis, gout, or anti-inflammatory syndromes has been extensively studied (Dinarello, 2009), its role in non-sterile inflammatory conditions, such as inflammatory bowel disease, has not been clearly defined. *Citrobacter rodentium,* bacteria classified based on their ability to adhere to the intestinal epithelium cause a breach of the intestinal epithelial barrier, leading to colitis via a vigorous

inflammatory response resulting in diarrhea and a protective antibody response that clears the pathogen. IL-1R signaling was shown to protect mice following infection with *C. rodentium*. Upon infection, mice lacking the type I IL-1R exhibit increased mortality together with severe colitis (Lebeis, 2009).

DSS (Dextran sulfate sodium)-induced colitis in mice is a model that exhibits symptoms similar to those observed clinically in human ulcerative colitis. Studies using this model are used to give information about the efficacy of drugs for the therapy of ulcerative colitis. Neutralization of IL-1 in acute DSS-colitis aggravated the symptoms, indicating that IL-1 may play a protective role in acute colitis (Kajouharoff, 1997).

In addition, mucosal macrophages secrete abundant IL-10 in response to bacterial stimulants and proinflammatory molecules, serving to down-regulate the inflammatory response. Accordingly, elimination of mucosal macrophages (with rectal microspheres) and the associated depletion of IL-10, was shown to attenuate experimental colitis (Watanabe *et al*, 2003).

RESULTS

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

In the DSS model of colitis, mice are subjected several days to drinking water supplemented with DSS, which seems to be directly toxic to colonic epithelial cells of the basal crypts. The procedures for acute DSS colitis can be accomplished in about 2 weeks (Wirtz, 2007). Mice exposed to orally delivered DSS develop acute colitis characterized by bloody diarrhea, ulcerations rectal bleeding, weight loss and inflammatory infiltration of the colon, due to the DSS toxicity on gut epithelial cells and distortion of the integrity of the mucosal barrier. We exposed C567BL/6 (B6 and WT) and *Il1r1*$^{-/-}$ mice to 2% DSS in the drinking water for 7 days. Surprisingly, *Il1r1*$^{-/-}$ 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 and Graph 1).

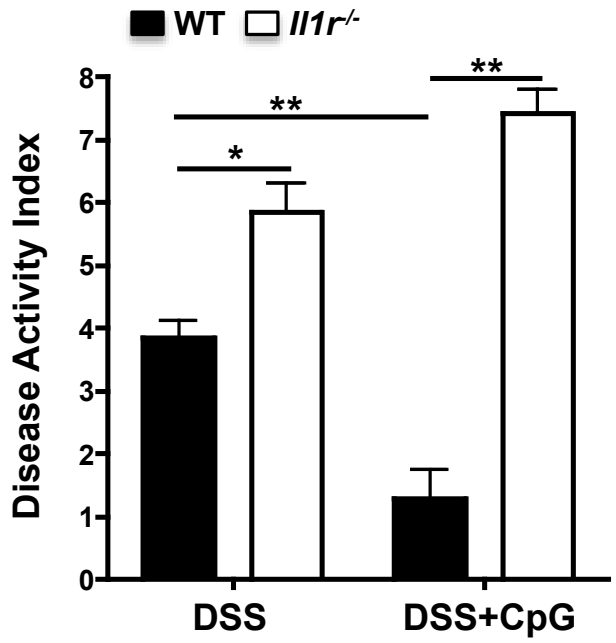
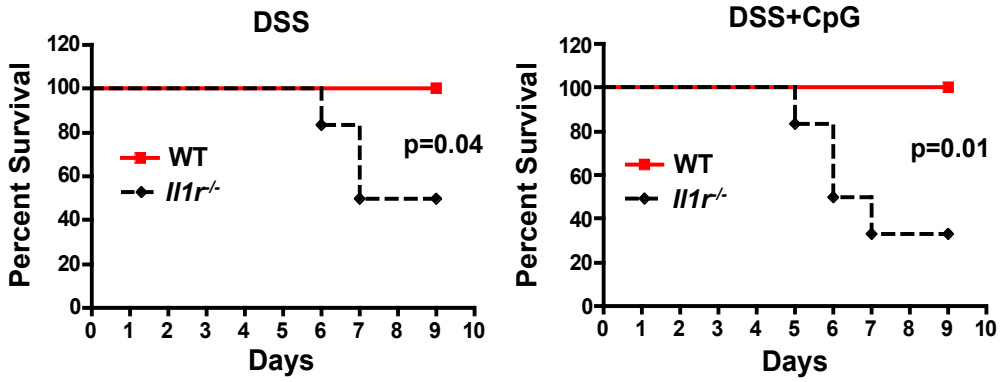


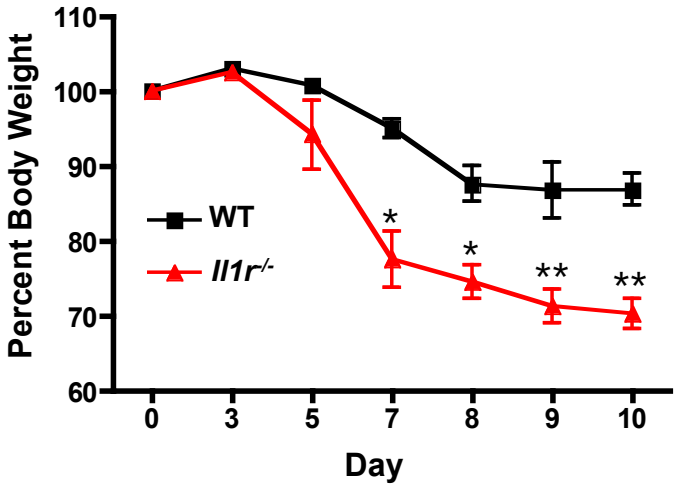
Figure 1. Il1r1$^{-/-}$ mice are more susceptible to DSS-induced colitis than WT mice. DAI score in WT and *Il1r1*$^{-/-}$ mice. Mice were given DSS (2%) in their drinking water for 7 d with or without pretreatment with CpG oligonucleotides (10 µg/mouse) 2 h before DSS administration.

Graph 1. Il1r1$^{-/-}$ mice are more susceptible to DSS-induced colitis than WT mice. Survival ofWT and *Il1r1*$^{-/-}$ mice treated as described in Figure 1.



Furthermore, *Il1r1*$^{-/-}$ mice showed an impaired ability to recover from DSS-induced colitis and kept losing weight after DSS removal at day 7 (Graph 2).

Graph 2. Anakinra treatment exacerbates intestinal inflammation after DSS injury. WT **(**B6) and *Il1r1*$^{-/-}$ mice were exposed to 2% DSS in the drinking water for 7 d. At day 7, DSS was replaced with normal water and the mice were followed for additional 3 d. Although the body weight of WT mice was stabilized, *Il1r1*$^{-/-}$ mice kept losing weight during this period. Data are representative of two independent experiments.



Previous studies showed observations of CpG-driven attenuation of DSS-induced colitis in mice, mainly via the induction of a type I IFN response. Accordingly, intraperitoneal (i.p.) injection of CpG (before DSS administration) efficiently ameliorated the severity of colonic inflammation in WT mice (Fig. 2). In contrast, CpG administration resulted in a higher DAI score and further increased mortality in *Il1r1*$^{-/-}$ mice (Fig. 1 and Graph 1).

