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Cellular Inhibitors of Apoptosis cIAP1 and cIAP2 Are Required for Innate Immunity Signaling by the Pattern Recognition Receptors NOD1 and NOD2

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SUMMARY

Cellular inhibitor of apoptosis proteins (cIAPs) block apoptosis, but their physiological functions are still under investigation. Here, we report that cIAP1 and cIAP2 are E3 ubiquitin ligases that are required for receptor-interacting protein 2 (RIP2) ubiquitination and for nucleotide-binding and oligomerization (NOD) signaling. Macrophages derived from *Birc2*$^{-/-}$ or *Birc3*$^{-/-}$ mice, or colonocytes depleted of cIAP1 or cIAP2 by RNAi, were defective in NOD signaling and displayed sharp attenuation of cytokine and chemokine production. This blunted response was observed in vivo when *Birc2*$^{-/-}$ and *Birc3*$^{-/-}$ mice were challenged with NOD agonists. Defects in NOD2 signaling are associated with Crohn's disease, and muramyl dipeptide (MDP) activation of NOD2 signaling protects mice from experimental colitis. Here, we show that administration of MDP protected wild-type but not *Ripk2*$^{-/-}$ or *Birc3*$^{-/-}$ mice from colitis, confirming the role of the cIAPs in NOD2 signaling in vivo. This discovery provides therapeutic opportunities in the treatment of NOD-dependent immunologic and inflammatory diseases.

INTRODUCTION

Innate immunity serves as the first line of defense against invading microbial pathogens by activating pathways that mediate inflammation and pathogen clearance. Detecting pathogenic invasion and activating innate immune responses are crucial for host survival. However, dysregulation of these pathways can lead to severe immunologic and inflammatory diseases. Understanding the biochemical mechanisms that regulate pathogen recognition, and the strength and duration of the innate immune response, is therefore an important priority.

The arsenal of innate immunity includes pattern recognition receptors (PRRs) that sense conserved microbial components called pathogen-associated molecular patterns (PAMPs) and induce the initial antimicrobial response. PRRs include membrane-bound Toll-like receptors (TLRs) that survey the extracellular environment for pathogenic assault and cytosolic nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) that function as intracellular sentinels. NOD1 (CARD4) and NOD2 (CARD15) proteins act as cytosolic PRRs for bacterial peptidoglycan and are essential regulators of both innate and adaptive immunity (Tattoli et al., 2007). NOD1 confers responsiveness to peptidoglycan found primarily in Gram-negative bacteria, which contains meso-diaminopimelic acid (DAP), whereas NOD2 detects the peptidoglycan derivative muramyl dipeptide MurNAc-L-Ala-D-isoGln (MDP) (Tattoli et al., 2007). Similar to TLR ligands, NOD agonists are shed and recycled during bacterial growth and infection. It is now established that PRR pathways synergize in their response to pathogens. They utilize a plethora of molecular effectors including adaptors, kinases, E3 ubiquitin ligases, and proteases to transduce the "danger" signal (Kawai and Akira, 2007; Shaw et al., 2008; Sirard et al., 2007). The complexity of crosstalk in innate immunity extends beyond these interactions given that it has been recently shown that the pathways activated downstream of NOD1 and NOD2 are closely related to those activated by TNF receptors. Because NOD receptors and TNFR1 use structurally and functionally similar components, they may be subject to common regulatory mechanisms and feed-forward interactions (Chen et al., 2004; Hitotsumatsu et al., 2008; Kim et al., 2008; Kobayashi et al., 2002; Kobayashi et al., 2005). Upon NOD1 or NOD2 stimulation, the receptor-interacting protein 2, RIP2, which is also referred to as RICK, CARDIAK, and RIPK2 and is a RIP family member that is homologous to RIP1, is conjugated with K63 ubiquitin chains. Polyubiquitinated RIP2 then serves as a scaffold for the binding of TAK1 and for the activation of the MAPK and NF-κB pathways leading to cytokine, chemokine, and antimicrobial peptide production (Hasegawa et al., 2008; Park et al., 2007; Yang et al., 2007). Ubiquitination involves the concerted action of an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase. Ubc13 and Uev1a form the E2 complex responsible for K63 ubiquitination of RIP2, but the obligate E3 ubiquitin ligase is still under debate (Hasegawa et al., 2008; Yang et al., 2007).

Identifying the physiological E3 ligases for RIP2 is an important advancement with potentially important therapeutic implications.

Inhibitor of apoptosis proteins (IAPs) form a family of genetically conserved proteins characterized by the presence of 1-3 baculovirus IAP repeat (BIR) motifs. Previous studies have shown that three family members (termed XIAP, cIAP1, and cIAP2) are potent suppressors of cell death (Salvesen and Duckett, 2002). XIAP, the most-studied IAP, inhibits apoptosis by binding and inhibiting caspases, and it has been broadly assumed that cIAP1 and cIAP2 block apoptosis through a similar mechanism. However, recent structure-function analyses have indicated that these IAPs are not direct caspase inhibitors, suggesting that their antiapoptotic function must involve alternative mechanisms (Eckelman and Salvesen, 2006). An interesting feature of several IAPs, including cIAP1 and cIAP2, is the presence of a C-terminal RING-finger domain with E3 ubiquitin ligase activity. We recently demonstrated that cIAP1 and cIAP2 are key players in the TNFR1 signaling pathway that act as K63 E3 ubiquitin ligases for RIP1 (Bertrand et al., 2008). cIAP-mediated polyubiquination of RIP1 allows it to bind to the prosurvival kinase TAK1 and facilitates activation of NF-κB to promote cell survival.

The IAPs play a critical role in innate immunity signaling in *Drosophila*. Flies express two IAPs and one of these, dIAP2, is required for innate immunity to Gram-negative bacteria (Gesellchen et al., 2005; Kleino et al., 2005; Leulier et al., 2006; Valanne et al., 2007). When flies are exposed to bacterial challenge, the PGRP-Lc receptor activates a signaling cascade involving IMD, dTAB2, and dTAK1 (the fly orthologs of RIP1 and 2, TAB2 and 3, and TAK1, respectively) to activate JNK- and NF-κB-dependent expression of genes encoding antimicrobial peptides. It is not certain whether K63 ubiquitination of IMD is required for this response, but it seems likely given that the *Drosophila* ortholog of Ubc13 is necessary for pathway activation (Zhou et al., 2005). Epistasis analyses have placed Ubc13 function at the level of IMD and placed dIAP2 downstream of IMD but upstream of TAB and TAK1 (Kleino et al., 2005; Leulier et al., 2006). Therefore, it is likely that dIAP2 functions as an E3 ligase that targets IMD for K63-linked polyubiquination.

In this report, we demonstrate that cIAP1 and cIAP2 function in innate immunity signaling activated by NOD1 and 2 receptors. We report that cIAP1 and cIAP2 are E3 ubiquitin ligases required for K63 ubiquitination of RIP2. cIAP1 or cIAP2 null cells or cells depleted of cIAP1 or cIAP2 by RNAi depletion showed profound defects in RIP2-dependent activation of MAPK and NF-κB signaling pathways in response to diaminopimelic acid (DAP) or muramyl dipeptide (MDP), the NOD1 and NOD2 agonists, and display sharp attenuation of NOD-dependent proinflammatory cytokine and chemokine production. This blunted inflammatory response translated in vivo to resistance of *Birc2*$^{-/-}$ and *Birc3*$^{-/-}$ mice to peritonitis induced by NOD agonists and to dampened serum cytokine titers. MDP activation of NOD2 has been previously reported to protect mice from dextran sulfate sodium (DSS) colitis (Watanabe et al., 2008) and we report here that this protective effect was lost in *Ripk2*$^{-/-}$ or *Birc3*$^{-/-}$ mice. Together, our results demonstrate that cIAPs are key regulators of NOD innate immunity signaling.

RESULTS

cIAPs Regulate NOD1 and NOD2 Signaling Pathways Leading to Cytokine and Chemokine Production

The RIP kinases have emerged as essential sensors of cellular stress, integrating both extracellular signals emanating from various cell-surface receptors and signals emerging from intracellular pattern recognition receptors (Festjens et al., 2007). Recent studies have highlighted the importance of RIP1 in TNF receptor signaling, and we recently demonstrated an unexpected function for cIAP1 and cIAP2 as K63 ubiquitin ligases for RIP1 (Bertrand et al., 2008).

