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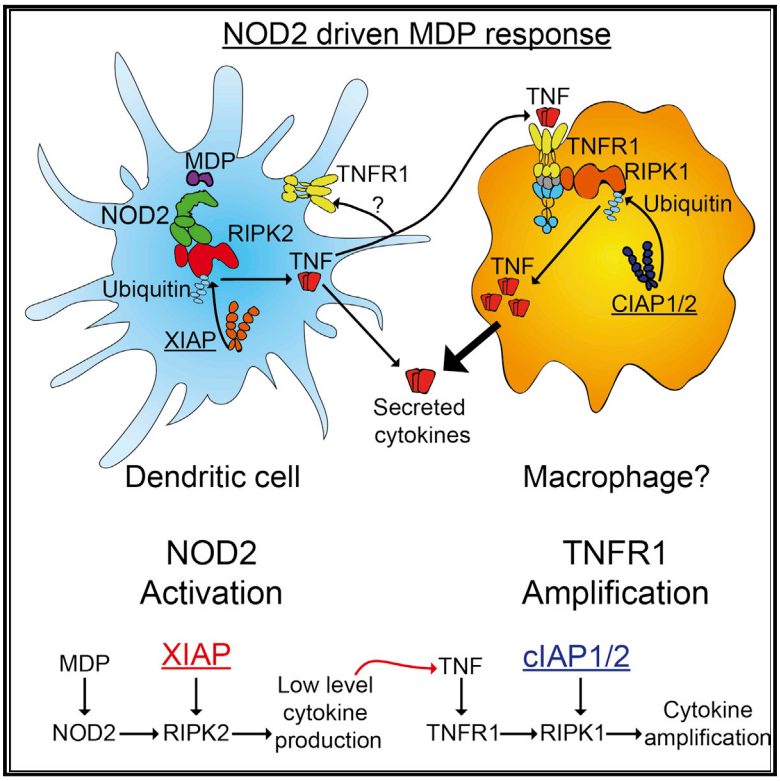
IAPs Regulate Distinct Innate Immune Pathways to Co-ordinate the Response to Bacterial Peptidoglycans

Graphical Abstract



Highlights

- XIAP is the only IAP required for NF-κB and MAPK activation downstream of NOD2
- NOD2-driven cytokine production relies on a TNF-dependent amplification loop
- Dendritic-like cells are the first responders to MDP stimulation *in vivo*

Authors

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In Brief

Stafford et al. show that XIAP is the only IAP required for the initial RIPK2/NOD2-dependent response to MDP, while autocrine TNF is required to amplify cytokine production in a cIAP1-dependent manner.

Stafford et al., 2018, Cell Reports *22*, 1496–1508
February 6, 2018
https://doi.org/10.1016/j.celrep.2018.01.024

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IAPs Regulate Distinct Innate Immune Pathways to Co-ordinate the Response to Bacterial Peptidoglycans

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https://doi.org/10.1016/j.celrep.2018.01.024

SUMMARY

Inhibitors of apoptosis (IAPs) proteins are critical regulators of innate immune signaling pathways and therefore have potential as drug targets. X-linked IAP (XIAP) and cellular IAP1 and IAP2 (cIAP1 and cIAP2) are E3 ligases that have been shown to be required for signaling downstream of NOD2, an intracellular receptor for bacterial peptidoglycan. We used genetic and biochemical approaches to compare the responses of IAP-deficient mice and cells to NOD2 stimulation. In all cell types tested, XIAP is the only IAP required for signaling immediately downstream of NOD2, while cIAP1 and cIAP2 are dispensable for NOD2-induced nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) activation. However, mice lacking cIAP1 or TNFR1 have a blunted cytokine response to NOD2 stimulation. We conclude that cIAPs regulate NOD2-dependent autocrine TNF signaling *in vivo* and highlight the importance of physiological context in the interplay of innate immune signaling pathways.

INTRODUCTION

Nucleotide-binding oligomerization domain-containing (NOD) receptors NOD1 and NOD2 are intracellular sentinels for Gram-positive and Gram-negative bacteria. NOD1 and NOD2 recognize the bacterial cell wall peptidoglycan components γ-D-Glu-m diaminopimelic acid (DAP) and muramyl dipeptide (MDP), respectively (Chamaillard et al., 2003; Girardin et al., 2003; Inohara et al., 2003). NOD1 and NOD2 play an essential role in the clearance of bacterial pathogens, including *Bacillus subtilis*, *Listeria monocytogenes*, and *Mycobacterium tuberculosis* (Lee et al., 2016; Jeong et al., 2014; Inohara et al., 2003). Engagement of NOD1 or NOD2 drives a conformational change that allows ATP-dependent self-oligomerization via its NAIP, C2TA, HET-E, and TP-1 (NACHT) domain and recruitment of downstream components (Maharana et al., 2015b). The discovery of peptidoglycan-independent activators of the pathway, such as endoplasmic reticulum (ER) stress or small Rho guanosine triphosphatases (GTPases) (Keestra and Bäumler, 2014; Keestra-Gounder et al., 2016), suggest a role for the NOD signaling pathway in scenarios other than bacterial recognition.

Inhibitors of apoptosis (IAPs) were initially thought to exert their main activity by binding and inhibiting caspases (Eckelman et al., 2006). However, it has become clear that another important role is to regulate the activation of nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways via their E3 ubiquitin ligase activity and thereby regulate cell death indirectly (Li et al., 2002; Yang et al., 2000). The best-studied ubiquitin-dependent signaling pathway involving IAPs is signaling via tumor necrosis factor (TNF) receptor 1 TNFR1, in which cellular IAP1 and IAP2 (cIAP1 and cIAP2) ubiquitylate receptor-interacting protein kinase 1 (RIPK1) to facilitate activation of NF-κB and limit the formation of a death-inducing signaling complex (Varfolomeev et al., 2008; Mahoney et al., 2008; Feltham et al., 2010). In addition to their roles in TNFR1 signaling, cIAP1 and cIAP2, as well as X-linked IAP (XIAP) have been reported to be required for NOD signaling (Krieg et al., 2009; Bertrand et al., 2009; Damgaard et al., 2013). All of these E3 ligases are able to bind to the kinase domain of receptor-interacting protein kinase 2 (RIPK2), a vital adaptor of the NOD signaling pathway, via their respective baculovirus IAP repeat 2 (BIR2) domains (Bertrand et al., 2011).

With their central role in promoting cell survival, it comes as no surprise that some cancers express IAPs at high levels. Well-tolerated drugs with differential activity against IAPs have been developed and trialed in the clinic to treat both cancer and infectious diseases (Fulda, 2015; Ebert et al., 2015). It is therefore relevant to understand the precise roles of individual IAPs during homeostasis and disease and in particular during NOD signaling for which IAP activity is required.

RIPK2 is the first component recruited to NOD1 or NOD2 via homotypic caspase recruitment domain (CARD)-CARD interactions (Maharana et al., 2015a, Inohara et al., 2000). RIPK2 has an N-terminal serine/threonine/tyrosine kinase domain with high homology to other RIPKs and a C-terminal CARD domain, linked by an intermediate domain of unknown function (Inohara et al., 1998). RIPK2 is an essential adaptor molecule in the NOD signaling pathway and drives NF-κB and MAPK activation (Park et al., 2007; Chin et al., 2002), resulting in the

transcriptional upregulation and release of pro-inflammatory cytokines (Ogura et al., 2001). The kinase activity of RIPK2 has been reported to be essential for NOD signaling; however, its downstream substrates in the NOD pathway have not been identified (Dorsch et al., 2006; Tigno-Aranjuez et al., 2010). After recruitment to NOD1 or NOD2, RIPK2 dimerizes and autophosphorylates on Ser176 and is ubiquitylated on Lys209 (Hasegawa et al., 2008; Pellegrini et al., 2017). All these steps are required for the recruitment of downstream signaling components. Upon NOD activation, RIPK2 is modified with Lys63 and Met1 polyubiquitin chains (Hrdinka et al., 2016), and several E3 ligases, including IAPs (Bertrand et al., 2011; Krieg et al., 2009), the linear ubiquitin assembly complex (LUBAC) (Damgaard et al., 2012), Pellino3 (Yang et al., 2013), and ITCH (Tao et al., 2009), have been implicated in this process.