Histological analysis of the colonic tissues from the DSS-treated mice revealed that both WT and *Il1r1*$^{-/-}$ mice developed mucosal inflammation with epithelial ulceration, crypt loss, depletion of goblet cells, and marked infiltration of mononuclear cells in the colonic lamina propria (Fig. 2). The extent of epithelial damage was more severe in *Il1r1*$^{-/-}$ mice in which DSS administration (with or without pretreatment with CpG) caused almost complete ablation of colonic

epithelium (Fig. 2). 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. 2).

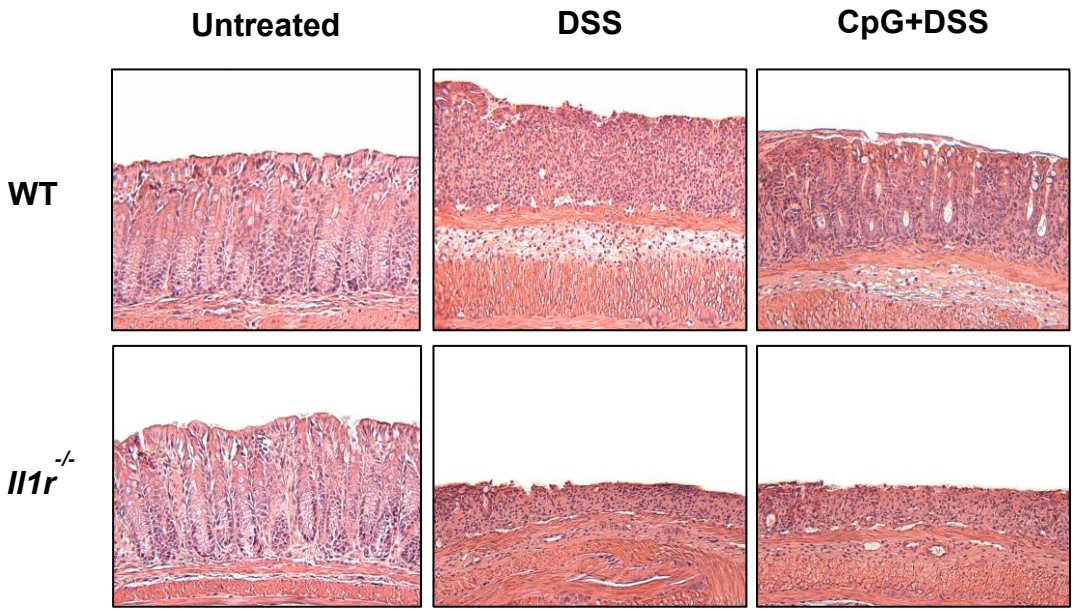


Figure 2. Il1r1$^{-/-}$ mice show a higher degree of intestinal inflammation. Hematoxylin and eosin staining of colon sections from untreated mice or WT and *Il1r1*$^{-/-}$ on day 7 of DSS treatment.

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 anti-inflammatory cytokines in colonic homogenates obtained from each group after 7 days of DSS, with or without CpG treatment. IL-6 and TNFα are key players in the pro-inflammatory cytokine profile of developing DSS colitis. TNFα is a proinflammatory mediator that plays an integral role in the pathogenesis of inflammatory bowel disease. In addition, mounting evidence indicates a genetic association between TNFα and ulcerative colitis

(Sands and Kaplan, 2007). Related studies show that blockade of IL-6 signaling enhance the generation of IL-10-producing $CD4^+$ T cells, and inhibited up-regulation of TNF-α mRNA expression in the colon. These findings clearly demonstrated that IL-6 is a critical factor for the induction of colitis (Noguchi, *et al*., 2007).

In contrast, IL-10 and IFN-β are key anti-inflammatory cytokines that assist in the recovery process following DSS-mediated destruction of the intestinal epithelium. interleukin-10 (IL-10) plays a critical role in the regulation of mucosal immune reactivity. Administration of recombinant IL-10 (rIL-10) shows beneficial effects on the incidence of colitis. However, withdrawal of the rIL-10-treatment 8 weeks after transfer results in severe colitis 4 weeks later (*Annacker et al.,* 2003). Similarly, IFN-β induces a clinical response and remission in a large subset of patients with ulcerative colitis (Mannon, *et al.,* 2010).

In our experiment, CpG administration decreased the mRNA levels of inflammatory cytokines IL-6 and TNFα in WT but not in *Il1r1*$^{-/-}$ mice (Fig. 3). More importantly, the administration of CpG resulted in increased mRNA levels for the anti-inflammatory mediators IL-10 and IFN-β in the colonic tissues obtained from WT mice but not in those obtained from *Il1r1*$^{-/-}$ mice (Fig. 3). This supports the increased inflammatory profile and lack of proper rehabilitation we observed in the histological and DAI analysis.

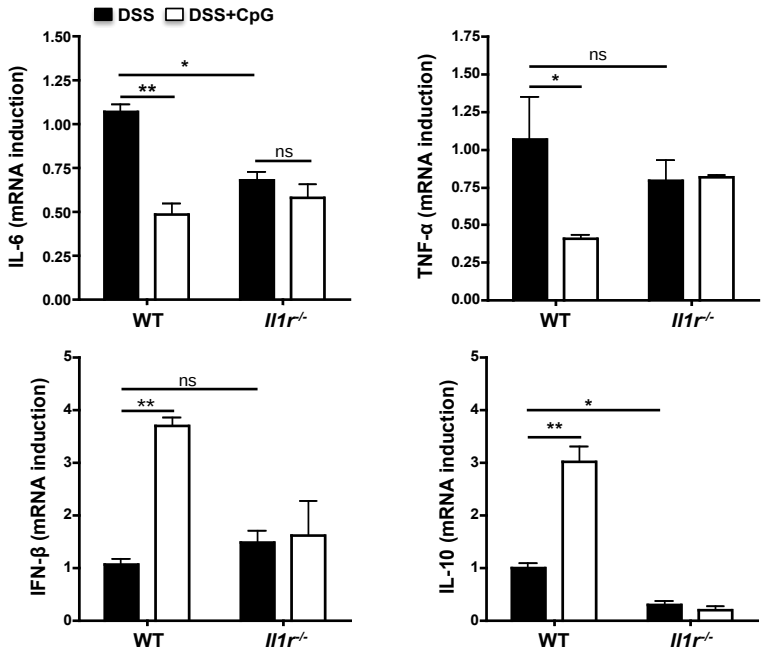


Figure 3. Il1r1$^{-/-}$ colon show degreased cytokine response in CpG-driven attenuation of colitis. Quantitative PCR analysis of pro- and antiinflammatory mediators in colonic homogenates from WT and *Il1r1*$^{-/-}$ mice on day 7 of DSS treatment. Data are representative of four different experiments (*n* = 6). Error bars represent mean ± SEM. ns, not significant. *, P < 0.05; **, P < 0.01.

To study the impact of IL-1Ra on the course of experimental colitis, we treated WT mice i.p. with either anakinra (1mg/mouse, twice a day) or vehicle during 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 more severe epithelial damage (Fig. 4, A and C). Anakinra treatment also resulted in increased levels of proinflammatory cytokine IL-6 and reduced anti-inflammatory cytokine IFN-β production in supernatants from ex vivo-cultured colonic tissues (Fig. 4, B). Collectively, these results correlated 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.