TNFR1 and NOD1 and NOD2 receptors activate structurally and functionally similar signaling components. We initially investigated whether cIAP1 and cIAP2 are part of the NOD1 signaling complex by using an RNAi-based approach to deplete cIAP expression in HT29 colonic cells. HT29 cells express NOD1 and signaling through this receptor can be activated with the purified NOD1 agonist DAP (LeBlanc et al., 2008). We tested whether cIAP1 or/and cIAP2 depletion had any effect on NOD1-dependent cytokine and chemokine production. Figure 1A shows that exposure of HT29 cells to the NOD1 agonist DAP induced expression of IL-8, TNFα, and MCP-1. As expected, siRNA targeting of RIP2 almost completely blocked the production of these factors. Remarkably, cIAP2 RNAi inhibited DAP-dependent induction of IL-8, TNFα, and MCP-1 to the same extent as RIP2 depletion, whereas cIAP1 depletion blocked the induction of TNFα and MCP-1 but had no effect on IL-8 production (Figure 1A). To begin to decipher the relationship between RIP2, cIAP1, and cIAP2, we investigated whether cells depleted in these proteins by RNAi show similar deficiencies in signaling pathways activated by NOD1. As previously reported, DAP stimulation of NOD1 induced RIP2-dependent activation of the MAP kinases p38 and JNK, as well as of the transcription factor NF-κB, shown by the degradation of IκBα (Figure 1B). Strikingly, repression of cIAP2 levels by RNAi prevented DAP-dependent phosphorylation of JNK and p38 and the degradation of IκBα (Figure 1C), whereas cIAP1 depletion reduced DAP-dependent activation of JNK and NF-κB but had no effect on p38 activation (Figure 1D). Taken together, our results suggest that cIAP1 and cIAP2 function in NOD1 innate immunity by regulating the signaling pathways leading to cytokine and chemokine production.

To test the physiological relevance of this finding, we isolated bone-marrow-derived macrophages (BMDM) from *Birc2*$^{-/-}$ and *Birc3*$^{-/-}$ mice and compared their innate immune responses to BMDMs derived from *Ripk2*$^{-/-}$ or wild-type mice. DAP stimulation of BMDMs did not induce production of cytokines and chemokines, probably because of the very low expression of Nod1 in these cells (data not shown). Nod2 is highly expressed in BMDMs and Figure 2A shows that wild-type BMDMs responded to MDP exposure by inducing production of IL-6, IL-1β, IL-10, and TNFα, whereas *Ripk2*$^{-/-}$, *Birc2*$^{-/-}$, and *Birc3*$^{-/-}$ macrophages were completely defective in MDP-dependent production of these factors.

Birc3$^{-/-}$ mice were reported to be resistant to lipopolysaccharide (LPS)-induced septic shock, specifically because of an attenuated inflammatory response (Conte et al., 2006). A synergy between TLR and NOD receptor pathways has recently been

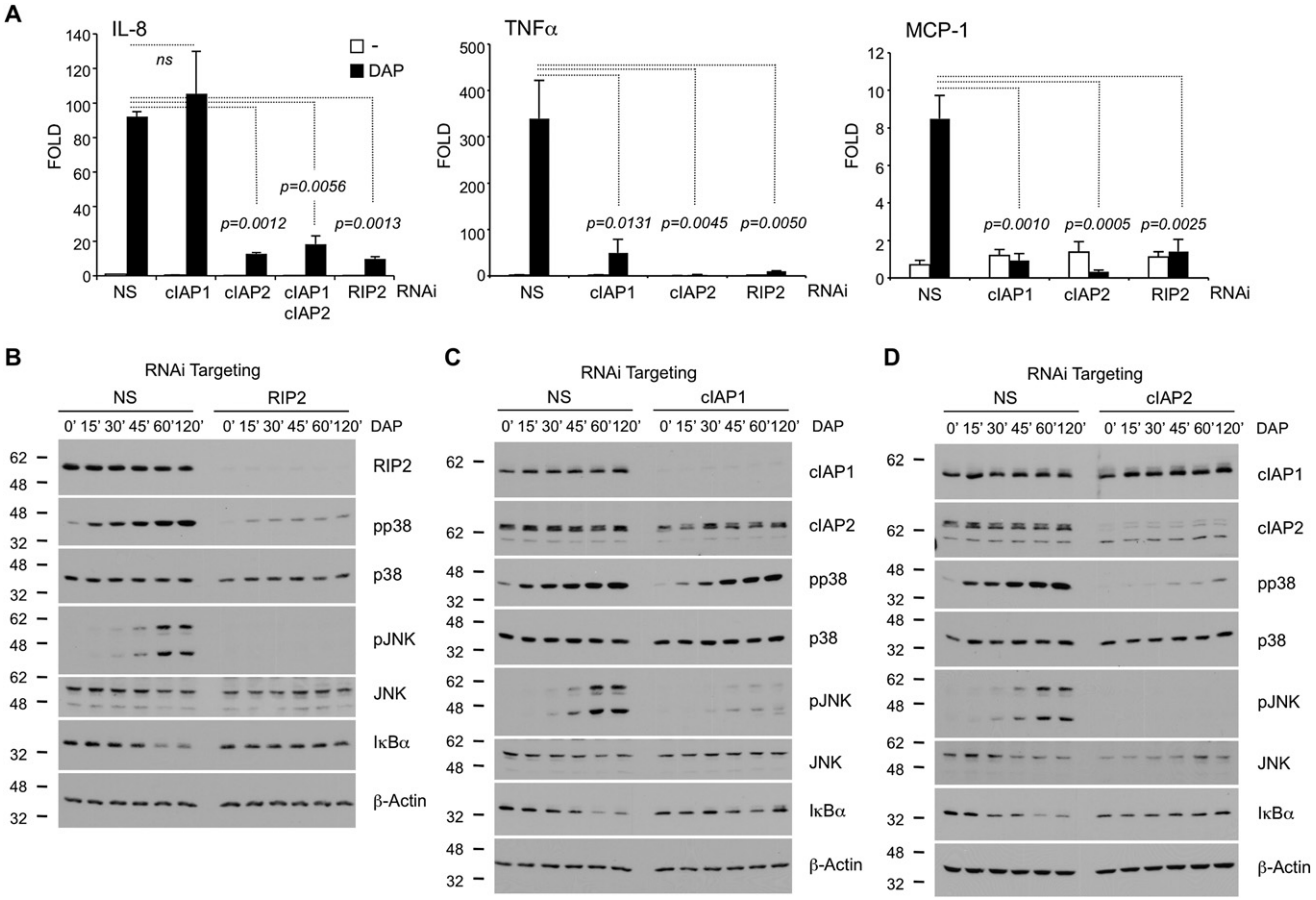

Figure 1. cIAP1 and cIAP2 Regulate RIP2-Dependent Innate Immune Response to the NOD1 Agonist DAP

(A) HT29 cells were transfected with siRNAs targeting cIAP1, cIAP2, RIP2, or nonspecific scramble RNA (NS) for 42 hr and were then left untreated or stimulated with DAP (10 μg/ml) for 2 hr. RNA was extracted and IL-8, TNFα, and MCP-1 levels were measured by quantitative real-time PCR. Data represent average expression ± SEM from four independent experiments.

(B–D) HT29 cells were transfected as in (A) and exposed to DAP (10 μg/ml) for the indicated period, and cell lysates were immunoblotted as mentioned.

established. MDP and LPS exert synergistic effects on cytokine production, and pretreatment with MDP sensitizes mice to endotoxic shock induced by LPS injection (Takada et al., 2002). Many preparations of bacterial components contain NOD1 and NOD2 agonists and using highly purified TLR ligands, Nunez and colleagues ruled out a role for RIP2 in TLR signaling (Park et al., 2007). To investigate whether LPS resistance in *Birc3*$^{-/-}$ mice is mediated by a defect in NOD signaling, we treated wild-type and *Birc3*$^{-/-}$ BMDMs with MDP and LPS, alone or in combination, and quantified IL-6 concentrations. Both wild-type and *Birc3*$^{-/-}$ macrophages produced equivalent amounts of IL-6 in response to LPS treatment alone. At the low concentration of MDP used in this experiment, treatment with MDP alone did not induce IL-6 production. However, when combined with LPS, MDP greatly enhanced the production of IL-6 in wild-type BMDMs but not in *Birc3*$^{-/-}$ cells (Figure 2B). Together, these data indicate that cIAP2 plays an obligate role in NOD2 signaling but is dispensable for TLR4 signaling.

We then compared the signaling pathways activated by MDP in wild-type, *Ripk2*$^{-/-}$, *Birc2*$^{-/-}$, and *Birc3*$^{-/-}$ BMDMs. Figure 2C shows that MDP-dependent activation of the MAP kinases p38 and JNK, and of the transcription factor NF-κB (shown by the phosphorylation of IKKα/β and the degradation of IκBα), did not occur in BMDMs derived from *Ripk2*$^{-/-}$ mice. Similarly, *Birc2*$^{-/-}$ and *Birc3*$^{-/-}$ BMDMs stimulated with MDP showed strong attenuation of p38, JNK, and NF-κB compared to wild-type cells (Figure 2D). Nod2 and Rip2 levels are unaffected in *Birc2*$^{-/-}$ and *Birc3*$^{-/-}$ macrophages (Figures 2D and 2E) and we conclude that cIAP1, cIAP2, and RIP2 lie in the same NOD-activated signaling path.

cIAP1 and cIAP2 Physically Interact with RIP2

NOD1 and NOD2 have a caspase recruitment domain (CARD) in their N-terminal regions, a central nucleotide-binding and oligomerization domain (NOD), and a C-terminal leucine-rich repeat domain (LRR). RIP2 is composed of N-terminal kinase and C-terminal CARD domains linked via an intermediate region, and it physically associates with NOD1 and NOD2 through CARD-CARD interactions. cIAP1 and cIAP2 are composed of three baculovirus IAP repeat (BIR) domains followed by a CARD and a RING finger domain. We tested whether cIAP1 and cIAP2 directly bind RIP2 by performing pull-down