The requirement of individual IAPs for signaling downstream of NOD2 is controversial. It was shown that cIAP1 and cIAP2 are required for signaling downstream of NOD2 in bone marrow-derived macrophages (BMDMs) from mice (Bertrand et al., 2009). In addition, we and others have shown that XIAP was essential for NOD signaling in several cellular systems (Krieg et al., 2009; Damgaard et al., 2012). Reduced ubiquitylation of RIPK2 occurred in the absence of XIAP and depended on cIAPs, because treatment of XIAP-deficient cells with the IAP antagonist LBW-242 abrogated RIPK2 ubiquitylation (Damgaard et al., 2012). Treatment of wild-type cells with LBW-242 had only a minor effect on RIPK2 ubiquitylation. These results suggest that XIAP is the predominant E3 ligase regulating NOD signaling; however, the relative importance of XIAP, cIAP1, and cIAP2 have not been studied in an endogenous and side-by-side setting that takes into account the major cell types that respond to the initial bacterial stimulus.

Underpinning the importance of such a study, we observed that genetic removal of cIAP1 and cIAP2 had no impact on RIPK2 ubiquitylation or early activation of NF-κB and MAPK after NOD stimulation. The cytokine response of BMDMs was also unaffected by loss of cIAPs, yet cIAP1-deficient mice and dendritic cells were compromised. Using *Tnfr1*$^{-/-}$ mice, we show that the reduced response was due to a TNF-TNFR1 amplification loop in which cIAP1 and cIAP2 play essential roles.

RESULTS

cIAP1 and cIAP2 Are Dispensable for NOD2-Induced NF-κB and MAPK Activation

Given the reported roles of IAPs for signaling downstream of NOD receptors, we predicted that BMDMs deficient in XIAP, cIAP1, or cIAP2 would exhibit defective activation of NF-κB and MAPK upon MDP stimulation. To explore, this we generated BMDMs from wild-type, *clap1*, *clap2*, *Xiap*, and *Ripk2* knockout mice. Wild-type BMDMs primed with interferon-γ (IFNγ) activated NF-κB and MAPK in response to MDP, and neither RIPK2- nor XIAP-deficient cells responded in this way (Figure 1A). However, cIAP1- or cIAP2-deficient cells displayed normal levels of NF-κB activation, measured by phosphorylation of the NF-κB subunit p65 or degradation of IκBα. Markers of MAPK activation, such as phosphorylation of p38 and MK2, were also normal (Figure 1A). Although these results contradict previously published results (Bertrand et al., 2009), we suspected that cIAP1 and cIAP2 played redundant roles. To test this hypothesis, we generated BMDMs from mice lacking cIAP2 ubiquitously and cIAP1 in myeloid cells (Clausen et al., 1999; Wong et al., 2014), because *clap1.clap2* double-knockout mice are embryonic lethal (Moulin et al., 2012). In BMDMs from these mice, cIAP1 levels were significantly reduced but not absent (Figure 1B). Even in the absence of cIAP2 and marked reduction in cIAP1 levels, signaling downstream of NOD2 occurred normally in BMDMs independently generated from three mice. Additional deletion of XIAP rendered cells unresponsive to MDP stimulation. One difference between our results and previously reported findings is the protocol for NOD2 stimulation: we primed our BMDMs with IFNγ to allow uptake of MDP and avoid MDP transfection. To examine whether IFNγ stimulation resulted in a change in the dependence of cIAPs, we used L18-MDP, a cell-permeable form of MDP, to activate the NOD2 pathway. However, as before, *clap1*, *clap2*, and even *clap1.clap2* double-knockout BMDMs responded like wild-type cells (Figure S1).

To examine the role of IAPs in NOD signaling in human cells, we generated clones from the human monocytic cell line THP-1 that were deficient in cIAP1, cIAP2, or XIAP using CRISPR/Cas9. As in BMDMs, *cIAP1* and *cIAP2*, as well as *cIAP1.cIAP2* double-knockout THP-1 cells, displayed normal markers of NF-κB and MAPK activation after NOD2 stimulation with L18-MDP (Figure 1C). However, in XIAP-deficient cells, phosphorylation of p65 and p38 was almost absent. The slight activation can be accounted for by the nonspecific activation by L18-MDP that we previously reported (Nachbur et al., 2012).

XIAP and RIPK2 Is Polyubiquitylated upon NOD2 Stimulation

Upon NOD stimulation, RIPK2 is ubiquitylated with Lys63- and Met1-linked chains, and cIAP1, cIAP2, and XIAP have all been shown to be able to ubiquitylate RIPK2 (Hrdinka et al., 2016; Bertrand et al., 2011). To interrogate ubiquitylation events upon NOD2 stimulation, we isolated ubiquitylated proteins from THP-1 cells using tandem ubiquitin binding entities (TUBEs) after L18-MDP stimulation and analyzed ubiquitylation of RIPK2, XIAP, and cIAP1 by western blot (Figures 2A and 2B). Together with the strong ubiquitylation of RIPK2 that occurred 20 min after MDP addition, we detected a time-dependent increase in ubiquitylation of XIAP (Figure 2A). The strongest ubiquitylation of both RIPK2 and XIAP occurred 30 min after stimulation, and this gradually declined over 2 hr (Figure 2B).

Because NOD2-dependent XIAP ubiquitylation has not been previously reported, we sought to determine the ubiquitin chain types that occurred on XIAP after stimulation of NOD2. We stimulated THP-1 cells for 30 min with L18-MDP and purified ubiquitylated proteins using glutathione S-transferase (GST)-ubiquitin associated domain (UBA) bound to Sepharose beads (Fiil et al., 2013) and performed a ubiquitin chain restriction assay (Hospenthal et al., 2015) using linkage-specific recombinant deubiquitinases (DUBs) (Figure 2C). The non-selective DUB, USP2, removed the

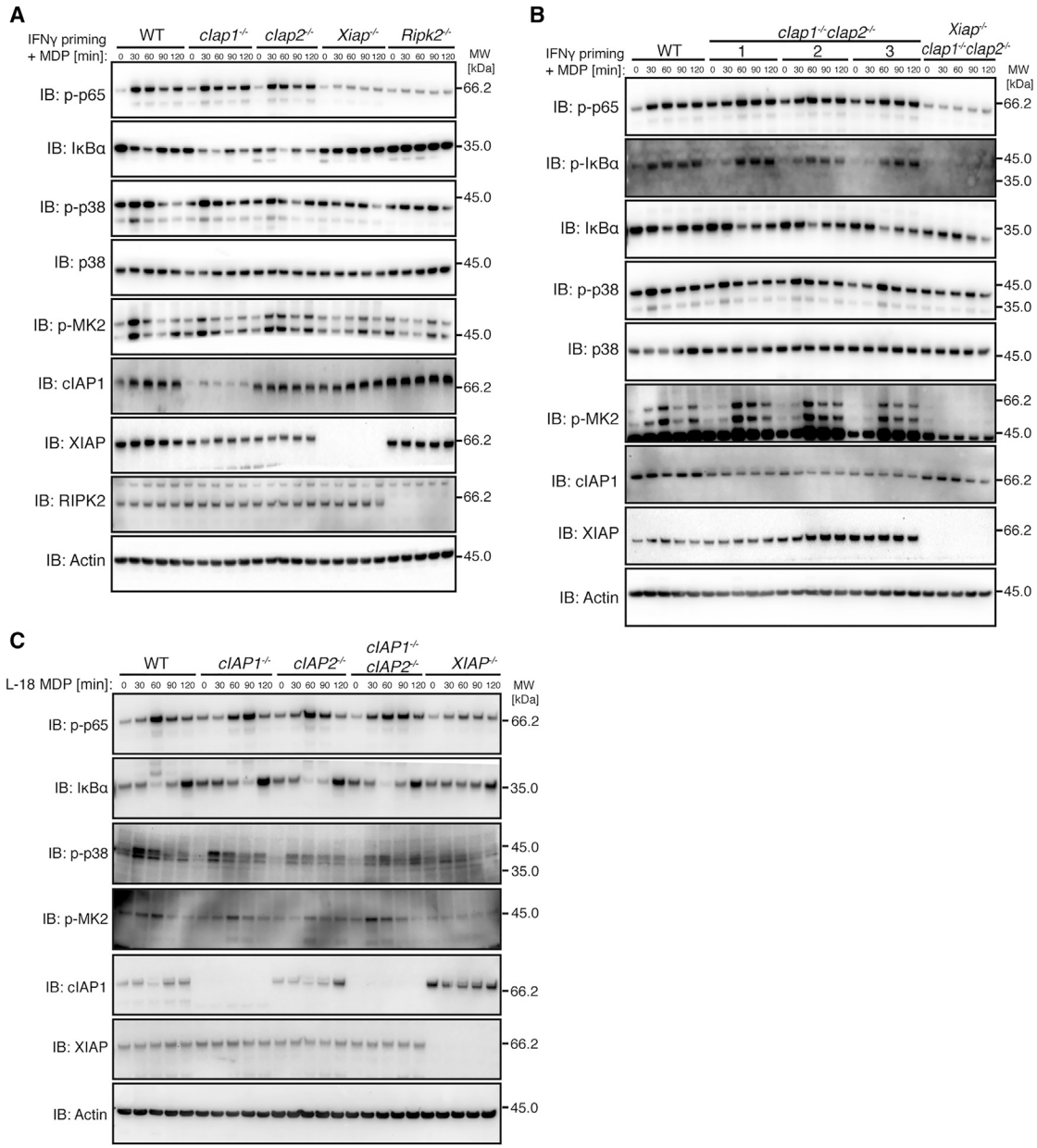

Figure 1. XIAP Is Essential, but cIAP1 and cIAP2 Are Not Required, for NOD2 Signaling

(A) Interferon-γ (IFNγ)-primed (5 ng/mL, 2 hr) BMDMs from wild-type (WT), *clap1*, *clap2*, *clap1.clap2*, *Xiap*, or *Ripk2* knockout mice were either left untreated or treated with MDP (10 μg/mL) for the indicated times. Cell lysates were separated on SDS-PAGE gels, western blotted, and probed with the indicated antibodies. Data are representative of at least three independent experiments.