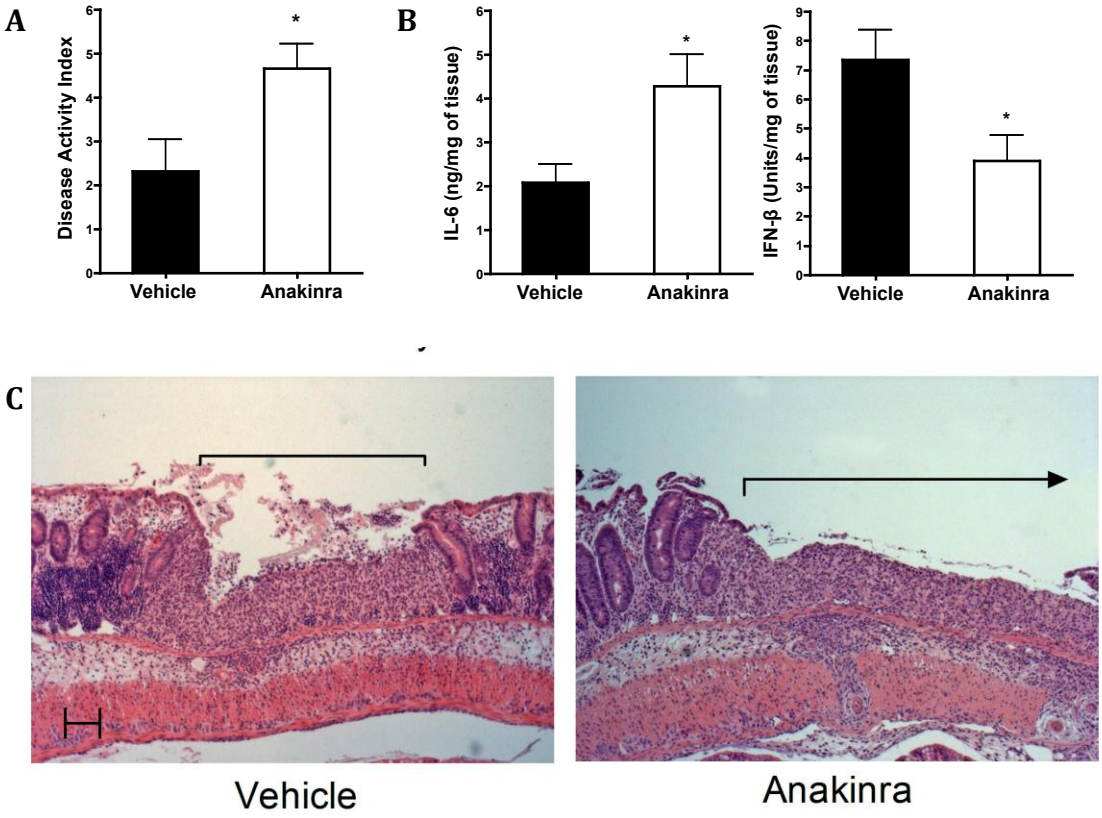


Figure 4. Anakinra treatment exacerbates intestinal inflammation after DSS injury. (A) The administration of anakinra increases DAI score in WT mice. Mice were exposed to 2% DSS in the drinking water for 7 d. Starting 1 d before DSS exposure, mice were treated with two daily injections of anakinra (1 mg/mouse) or saline solution (vehicle). (B) Hematoxylin and eosin staining of colon sections from WT mice treated as in A, displaying severe mucosal ulcerations. The bracket shows the size of the ulcer in vehicle-treated mice. The arrow shows the bigger size of the ulcers in anakinra-treated mice that continue outside the picture field. Bar, 50 μm. (C) Cytokine production from colonic explants of WT mice treated with anakinra. Colonic tissue was removed, weighted, and thoroughly washed in media containing antibiotics. Tissue was then cultured in complete RPMI for 36 h. Supernatants from these cultures were then collected and cytokine levels were determined by ELISA. (A-B) Data are representative of three independent experiments. Error bars represent SEM. *, $P < 0.05$; **, $P < 0.01$.

Il1r1$^{-/-}$ 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. Type I IFN production by CDllc^{+} DCs is partially responsible for CpG-dependent attenuation of colitis in WT mice in this model (Katakura et al., 2005). cDCs can enhance or attenuate the severity of DSS-induced colitis. The latter beneficial effect was achieved, in part, by IFN1 induced by TLR9 –activated cDCs. IFN-1 inhibits colonic inflammation by regulating neutrophil and monocyte trafficking to the inflamed colon and restraining the inflammatory products of tissue macrophages (Abe et al., 2007).

We reasoned that the impaired ability to produce type I IFN in response to TLR9 stimulation in *Il1r1*$^{-/-}$ mice facilitated colonic inflammation. Thus, we figured a dose of recombinant mouse (rm) IFN-β would decrease the severity of intestinal inflammation these *Il1r1*$^{-/-}$ mice. Indeed, administration of rm IFN-β ameliorated the severity of DSS- induced colitis (Fig. 5 A) and suppressed the inflammatory cytokine production in the colonic tissue of *Il1r1*$^{-/-}$ mice (Fig. 5 B).

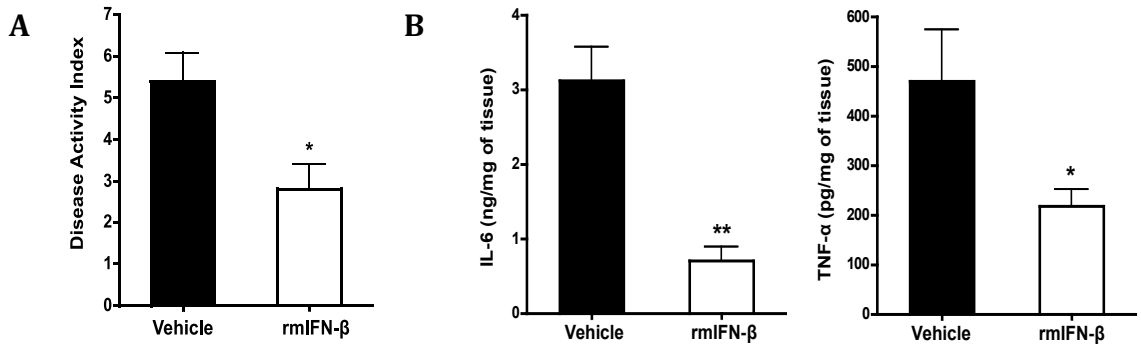


Figure 5. Treatment with rmIFN-β ameliorates colitis in Il1r1$^{-/-}$ mice. (A) Type I IFN ameliorates colitis in *Il1r1*$^{-/-}$ mice. Mice were exposed to 2% DSS in the drinking water for 7 d and treated with daily injections of rmIFN–β (1,000 U/mouse) or vehicle. (B) Cytokine production from colonic explants

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 intravenously (i.v.) and measured the serum levels of these cytokines. Indeed, we observed lower levels of these anti-inflammatory cytokines in the serum of *Il1r1*$^{-/-}$ as compared with WT mice (Fig 6 A). Furthermore, bone marrow-derived dendritic cells (BMDCs) from *Il1r1*$^{-/-}$ mice produced lower levels of 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 6 B). In contrast, we observed increased levels of IL-6 and TNF-α (pro-inflammatory cytokines) in response to CpG but not to P(I:C) stimulation (Fig 6 B).