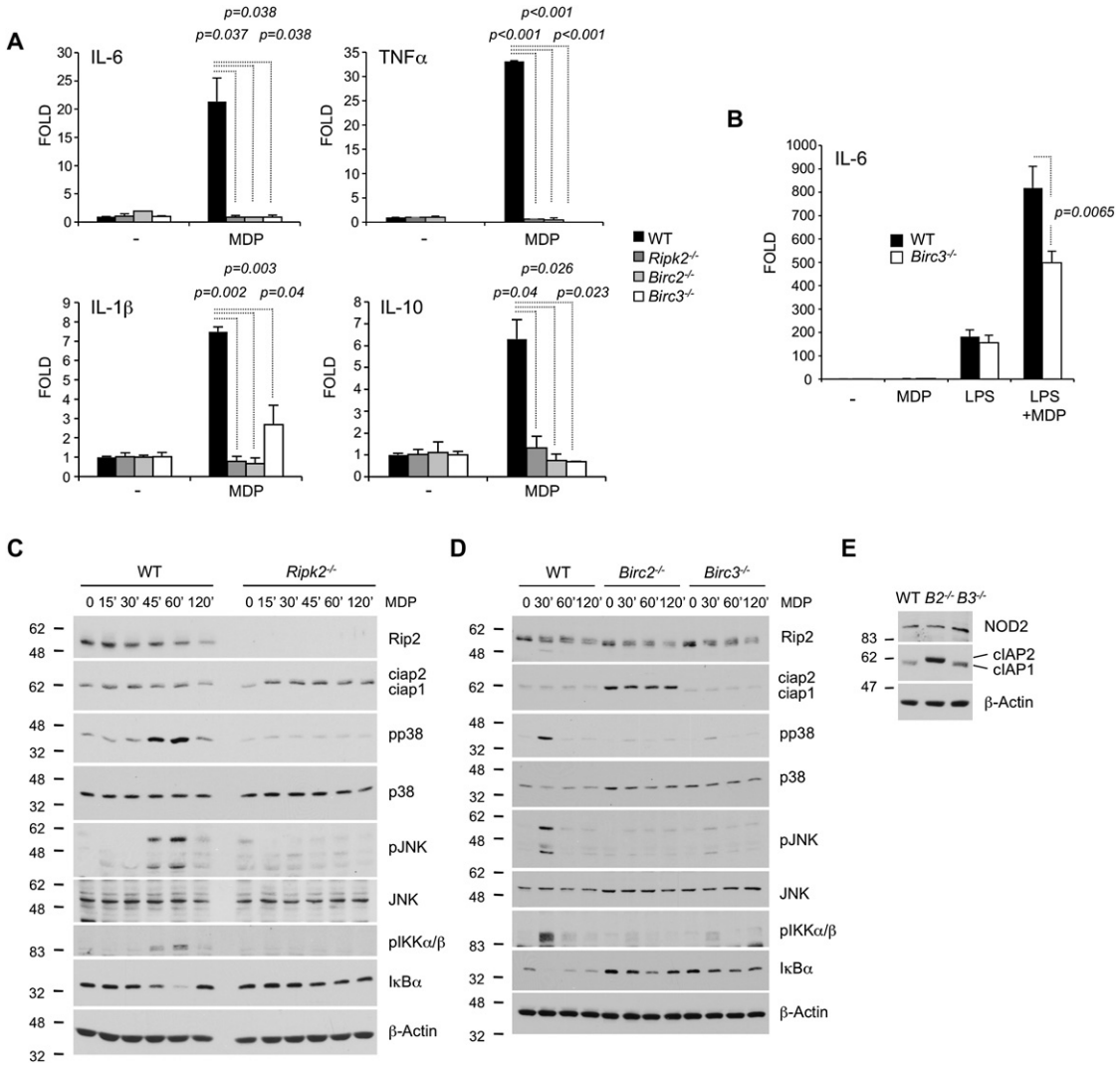

Figure 2. cIAP1 and cIAP2 Mediate RIP2-Dependent Innate Immune Response to the NOD2 Ligand MDP

(A) BMDMs isolated from wild-type, *Ripk2*$^{-/-}$, *Birc2*$^{-/-}$, and *Birc3*$^{-/-}$ mice were left untreated or stimulated with MDP (100 μg/ml) for 2 hr, RNA was extracted, and IL-6, TNFα, IL-1β and IL-10 levels were measured by quantitative real-time PCR. Data represent average expression ± SEM from three independent experiments.

(B) Wild-type and *Birc3*$^{-/-}$ BMDMs were left untreated or stimulated for 2 hr with MDP (10 ug/ml) and LPS (1 μg/ml) added either alone or together, RNA was extracted, and IL-6 production was measured by quantitative real-time PCR. Data represent average expression ± SEM from four experiments.

(C and D) Wild-type, *Ripk2*$^{-/-}$, *Birc2*$^{-/-}$, and *Birc3*$^{-/-}$ BMDMs were stimulated with MDP (100 μg/ml) for the periods indicated, and cell lysates were immunoblotted as mentioned.

(E) Immunoblot showing that Nod2 expression is not affected in Birc2 (*c1*$^{-/-}$)- or Birc3 (*c2*$^{-/-}$)- deficient BMDMs.

experiments using bacterially produced GST-cIAP1 and GST-cIAP2 fusion proteins and in vitro-transcribed and -translated RIP2 labeled with ^{35}S-Methionine. Figure 3A shows that RIP2 does not bind GST alone but does interact with GST-cIAP1 and GST-cIAP2. To address whether binding occurs through CARD-CARD interactions, we transfected HEK293T cells with cIAPs and RIP2 deletion mutants. A RIP2 mutant lacking the CARD domain (RIP2ΔCARD), but not the CARD-only mutant (CARD), coimmunoprecipitated full-length cIAP2 (FL), indicating that RIP2 binds cIAP2 independently of its CARD domain (Figure 3B). We next mapped the regions of cIAP2 involved in RIP2 binding by using cIAP2 mutants containing either the BIR domains (BIR) or the CARD-RING portion of the protein (CARD-RING). We found that both fragments could bind full-length RIP2, indicating a two-site interaction (Figure 3B). Interestingly, expression of full-length RIP2 consistently increased levels of cIAP2 and cIAP2 fragments. In a similar set of experiments, we found that full-length cIAP1, or a mutant containing the BIR domains, coimmunoprecipitated full-length RIP2 but not the CARD-only mutant (Figure 3C), indicating that cIAP1 does not rely on a CARD-CARD interaction to bind RIP2.

RIP2, cIAP1, or cIAP2 deletion causes similar defects in innate immune responses and experiments above show that RIP2 binds to both cIAP1 and cIAP2. These findings raised the