(B) BMDMs were generated from three mice to generate independent *clap1.clap2* double-knockout cells and treated as in A. BMDMs from one WT and one XIAP$^{-/-}$ mouse were loaded on the same gel as controls.

(C) WT and IAP-deficient THP-1 knockout cells were either left untreated or treated with L-18 MDP (200 ng/mL) for the indicated times. Western blots of cell lysates were probed with the indicated antibodies. A representative experiment of three independent repeats is shown.

See also Figure S1.

laddering of RIPK2 and XIAP and collapsed the high-molecular-weight bands to a single band, confirming that the higher-molecular-weight forms of these proteins are ubiquitylated (Figure 2C, lane 1). The Lys63-specific DUB AMSH needed to be combined with the Met1/linear-specific OTULIN to observe a significant shift in the ubiquitylation pattern of RIPK2. In contrast, the ubiquitylation pattern on XIAP was altered in the presence of AMSH alone, and addition of OTULIN made no difference (Figure 2C, lane 9 versus lanes 2 and 8). No other linkage-specific DUB made a notable

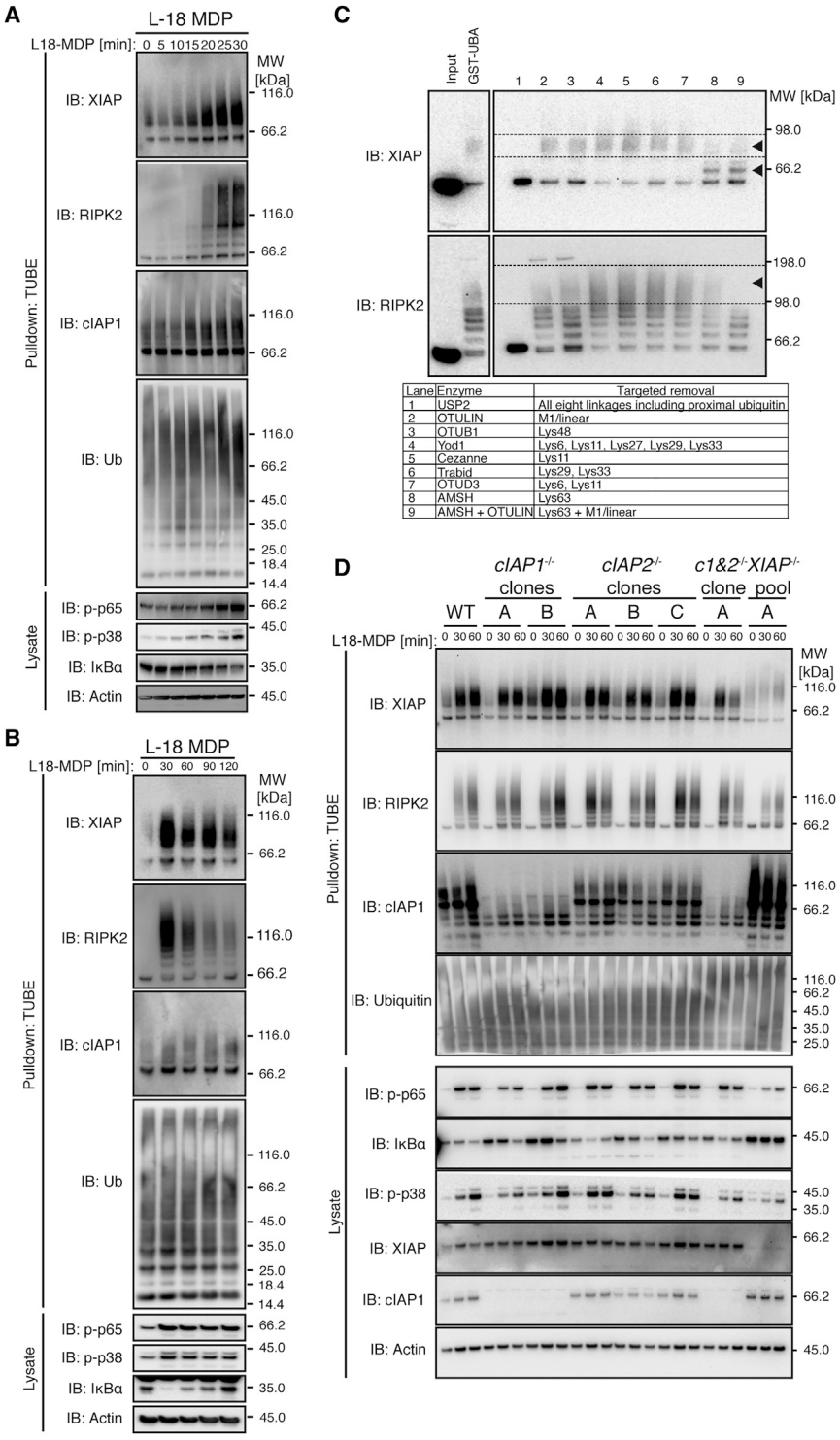

Figure 2. RIPK2 and XIAP Are Ubiquitylated upon NOD2 Signaling

(A and B) THP-1 cells were stimulated for a short (A) or long (B) time course with L18-MDP (200 ng/mL). Endogenous ubiquitylated proteins were purified using TUBEs separated on SDS-PAGE gels, western blotted, and probed using the indicated antibodies. A representative experiment of three independent repeats is shown.

(C) THP-1 cells were stimulated with L18-MDP (200 ng/mL), and ubiquitylated proteins were isolated using GST-UBA. Ubiquitin linkages were determined using specific DUBs. Lane numbers on autoradiographs correspond to the table indicating the specific DUBs used. One experiment from four independent repeats is shown.

(D) Wild-type, *cIAP1*, *cIAP2*, *cIAP1.cIAP2*, or *XIAP* knockout THP-1 cells were either left untreated or treated with L18-MDP (200 ng/mL) for the indicated times. Ubiquitylated proteins were isolated using TUBEs and analyzed as before. Data are representative of three independent experiments. See also Figure S2.

cIAP1 and cIAP2 Are Not Involved in NOD2-Dependent Ubiquitylation of RIPK2 or XIAP

To determine whether cIAPs are involved in NOD2-induced RIPK2 or XIAP ubiquitylation in human cells, we stimulated THP-1 cells deficient in cIAP1, cIAP2, cIAP1&2, or XIAP with L18-MDP and isolated ubiquitylated proteins using TUBEs. As before, we observed an increase in ubiquitylation of RIPK2 and XIAP in wild-type cells, while cIAP1 remained unmodified (Figure 2D). Equivalent ubiquitylation patterns for RIPK2 and XIAP were observed in *cIAP1*$^{-/-}$, *cIAP2*$^{-/-}$, and *cIAP1*$^{-/-}$.*cIAP2*$^{-/-}$ cells compared to wild-type cells. RIPK2 ubiquitylation was strongly reduced, and high-molecular-weight species were lost in *XIAP*$^{-/-}$ THP-1 cells (not a clonal population). This was repeated using a clonal *XIAP*$^{-/-}$ cell line, with a more pronounced reduction in RIPK2 ubiquitylation and decreased NF-κB and MAPK activation (Figure S2).

Pharmacological Targeting of XIAP, but Not cIAP1 or cIAP2, Impairs NOD2 Signaling

Based on our observations with knockout cells, we hypothesized that small molecules targeting cIAP1 and cIAP2 would have no effect, while inhibitors that targeted XIAP would greatly reduce NOD2 signaling. To test this, THP-1 cells were stably infected with a lentiviral NF-κB GFP reporter and pretreated for 30 min with limiting doses of birinapant (inhibits

change to the ubiquitylation pattern of XIAP. These results show that XIAP is modified with Lys63-linked ubiquitin chains after MDP stimulation, suggesting a role as a signaling platform, together with RIPK2, to assemble further components that regulate signaling downstream of NOD2.