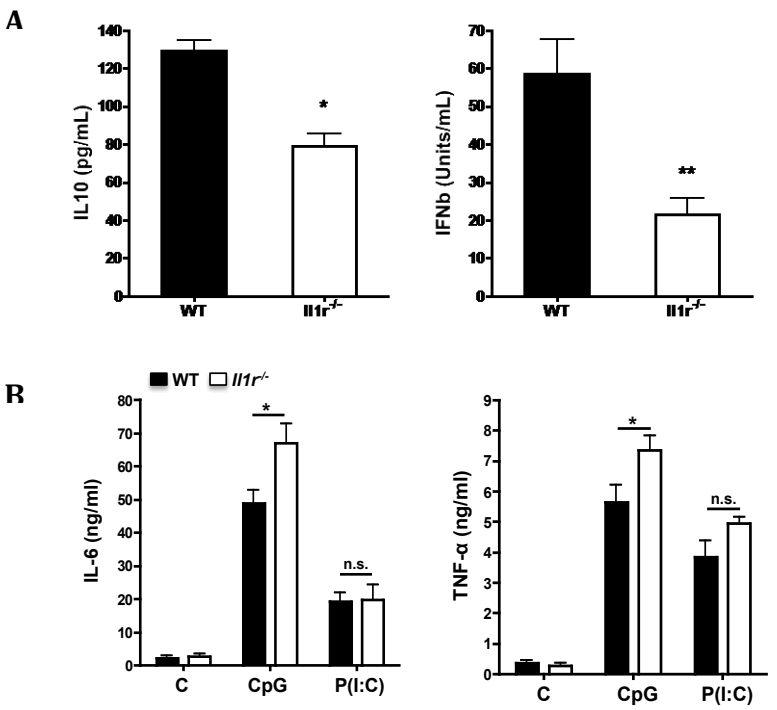


Figure 6. Il1r1$^{-/-}$ mice have impaired IL-10 and type I IFN response. (A) Cytokine levels in serum of WT and *Il1r1*$^{-/-}$ mice 2 h after i.v. administration of 50 μg CpG oligonucleotides or vehicle (C). Data are representative of two independent experiments (*n* = 4). (B) Cytokine levels in supernatants from WT and *Il1r1*$^{-/-}$ CD11c+ BMDCs 24 h after stimulation with 10 μg/ml CpG, 10 μg/ml Poly(I:C), or vehicle (C).

In order to further illustrate these observations, we performed in vitro blockade of IL-1RI signaling in WT BMDCs, with either anti-IL-1RI blocking on anti-IL-1β neutralizing antibodies. As expected, these IL-1RI signaling impediments resulted in reduced TLR9-dependent production of IL-1 and IFN-β level and in augmented levels of TNF and IL-6 (Fig. 7 A).

To further elucidate the anti-inflammatory 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. 7

B). However, when IL-1β pre-treated cells were restimulated with CpG, we observed a significant reduction in TLR9-dependent production of IL-6 and TNF and an increased production of IL-10 and IFN–β (Fig. 7B).

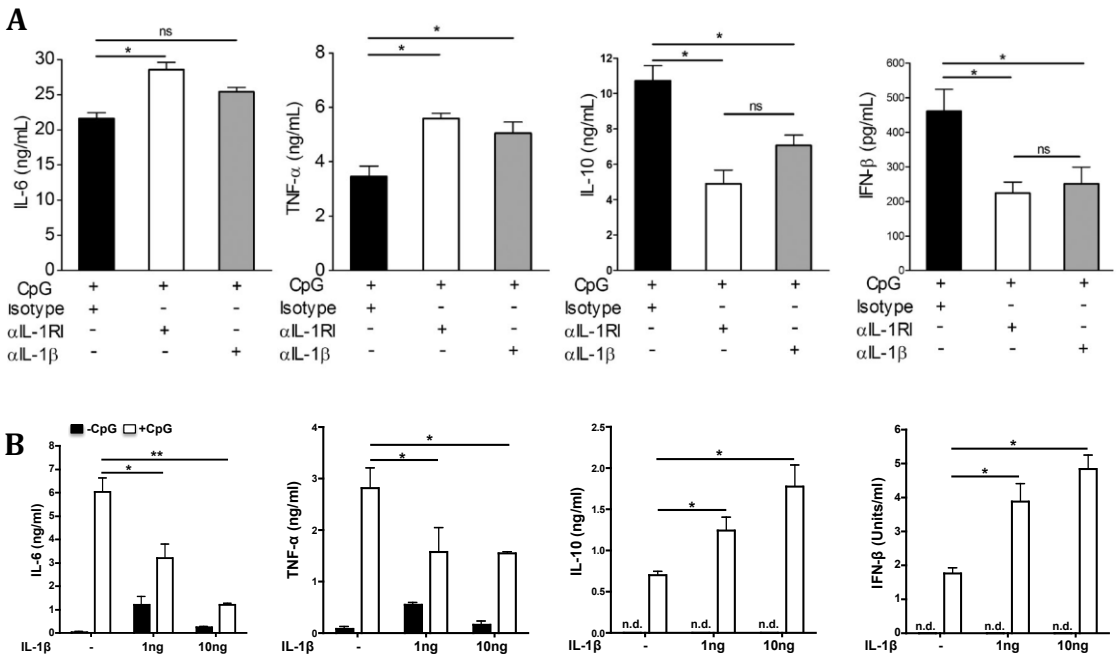


Figure 7. Mice deficient in Il1r1-signaling have impaired IL-10 and type I IFN response. (A) WT BMDCs were incubated with isotype control antibody, αIL-1RI blocking antibodies, or αIL-1β neutralizing antibody for 2 h. Cells were then stimulated with CpG for 24 h and cytokine levels were determined in the supernatants. (B) 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. (A and B) Data are representative of three different experiments. Error bars represent mean ± SEM. ns, not significant. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Collectively, these in vitro results further support the anti-inflammatory role of IL-1RI signaling observed in vivo (Fig. 3), 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-κB, MAPKs, and IRFs. The augmented production of TNF and IL-6 in *Il1r1*$^{-/-}$ BMDCs in response to TLR9 stimulation caused us to ask whether these MAPK, NF-κB and IRF pathways had a role in the deviating *Il1r1*$^{-/-}$ inflammatory profile we observe. In vitro activation of BMDCs from *Il1r1*$^{-/-}$ mice by CpG led to a modest increase in the activation of NF-κB and NK-κB target genes (TNFα and IL-12p40) compared with WT BMDCs (Fig. 8 A and B).