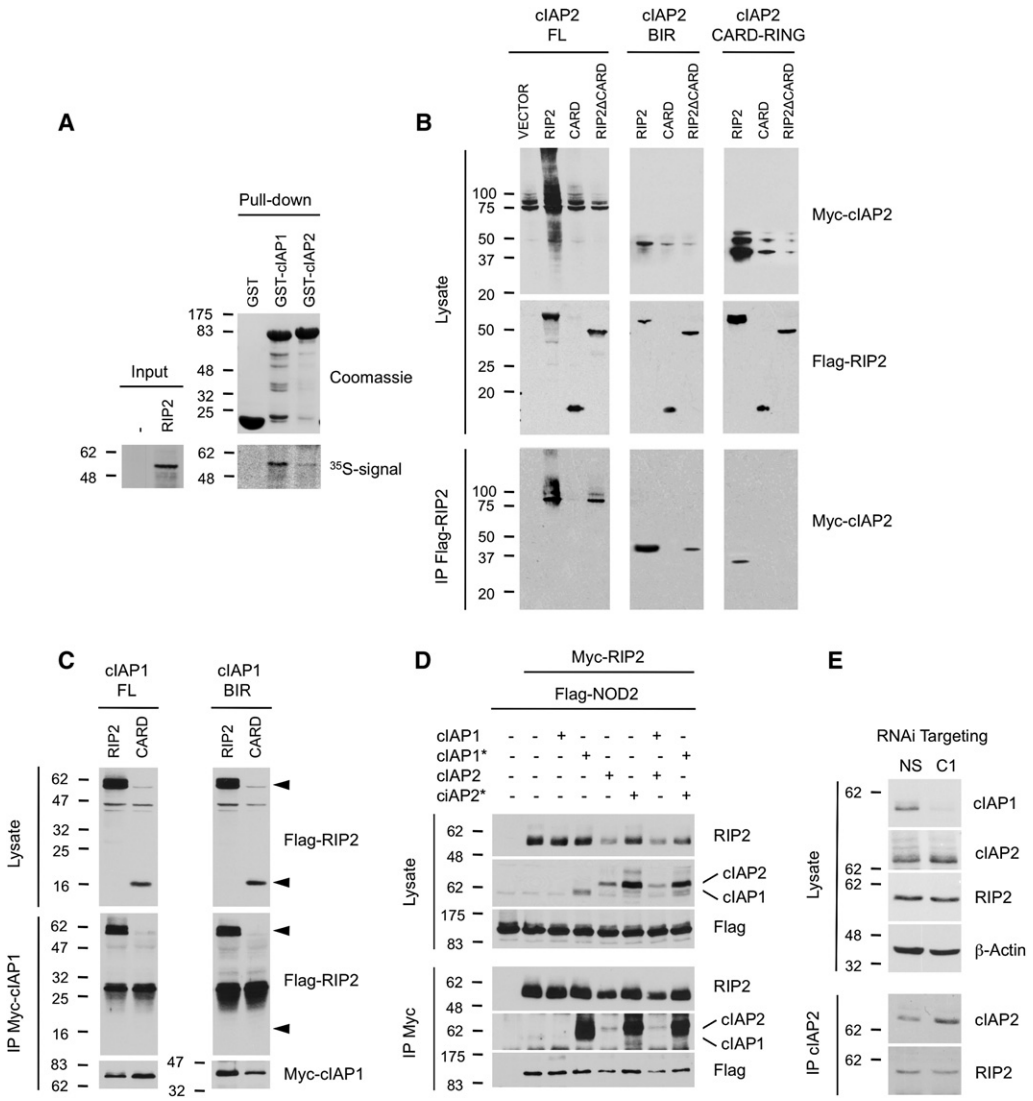

Figure 3. cIAP1 and cIAP2 Bind RIP2 through a Non-CARD-CARD Interaction

(A) In vitro-transcribed and -translated RIP2 labeled with ^{35}S-Methionine (Input) was incubated overnight with bacterially produced GST, GST-cIAP1, or GST-cIAP2 bound to Sepharose beads. The beads were washed extensively and ran on an acrylamide gel, and RIP2 binding was revealed by autoradiography (Pull-down).

(B) We analyzed the mapping of cIAP2-RIP2 interaction in HEK293T cells by coexpressing Flag-tagged RIP2 and Myc-tagged cIAP2 deletion mutants. RIP2 was immunoprecipitated with a Flag antibody and coimmunoprecipitated cIAP2 was revealed by immunoblotting with a Myc antibody.

(C) The mapping of cIAP1-RIP2 interaction was analyzed in HEK293T cells by coexpressing Flag-tagged RIP2 and Myc-tagged cIAP1 deletion mutants. cIAP1 was immunoprecipitated with a Myc antibody and coimmunoprecipitated RIP2 was revealed by immunoblotting with a RIP2 antibody. Expression of exogenous RIP2 in the lysate was monitored with a Flag antibody.

(D) HEK293T cells were transfected with the indicated plasmids and RIP2 was immunoprecipitated using a Myc antibody. Coimmunoprecipitated cIAP1, cIAP2 and FLAG-NOD2 were revealed by immunoblotting. For (B)–(D), RIP2 indicates full-length RIP2, RIP2ΔCARD indicates RIP2 mutant lacking the CARD domain, CARD indicates the CARD only RIP2 mutant, and CIAP1* and CIAP2* refer to forms of cIAP1 and cIAP2 lacking E3 ligase activity.

(E) HT29 cells were transfected with siRNAs targeting cIAP1 (C1) or nonspecific scramble RNA (NS) and after 48 hr, endogenous cIAP2 was immunoprecipitated and endogenous coimmunoprecipitated RIP2 was revealed by immunoblotting with a RIP2 antibody.

possibility that a heteromeric complex of these proteins may be required for normal signaling, as indicated by others (Rajalingam et al., 2006). To test this, we transfected HEK293T cells with RIP2 and NOD expression plasmids in the presence and absence of wild-type or RING-finger-mutated cIAP1 and cIAP2. Figure 3D shows that NOD2, cIAP1, and cIAP2 can be coimmunoprecipitated with RIP2 and that mutation of the RING domain enhances the association of cIAP1 and cIAP2 with RIP2. Expression of cIAP1 or cIAP2 did not alter the coimmunoprecipitation of NOD2 with RIP2, suggesting that the IAP-RIP2 and NOD2-RIP2 interactions occur independently. We then investigated whether a complex is formed between these proteins in HT29 cells, in which they are endogenously expressed. RIP2 and cIAP2 can be coimmunoprecipitated from these cells, and

interestingly cIAP1 depletion reduces the amount of RIP2 protein coimmunoprecipitated with cIAP2, suggesting that an oligomeric complex of cIAP1 and cIAP2 collaborate to bind RIP2 in this cell type (Figure 3E). We also examined whether the subcellular location of NOD2, RIP2, cIAP1, or cIAP2 was altered in HT29 cells treated with MDP or in transfected 293T cells but did not observe translocation of these components in settings where the NOD agonist activates NFkB and MAPK pathways and induces cytokine and chemokine production (Figure S2 available online and data not shown).

cIAP1 and cIAP2 Are E3 Ubiquitin Ligases Required for K63 Ubiquitination of RIP2

A recent study has reported that RIP2 is conjugated with K63-linked polyubiquitin chains at lysine 209 upon NOD1 or NOD2 activation (Hasegawa et al., 2008). This ubiquitination step is crucial for activating the NOD-dependent immune response because K63-ubiquitinated RIP2 serves as a scaffold that assembles signaling complexes to activate the NF-κB pathway, and presumably, the MAPK pathway. A20, a deubiquitinase initially reported to remove K63-linked polyubiquitin chains on RIP1 in the TNFR1 pathway, acts as a negative regulator of the NOD2 immune response by editing RIP2-K63 ubiquitin chains (Hasegawa et al., 2008; Hitotsumatsu et al., 2008). With an RNAi-based approach, Ubc13-Uev1a was identified as the E2 component required for RIP2 ubiquitination (Yang et al., 2007), but the physiological E3 ubiquitin ligase for RIP2 remained under debate. We recently reported that Ubc13-Uev1a and cIAP1 and cIAP2 function, respectively, as E2 and E3s for RIP1-K63 ubiquitination in response to TNFR1 activation.

Hitotsumatsu and colleagues (2008) have shown that MDP-induced RIP2 ubiquitination in BMDMs can only be detected in an A20-deficient background because of the rapid editing of RIP2 by A20. However, other groups have reported K63 ubiquitination of RIP2 in HEK293 cells (Hasegawa et al., 2008; Yang et al., 2007). We therefore tested whether cIAP1 and cIAP2 could function as E3s for RIP2 by examining the effect of cIAP1 and cIAP2 on RIP2 ubiquitination in transfected HEK293 cells. TRAF6 has been suggested to function as an E3 for RIP2 (Yang et al., 2007), and we therefore used TRAF6 as a control in these experiments. Surprisingly, expression of cIAP1 and cIAP2 induced ubiquitination of RIP2 but expression of TRAF6 did not (Figure 4A). The stronger effect observed with cIAP2 may reflect the fact that cIAP1 is more difficult to overexpress than cIAP2 (Figure S1). We next asked whether cIAP1 and cIAP2 E3 ligase activity was required for RIP2 ubiquination. Remarkably, cIAP1- and cIAP2-induced ubiquitination of RIP2 was not observed when RIP2 was coexpressed with E3 inactive forms of the IAPs (Figure 4B). To determine the type of ubiquitin chains the IAPs added to RIP2, we expressed RIP2 with wild-type or mutant forms of ubiquitin that can only be K48 or K63 linked and found that RIP2 was conjugated with both K48 and K63 polyubiquitin chains (Figure 4C). In HEK293T cells, RIP2 is very weakly ubiquitinated when expressed by itself but is highly ubiquinated when coexpressed with NOD2 (Figure 4D). We used that property to test whether endogenous cIAP1 and cIAP2 in HEK293T cells were required for NOD2-induced RIP2 ubiquination. Figure 4E shows that RNAi targeting cIAP1 or cIAP2 (C1 and C2) reduced RIP2 ubiquitination, whereas nonspecific scrambled siRNA (NS) did not. This effect was even more potent when cIAP1 and cIAP2 RNAi were combined, suggesting a cooperative function for these IAPs (Figure 4E). Interestingly, endogenous cIAP2 protein levels are below our immunoblot detection limit in HEK293T cells, but these experiments demonstrate that the protein is expressed and is functional.

Together, our results demonstrate that cIAP1 and cIAP2 have a physiological role in NOD innate immunity pathways, that their E3 ligase activity is required for RIP2 ubiquitination, and that cIAP1 and cIAP2 collaborate to regulate RIP2 signaling.