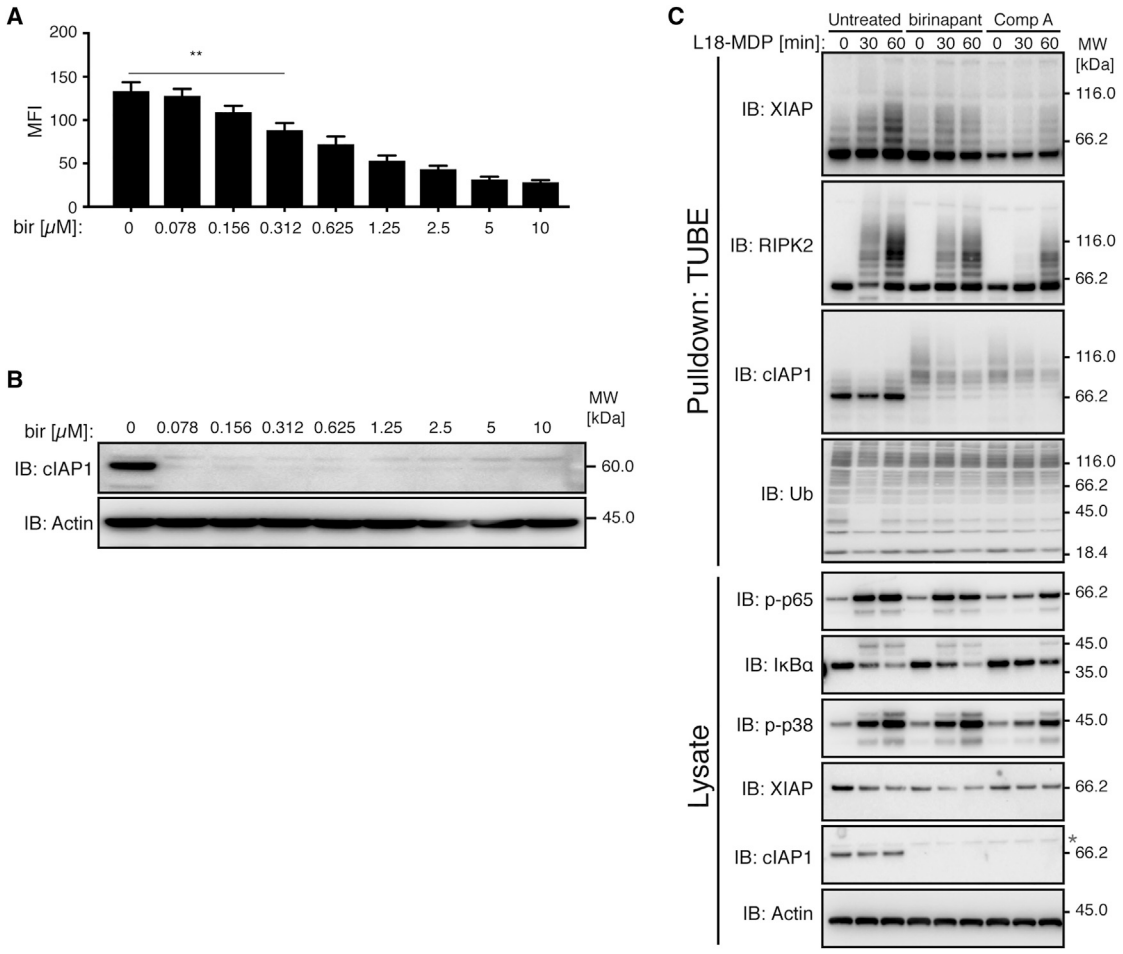

Figure 3. Targeting of XIAP, but Not cIAP1 and cIAP2, Impairs NOD2 Signaling

(A) THP-1 NF-κB GFP reporter cells were either left untreated or pretreated for 30 min with the indicated doses of birinapant before the addition of L18-MDP (200 ng/mL). GFP mean fluorescent intensity (MFI) was measured after 7 hr using flow cytometry (n = 4). Error bars are SEM.

(B) WT THP-1 cells were treated with the indicated doses of birinapant for 7 hr, and cell lysates were analyzed by western blot. A representative of three experiments is shown.

(C) THP-1 cells were either left untreated or pretreated with 500 nM of the specified Smac-mimetic compounds for the indicated times. Lysates were analyzed as before with the indicated antibodies. A representative experiment from three independent repeats is shown.

See also Figure S3.

cIAP1 with ~1 nM K_i but is 30- and 50-fold less effective against cIAP2 and XIAP, respectively) (Condon et al., 2014), before stimulation with L18-MDP for 7 hr (Figures 3A and 3B). The cIAP1 levels in THP-1 cells treated with the same doses of birinapant were assessed in parallel by western blot. The lowest concentration of birinapant tested, 78 nM, was sufficient to degrade cIAP1 within 30 min (Figure 3B). At this concentration, no significant decrease in GFP, the measure of NOD-induced NF-κB activation, was observed. Birinapant only reduced L18-MDP-induced GFP levels at concentrations at which partial inhibition of XIAP is expected (312 nM and above). At these concentrations, birinapant did not reduce cell viability or activate NF-κB (Figures S3A and S3B). To compare the effects of targeting XIAP more effectively, we pretreated cells with compound A (K_i, in an IAP binding motif peptide binding competition assay for cIAP1, cIAP2, and XIAP of ~1 nM) or birinapant (Condon et al., 2014), stimulated them with L18-MDP for 30 and 60 min, and assayed for XIAP and RIPK2 ubiquitylation and for NF-κB and MAPK activation (Figure 3C). While birinapant-treated cells responded like wild-type cells, compound A treatment resulted in a loss of XIAP ubiquitylation and in delayed and reduced ubiquitylation of RIPK2. Consistently, altered XIAP and RIPK2 ubiquitylation resulted in delayed and reduced NF-κB and MAPK activation.

cIAP1 and TNFR1 Are Required to Amplify the Response to MDP

To test the role of IAPs in NOD2 signaling *in vivo*, we intraperitoneally injected MDP into wild-type, *clap1*, *clap2*, *Xiap*, and *Ripk2* knockout mice and measured cytokine levels in their serum by ELISA. As an additional control, we included *Tnfr1* knockout mice because of the known roles of cIAPs in the TNFR1 signaling pathway. MDP challenge caused a reproducible increase in the

levels of TNF and interleukin (IL)-6 in wild-type mice, which was absent both in *Ripk2* and in *Xiap* knockout mice (Figures 4A and 4B). Intriguingly, given the unambiguous results of our *in vitro* experiments, TNF and IL-6 levels were also significantly reduced in *clap1* knockout mice. We observed similarly reduced levels of TNF and IL-6 in TNFR1-deficient mice.

This result indicated that TNF signaling in response to NOD stimulation was important for the overall cytokine response *in vivo*. To test this *in vitro*, we stimulated wild-type and *Tnfr1*$^{-/-}$ BMDMs with IFNγ and MDP and examined mRNA levels of the cytokines IL-6 and IL-1β (Figures 4C and 4D). We observed no difference in cytokine mRNA levels 1 hr after stimulation, suggesting equivalent activation of the NOD2 pathway. However, at later time points, cytokine mRNA levels were higher in wild-type cells compared to *Tnfr1*$^{-/-}$ cells, supporting a role of TNF amplification in the overall cytokine response to MDP. We also examined markers for NOD2 and TNFR1 complex activation at an early (30 min) and at a late time point (240 min) by western blot (Figure 4E). As before, we isolated ubiquitylated proteins using TUBEs and probed for ubiquitylation of RIPK1, a specific marker of TNFR1 signaling, as well as XIAP and RIPK2, two markers for NOD2 activation. XIAP and RIPK2 were ubiquitylated in wild-type and *Tnfr1*$^{-/-}$ cells after 30 min, correlating with activation of the NF-κB and MAPK pathways (Figure 4E). However, RIPK1 was ubiquitylated much later in wild-type BMDMs, but not in *Tnfr1*$^{-/-}$ BMDMs, and late activation of NF-κB signaling was absent in *Tnfr1*$^{-/-}$ cells (Figure 4E). In THP-1 cells, which secrete minuscule amounts of TNF in response to MDP, we did not observe RIPK1 ubiquitylation at late time points, although these cells are competent to respond to TNF (Figure 4F). These results are consistent with the idea that TNFR1 can be activated by autocrine TNF following NOD stimulation.