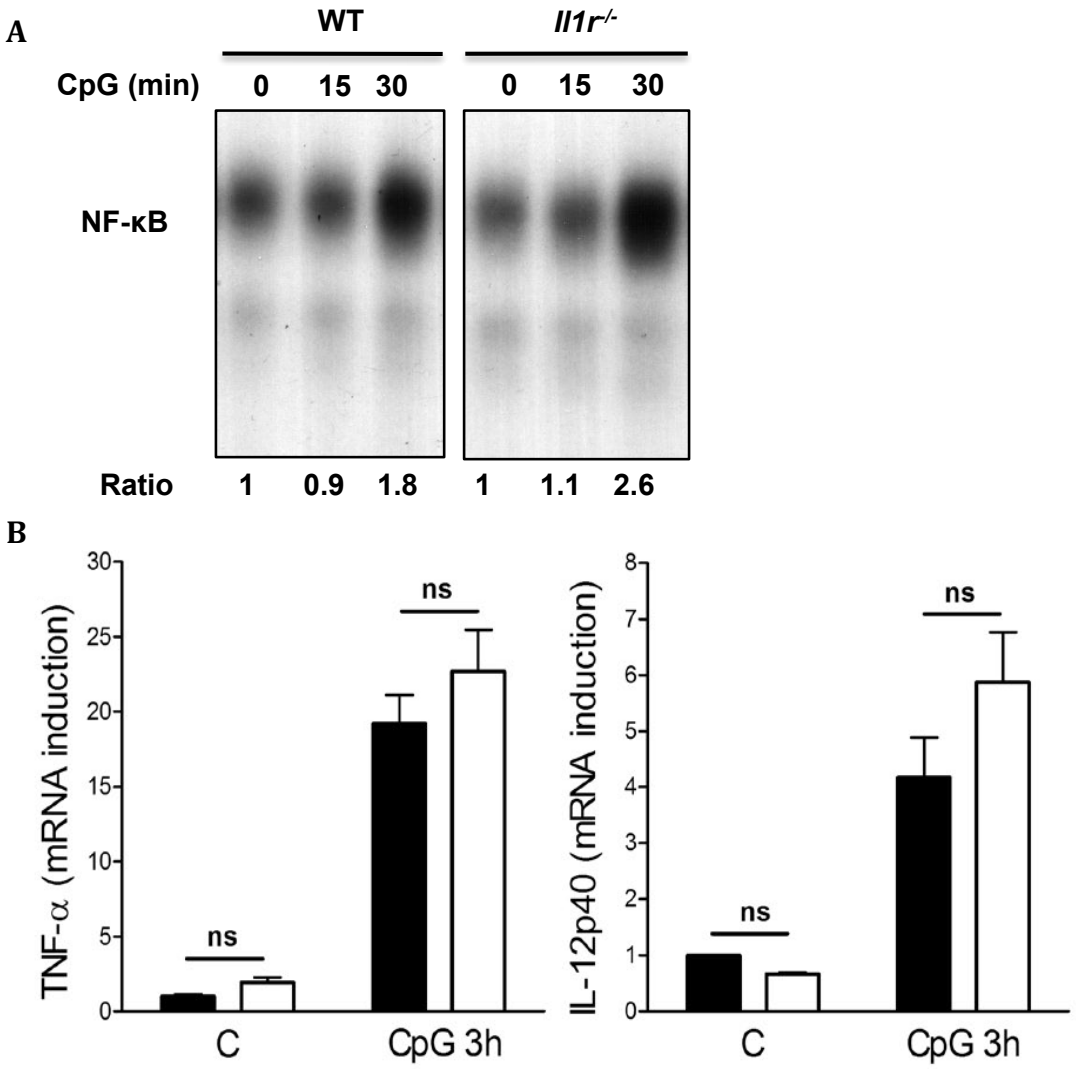


Figure 8. Il1r1$^{-/-}$ BMDCs display an increased activation of NF-κb and NK-κb target genes. (A) Electrophoretic mobility shift assay (EMSA) analysis of NF-κB activation in WT and *Il1r1*$^{-/-}$ BMDCs after CpG stimulation (10 μg/ml) for the indicated time periods. (B) BMDCs from WT and *Il1r*$^{-/-}$ 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. (A and B) Data are representative of three independent experiments. Error bars represent mean ± SEM. ns, not significant.

The MAPK family members, p38, JNK and ERK are all activated downstream of innate immunity's TLR to induce the production of cytokines and

inflammatory mediators. However, the relative intensity and duration of the activation of different MAPK appears to determine the type of immune response. MAPK activation is important in cytokine and chemokine gene expression. For example, IL-10 production was inhibited by the Map2k1/MEK1 (upstream of MAPK) U0126 (Lang, Hammer and Mages, 2006). CpG activation resulted in a marked increase in the phosphorylation (activation) levels of ERK and a mild increase in the activation of p38 and JNK MAPKs (Fig. 9). The increase in NF-κb and MAPK activation probably explain the augmented production of TNF and IL-6 by *Il1r1*$^{-/-}$ BMDCs in response to TLR9 stimulation.

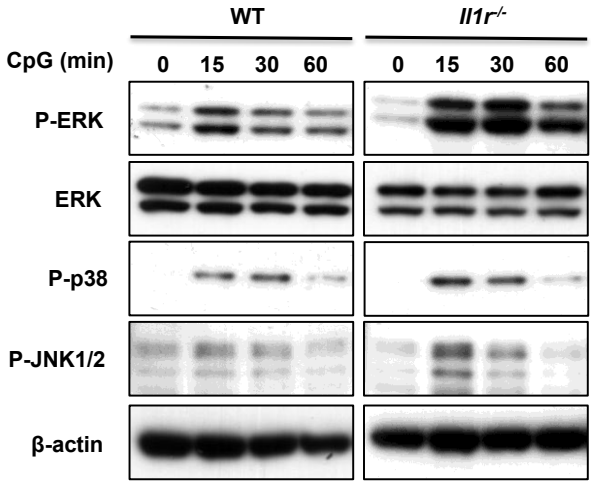


Figure 9. Il1r1$^{-/-}$ BMDCs display increased MAPK activity. Immunoblot analysis of MAPK activation in BMDCs from WT and *Il1r1*$^{-/-}$ stimulated with 10 μg/ml CpG oligonucleotides for the indicated time periods.

Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF) is an adaptor molecule that is recruited to TLR9 agonist stimulation and triggers activation of IFN regulatory factor 3 (IRF3) and IRF7 and expression of type I IFNs.

The activation and nuclear translocation of IRF3 and IRF7, under the same conditions, were greatly reduced in *Il1r1*$^{-/-}$ BMDCs (Fig. 10 A). Importantly, freshly isolated splenocytes from *Il1r1*$^{-/-}$ mice also showed reduced nuclear translocation of IRF3 and IRF7 (Fig. 10 B).

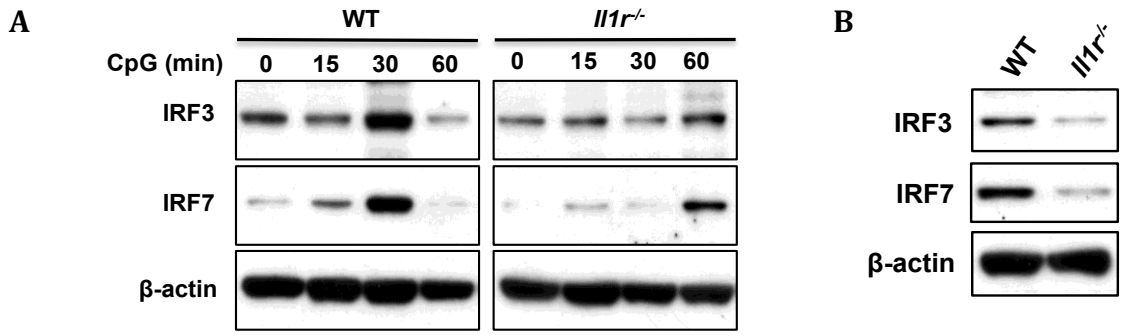


Figure 10. Il1r1$^{-/-}$ BMDCs display impaired IRF3 and IRF7 translocation responses. (D) Immunoblot analysis of IRF3 and IRF7 in nuclear extracts of BMDCs from WT and *Il1r1*$^{-/-}$ mice stimulated as in C. (E) Baseline levels of IRF3 and IRF7 in nuclear extracts from freshly isolated unstimulated splenocytes from WT and *Il1r1*$^{-/-}$ mice.