Birc2$^{-/-}$ and *Birc3*$^{-/-}$ Mice Exhibit a Blunted Inflammatory Response to NOD Stimulation

We have shown that cIAP1 and cIAP2 participate in NOD biological responses in murine macrophages and human intestinal epithelial cells. To specifically investigate the role of these proteins in NOD activation in vivo, we first challenged wild-type, *Ripk2*$^{-/-}$, *Birc2*$^{-/-}$, and *Birc3*$^{-/-}$ mice with intraperitoneal injection of the purified Nod1 ligand DAP. IP injection of DAP or synthetic NOD1 agonists (KF1B and FK156) has been shown to stimulate chemokine production and neutrophil infiltration in the peritoneum in wild-type but not in Nod1-deficient mice (Inohara et al., 2003; Lysenko et al., 2007; Masumoto et al., 2006). We therefore tested these parameters in the four mouse genotypes under study. Intraperitoneal injection of DAP resulted in a substantial release of the chemokines KC and MCP-1 and the cytokine IL-6 in the bloodstream of wild-type mice 2 hr after DAP injection (Figure 5A). This DAP response was blocked in *Ripk2*$^{-/-}$ mice and, interestingly, was also abrogated in *Birc2*$^{-/-}$ and *Birc3*$^{-/-}$ mice (Figure 5A). The impaired chemokine production in *Ripk2*$^{-/-}$, *Birc2*$^{-/-}$, and *Birc3*$^{-/-}$ mice translates into profound defects in immune cell migration given that the recruitment of neutrophils into the peritoneum 24 hr after DAP injection was sharply reduced in *Ripk2*$^{-/-}$ and *Birc3*$^{-/-}$ mice compared to wild-type mice (Figure 5B), further confirming the requirement of cIAPs in NOD1 signaling in vivo. Importantly, we also found that IL-6 release in the bloodstream after IP injection of the NOD2 agonist MDP was abrogated in *Ripk2*$^{-/-}$ and *Birc3*$^{-/-}$ mice compared to wild-type animals (Figure 5C), demonstrating that cIAP proteins not only function in NOD1 signaling but is also required for NOD2 signaling in vivo.

MDP Protects Wild-Type Mice, but Not *Ripk2*$^{-/-}$ or *Birc3*$^{-/-}$ Mice, from DSS Colitis

MDP administration protects mice from experimental colitis and this protective effect is absent in mice with defective Nod2 signaling (Watanabe et al., 2008). To address the significance of the role of the cIAP proteins in transducing NOD2 signaling in vivo, we determined whether administration of MDP protects mice from DSS colitis in the absence of cIAP function. In these studies, wild-type, *Ripk2*$^{-/-}$, or *Birc3*$^{-/-}$ mice were treated with 4% DSS from day 0 to day 5 and were administered MDP (100 μg) or PBS, intra-peritoneally, for 3 consecutive days beginning at the time of colitis induction (days 0, 1, and 2). As shown in Figure 6A, DSS treatment induced drastic body weight loss in wild-type mice treated with PBS (white squares) but not those administered MDP (black squares). The protective effect of MDP was absent in *Ripk2*$^{-/-}$ mice because these mice exhibited equivalent body weight loss in response to DSS colitis

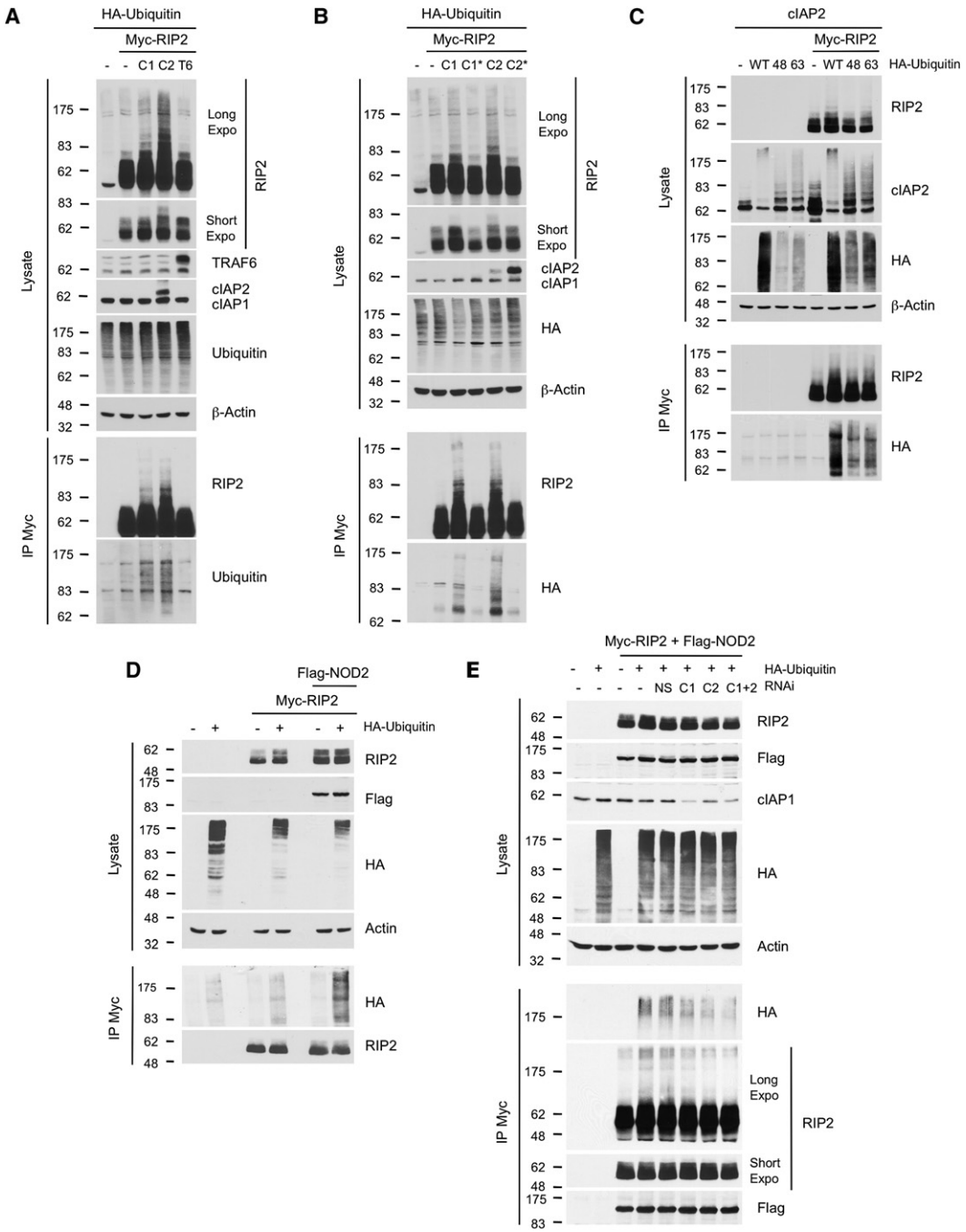

Figure 4. cIAP1 and cIAP2 Are E3 Ubiquitin Ligases for K63 Ubiquitination of RIP2

(A) HEK293T cells were transfected for 42 hr with HA-Ubiquitin, Myc-tagged RIP2 and cIAP1, cIAP2, or TRAF6. RIP2 was immunoprecipitated and ubiquitinated RIP2 was detected by immunoblotting as indicated.

(B) HEK293T cells were transfected for 42 hr with HA-tagged ubiquitin, Myc-tagged RIP2, and with E3 active (C1 and C2) or inactive (C1* and C2*) forms of cIAP1 and cIAP2, respectively. RIP2 was immunoprecipitated and ubiquitinated RIP2 was detected by immunoblotting as indicated.

(C) HEK293T cells were transfected for 42 hr with cIAP2 and wild-type (WT), K48-only (K48), or K63-only (K63) ubiquitin in the presence or absence of Myc-tagged RIP2. RIP2 was immunoprecipitated and ubiquitinated RIP2 was detected by immunoblotting as indicated.

(D) HEK293T cells were transfected for 42 hr with HA-tagged ubiquitin, Myc-tagged RIP2, and Flag-tagged NOD2. RIP2 was immunoprecipitated and ubiquitinated RIP2 was detected by immunoblotting as indicated.

(E) HEK293T cells were transfected with siRNAs targeting cIAP1 (C1), cIAP2 (C2), cIAP1+cIAP2 (C1+2), or nonspecific scramble RNA (NS) for 42 hr and with Myc-tagged RIP2, Flag-tagged NOD2, and HA-tagged ubiquitin for the last 30 hr. RIP2 was immunoprecipitated and ubiquitinated RIP2 was detected by immunoblotting as indicated.