To test whether the changes we observed in signaling and cytokine mRNA levels translated into reduced cytokine levels *in vitro*, we measured cytokine secretion from MDP-stimulated peritoneal cells, the first responders to MDP challenge in our *in vivo* model. Mirroring our *in vivo* results, peritoneal cells from wild-type mice secreted TNF and IL-6 in response to MDP, while *Xiap*$^{-/-}$ and *Ripk2*$^{-/-}$ peritoneal cells did not. Furthermore, peritoneal cells from *clap1* or *Tnfr1* knockout mice produced significantly less cytokines than wild-type cells (Figures 5A and 5B). This dependency on TNF signaling was also demonstrated using a TNF blocking antibody (Figure S4). IFNγ treatment affected the quantity, but not the quality, of the response to MDP, suggesting that different treatment conditions (i.e., ±IFNγ) do not alter the dependence of NOD signaling on cIAPs (Figures 5C and 5D). Cell death was not altered among the genotypes following *ex vivo* stimulation (Figure S5).

Dendritic Cells, but Not Macrophages, Require cIAP1 to Fully Respond to MDP

Given this TNF-dependent amplification loop and the known role of cIAPs in TNFR1 signaling, we hypothesized that MDP stimulation should lead to a reduced cytokine response in *clap1*$^{-/-}$ BMDMs. Contrary to our expectations, MDP-induced secretion of TNF, IL-6, and the C-C motif chemokine ligand 2 (CCL2), occurred in wild-type, *clap1*$^{-/-}$, and *clap2*$^{-/-}$ BMDMs but was absent from *Xiap*$^{-/-}$ and *Ripk2*$^{-/-}$ BMDMs (Figures 6A and 6B; Figure S6A). IFNγ priming was necessary, because MDP stimulation alone failed to produce a cytokine response in all BMDMs (Figures S6B and S6C).

This difference in the dependency on cIAP1 for the overall cytokine response between BMDMs and freshly isolated peritoneal cells suggested that responder cells *in vivo* are not equivalent to BMDMs but could be another type of leukocytes. We analyzed the composition of peritoneal cells from unchallenged wild-type and IAP knockout mice to determine whether loss of cIAP1 perturbs peritoneal leukocyte populations. We observed no significant alteration in the proportion of the most prevalent phagocytic cell, the large peritoneal macrophage (LPM) or detect major differences in the proportion of bone marrow-derived small peritoneal macrophages (SPMs) that are known to produce cytokines in response to inflammatory stimuli (Figures 6C and 6D) (Cassado et al., 2015).

The low abundance of SPMs precluded biochemical experiments, so to test the response of an analogous antigen-presenting cell type, we generated bone marrow-derived dendritic cells (BMDCs) from wild-type, *clap1*, *clap2*, *Xiap*, and *Ripk2* knockout mice and compared their response with BMDMs. Consistent with our results from peritoneal cells, wild-type BMDCs were able to generate a robust cytokine response to MDP alone (Figures 6E and 6F), and priming with IFNγ increased the quantity, but did not affect the quality, of the response (Figures 6G and 6H). Furthermore, mirroring our *in vivo* results, TNF and IL-6 levels were significantly decreased in *clap1* knockout BMDCs, while *clap2* deletion had no impact on the MDP cytokine response. However, and consistent with our BMDM results, loss of cIAP1, cIAP2, or TNFR1 had no negative impact on early MDP signaling in BMDCs (Figure 6I). Combining these different experimental strands, these observations suggest that dendritic-type cells are the primary responders to MDP in the peritoneum and that loss of cIAPs does not affect their ability to respond to MDP but does affect a second TNFR1-dependent response.

DISCUSSION

The NOD2 pathway is of great interest due to its potential role in cancer and inflammatory diseases (Udden et al., 2017; Singel et al., 2014; Maekawa et al., 2016). NOD2-induced NF-κB signaling, like that of many other inflammatory receptors, is reliant on post-translational regulation, particularly the phosphorylation and ubiquitylation of its adaptor protein RIPK2 (Dorsch et al., 2006; Pellegrini et al., 2017; Damgaard et al., 2012; Hasegawa et al., 2008). Ubiquitin forms different linkage chains that determine the duration and character of the signaling, and these are usually generated by distinct E3 ligases. This raises the question of how different E3 ligases co-ordinate ubiquitylation in signaling systems like NOD2. Three members of the IAP E3 ligase family, cIAP1, cIAP2, and XIAP, have all been shown to regulate NOD signaling and RIPK2 and are therefore an ideal starting point to address this fundamental question.

However, our genetic and biochemical analysis in human monocytes and murine macrophages did not provide support for the idea that cIAPs directly regulate NOD signaling. XIAP

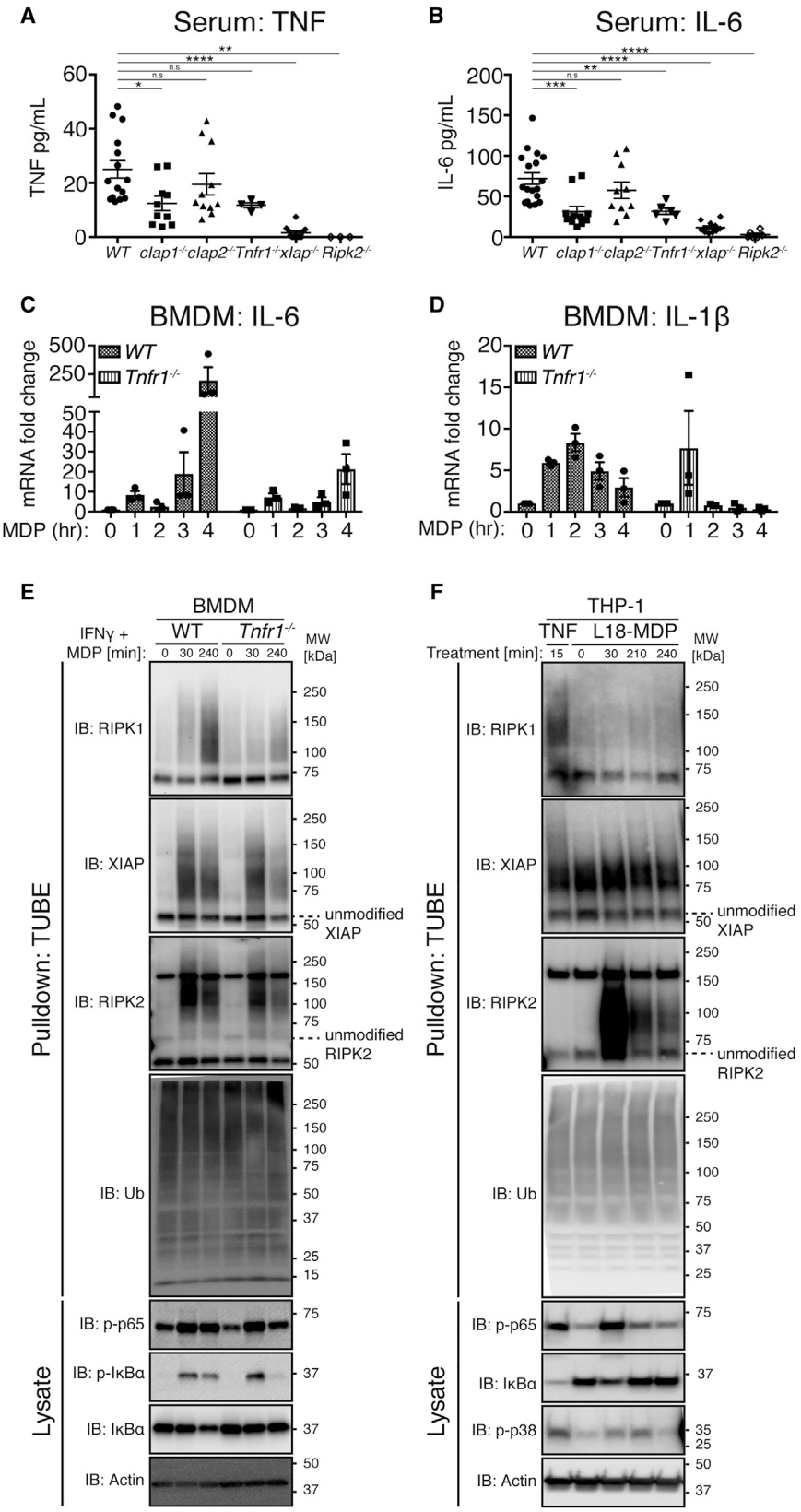

Figure 4. The TNFR1 Pathway Is Required for a Robust Cytokine Response to MDP

(A and B) WT and knockout (KO) mice were injected with MDP (100 μg i.p.), and serum levels of (A) TNF (WT, n = 15; *clap1*$^{-/-}$, n = 10; *clap2*$^{-/-}$, n = 11; *Tnfr1*$^{-/-}$, n = 4; *Xiap*$^{-/-}$, n = 12; *Ripk2*$^{-/-}$, n = 3) and (B) IL-6 (WT, n = 18; *clap1*$^{-/-}$, n = 11; *clap2*$^{-/-}$, n = 10; *Tnfr1*$^{-/-}$, n = 6; *Xiap*$^{-/-}$, n = 11; *Ripk2*$^{-/-}$, n = 6) after 4 hr were measured by ELISA. Bars are average ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; NS, not significant.