Furthermore, consistent with the stimulatory effect of IL-1β on the production of type I IFN (Fig. 7 B), IL-1β and CpG costimulation led to increased nuclear translocation of IRF7 when compared with CpG stimulation alone (Fig. 11). 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.

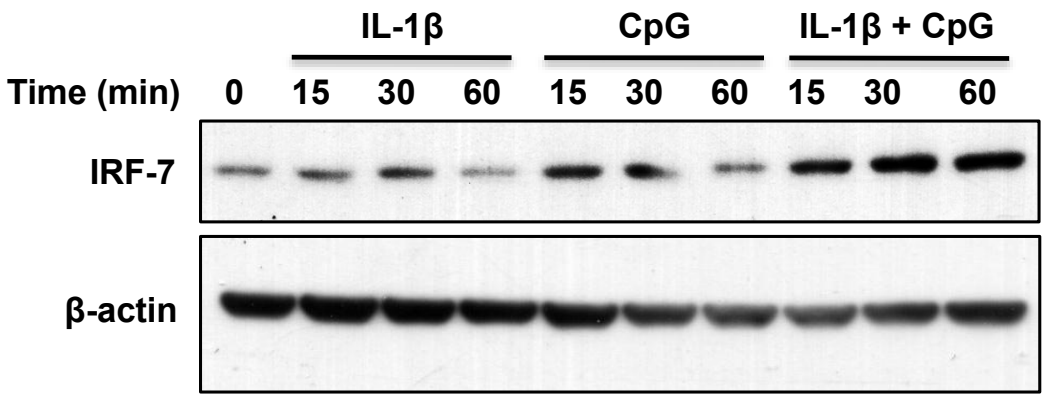


Figure 11. Il1r1$^{-/-}$ BMDCs exhibit rescued IRF3 and IRF7 responses with IL-1β pre-treatment. WT BMDCs were left unstimulated (0) or were stimulated with 10 ng/ml of recombinant IL-1β, 10 μg/ml CpG, or both for the indicated time periods. Nuclear extracts were then isolated and IRF7 translocation was analyzed by immunoblotting.

IL-1RI signaling modulates the ubiquitination profile of TRAF3

Balanced production of type I interferons and proinflammatory cytokines after engagement of TLRs, which signal through adaptors containing a Toll-interleukin 1 receptor (TIR) domain, such as MyD88 and TRIF, has been proposed to control the pathogenesis of autoimmune disease and tumor responses to inflammation. TRAF3, a ubiquitin ligase that interacts with both MyD88 and TRIF, regulates the production of interferon and proinflammatory cytokines in different ways. Lysine 48 (K48)-linked ubiquitination during MyD88-dependent TLR signaling leads to the degradation of TRAF3 and is essential for the activation of MAPKs and production of inflammatory cytokines. In contrast, TRIF-dependent signaling triggers noncanonical TRAF3 self-ubiquitination at lysine 63 (K63) which is essential for the activation of IRFs and subsequent type I IFN production (Tseng *et al.,* 2010).

To further elucidate the way in which IRFs and type I IFN production are regulated via the TRAF3 ubiquitination pathway, we investigated the fate of TRAF3 in these *Il1r1*$^{-/-}$ cells following 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. 12 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.12 B, top).

In contrast, K63-linked ubiquitination was greatly reduced in *Il1r1*$^{-/-}$ as compared with WT BMDCs (Fig. 12 B, bottom).

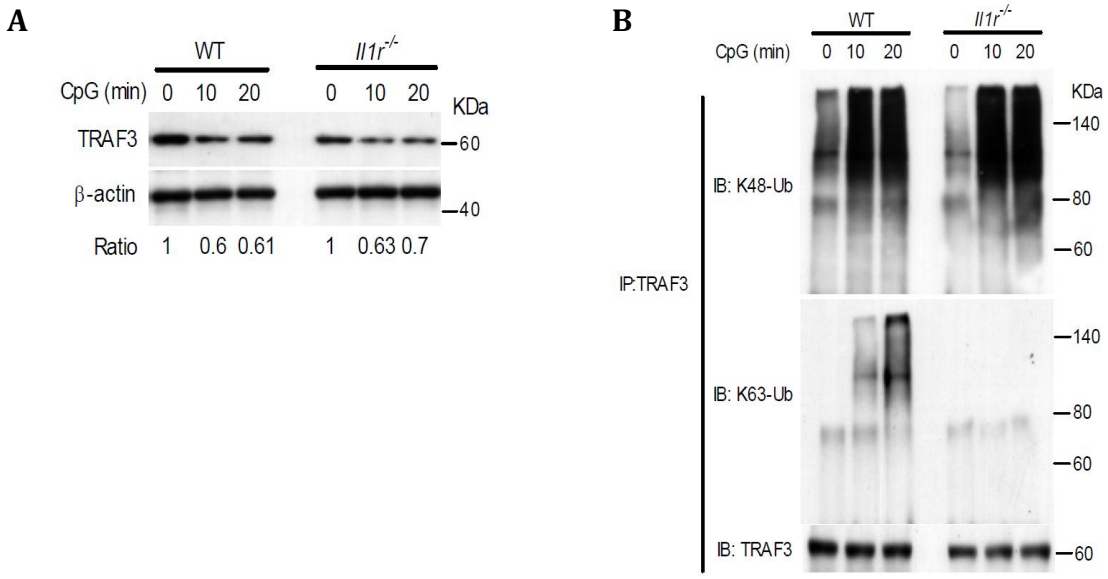


Figure 12. IL-1RI signaling modulates the ubiquitination profile of TRAF3. (A) Immunoblot analysis of TRAF3 in total cell lysates from WT and *Il1r1*$^{-/-}$ BMDCs stimulated with 10 μg/ml CpG for the indicated time periods. Ratio expresses the densitometry analysis of the TRAF3 bands normalized to the 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.

K63-linked ubiquitination in WT BMDCs was also shown in the absence of proteasomal inhibition (lack of MG132 in media), indicating that CpG induces K63 ubiquitination despite the K48-dependent degradation of TRAF3 (Fig. 13). Collectively, these results identified the role of IL-1RI signaling in the activation of TRAF3 and explain that *Il1r1*$^{-/-}$ BMDCs have lower production of IFN–β and IL-10

due to lower K-63 activity, but increased or normal production of pro-inflammatory cytokines under the experimental conditions specified in earlier.