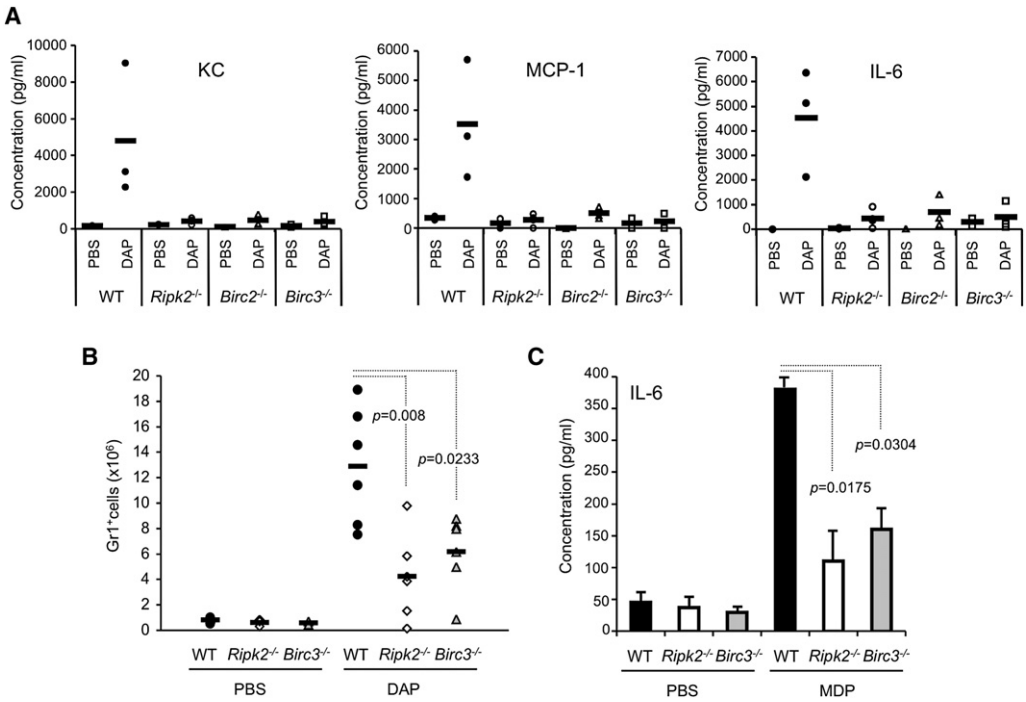

Figure 5. Defective NOD-Dependent Innate Immune Responses In Vivo in *Birc2*$^{-/-}$ and *Birc3*$^{-/-}$ Mice

(A) Wild-type, *Ripk2*$^{-/-}$, *Birc2*$^{-/-}$, and *Birc3*$^{-/-}$ mice (n = 3/genotype/treatment) were injected intraperitoneally with DAP (2 mg/Kg) or endotoxin-free PBS. Blood was collected from animals 2 hr after injection and KC, MCP-1, and IL-6 in the serum were measured with bead-based immunoassays.

(B) Wild-type, *Ripk2*$^{-/-}$, and *Birc3*$^{-/-}$ mice (n = 6 per genotype/treatment) were injected intraperitoneally with DAP (1 mg/kg) or endotoxin-free PBS. The number of Gr1+ cells in the peritoneal lavage was quantified 24 hr later by FACS analysis. Averages are represented by straight bars.

(C) Wild-type, *Ripk2*$^{-/-}$, and *Birc3*$^{-/-}$ mice (n = 4 per genotype/treatment) were injected intraperitoneally with 25 mg/kg of MDP or endotoxin-free PBS. Blood was collected from animals 4 hr after injection and IL-6 in the serum was measured by bead-based immunoassays. Error bars represent SEM.

irrespective of whether they received MDP or PBS (white and black circles). Furthermore, the MDP protective effect was completely lost in mice deficient in ciap2 (white and black triangles), strongly indicating a critical role of this protein in Nod2-dependent biological responses in the gut. Figure 6B shows that the body weight data correlated with expression of serum amyloid A (SAA), an acute phase protein that reflects colitis severity: wild-type mice treated with MDP had significantly lower SAA amounts compared to PBS-treated controls ($p = 0.001$), and no difference in SAA amounts was seen in *Ripk2*$^{-/-}$ or *Birc3*$^{-/-}$ mice treated with either PBS or MDP. Histological examination of colon sections derived from wild-type, Rip2-, or ciap2-deficient mice further supported the body weight loss and SAA measurements, showing a conserved intestinal tissue architecture in wild-type mice treated with DSS and MDP but not in DSS-treated mice exposed to vehicle or in either Rip2- or ciap2-deficient mice, which exhibited a severe colitis phenotype characterized by loss of goblet cells, inflammatory infiltrates, and intestinal epithelial lesions (Figure 6C). Altogether, these data suggest that loss of cIAP2 function impairs the ability of the NOD2-RIP2 complex to control colonic inflammation upon systemic administration of MDP.

DISCUSSION

It has long been assumed that IAPs promote cell survival by acting as direct caspase inhibitors. However, recent structure-function analysis revealed that, out of the eight IAPs found in the human genome, XIAP is the only family member capable of direct caspase inhibition (Eckelman and Salvesen, 2006). The finding that a subset of IAPs contains a RING finger domain with E3 ubiquitin ligase activity has implied the presence of alternative functions of these IAPs. We recently reported that cIAP1 and cIAP2 promote cancer cell survival by functioning as K63 ubiquitin ligases for RIP1, which allows RIP1 to activate the pro-survival NF-κB pathway, while preventing it from binding to caspase-8 and inducing apoptosis (Bertrand et al., 2008). This function appears to be evolutionarily conserved and physiologically relevant as primary cultures of cells deficient in cIAPs are defective in TNF-induced NF-κB (Mahoney et al., 2008; Varfolomeev et al., 2008). Similarly, the sole ciap family member expressed in zebrafish maintains blood vessel integrity by facilitating the formation of a TNFR complex I, promoting NF-κB activation and preventing caspase-8-dependent apoptosis that leads to vessel regression (Santoro et al., 2007). The TNFR signaling pathway has similarities with the innate immune pathways activated by NOD1 and NOD2 receptors in mammals and by PGRP-Lc receptor in the fly, given that they all lead to NF-κB and MAP kinases activation using similar components.

Interestingly, in vivo and in vitro studies in *Drosophila* have identified the fly IAP dIAP2 as an important regulator of the innate immune response downstream of the PGRP-Lc receptor (Gesellchen et al., 2005; Kleino et al., 2005; Leulier et al., 2006; Valanne et al., 2007), and available data suggest that dIAP2 may be a K63

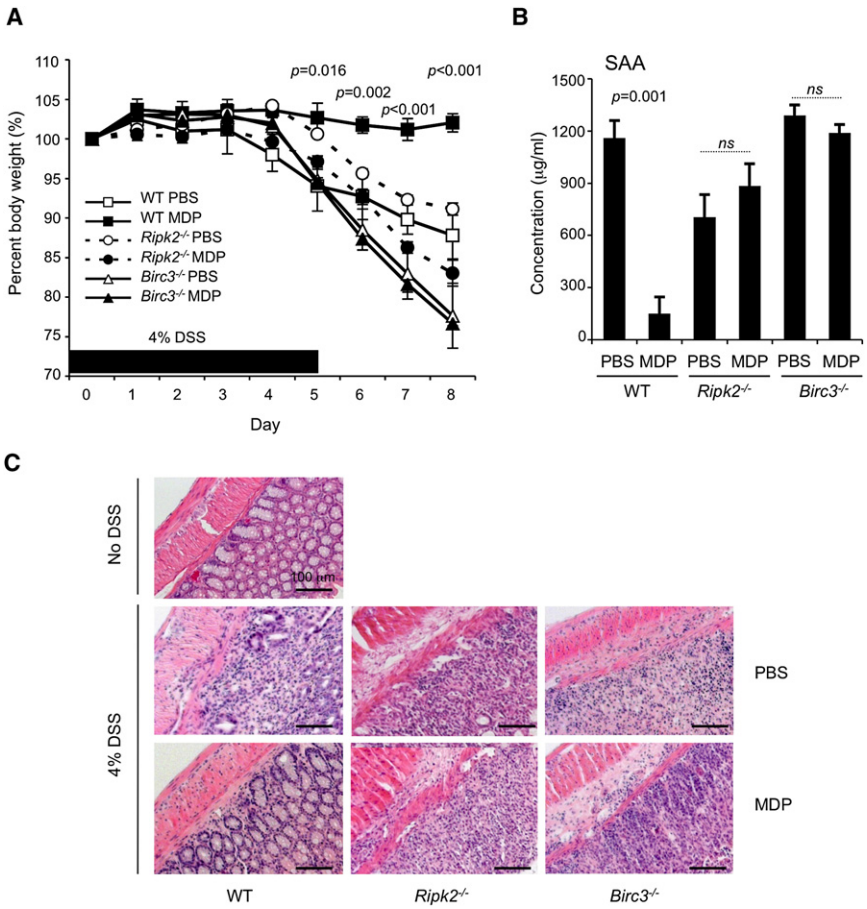

Figure 6. MDP Administration Prevents DSS Colitis in Wild-Type but Not in *Ripk2*$^{-/-}$ or *Birc3*$^{-/-}$ Mice

Wild-type (n = 8), *Ripk2*$^{-/-}$ (n = 6), and *Birc3*$^{-/-}$ (n = 6) mice were treated with drinking water containing 4% DSS for 6 days (day 0–5) and were consequently given regular drinking water (DSS-free) for an additional 3 days (day 6–8). On days 0, 1, and 2, mice were injected intraperitoneally with either MDP (100 μg; 500 μl per mouse) or an equivalent volume of endotoxin-free PBS, every day.

(A) Body weight loss of mice treated with MDP or PBS. Error bars represent SEM.

(B) Serum amyloid A (SAA) levels in the serum of mice on day 8 were determined by ELISA. Error bars represent SEM.

(C) H&E-stained colonic tissue sections of the mice harvested on day 8. Original magnification ×50 is shown.

ubiquitin ligase for IMD, the fly ortholog of RIP1 and RIP2. Here, we demonstrate a physiological role for cIAP1 and cIAP2 as regulators of innate immune signaling in mammals. We show that these cIAPs function as E3 ubiquitin ligases required for RIP2 ubiquitination that is induced by triggers that activate the cytosolic PRRs NOD1 and NOD2. Taken together with other recent findings, our data indicate that these IAPs function downstream of the TNFR1 and of the NOD1 and NOD2 receptors to produce RIP1 ubiquination in the former and RIP2 ubiquination in the latter.