(C and D) BMDMs from WT and *Tnfr1*$^{-/-}$ knockout mice were primed with IFNγ (2 hr, 5 ng/mL) and then treated with MDP (10 μg/mL) for the indicated times. mRNA levels for IL-6 (C) and IL-1β (D) was measured using qPCR. All samples were normalized to GAPDH (WT, n = 3; *Tnfr1*, n = 3).

(E) BMDMs from WT or *Tnfr1*$^{-/-}$ mice were primed with IFNγ (2 hr, 5 ng/mL) and then treated with MDP (10 μg/mL) for the indicated times. Ubiquitylated proteins were isolated using TUBEs, western blotted, and probed with the indicated antibodies. Data are representative of two independent experiments.

(F) WT THP-1s were treated with either TNF (100 ng/mL) or L18-MDP (200 ng/mL) for the indicated times. Ubiquitylated proteins were isolated using TUBEs, western blotted, and probed with the indicated antibodies. Data shown are representative of two independent experiments.

See also Figure S4.

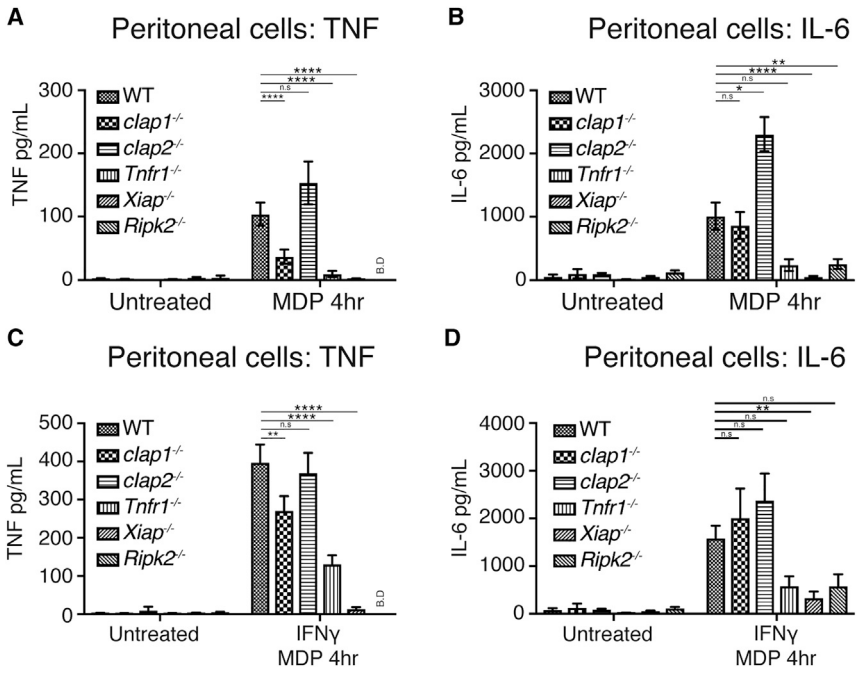

Figure 5. Peritoneal Cells Require cIAP1, TNFR1, XIAP, and RIPK2 to Respond Fully to MDP *Ex Vivo*

(A and B) Cells from wild-type and knockout mice were extracted by peritoneal lavage, left untreated, or treated with MDP (10 μg/mL) for 4 hr. Levels of TNF (A) and IL-6 (B) in cleared supernatant were measured using ELISA (WT, n = 11; *clap1*, n = 12; *clap2*, n = 3; *Tnfr1*, n = 8; *Xiap*, n = 6; *Ripk2*, n = 3). (C and D) Peritoneal cells from WT and knockout mice were left untreated or primed with IFNγ (2 hr, 5 ng/mL) and then treated with MDP (4 hr, 10 μg/mL). Levels of TNF and IL-6 in the cleared supernatant were measured using ELISA (WT, n = 22; *clap1*, n = 19; *clap2*, n = 9; *Tnfr1*, n = 11; *Xiap*, n = 15; *Ripk2*, n = 7). Bars are average ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$; NS, not significant. See also Figure S5.

loss had as profound an effect on NOD2 signaling as loss of RIPK2. XIAP loss cannot be compensated for by cIAPs, which already shows that if they have a role, it must be a subordinate one. Among other experiments, Bertrand et al. (2009) monitored activation of MAPKs and NF-κB in *clap1*$^{-/-}$ and *clap2*$^{-/-}$ BMDMs in response to MDP treatment *in vitro* and measured transcript levels of the same key cytokines (IL-6 and TNF) that we assessed by ELISA. Although close inspection of their data in *cIAP1* and *cIAP2* knockout BMDMs indicates that signaling was not completely ablated, their qPCR data showed an absence of NOD-stimulated transcripts. These authors used different strains of cIAP-deficient mice, and it has been suggested that the *clap1*$^{-/-}$ mice we used might have lower levels of cIAP2 than wild-type mice (Moulin et al., 2015; Heard et al., 2015). Although we do not think this is the case, it only underlines our argument that cIAPs are not required for proximal NOD signaling, and even our *clap1*$^{-/-}$.*clap2*$^{-/-}$ BMDMs responded like wild-type cells to MDP. Furthermore, the strains of knockout mice used by Bertrand et al. (2009) were generated using 129/Sv embryonic stem cells (ESCs), and because the *clap* genes are on the same chromosome and closely linked to *casp11*, these mice originally contained a *casp11* deletion mutation (Kenneth et al., 2012). The *clap1*$^{-/-}$ mice used by Bertrand et al. (2009) have retained the caspase-11 defect, while the *clap2*$^{-/-}$ mice appear to have gained the wild-type version; however, it is unclear whether this happened before or after the Bertrand et al. (2009) study. Although a link between caspase-11 and NOD signaling has not been proposed or investigated, it remains conceivable that this additional genetic defect affected the response of the *clap*-deficient mice used by Bertrand et al. (2009).

Our results showed that XIAP was the only IAP required for signaling downstream of NOD2 *in vitro*, however, and in accordance with Bertrand et al. (2009), when we tested *clap1*$^{-/-}$ mice, their response to MDP injection into the peritoneal cavity was reduced compared to that of wild-type mice. This result emphasizes that the physiological role of a protein maybe different from its role in the controlled environment of biochemical experiments and prompted us to examine the issue further.

We tested three possible explanations for the discrepancy between the *in vitro* systems in which cIAP1 was not required for NOD2 signaling and the *in vivo* model in which it was. First, *clap1*$^{-/-}$ mice might be deficient in a cell type required for the *in* vivo response. Second, the cell type required might have different signaling componentry from that of the BMDMs that we tested. Third, the experimental setting in the mice was not faithfully reproduced *in vitro*. We were particularly intrigued by *Tnfr1*$^{-/-}$ mice also being deficient in their response to MDP, similar to *clap1*$^{-/-}$ mice. Because cIAPs are known to regulate TNFR1 signaling, we suspected that a TNFR1 amplification step was required for full NOD signaling. We showed that TNFR1 was required for a complete response to MDP; however, its contribution to that response is still not clear. In this regard, in *Tnfr1*-deficient BMDMs, we also observed a reduction of XIAP and RIPK2 ubiquitylation at later time points. Thus, rather than TNF signaling taking over from NOD2, this suggests that it may contribute to cytokine production by sustaining the NOD2 signal. This conclusion is in line with data showing that NOD2 and RIPK2 may be upregulated by TNF (Chen et al., 2017).

