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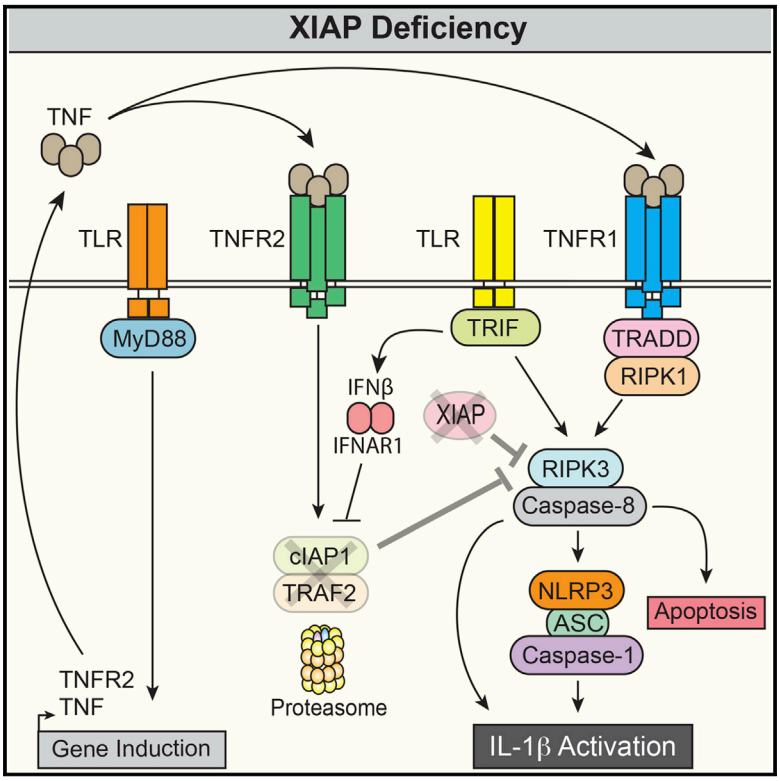
XIAP Loss Triggers RIPK3- and Caspase-8-Driven IL-1β Activation and Cell Death as a Consequence of TLR-MyD88-Induced cIAP1-TRAF2 Degradation

Graphical Abstract



Highlights

- TLR-MyD88 engagement triggers cIAP1 and TRAF2 degradation
- TLR-induced cIAP1 and TRAF2 degradation results from TNF and TNFR2 induction
- Upon XIAP loss, TNFR2-induced cIAP1 removal induces RIPK3-caspase-8-induced IL-1β
- A TLR-TRIF-IFNβ axis protects from cIAP1 degradation and consequent cell death

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In Brief

Lawlor et al. find that TLR signaling induces TNF and TNFR2 to trigger degradation of the IAP family member cIAP1. In the absence of XIAP, these events induce TLR-RIPK3-caspase-8-driven NLRP3 and IL-1β activation and cell death.

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XIAP Loss Triggers RIPK3- and Caspase-8-Driven IL-1β Activation and Cell Death as a Consequence of TLR-MyD88-Induced cIAP1-TRAF2 Degradation

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SUMMARY

X-linked Inhibitor of Apoptosis (XIAP) deficiency predisposes people to pathogen-associated hyper-inflammation. Upon XIAP loss, Toll-like receptor (TLR) ligation triggers RIPK3-caspase-8-mediated IL-1β activation and death in myeloid cells. How XIAP suppresses these events remains unclear. Here, we show that TLR-MyD88 causes the proteasomal degradation of the related IAP, cIAP1, and its adaptor, TRAF2, by inducing TNF and TNF Receptor 2 (TNFR2) signaling. Genetically, we define that myeloid-specific cIAP1 loss promotes TLR-induced RIPK3-caspase-8 and IL-1β activity in the absence of XIAP. Importantly, deletion of TNFR2 in XIAP-deficient cells limited TLR-MyD88-induced cIAP1-TRAF2 degradation, cell death, and IL-1β activation. In contrast to TLR-MyD88, TLR-TRIF-induced interferon (IFN)β inhibited cIAP1 loss and consequent cell death. These data reveal how, upon XIAP deficiency, a TLR-TNF-TNFR2 axis drives cIAP1-TRAF2 degradation to allow TLR or TNFR1 activation of RIPK3-caspase-8 and IL-1β. This mechanism may explain why XIAP-deficient patients can exhibit symptoms reminiscent of patients with activating inflammasome mutations.

INTRODUCTION

Mammalian Inhibitor of Apoptosis (IAP) proteins include the RING domain ubiquitin E3 ligases X-linked IAP (XIAP), cellular IAP1 (cIAP1), and cIAP2 (Vaux and Silke, 2005). Despite XIAP being best characterized for its role in inhibiting apoptosis, mutations in XIAP, which result in XIAP deficiency, cause a hereditary disorder classified as X-linked lymphoproliferative syndrome type 2 (XLP-2) (Rigaud et al., 2006). Clinically, XIAP-deficient patients can present with a range of symptoms, including fevers, hepatosplenomegaly, cytopenia, and inflammatory bowel disease. Within their first decade of life, many XIAP-deficient patients also develop hemophagocytic lymphohistiocytosis (HLH), a potentially fatal hyperinflammatory disease that is often triggered by an infectious agent, such as Epstein-Barr virus (Aguilar and Latour, 2015; Marsh et al., 2010; Rigaud et al., 2006). In view of the lack of monoclonal lymphoproliferation and the prevalence of HLH that is associated with XIAP loss, it has been suggested that XIAP deficiency should be reclassified as an X-linked HLH disease (Marsh et al., 2010). Intriguingly, recurrent X-linked HLH is accentuated by dramatically elevated and sustained levels of the cytokine interleukin (IL)-18, which are comparable to the levels of IL-18 observed in other inflammatory conditions featuring the related entity, macrophage activation syndrome (Wada et al., 2014).