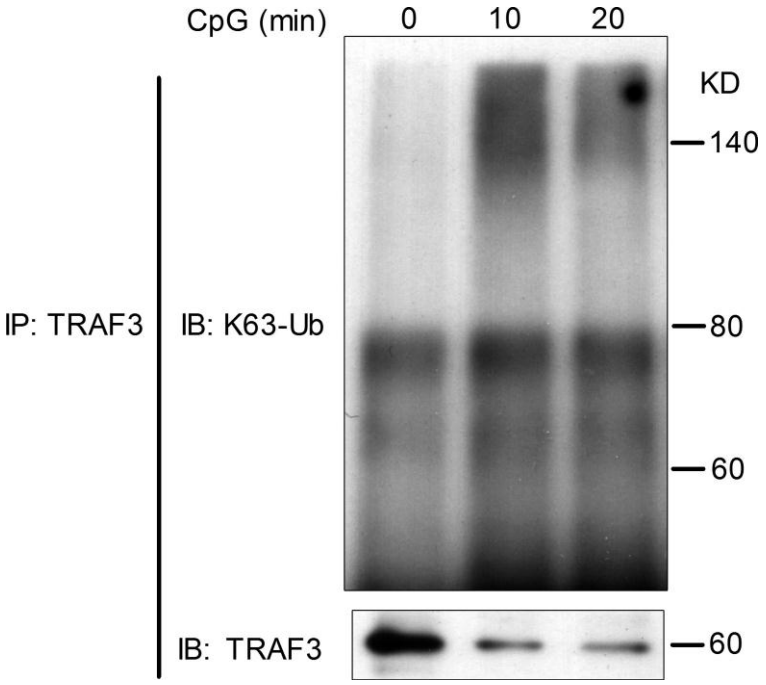


Figure 13. CpG triggers the K63-linked ubiquitination of TRAF3 in the absence of proteasomal inhibition. CD11c+ cells from WT mice were stimulated with 10 μg/ml CpG in the absence of MG132. Cells were then lysed and K63-linked ubiquitination of TRAF3 was determined as described in Materials and methods. Data are representative of two independent experiments.

DUBA inhibits type I IFN production in the absence of IL-1R signaling via TRAF3 ubiquitination regulation

Production of type I IFN is a critical host defense triggered by pattern-recognition receptors (PRRs) of the innate immune system. Deubiquitinating enzyme A (DUBA), an ovarian tumor domain-containing deubiquitinating enzyme, was discovered in a small interfering RNA(siRNA)-based screen as a regulator of IFN-I production. DUBA selectively cleaves the K63-linked polyubiquitin chains on TRAF3, resulting in its dissociation from the downstream signaling complex containing TANK-binding kinase 1. DUBA has been identified as a negative regulator of innate immune responses (Kayagaki, 2007).

To study the role of DUBA in the differential ubiquitination profile of TRAF3 seen, we stimulated BMDCs from WT and *Il1r1*$^{-/-}$ mice with CpG and assayed its impact on DUBA protein levels. CpG induced higher levels of DUBA protein in *Il1r1*$^{-/-}$ as compared with WT BMDCs (Fig. 14 A). Importantly, steady-state levels of DUBA protein were increased in BMDCs (Fig. 14 A) as well as in freshly isolated splenocytes from *Il1r1*$^{-/-}$ mice (Fig. 14 B). Consistent with the protein data, Duba mRNA levels were increased in freshly isolated splenocytes and mesenteric lymph node (MLN)- derived cells from *Il1r1*$^{-/-}$ mice (Fig. 14 C), suggesting that IL-1RI signaling negatively regulates DUBA transcription.

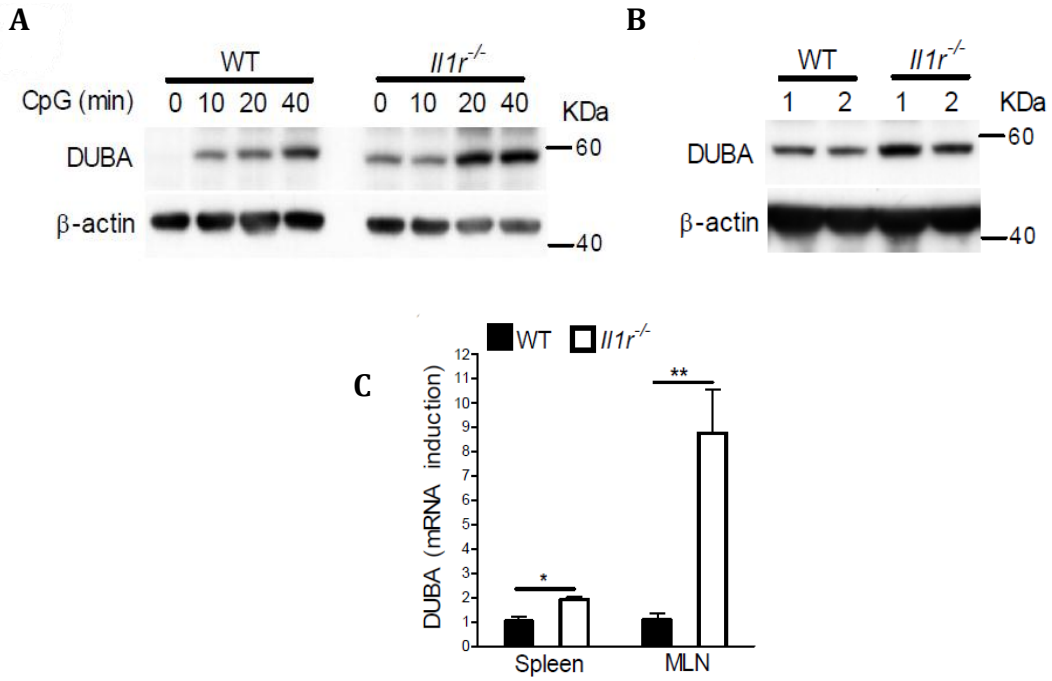


Figure 14. Il1r1$^{-/-}$ BMDCs show increased level of DUBA expression. (A) Immunoblot analysis of DUBA in total cell lysates from WT and *Il1r1*$^{-/-}$ BMDCs stimulated with 10 μg/ml CpG for the indicated time periods. (B) Immunoblot analysis of DUBA expression in total cell lysates from freshly isolated 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. (A–C) Data are representative of three different experiments. Error bars represent mean ± SEM. *, $P < 0.05$; **, $P < 0.01$.

As noted before, the IL-1R family has an intracellular TLR domain that allows them to communicate their immune responses. Therefore, we questioned whether or not the absence of other TLRs would cause a similar response. In fact, DUBA levels were not increased in splenocytes from *Tlr9*$^{-/-}$ or *Tlr3*$^{-/-}$ mice (Fig. 15), indicating that the dysregulation of DUBA expression is not a common characteristic in every TLR deficiency phenotype.