The cellular composition of the peritoneum was, as far as we could determine, unaltered in IAP-deficient animals, leaving us with the possibility that the cells responding to NOD *in vivo* behave differently to BMDMs and monocytes and have different IAP requirements. This idea was supported when we tested the peritoneal cells *ex vivo* and observed that they mimicked the *in vivo* need for a TNFR1 and cIAP1 contribution. One major difference between our experiments with macrophages and the experiments in mice is that macrophages require priming

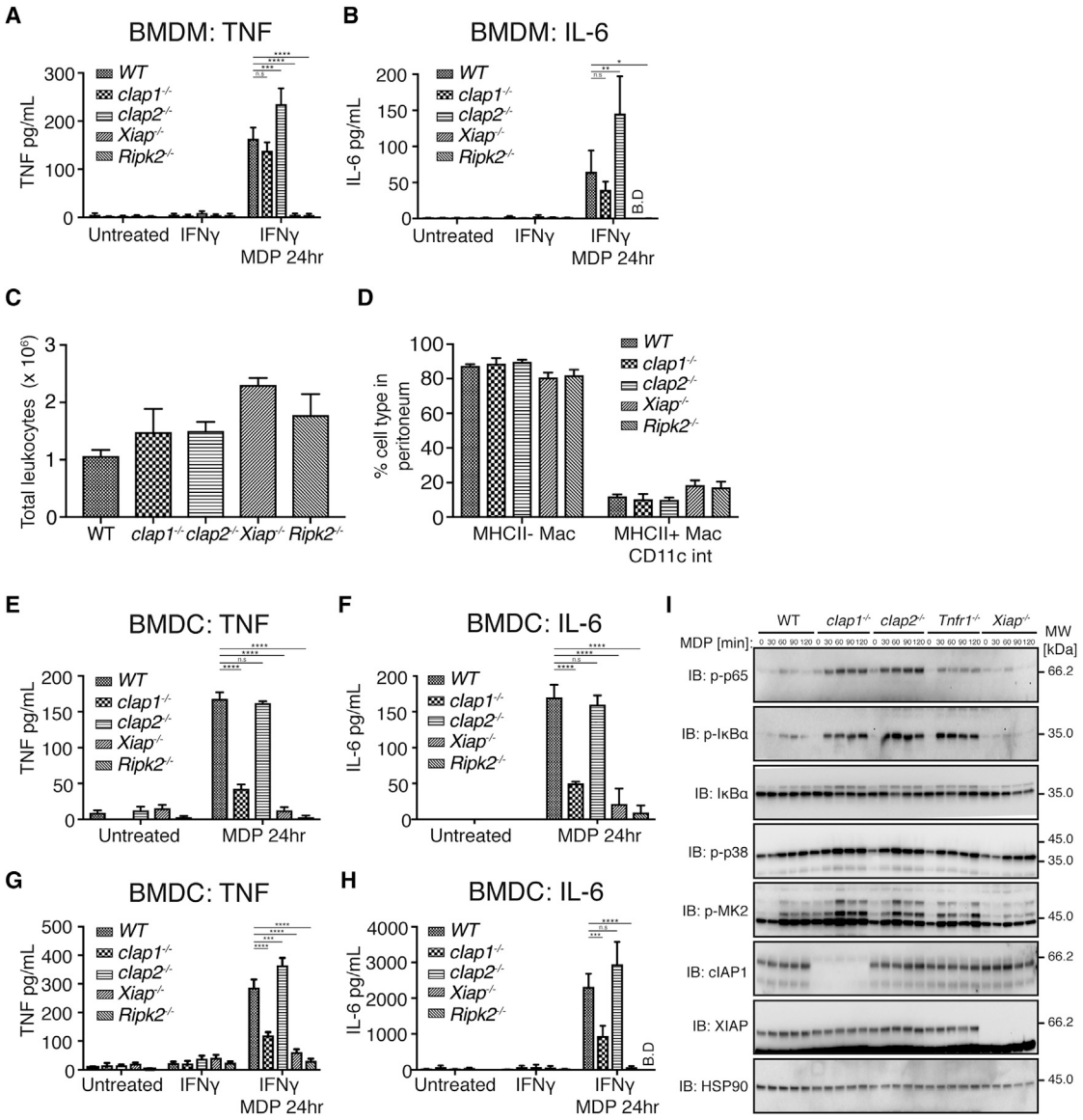

Figure 6. DCs Do Not Require IFNγ Priming but Do Require cIAP1 to Fully Respond to MDP

(A and B) BMDMs from wild-type and knockout mice were left untreated or primed with IFNγ (2 hr, 5 ng/mL) followed by MDP (10 μg/mL) treatment for 24 hr. Levels of TNF (A) (WT, n = 16; *clap1*, n = 16; *clap2*, = 16; *Xiap*, = 16; *Ripk2*, = 13) or IL-6 (B) (WT, n = 13; *clap1*, n = 13; *clap2*, = 13; *Xiap*, = 13; *Ripk2*, = 13) were measured from cleared supernatants.

(C and D) The cellular composition of the peritoneal lavage of untreated mice was assessed by flow cytometry. Total leukocytes (C) ($CD45^+$Propidiumlodide$^-$(live)) were gated on $CD11b^+F4/80^+MHCII^-$ or $CD11b^+F4/80^+MHCII^+CD11c^+$ (D), and percentages were plotted. Columns are average ± SEM, n = 3 mice/group.

(E and F) BMDCs from wild-type and knockout mice were left untreated or stimulated by MDP (10 μg/mL) treatment for 24 hr. Levels of TNF (E) and IL-6 (F) were measured from cleared supernatants (WT, n = 3; *clap1*, n = 3; *clap2*, n = 3; *Xiap*, n = 3; *Ripk2*, n = 3).

(G and H) BMDCs from wild-type and knockout mice were left untreated or primed with IFNγ (2 hr, 5 ng/mL) ± MDP (10 μg/mL) treatment for 24 hr. Levels of TNF (G) and IL-6 (H) were measured from cleared supernatants (WT, n = 6; *clap1*, n = 6; *clap2*, n = 6; *Xiap*, n = 6; *Ripk2*, n = 6).

(I) BMDCs from WT, *clap1*, *clap2*, *Tnfr1*, or *Xiap* KO mice were either left untreated or treated with MDP (10 μg/mL) for the indicated times. Cell lysates were western blotted and probed with the indicated antibodies. Columns are average ± SEM.

*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; NS, not significant. See also Figure S6.

with IFNγ to promote uptake of the NOD2 ligand. In contrast, MDP alone is sufficient for a complete NOD2 response *in vivo*. Reassuringly for our hypothesis, freshly isolated peritoneal cells did not require IFNγ priming, suggesting that the cell type responding *in vivo* was different from a macrophage. When we examined the cellular composition of the peritoneum, the majority of cells had a macrophage-like phenotype, while a minority had an antigen-presenting, dendritic cell (DC)-like phenotype. When we tested DCs *in vitro*, this cell type recapitulated the phenomena we observed *in vivo*; namely, signaling did not require

IFNγ priming and the cytokine response was reduced, but not completely absent in *clap1*$^{-/-}$ cells.

In our work, we show that TNF signaling is required for the sustained response to MDP. This is supported by previous observations and clinical data obtained from NOD-associated diseases, including sarcoidosis, uveitis (Milman et al., 2012), rheumatoid arthritis (Vieira et al., 2012), and multiple sclerosis (Shaw et al., 2011). All show potential to be controlled via inhibition of the NOD2 pathway. For instance, antigen-induced experimental autoimmune encephalomyelitis is critically regulated by the NOD-RIPK2 axis (Shaw et al., 2011), as well as the TNFR1 and TNFR2 pathways (Baker et al., 1994). However, in multiple sclerosis patients, global TNF inhibition is not only ineffective but also exacerbates the disease, most likely due to the role of TNFR2 in the regeneration of the damaged CNS (McCoy and Tansey, 2008; Arnett et al., 2001). Therefore, inhibition of NOD signaling could be a promising strategy to dampen neuroinflammation without affecting CNS regeneration. In other inflammatory diseases, TNF inhibition is often effective; however, not all patients respond and remission occurs frequently (Ma et al., 2015). Inhibition of NOD2 signaling might therefore represent an alternative treatment option in these situations. As we show here, inhibition of XIAP with IAP antagonists could be one approach to targeting this pathway; however, clinical IAP antagonists do not specifically target XIAP (Condon et al., 2014), and pan-IAP antagonists promote inflammation and are therefore inappropriate for treatment of inflammatory diseases (Lawlor et al., 2015). Specifically targeting the RIPK2:XIAP interaction could be a potential strategy for treatment. Various RIPK2 inhibitors such as Ponatinib or WEHI-345 have been shown to interfere with the function and structure of RIPK2 and thereby prevent XIAP from interacting and ubiquitylating RIPK2 (Canning et al., 2015; Nachbur et al., 2015).

In this work, we have revisited and re-evaluated the roles of cIAP1, cIAP2, and XIAP in NOD2 signaling. The picture that emerges is of an initial RIPK2- and XIAP-dependent response that drives autocrine TNF and a cIAP1-dependent pathway that contributes to the final response. The initial response is probably driven by a specific and small subset of peritoneal cells that are able to rapidly react to a physiological signal without prior stimulation or priming. cIAPs have a separate role and regulate the secondary TNF-dependent response; however, it appears that there is also an interplay between these signaling pathways. Our results therefore indicate that effective therapeutic targeting in inflammatory diseases will require a thorough understanding of this interplay and lay down an initial framework for future work.