Both IL-18 and IL-1β secretion are markers of inflammasome activation. Inflammasomes are large protein complexes that recruit and activate caspase-1 to cleave precursor IL-1β and IL-18 to their mature bioactive fragments and induce a lytic form of cell death, termed pyroptosis (de Zoete et al., 2014). Other than the pathological association of X-linked HLH with IL-18, a recent case report documented use of anti-IL-1 therapy in an XIAP-deficient patient (Christiansen et al., 2016). Consistent with these studies, we and others have reported how the inflammasome sensor protein NOD-like Receptor Protein 3 (NLRP3) can spontaneously form in the absence of IAPs, and in particular XIAP, to activate caspase-1 upon pathogen ligand-induced Toll-like receptor (TLR) stimulation (Lawlor et al., 2015; Vince et al.,

2012; Wicki et al., 2016; Yabal et al., 2014). In macrophages, dendritic cells, and neutrophils, TLR ligation and IAP loss stimulate the formation and activation of a large receptor-interacting protein kinase (RIPK) complex, termed the ripoptosome (Feoktistova et al., 2011; Tenev et al., 2011), that is responsible for triggering NLRP3 and IL-1β activity.

The ripoptosome complex is principally comprised of RIPK1, FADD, and caspase-8, and it was first described to induce apoptotic cell death upon TLR activation, tumor necrosis factor (TNF) receptor 1 (TNFR1) ligation, or genotoxic stress (Feoktistova et al., 2011; Tenev et al., 2011). The cIAPs act to inhibit this complex by ubiquitylating RIPK1 and recruiting LUBAC to induce a pro-survival nuclear factor κB (NF-κB) signal. In some cell types, including macrophages, XIAP prevents ripoptosome formation and caspase-8 activation (Lawlor et al., 2015; Yabal et al., 2014), although the mechanism remains elusive. However, what is clear is that macrophages and dendritic cells lacking IAPs, including XIAP, undergo caspase-8-mediated apoptotic cell death upon TLR ligand or TNF exposure in a largely RIPK3-dependent manner (Lawlor et al., 2015; Vince et al., 2012; Yabal et al., 2014). This is in line with recent reports indicating that RIPK3 can recruit RIPK1 to activate caspase-8 (Mandal et al., 2014; Newton et al., 2014). In situations where ripoptosome-induced caspase-8 activity is insufficient, RIPK1 associates with RIPK3, triggering RIPK3 phosphorylation of mixed-lineage kinase domain-like (MLKL) and necroptosis (Murphy and Vince, 2015), as well as NLRP3 inflammasome activation (Conos et al., 2017; Gutierrez et al., 2017; Kang et al., 2013, 2015; Lawlor et al., 2015).

Recently, we documented how, upon IAP antagonist treatment or IAP deficiency, TLR and TNFR1 signal to ripoptosome-associated RIPK3 to promote caspase-8 auto-proteolysis and drive IL-1β and IL-18 activation in macrophages (Lawlor et al., 2015; Vince et al., 2012). Activated caspase-8 triggered IL-1β secretion by inducing both NLRP3 inflammasome formation and by directly processing precursor IL-1β. Importantly, XIAP deficiency alone was sufficient to allow ripoptosome-mediated IL-1β activation in response to TLR ligands. The mechanism by which this occurs remains of outstanding interest, and it is considered important for not only defining the causes of X-linked HLH but also how pathogen ligands can act to modulate innate immune cell inflammatory and cell death responses.

Here we now document that multiple pathogen TLR ligands that signal through MyD88 induce TRAF2-dependent cIAP1 degradation that can activate non-canonical NF-κB signaling. More importantly, we demonstrate that XIAP deficiency results in enhanced TLR-induced cIAP1 loss, leading to heightened IL-1β production and cell death signaling. TLR-induced degradation of cIAP1 and TRAF2 was critically dependent on TLR-induced TNFR2 signaling, as TNFR2 loss blocked cIAP1 degradation, apoptotic cell death, and NLRP3-driven inflammatory responses. These results show how TLR and TNF receptor (TNFR) superfamily members can cooperate to regulate cell death and inflammation generated by pathogen ligands, and they document how this crosstalk might contribute to the autoinflammatory disease observed in XIAP-deficient patients.

RESULTS

The Loss of cIAPs Enhances IL-1β Activation and Secretion When XIAP Is Absent

Consistent with previous findings (Lawlor et al., 2015; Vince et al., 2012; Yabal et al., 2014), we observed secretion of IL-1β in XIAP-deficient (*Xiap*$^{-/-}$), but not wild-type (WT), bone marrow-derived macrophages (BMDMs) upon stimulation with lipopolysaccharide (LPS) (Figure 1A). On the other hand, LPS-induced TNF levels were comparable between WT and *Xiap*$^{-/-}$ cells (Figure 1B). Treatment of *Xiap*$^{-/-}$ BMDMs with IAP antagonists (Smac-mimetics) that show functional affinity against the cIAPs (cIAP1 and cIAP2; 711, 851, and 883; Lawlor et al., 2015) resulted in enhanced LPS-induced IL-1β processing and secretion, and cell death, in XIAP-deficient cells but did not trigger IL-1β activation or death in WT BMDMs (Figures 1A, 1C, and 1D). In contrast, IAP antagonists that functionally inhibited all three IAPs, XIAP, cIAP1, and cIAP2 (030, 031, and Cp. A; Lawlor et al., 2015) elicited significant LPS-induced IL-1β processing and secretion, even from WT macrophages (Figure 1D).