RIP2 polyubiquitination is required for NOD-induced NF-κB activation and subsequent cytokine and chemokine production (Hasegawa et al., 2008; Hitotsumatsu et al., 2008), but the identity of the E3 ligase(s) that target RIP2 has been uncertain. RIP2 binds TRAF2, TRAF5, and TRAF6 proteins in vitro (McCarthy et al., 1998), and some studies have suggested that these E3 ligases may mediate K63 ubiquitination of RIP1. For example, Yang et al. (2007) showed that siRNA-mediated TRAF6 depletion reduced NOD2-dependent RIP2 ubiquitination in HEK293T cells and attenuated MDP-induced NF-κB activation in the RAW264.7 macrophage cell line. However, an earlier study from this group reported that inhibition of TRAF6 expression had no effect on NOD2-induced NF-κB activation in HEK293T cells, whereas RIP2 repression greatly decreased it (Abbott et al., 2007). This work is consistent with a recent report by Hasegawa et al. (2008), who found that mouse embryonic fibroblasts (MEFs) deficient in Traf6 show normal NF-κB activation upon Nod1 stimulation (Hasegawa et al., 2008).

Under conditions in which cIAP1 and cIAP2 induce robust RIP2 ubiquitination, TRAF6 did not. We, and others, have previously shown that TRAF6 binds to RIP2 and that TRAF6 is highly ubiquitinated upon NOD activation (Abbott et al., 2004; LeBlanc et al., 2008). It is conceivable that the reduction of RIP2 ubiquitination observed by Yang et al. (2007) in TRAF6-depleted cells represents a reduction in the amount of polyubiquitinated TRAF6 coimmunoprecipitated with RIP2. cIAP1 and cIAP2 were originally identified as TRAF1- and TRAF2-binding partners (Rothe et al., 1995), and one study shows that TRAF2 promotes the interaction of cIAP2 with RIP1 (Park et al., 2004). It is therefore possible that TRAF2, TRAF5, and TRAF6 do not function as E3 enzymes targeting RIP2 but rather serve as adaptor proteins that function in a cell-specific manner to facilitate the interaction of cIAP1 and cIAP2 with RIP2. This may explain the requirement of TRAF6, TRAF2, and TRAF5 for NOD-induced NF-κB activation in RAW cells and MEFs.

cIAP1 and cIAP2 were originally identified as antiapoptotic proteins, and we therefore determined whether they contribute to viability in cells exposed to NOD ligands. NOD agonists had no effect on the survival of wild-type, *Birc2*$^{-/-}$, or *Birc3*$^{-/-}$ BMDMs examined in vitro. Similarly, the survival of macrophages in the peritoneum of wild-type or *Birc3*$^{-/-}$ mice was not altered by MDP injection (data not shown).

The majority of polyubiquitin chains produced in vivo are either K48 or K63 linkages and the fate of polyubiquitinated proteins is in large part determined by the nature of these linkages. Generally, addition of K48 polyubiquitin chains allows recognition and degradation by the proteasome, whereas K63 ubiquitination has emerged as a key regulatory event that conveys signals from receptors to intracellular kinase cascades. K63 ubiquitin chains

on RIP1 and RIP2 facilitate binding of TAB adaptor proteins, the recruitment of the TAK1 kinase to the complex, and activation of MAPK and NF-κB pathways. Upon cell stimulation, RIP1 is initially K63 polyubiquitinated but with time, this is converted to K48-linked polyubiquitination through the action of the A20 protein (Newton et al., 2008). Importantly, cIAP2 increases K48 and K63 ubiquitination of RIP2, and cells lacking cIAP1 or cIAP2 fail to activate NOD-induced MAPK and NF-κB activation. Previous studies from ourselves and others have demonstrated that IAPs can function as K48 or K63 E3 ligases, depending on target substrates and the availability of specific E2s (Bertrand et al., 2008; Park et al., 2004). It is thus possible that cIAP1 and cIAP2 directly mediate K48 and K63 ubiquitination of RIP2. Alternatively, the cIAPs may only induce K63 ubiquitination, which is subsequently edited to K48 by A20 or other enzymes. The rapid K63 deubiquination of RIP2 controls the duration and strength of inflammatory and antimicrobial responses. Indeed, targeted gene deletion of either of the two known K63 deubiquitinases, A20 and CYLD, results in uncontrolled inflammation and inflammatory bowel disease (Abbott et al., 2007; Zhang et al., 2006).

The activities of cIAP1 and cIAP2 are largely, but not entirely, overlapping. For example, in HT29 cells, knockdown of cIAP1 sharply reduced DAP-induced production of TNFα and MCP-1 but had no effect on IL-8 production. The depletion of cIAP2, on the other hand, inhibited production of all three cytokines. Correspondingly, p38 activation induced by DAP was blocked by cIAP2 depletion but not by cIAP1 depletion, raising the possibility that p38 activation is crucial for IL-8 mRNA production and/or stabilization (Berube et al., 2009; Winzen et al., 1999). The molecular basis for differences in downstream signaling activities supported by cIAP1 versus cIAP2 is not known at present. However, it has been recently shown that regulation of p38 activation downstream of NOD signaling is distinct from that of NF-κB and JNK pathways, in that it involves CARD9 and MEKK4. Both *Card9*$^{-/-}$ macrophages and *Map3k4*$^{-/-}$ cells (MEFs or T cells) show defective p38 activation in response to Nod stimulation and retain normal NF-κB and JNK signaling (Abell et al., 2005; Chi et al., 2005; Hsu et al., 2007). It will be therefore important to explore whether cIAP1 and cIAP2 differentially interact with CARD9 and MEKK4 to coordinate NOD signaling. Intriguingly, a recent report has shown that mice lacking the IAP family member Xiap are resistant to infection with *Listeria monocytogenes*, raising the possibility that other IAPs may participate in these pathways (Bauler et al., 2008).

Mutations in NOD1 and NOD2 genes are linked to inflammatory bowel diseases and polymorphisms in the *NOD2* gene are tightly associated with Crohn's disease (CD) and Blau's syndrome in human patients (Tattoli et al., 2007). The *NOD2* polymorphisms in CD are believed to confer NOD2 loss of function, yet CD is associated with production of IL-1β, IL6, IL-12, and TNFα, which are inflammatory cytokines derived from genes that are strongly upregulated by NF-κB. Loss of NOD2 function was hypothesized to result in bacterial overgrowth and spontaneous colitis, but Nod2-deficient mice do not show any sign of spontaneous intestinal inflammation and therefore do not support this hypothesis (Kobayashi et al., 2005). Chronic NOD activation leads to tolerance and crosstolerance to bacterial products in the intestine (Hedl et al., 2007; Kullberg et al., 2008), and NOD2 stimulation by MDP protects mice from experimental colitis by attenuating TLR responses (Watanabe et al., 2008). Therefore, an alternative hypothesis is that under normal circumstances, NOD proteins regulate innate responses to intestinal microflora by suppressing TLR responses and that absence of this negative crossregulation increases susceptibility to Crohn's disease. Interestingly, in the DSS model of experimental colitis, *Birc3*$^{-/-}$ mice do not display the MDP-induced protection that is observed in wild-type mice. This suggests that IAPs may be part of a signaling complex that normally helps balance innate immune signaling responses to intestinal microflora. The identification of cIAP1 and cIAP2 as regulators of the innate immune response provides new therapeutic opportunities for the treatment of immunologic and inflammatory diseases.

EXPERIMENTAL PROCEDURES

Animal Strains

Birc2$^{-/-}$, *Birc3*$^{-/-}$, and *Ripk2*$^{-/-}$ animals have been previously described (Conte et al., 2006; Conze et al., 2005; Kobayashi et al., 2005). All these mice lines were maintained in a pure C57Bl/6J genetic background and all animal procedures were performed under guidelines of the animal ethics committees of McGill University (Canada).