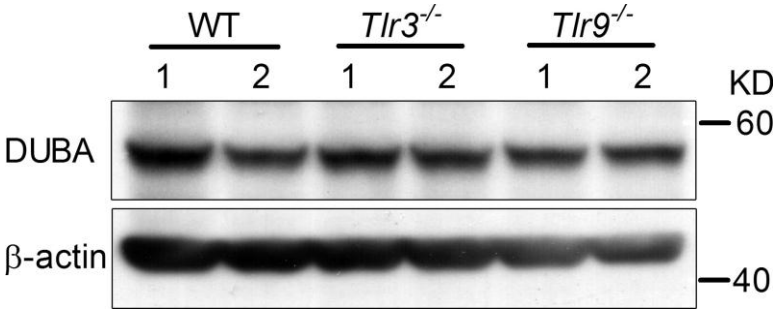


Figure 15. DUBA is not overexpressed in Tlr3$^{-/-}$ or Tlr9$^{-/-}$ mice. Immunoblot analysis of DUBA levels in total cell lysates from freshly isolated splenocytes obtained from WT (b6), *Tlr3*$^{-/-}$ and *Tlr9*$^{-/-}$ mice. Data are representative of two different experiments.

Finally, we wanted to determine whether DUBA is responsible for the reduced production of IL-10 and IFN-β anti-inflammatory cytokines seen in *Il1r1*$^{-/-}$ BMDCs. We performed DUBA knockdown by siRNA transfection in *Il1r1*$^{-/-}$ BMDCs (confirmed in Fig. 16 A). siRNA transfection, followed by CpG stimulation of *Il1r1*$^{-/-}$ BMDCs, resulted in significantly increased IL-10 and IFN-β production and decreased secretion of IL-6, but not TNF (Fig. 16 B).

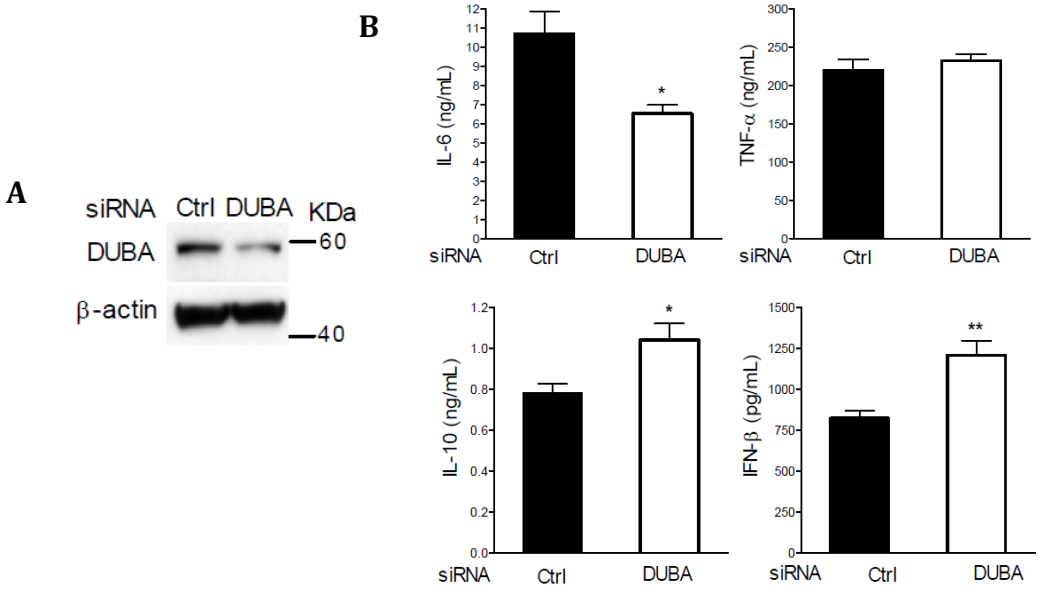


Figure 16. DUBA inhibits type I IFN production by Il1r1$^{-/-}$ BMDCs.
(A) Immunoblot analysis of DUBA expression in total cell lysates from *Il1r*$^{-/-}$ BMDCs transfected with 0.5 μM of either control (Ctrl) or DUBA siRNAs. (B) Pro- and antiinflammatory cytokine production in *Il1r1*$^{-/-}$ BMDCs after DUBA knockdown. *Il1r1*$^{-/-}$ BMDCs were transfected. 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 and B) Data are representative of three different experiments. Error bars represent mean ± SEM. *, $P < 0.05$; **, $P < 0.01$.

Results, in part, have been published of the material as it appears in the Journal of Experimental Medicine, 2010. González-Navajas, Jose M.; Law, Jason, Phung Nguyen, Kim; Bhargava, Meha; Corr, Mary Patricia; Varki, Nissi; Eckmann, Lars; Hoffman, Hal M.; Lee, Jongdae, Lee; Raz, Eyal. The Thesis author was a co-author of this paper.

DISCUSSION

The pro-inflammatory cytokine interleukin-1 (IL-1) signals via IL-1RI, inducing an increase in the expression of many genes with roles in immunity and inflammation. IL-1 is an endogenous cytokine that utilizes TLR signaling pathways and plays an essential role in the maintenance of skin barrier function, suggesting its close relation to innate defense networks against microbial threats.

Upon obstruction of IL-1 signaling, via IL-1RI knockout, anti-IL-1RI blocking or anti-IL-1β neutralizing antibodies, there was a reduced TLR9-dependent production of IL-10 and IFN-β. In addition, when cells were pretreated with IL-1β followed with CpG stimulation there was a significant increase in IL-10 and IFN-b production. These two findings suggest that IL-1RI signaling positively regulates TLR-dependent anti-inflammatory cytokine production, mainly type I IFN.

In the absence of IL-1 signaling, levels of K63- linked ubiquitination of TRAF3, essential for type I IFN production (Tseng, *et al*), decreased. In contrast, K48-linked ubiquitination, which leads to the degradation of TRAF3 and production of profinflammatory cytokines, showed no significant change. DUBA, a deubiquitinase that removes K63-linked ubiquitin chains off TRAF3, showed increased basal levels in IL-1RI$^{-/-}$ as compared with WT BMDCs. 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.

By regulating DUBA levels, IL-1RI signaling equilibrates the pro- and anti-inflammatory cytokine production in response to exogenous microbial TLR stimulation. Our results have uncovered new anti-inflammatory 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.

Pro-inflammatory cytokines are an essential component of host defense in patients who are susceptible to various inflammatory diseases. The effects of IL-1 are tightly controlled by several naturally occurring inhibitors, such as IL-1 receptor antagonist (IL-1Ra), IL-1 receptor type II (IL-1RII), and other soluble receptors. Numerous IL-1 inhibitors have been developed and tested primarily in rheumatoid arthritis, with modest effects (Gabay, *et al*., 2010). The use of IL-1 antagonists has been uniformly associated with beneficial effects in patients with hereditary autoinflammatory conditions associated with excessive IL-1 signaling, such as cryopyrinopathies and IL-1Ra deficiency. Successful treatment with IL-1 blockers has also been reported in other hereditary autoinflammatory diseases, as well as in nonhereditary inflammatory diseases, such as Schnizler syndrome, systemic-onset juvenile idiopathic arthritis and adult Still disease.

However, this inhibitory tactic has not proved successful in every inflammatory disease model. Several clinical trials have attempted to treat sepsis by blocking certain aspects of the inflammatory response. Interleukin 1, through IL-1RA, has been a specific target for inhibition but none of the trials have been successful (Remick, 2003). Our findings may help to explain these clinical

observations and suggest that IL-1R blockade can be harmful in certain infections or in non-sterile 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-γ 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-1β (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., antiubiquitin

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 realtime 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 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 IgGHRP 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 antiubiquitin 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. 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-γ 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.

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