TLR engagement and IAP loss activate caspase-8, which can directly process IL-1β and induce formation of the NLRP3-caspase-1 inflammasome (Lawlor et al., 2015; Vince et al., 2012; Yabal et al., 2014). Consistent with this, the secretion of IL-1β upon LPS stimulation and XIAP loss coincided with caspase-8 and caspase-1 processing (Figure 1D). These results show that XIAP cooperates with cIAPs to prevent spontaneous TLR-induced NLRP3 inflammasome and IL-1β responses (Figure 1E).

TLRs Induce the Degradation of cIAP1 and TRAF2 in a Proteasomal-Dependent Manner

In light of the results using IAP antagonist compounds, we considered whether TLR signaling might deplete cellular cIAP1 to promote inflammatory responses. In line with this idea, stimulation of BMDMs with Pam3CSK4 (P_3Cys; TLR1/2), CpG (TLR9), or R837 (TLR7) induced a substantial reduction in total cellular cIAP1, as well as its binding partner TRAF2, within 4–8 hr (Figures 2A–2D and S1A). In comparison, LPS (TLR4) was less efficient at inducing cIAP1 loss, particularly when cells were treated with higher concentrations (Figures 2A–2D and S1A). However, despite this less prominent cIAP1 degradation in response to LPS treatment, LPS-induced degradation of TRAF2 was comparable to the other TLR ligands. Therefore, multiple TLR family members are able to induce cIAP1 and TRAF2 depletion.

TRAF2 helps recruit cIAP1 into TNFR superfamily (TNFRSF) complexes, such as TNFR2 and FN14 (Brown et al., 2003; Rothe et al., 1995). Signaling from TNFR2 and FN14 also promotes the degradation of TRAF2 and cIAP1 in a proteasomal and/or lysosomal manner to trigger activation of non-canonical NF-κB (Fotin-Mleczek et al., 2002; Li et al., 2009; Varfolomeev et al., 2012; Vince et al., 2008). Consistent with TRAF2 being required for the recruitment of cIAP1 into receptor-signaling complexes, we observed that cIAP1 levels in TRAF2-deficient BMDMs stimulated with the TLR ligands LPS or P_3Cys did not decline, but rather they increased over time (Figure 2E). Treatment of BMDMs with the proteasomal inhibitor MG132 also prevented

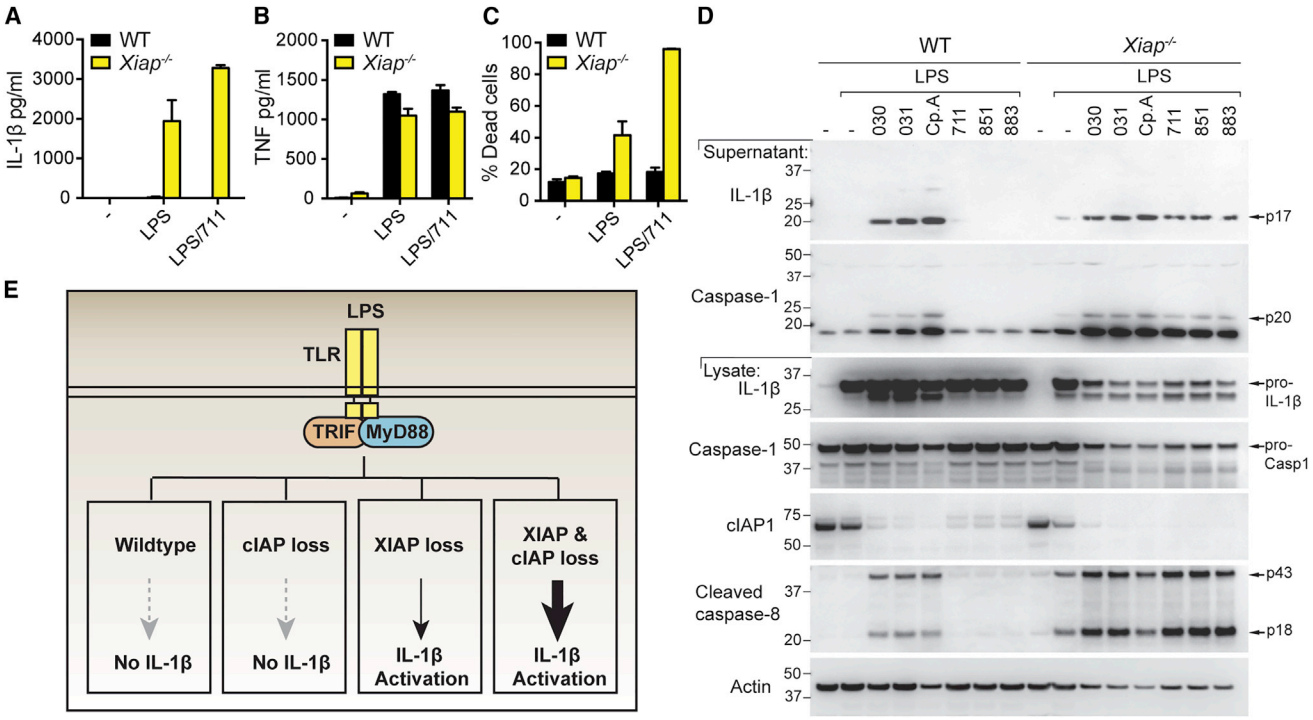


Figure 1. LPS-Induced Spontaneous IL-1β Activation and Cell Death in the Absence of XIAP Is Enhanced by cIAP Depletion

(A–D) WT and *Xiap*$^{-/-}$ BMDMs were primed with or without LPS (100 ng/mL) for 2 hr, as indicated, prior to stimulation with 1 μM IAP antagonists (cIAP1/2 selective: 711 [Birnapant], 851, and 883; XIAP/cIAP1/2 selective: 030, 031, and Cp. A.). (A) IL-1β and (B) TNF levels were assayed in supernatants by ELISA after 24 hr (mean + SEM; n = 3 mice per group, one of two experiments). (C) Cell death was measured by propidium iodide (PI) uptake and flow cytometric analysis after 24 hr (mean + SEM; n = 3 mice per group, one of two experiments). (D) Supernatants and cell lysates were analyzed by immunoblot after 8 hr. Data are representative of one of two experiments.