Reagents

Cell-culture supplies were obtained from Hyclone. Antibodies for pJNK (cat# 9255), pp38 (cat# 9211), p38 (cat# 9212), and pIKKa/b (cat# 2697) were purchased from Cell Signaling Technologies and those for actin (cat# 69100) were from MP Biomedicals. The monoclonal antibodies directed against ubiquitin (cat# MMS-257P) and HA (cat# MMP-101P) were obtained from Covance; polyclonal and monoclonal antibodies against RIP2 were obtained from Santa Cruz Biotechnology (cat# sc-22763) and BD Bioscience (cat# 612349), respectively. TRAF6 antibodies were obtained from Stressgen (cat# AAP-426). The RIAP1 antibody used for detecting murine cIAP1 and cIAP2 was previously described (Bertrand et al., 2008), and cIAP1- and cIAP2-specific antibodies were obtained from R&D systems (cat# AF8171 and AF8181). JNK1 (cat# sc-474) and IkBα (cat# sc-969) antibodies were purchased form Santa Cruz Biotechnology; the Flag antibody was obtained from Sigma (cat# F3165), and the 9E10 monoclonal antibody directed against Myc was produced in the lab. Antibodies for NOD2 were obtained from D. Podolsky (Harvard Medical School, USA). Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories. siRNA directed against human cIAP1, cIAP2, and RIP2 and corresponding control RNAs were all Stealth siRNAs purchased from Invitrogen Canada. siRNA targeting two distinct regions of the mRNA were chosen and typically used in parallel experiment together with matched controls. Specific siRNA sequences are available on request. Tri-DAP, L-Ala-γ-D-Glu-mDAP (cat# 60774), was from Anaspec; and MDP, Ac-muramyl-Ala-D-Glu-NH2 (cat# G-1055), was from Bachem. DOTAP transfection reagent (cat# 11 202 375 001) was from Roche, and DSS, dextran sulfate sodium (MW 36,000-50,000), was from MP Biomedicals (cat# 160110). The Milliplex kit for IL-6, KC, and MCP-1 was purchased from Millipore (cat# MPXMCYTO-70K), and Serum Amyloid A (SAA) ELISA was from Biosource (cat# KMA0011). The plasmid expressing Myc-tagged human RIP2 was a kind gift from M. Chao (NYU School of Medicine, USA); those encoding HA-tagged WT, K48, and K63 ubiquitin were obtained from Y. Zhang (Howard Hughes Medical Institute, USA). We obtained Flag-tagged NOD2 construct by inserting a Flag sequence in frame with the human NOD2 sequence contained within a pCDNA3 plasmid originally produced by G. Nunez (University of Michigan, USA). The E3 inactive forms of cIAP1 and cIAP2 (producing H588A and H574A substitutions, respectively) were obtained with a site-directed mutagenesis kit from Stratagene (cat# 200519-5) and cloned, in parallel with the E3 active forms, into pDEST12.2. The plasmid expressing Myc-tagged TRAF6 was kindly provided by S. Baksh (University of Alberta, Canada). The construct expressing Flag-tagged RIP2 was a kind gift of G. Nunez (University of Michigan); Flag-RIP2-CARD and Flag-RIP2-ΔCARD

were obtained by PCR subcloning into pcDNA3.1 with the following primers: 5′-GCGG CTC GAG TTA CAT GCT TTT ATT TTG AAG-3′ (forward) and 5′-GCGG AAGCTT CCACC ATG GAT TAC AAG GAT GAC GAC GAT AAG CCT GGT ATA GCC CAG CAG-3′ (reverse), and 5′-GCGG CTCGAG TTA CTG CAG ACG TTC TGA GTT TCC-3′ (forward) and 5′-GCGG AAGCTT CCACC ATG GAT TAC AAG GAT GAC GAC GAT AAG GAT CAC AAG ACC ACT CC-3′ (reverse), respectively. The plasmid expressing Myc-tagged cIAP2 was obtained from R. Korneluk (University of Ottawa, Canada); Myc-cIAP2-BIR and Myc-cIAP2-CARD-RING were obtained by PCR subcloning with the following primers: 5′-GCGG AAGCTT CC ACC ATG GAA CAA AAA CTT ATT TCT GAA GAA GAT CTG GTA GAA AAC AGC ATA TTC-3′ (forward) and 5′-GCGGC TCGAGTCA GTCTG ATG TGG ATA GCA GCT G-3′ (reverse) and 5′-GCGG AAGCTT CCACC ATG GAA CAA AAA CTT ATT TCT GAA GAA GAT CTG CAG CTG CTA TCC ACA TCA GAC-3′ (forward) and 5′-GCGG CTCGAGTC ATG AAA GAA ATG TAC G-3′ (reverse), respectively.

Cell Culture

HEK293T and HT29 cells were maintained in 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (HyClone) and McCoy's 5A modified medium (Sigma), respectively, supplemented with 10% fetal calf serum (HyClone), 2 mM L-glutamine, and 100 mg/mL penicillin/streptomycin. siRNA were transfected with lipofectamine 2000 (Invitrogen, cat# 11668-19) and plasmid transient expression was obtained with either lipofectamine 2000 or calcium phosphate transfections. We treated HT29 cells for different time points (0, 15 min, 30 min, 45 min, 1 hr, and 2 hr) with Tri-DAP at 10 μg/mL transfected into the cells by using the DOTAP cationic lipid transfection reagent in accordance with the manufacturer's instructions.

Primary Culture of Bone-Marrow-Derived Macrophages

Femurs and tibias of wild-type (C57BL6), *Ripk2*$^{-/-}$, *Birc2*$^{-/-}$, and *Birc3*$^{-/-}$ mice were flushed with a 25G needle and cold RPMI 1640 (GIBCO) into a 15 mL tube. The suspension was then filtered through a 70 μm cell strainer and spun for 5 min at 400 g. Pellets were resuspended in complete RPMI 1640 supplemented with 15% L929 cell-conditioned media and plated in non-cell-culture-treated 10 cm dishes. Cells were cultured at 37°C in a 10% CO2 tissue culture incubator for 5–6 days, with media changed on day 3. MDP (at 10 or 100 μg/mL) was transfected into cells for different time points (0, 15 min, 30 min, 45 min, 1 hr, or 2h) with DOTAP according to the manufacturer's instructions.

Quantitative Real-Time PCR

Total RNA was extracted from cells with the Trizol reagent (Invitrogen), and this was followed by isopropanol precipitation. We reverse-transcribed 2 μg total RNA to cDNA by using random hexamers and the M-MLV Reverse transcriptase (Invitrogen) in a volume of 20 μl according to the manufacturer's protocol. Quantitative RT-PCR primers for different cytokines and chemokines used in this study are described in Table S1 and S2.

Immunoblotting, Immunoprecipitation, and Pull-Down Experiments

Cells were lysed either directly in Laemmli sample buffer, in RIPA lysis buffer (10 mM Tris [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, and 0.5% deoxycholate, supplemented with a protease inhibitor tablet [Roche]), in NP40 buffer (10 mM Tris [pH 8.0], 150 mM NaCl, and1% Nonidet P-40, supplemented with a protease inhibitor tablet [Roche]), or in buffer B150 (20 mM Tris-HCl [pH 8.0], 150 mM KCl, 10% glycerol, 5 mM MgCl2, and 0.1%). SDS-polyacrylamide gel electrophoresis and immunoblotting were performed in accordance with standard protocols. Direct immunoprecipitations were performed in RIPA; coimmunoprecipitations were performed in NP-40 or B150 buffer and pull-down assays were performed in NP40 buffer. Myc-tagged RIP2 was produced in vitro with a TnT T7 quick coupled transcription and translation system (Promega Cat# L1170) and GST, GST-cIAP1, and GST-cIAP2 fusion proteins were produced in bacteria from pGEX4T2 constructs.

Intraperitoneal Injection of MDP and DAP

Wild-type, RIP2, and ciap2-deficient mice were injected intraperitoneally with 2 mg/kg of DAP. Twenty-four hours after injection, infiltrating cells were collected in PBS by peritoneal lavage, counted, and labeled for 30 min in the dark with Gr-1 antibodies coupled with Alexa Fluor 488 (Serotec). Gr-1-positive cells were enumerated by FACSCalibur flow cytometery. DAP (2 mg/kg) or MDP (25 mg/kg) were injected intraperitoneally (i.p.). Blood was collected by heart puncture 2 hr or 4 hr, respectively, after agonist injection. Serum was used to quantify IL-6, KC, and MCP-1 levels by bead-based immunoassays with a kit from Lincoplex (Millipore).

DSS Colitis

C57BL6, Rip2-deficient, and ciap2-deficient mice were given 4% DSS (MW 36,000-50,000; MP Biomedicals) in drinking water for 6 days (days 0–5) and then placed on regular water for 3 days (days 6–8). Mice were administered MDP (100 μg, i.p.) or PBS early during colitis on days 0, 1, and 2. Mice were monitored daily for body weight loss. Blood was collected by heart puncture on day 8 and serum was used to quantify serum amyloid A (SAA) levels by ELISA (Biosource).

Histopathological Analysis and Immunohistochemistry

Colons were fixed in 10% formalin and embedded in paraffin. Serial 5-μm-thick sections were cut onto glass slides and stained with hematoxylin and eosin (H&E). Sections were blindly assessed for signs of colitis: tissue morphology, epithelial erosion, loss of goblet cells, loss of crypts, presence of inflammatory infiltrate, and thickening of the colon wall.

Statistical Analysis

Two-tailed Student t test was used to evaluate significant differences among two groups. In Figures 1, 2, and 5, two-tailed Student's t test was used for statistical analysis of cytokine and chemokine production comparing the agonist-treated group of each deficient genotype to the agonist-treated group of wild-type controls. In Figure 6, two-tailed Student's t test was used for statistical analysis of SAA levels, comparing the PBS-treated group to the MDP-treated group within each genotype. One-way ANOVA was used for evaluating significant differences among multiple groups in Figure 6A. Two-tailed Student's t test was also used to compare PBS-treated group to the MDP-treated group within each genotype.

SUPPLEMENTAL DATA

Supplemental Data include two figures and two tables and can be found with this article online at http://www.cell.com/immunity/supplemental/S1074-7613(09)00203-9.

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