EXPERIMENTAL PROCEDURES

Cell Culture and Generation of BMDMs and BMDCs

Wild-type THP-1 cells were sourced from ATCC. THP-1 cells were cultured in RPMI supplemented with 8% fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin; Gibco) at 37°C with 10% CO_2 in a humidified incubator. BMDMs were generated from the femur and tibiae of C57BL/6 mice aged 6–12 weeks and cultured for 6 days in DMEM (InvivoGen) supplemented with 8% FBS (Gibco) and 20% L929 supernatant and antibiotics (penicillin and streptomycin). After 6 days, cells were detached using trypsin-EDTA and re-plated in 12- and 24-well tissue culture plates. BMDCs were generated from the femur and tibiae of mice and cultured for 3 days in Iscove's Modified Dulbecco's Medium (IMDM) (InvivoGen) supplemented with 10% FBS (Gibco) and GM-CSF (10 ng/mL, Lonza); half the media was replenished on day 3, and experiments were begun on day 7. Harvesting bone marrow for the generation of BMDMs and BMDCs was approved by the animal ethics committee of WEHI, ethics approval numbers 2011.013, 2014.004, and 2017.004.

Stimulation Protocols

BMDMs were either primed with murine IFNγ (5 ng/mL, R&D Systems) for 2 hr before stimulation with MDP (10 μg/mL, InvivoGen) or directly stimulated with L18-MDP (200 ng/mL, InvivoGen). THP-1 cells were stimulated directly with L18-MDP (200 ng/mL, InvivoGen). Fc-TNF was generated in house as described (Bossen et al., 2006). TNF was used at 100 ng/mL, and TNF blocking antibody was used at 1 μg/mL 30 min before treatment (Abcam).

Western Blotting

Following stimulation, cells were lysed in 2× SDS lysis buffer (126 mM Tris-HCl [pH 8], 20% v/v glycerol, 4% w/v SDS, 0.02% w/v bromophenol blue, 5% v/v 2-mercaptoethanol) and subjected to repeated freeze-boil cycles. Samples were separated using SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membranes, and probed with antibodies against phospho-p65, total-p65, phospho-IκBα, total-IκBα, total-p38, phospho-MK2, ubiquitin, phospho-JNK, phospho-ERK (all Cell Signal), phospho-p38 (Assay Biotech), cIAP1 (Enzo), XIAP (Medical & Biological Laboratories), RIPK2 (Santa Cruz and BD Transduction Laboratories), RIPK1(BD Transduction Laboratories), and actin (Sigma).

Cytokine Measurement by ELISA

Cytokines from mouse serum or cell culture supernatant were measured by Ready-Set-Go! ELISA kits from eBioscience according to the manufacturer's instructions. Sera and supernatants were diluted 1:10 for CCL2 measurements.

NF-κB GFP Activity Assay

THP-1 cells were stably transfected with an NF-κB-GFP reporter (pTRH NF-κB, System Biosciences). Cells were left untreated or pretreated with 500 nM of birinapant, compound A (TetraLogic Pharmaceuticals) and then stimulated with 200 ng/mL L18-MDP (InvivoGen). Mean fluorescent intensity was measured by flow cytometry and analyzed using Weasel 3.1 software (WEHI).

In Vivo MDP Challenge

All *in vivo* experiments were approved by the animal ethics committee of WEHI, ethics approval numbers 2014.004 and 2017.004. Sex-matched 6- to 8-week-old C57BL/6 mice were used within each experiment. For *in vivo* MDP challenge, C57BL/6, *clap1*, *clap2*, *Tnfr1*, *Xiap*, or *Ripk2* knockout mice were treated with MDP (100 μg intraperitoneally [i.p.]) and sacrificed 4 hr later. Peripheral blood was collected for serum by cardiac puncture, and peritoneal cells were harvested by lavage with 1.5 mL of 5 mM EDTA in PBS. Peritoneal cells were subjected to red blood cell lysis before staining with Fc block (anti-mouse CD16/32, 2.4G2) and fluorescently labeled anti-mouse immunoglobulin G (IgG) antibodies to CD45.2 (Ly5.2), CD11b (Mac-1), Ly6G (1A8), CD11c (N418), major histocompatibility complex class II (MHC class II) (M5/114.15.2), and F4/80 (BM8) from eBioscience, BD Biosciences, BioLegend, and WEHI monoclonal laboratories. Viable cell (propidium iodide [PI] negative) populations were quantified using counting beads (eBioscience/BD Biosciences) on a BD LSR-Fortessa Cell Analyzer (BD Biosciences). Data were analyzed using Weasel 3.1 software (WEHI).

Purification of Ubiquitin Conjugates

GST-TUBEs (TUBE1, Lifesensors) were used to purify ubiquitin conjugates from either BMDMs or THP-1 cells. 2 × 10^7 cells/condition were lysed in 1 mL of DISC buffer (1% Triton X-100, 150 mM NaCl, 20 mM Tris [pH 7.5], 10% glycerol, 2 mM EDTA) + 10 mM N-ethylmaleimide and cOmplete Protease Inhibitor Cocktail (Roche) for 20 min on ice. Lysates were clarified by centrifugation, at 15,000 × *g*, at 4°C for 10 min. Lysates were incubated with 20 μL of packed TUBE1 beads at 4°C and rotated overnight. Beads

were then washed 5× in lysis buffer, resuspended in 60 μL of 1× SDS sample buffer, and subjected to western blot analysis.

DUB Assay

Purified GST-UBA was prebound to glutathione Sepharose high-performance beads (10 μL/condition) at 4°C for 30 min. After washing beads in PBS-Tween (0.1% Tween 20), lysates from THP-1 cells (2×10^7 cells/DUB treatment) stimulated with L18-MDP (200 ng/mL, InvivoGen) were incubated overnight (O/N) at 4°C. Beads were then treated according to the UbiCREST DUB Enzyme Kit (Boston Biochem) protocol and subject to western blot analysis.

Generation of THP-1 IAP Cell Lines

CRISPR/Cas9 THP-1 cell lines were generated using pFU Cas9 Cherry, which allows constitutive expression of the Cas9 protein, and the pF GH1t UT GFP vector, which allows doxycycline-inducible expression of guide RNA sequences. Single cells were sorted for GFP and mCherry into 96-well plates. Guide RNAs (gRNAs) were induced with 1 μg/mL of doxycycline, and single-cell clones were tested for absence of expression of cIAP1 or XIAP by western blot and cIAP2 by next-generation sequencing.

RNA Isolation, cDNA Synthesis, and qPCR Gene Analysis

Total RNA was isolated using the Isolate II RNA Micro Kit, and cDNA was generated using a Tetro cDNA synthesis kit (both Bioline). qPCR was performed using TaqMan probes for IL-6 (Mm00446190_m1; Thermo Fisher Scientific) and IL-1β (Mm00434228_m1) on a ViiA 7 qPCR system. All samples were normalized to GAPDH (Mm99999915_g1) mRNA levels.

Statistical Analysis

The p values were calculated using one-way ANOVA (Figures 4A and 4B) or two-way ANOVA (all other figures) using Prism v.7 (GraphPad). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$; p values > 0.05 are indicated as not significant (NS).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.01.024.

ACKNOWLEDGMENTS

We thank Prof. R. Flavell for the gift of *Ripk2*$^{-/-}$ mice, Dr. Marco Herold for the CRISPR/Cas9 constructs, Dr. Angus Stock for antibodies, Prof. C. Day for the GST-BIR2 constructs, and C. Stivala, N. Lynch, and S. Stoev for assistance with animal work. Thanks also to D. Vaux and J. Vince for suggestions and support, as well as Julie Stafford for support and proofreading. The work was supported by NHMRC grants 1046986 and 1057888 and fellowships 541901 and 1058190 to J.S. and an ARC fellowship FT130100166 to U.N. U.N. was also supported by the Swiss National Science Foundation (SNSF, fellowship PA00P3_126249). This work was made possible through Victorian State Government Operational Infrastructure Support and Australian Government NHMRC IRIISS (9000220).

AUTHOR CONTRIBUTIONS

C.A.S., A.B., K.E.L., V.J.H., J.P.B., and U.N. designed and performed experiments. C.A.S., J.S., and U.N. analyzed data, designed the study, and wrote the manuscript.

DECLARATION OF INTERESTS

J.S. was on the Scientific Advisory Board (SAB) of TetraLogic Pharmaceuticals while some of this work was generated.

Received: July 27, 2017
Revised: November 2, 2017
Accepted: January 8, 2018
Published: February 6, 2018

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