(E) Schema depicting how the loss of different IAPs impacts LPS-induced IL-1β activation.

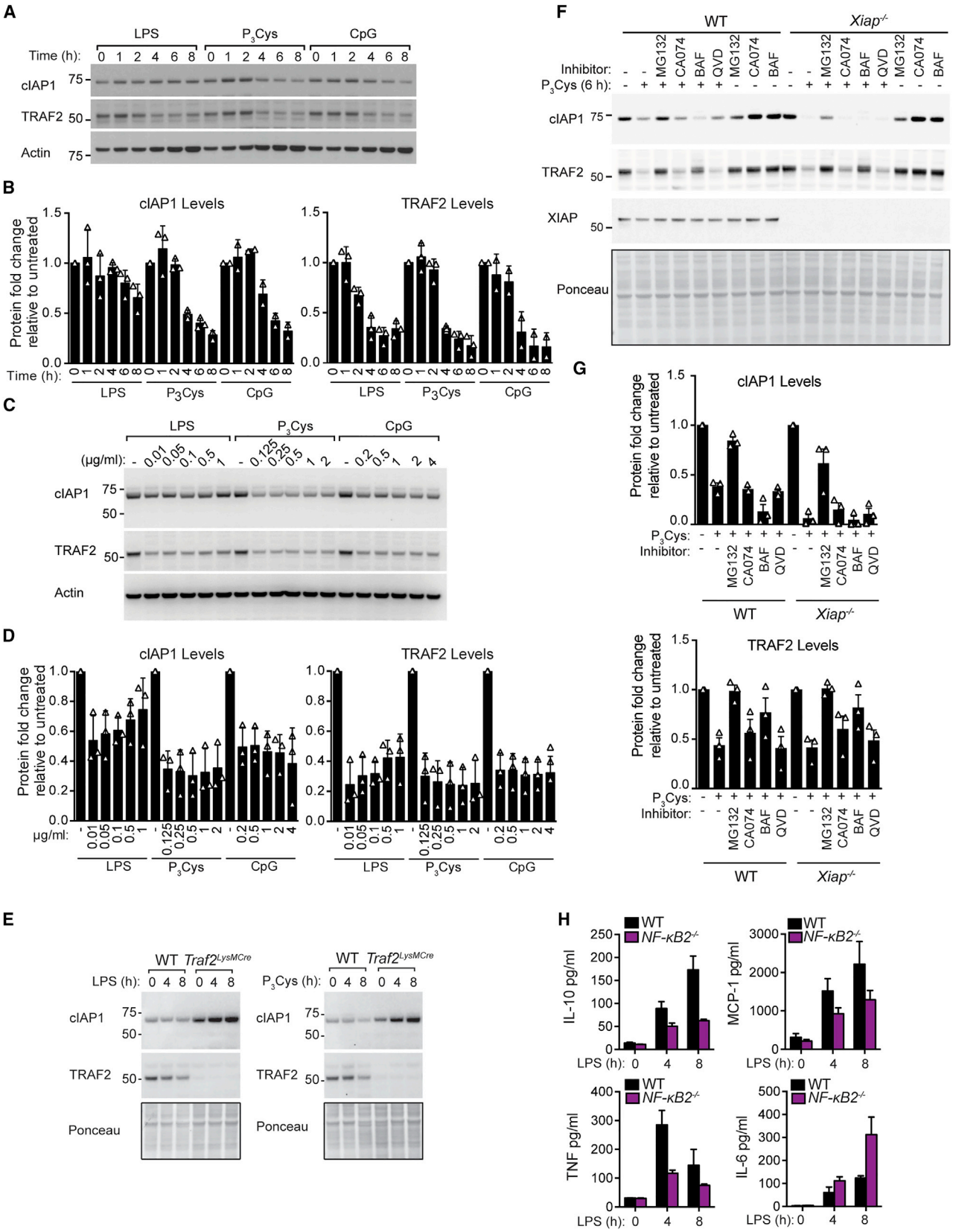
P_3Cys-mediated cIAP1 and TRAF2 depletion, whereas the caspase inhibitor QVD-OPh was not sufficient to prevent their loss (Figures 2F and 2G). The inhibitor of lysosomal function, Bafilomycin A1, partly reduced the loss of TRAF2, but it had no impact on cIAP1 degradation (Figures 2F and 2G). Therefore, TLR ligation stimulates the degradation of cIAP1 and TRAF2 in a manner that is largely dependent on the proteasome.

Optimal TLR-Induced Cytokine Production Requires Non-canonical NF-κB Signaling

In unstimulated cells, a cIAP/TRAF2/TRAF3 complex associates with NF-κB-inducing kinase (NIK), and it allows cIAPs to target NIK for proteasomal degradation, thereby preventing non-canonical NF-κB2/p100 processing to the active p52 subunit. Hence, TNFRSF-induced cIAP1-TRAF2 loss, or IAP antagonist treatment, can trigger non-canonical NF-κB (Sun, 2012; Varfolomeev et al., 2007; Vince et al., 2007). Our results therefore suggested that NF-κB2/p100 might be involved in TLR-induced cytokine production. Consistent with this idea, the production of a subset of cytokines was diminished in NF-κB2-deficient BMDMs compared to WT cells in response to LPS or P_3Cys treatment (Figures 2H, S1B, and S1C). Immunoblotting also revealed increased non-canonical NF-κB activation upon LPS or P_3Cys treatment, as measured by p100 processing to p52 and stabilization of NIK (Figures S1D and S1E). These data imply that TLR-induced non-canonical activity in macrophages is important for optimal cytokine production.

LPS-Induced cIAP1 Degradation Is Enhanced in the Absence of XIAP and Is Associated with Caspase-8-Driven NLRP3-Caspase-1 and IL-1β Processing and Cell Death

Based on the above data, we hypothesized that TLR-induced depletion of cIAP1 is required for efficient NLRP3 inflammasome activation and cell death in the absence of XIAP. However, our results demonstrated that LPS treatment in WT BMDMs is often less efficient at degrading cIAP1 when compared to other TLR ligands, even though it can trigger IL-1β secretion in *Xiap*$^{-/-}$ BMDMs (Figures 1 and 2). We therefore compared cIAP1 levels in TLR-stimulated WT and XIAP-deficient BMDMs. Remarkably, we observed that LPS-induced cIAP1 degradation was typically enhanced in XIAP-deficient macrophages compared to WT cells and this correlated with caspase-8 processing and cell death, while P_3Cys-induced cIAP1 loss was moderately accelerated in *Xiap*$^{-/-}$ BMDMs (Figures 3A–3C and S2A–S2C). We also observed that, in the absence of XIAP, IL-1β secretion and cell death occurred more efficiently at lower cell densities (Figures S3A–S3E). Higher concentrations of LPS (100–1,000 ng/mL) did not significantly alter TNF secretion by *Xiap*$^{-/-}$ BMDMs, but it did result in reduced levels of precursor, cleaved, and



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secreted IL-1β (Figures 3B and 3D–3F). This phenomenon probably reflects increased TRIF-induced type I interferon (IFN) production and the inhibition of IL-1β gene transcription (Guarda et al., 2011; Masters et al., 2010).

Interestingly, the TLR3 ligand Poly(I:C) failed to induce cIAP1 loss in either WT or XIAP-deficient BMDMs, and it did not result in detectable IL-1β secretion or cell death, thus correlating an inability to degrade cIAP1 with a lack of NLRP3 and cell death responses (Figures 3A, 3D, and S2A). In contrast to IL-1β secretion, TNF secretion in response to all TLR ligands tested was comparable between WT and XIAP-deficient cells (Figures 3E and S3D). Hence, the loss of XIAP can promote LPS-induced cIAP1 degradation, which correlates with IL-1β maturation, caspase-8 cleavage, and enhanced cell death.

Genetic Deletion of XIAP and cIAP1 in Myeloid Cells Sensitizes Macrophages to LPS-Induced IL-1β Activation

The degradation of cIAP1 induced by TLR ligands was only partial (Figure 2). Therefore, prompted by our Smac-mimetic results (Figure 1), we reasoned that genetic loss of cIAP1 should cause more pronounced NLRP3 inflammasome activation upon TLR ligation than that observed upon XIAP loss alone. To test this idea, we generated mice lacking cIAP1 in myeloid cells on an XIAP-deficient background ($cIAP1^{LysMcre}Xiap^{-/-}$). Distinct from the defective generation of BMDMs from $cIAP1^{LysMcre}Xiap^{-/-}cIAP2^{-/-}$ mice (Lawlor et al., 2015; Wong et al., 2014), $cIAP1^{LysMcre}Xiap^{-/-}$ bone marrow yielded normal numbers of BMDMs (Figure 4A).

As predicted, based on our published results using chemical IAP antagonists (Conos et al., 2017; Lawlor et al., 2015), LPS treatment of $cIAP1^{LysMcre}Xiap^{-/-}$ BMDMs induced RIPK3-caspase-8 or RIPK3-MLKL (in the presence of low-dose QVD-OPh) activity, and it significantly exacerbated caspase-1 and IL-1β maturation and secretion, as well as cell death, when compared to XIAP-deficient cells (Figures 4B–4D). In contrast, TNF secretion was comparable among WT, $Xiap^{-/-}$, and $cIAP1^{LysMcre}Xiap^{-/-}$ BMDMs (Figure 4E). The LPS-induced caspase-1 activation observed in $cIAP1^{LysMcre}Xiap^{-/-}$ (and $Xiap^{-/-}$) BMDMs was driven by NLRP3 inflammasome signaling, as the NLRP3 inhibitors MCC950 or Glyburide blocked processing of caspase-1 into the active p10 fragment (Figure 4F). However, these NLRP3 inhibitors only partly reduced IL-1β cleavage and secretion in $Xiap^{-/-}$ or $cIAP1^{LysMcre}Xiap^{-/-}$ BMDMs following LPS treatment alone (Figures 4C, 4F, and S4A), which is likely to reflect direct caspase-8 processing of IL-1β (Vince et al., 2012; Yabal et al., 2014; Lawlor et al., 2015). Notably, akin to MLKL-driven IL-1β release (Conos et al., 2017; Gutierrez et al., 2017), upon IAP loss and TLR treatment Gasdermin D was not essential for NLRP3-independent caspase-8-directed release of IL-1β or cell death (Figures S4B and S4C). These results provide genetic proof that cIAP1 expression levels in macrophages critically influence TLR-induced NLRP3 and cell death responses resulting from XIAP deficiency.

Myeloid cell deletion of NF-κB-signaling components can promote TLR-induced IL-1β activation (Greten et al., 2007; Martin et al., 2014), as can loss of the NF-κB-inducible pro-survival gene cFLIP (Wu et al., 2014). We therefore examined if IAP loss, which in immortalized cell lines often results in defective NF-κB responses, impacted TLR-mediated transcription factor activation. However, no alteration in LPS-induced canonical NF-κB, p38, ERK, or JNK activation was detected in either $Xiap^{-/-}$ or $cIAP1^{LysMcre}Xiap^{-/-}$ BMDMs when compared to WT cells (Figure S4D). Interestingly, the anti-apoptotic cFLIP long isoform ($cFLIP_L$) failed to accumulate in LPS-treated $cIAP1^{LysMcre}Xiap^{-/-}$ cells, in contrast to $Xiap^{-/-}$ or WT BMDMs (Figure S4E). However, caspase inhibition restored c-$FLIP_L$ expression in $cIAP1^{LysMcre}Xiap^{-/-}$ BMDMs, indicating its loss occurred downstream of caspase activation (Figure S4F). These data indicate that the susceptibility of $Xiap^{-/-}$ or $cIAP1^{LysMcre}Xiap^{-/-}$ BMDMs to TLR-mediated IL-1β activation is unlikely to be a direct consequence of defective transcription factor signaling.

LPS-Induced IL-1β Production Is Enhanced In Vivo in the Absence of XIAP and cIAP1 in Myeloid Cells

Like XIAP-deficient mice, $cIAP1^{LysMcre}Xiap^{-/-}$ mice appeared overtly normal and did not display any gross signs of clinical disease (Figures 5A and 5B). Moreover, $cIAP1^{LysMcre}Xiap^{-/-}$ mice did not show signs of subclinical neutrophil infiltration, as measured using live in vivo imaging of myeloperoxidase (MPO) activity (Figures 5B and 5C). This is in stark contrast to the prominent TNF-driven IL-1-associated multi-organ autoinflammatory disease that develops from birth in mice lacking myeloid cell expression of cIAP1/2 and XIAP and that leads to premature morbidity (Lawlor et al., 2015; Wong et al., 2014). Further examination of $cIAP1^{LysMcre}Xiap^{-/-}$ mice revealed little evidence of splenomegaly or perturbation of leucocyte numbers in the blood, bone marrow, splenic, or peritoneal compartments compared to $Xiap^{-/-}$ and WT mice (Figures 5D and S5). A trend toward

Figure 2. TLR Ligands Trigger Proteasomal Degradation of the cIAP1-TRAF2 Complex

(A–D) WT BMDMs were treated with TLR ligands, LPS (100 ng/mL, TLR4), P_3Cys (500 ng/mL, TLR1/2), and CpG (2 μg/mL, TLR9) or as specified for up to 8 hr. (A and C) Immunoblots of total cell lysates and (B and D) degradation quantified from three independent experiments (mean ± SEM) are shown. Data reflect protein fold change relative to levels in untreated BMDMs.

(E) WT and TRAF2-deficient BMDMs were stimulated with LPS (100 ng/mL) or P_3Cys (2 μg/mL) for up to 8 hr, and immunoblots were performed (represents one of three independent experiments).

(F and G) WT and $Xiap^{-/-}$ BMDMs were pre-incubated with the proteasome inhibitor MG132 (5 μM), caspase inhibitor QVD-OPh (QVD, 20 μM), lysosome inhibitor Bafilomycin (BAF, 150 nM), and cathepsin inhibitor CA074-Me (CA074, 20 μM) for 1 hr. BMDMs were then treated with or without P_3Cys (500 ng/mL) for 6 hr, and (F) immunoblots were performed for the indicated proteins. (G) The degradation of cIAP1 and TRAF2 was quantified from three independent experiments. Data reflect protein fold change relative to levels in untreated BMDMs (mean ± SEM).

(H) WT and NF-κB2-deficient BMDMs were cultured with LPS (100 ng/mL) for the specified times, and supernatants were analyzed by Bioplex for cytokine and chemokine secretion. Data are the mean + SEM (n = 3 mice per group).

See Figure S1B for complete Bioplex results and Figure S1C for a representative example of a P_3Cys time course. In all cases, three independent experiments were performed to assess levels of the identified subset of NF-κB2-dependent cytokine/chemokines.

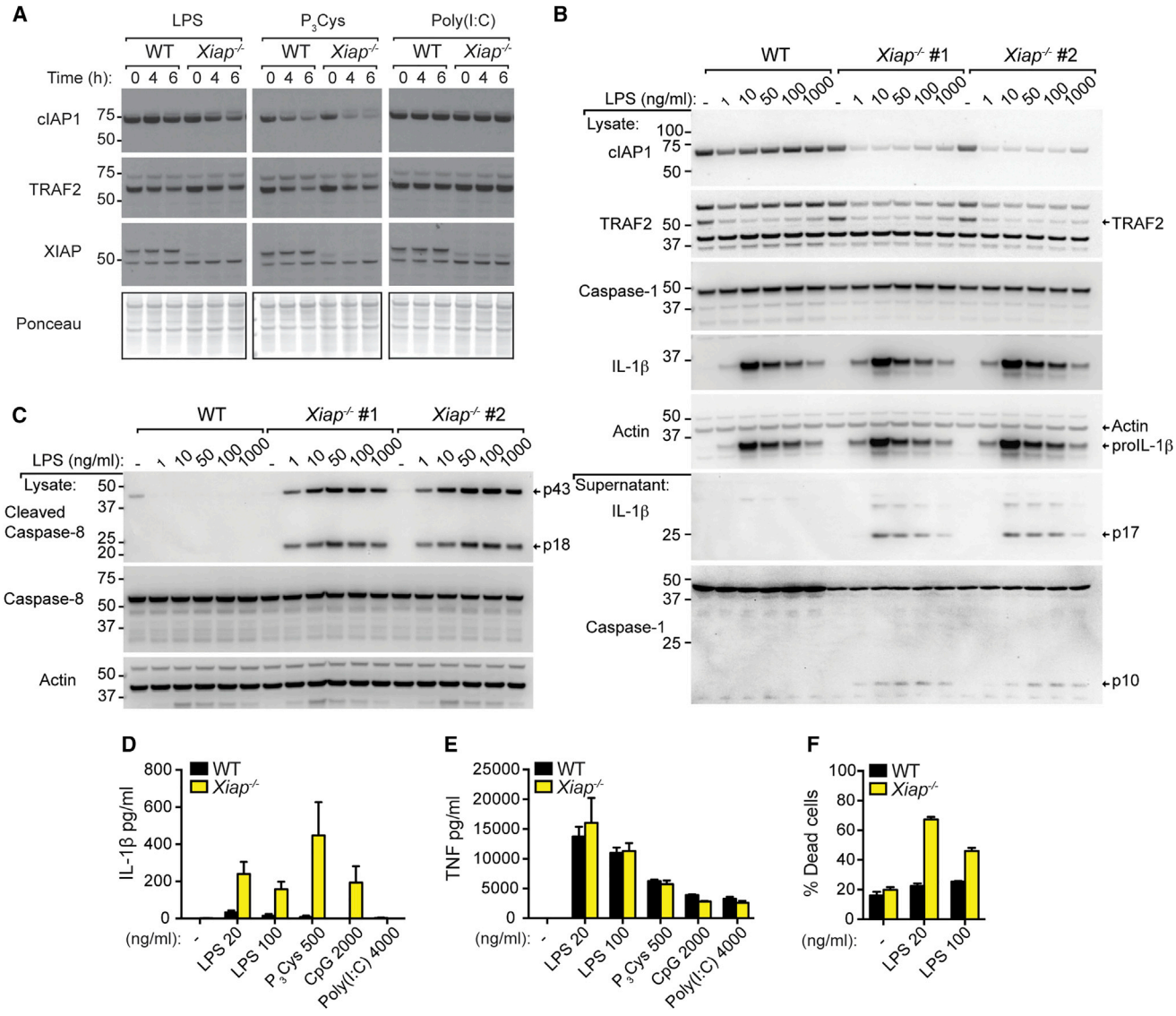

Figure 3. LPS-Induced Loss of cIAP1 Is Accelerated in XIAP-Deficient Macrophages and Is Associated with IL-1β Activation

(A–F) WT and *Xiap*$^{-/-}$ BMDMs were treated with LPS (100 ng/mL), P_3Cys (500 ng/mL), or Poly (I:C) (4 μg/mL), unless otherwise specified.

(A–C) After 8 hr, supernatants and total cell lysates were analyzed by immunoblot (cell lysates in B and C are from the same experiment; one of at least three experiments).

(D–F) After 24 hr, supernatants were harvested and analyzed for (D) IL-1β or (E) TNF levels, and (F) cell death was assessed by PI uptake and flow cytometry after 24 hr (n = 3 mice per group, one of two experiments). Data are the mean + SEM.

See also Figures S2 and S3.

increased peripheral blood neutrophil numbers was observed in *cIAP1*^{LysMcre}*Xiap*$^{-/-}$ animals, suggesting a possible predisposition to heightened granulopoiesis (Figure 5E). However, basal serum cytokine levels, including IL-1β, TNF, and IL-6, were similar in WT, *Xiap*$^{-/-}$, and *cIAP1*^{LysMcre}*Xiap*$^{-/-}$ mice (Figure 5F). These data indicate that the loss of XIAP and cIAP1 in myeloid cells is well tolerated.

Our in vitro results suggested that, although overtly normal, mice lacking XIAP or XIAP and cIAP1 in myeloid cells might be more susceptible to TLR-induced inflammasome responses. We therefore challenged WT, *Xiap*$^{-/-}$, and *cIAP1*^{LysMcre}*Xiap*$^{-/-}$ mice intraperitoneally with LPS (100 μg), and we measured the serum levels of inflammasome-associated IL-1β and IL-1α, as well as TNF and IL-6. Following LPS administration, enhanced serum IL-1α and IL-1β levels were observed after 2 hr in XIAP-deficient mice, and they were even further increased in both serum and peritoneal lavage fluid of *cIAP1*^{LysMcre}*Xiap*$^{-/-}$ mice (Figures 5G and 5H). In contrast, serum TNF and IL-6 levels were comparable among WT, *Xiap*$^{-/-}$, and *cIAP1*^{LysMcre}*Xiap*$^{-/-}$ animals following LPS challenge (Figures 5G and 5H). These data demonstrate that reduced cIAP1 in myeloid cells promotes IL-1β responses in vivo in XIAP-deficient mice.

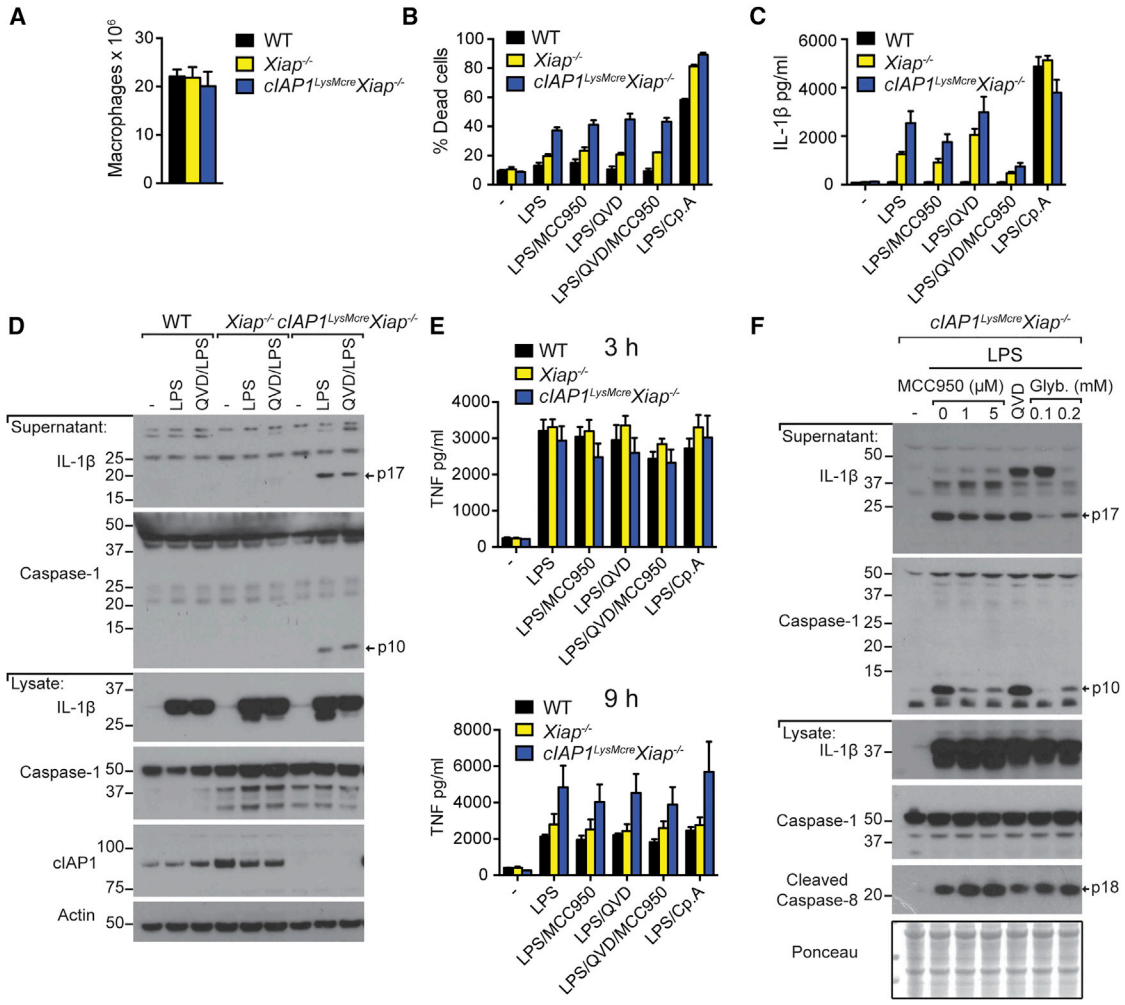

Figure 4. Genetic Deletion of cIAP1 Sensitizes XIAP-Deficient Macrophages to LPS-Induced NLRP3 Inflammasome Activation

(A) Yield of WT, *Xiap*$^{-/-}$, and *cIAP1*^{LysMcre}*Xiap*$^{-/-}$ BMDMs following 6 days of differentiation in 20% L929 cell-conditioned media (n = 3 mice per group). Data are the mean + SEM.

(B–F) WT, *Xiap*$^{-/-}$, and *cIAP1*^{LysMcre}*Xiap*$^{-/-}$ BMDMs were pre-treated as indicated with the pan-caspase inhibitor QVD-OPh (10–20 μM, blocks caspase-8, but not caspase-1) and NLRP3 inhibitors MCC950 (1 μM, unless specified) and Glyburide (Glyb 0.1 mM, unless specified) for 15–30 min prior to stimulation with LPS (100 ng/mL) for up to 9 hr. Alternatively, cells were treated LPS (100 ng/mL) for 3 hr prior to stimulation with the IAP antagonist Cp. A (inhibits XIAP and cIAPs) for 5–6 hr. (B) Apoptotic (LPS) and necroptotic (LPS/QVD) cell death was measured by PI uptake and flow cytometry. (C and E) Cell supernatants were analyzed by ELISA for levels of (C) IL-1β and (E) TNF. (D and F) Supernatants and total cell lysates were analyzed by immunoblot after 7–8 hr of stimulation, as indicated (one of two independent experiments).

In (B), (C), and (E), n = 3 mice per group, representative of two to three experiments. Data are the mean + SEM. See also Figure S4.

TLR-Induced cIAP1 Degradation Is Dependent on MyD88 Signaling and Is Prevented by TRIF-Induced Type I IFN Production

We were intrigued by the fact that Poly(I:C) stimulation failed to induce proteasomal cIAP1-TRAF2 degradation (Figure 3). Unlike the other TLRs, TLR3 signals exclusively via TRIF and does not utilize the adaptor protein MyD88. We therefore examined whether MyD88 was required for cIAP1-TRAF2 loss. Consistent with this idea, P_3Cys-induced TRAF2 and cIAP1 degradation was not observed in MyD88-deficient BMDMs, but it still occurred upon deletion of the TLR3 and TLR4 adaptor TRIF (Figure 6A). Notably however, the loss of TRIF appeared to enhance the degradation of cIAP1 upon LPS exposure (Figure 6B).

LPS-TLR4 complexes uniquely engage both MyD88 to activate NF-κB and TRIF to induce IRF3-regulated type I IFNs. Considering that LPS is less efficient at degrading cIAP1 and that the loss of TRIF enhanced LPS-induced cIAP1 loss, we questioned whether TLR-TRIF-signaling-induced type I IFN production might limit cIAP1 degradation. In support of this hypothesis, priming WT and *Trif*$^{-/-}$ BMDMs for 1 hr with either Poly(I:C) or LPS to trigger TRIF signaling blocked P_3Cys-induced cIAP1 degradation in WT cells, but it afforded no protection from cIAP1 degradation in *Trif*$^{-/-}$ cells (Figure 6C). On the other

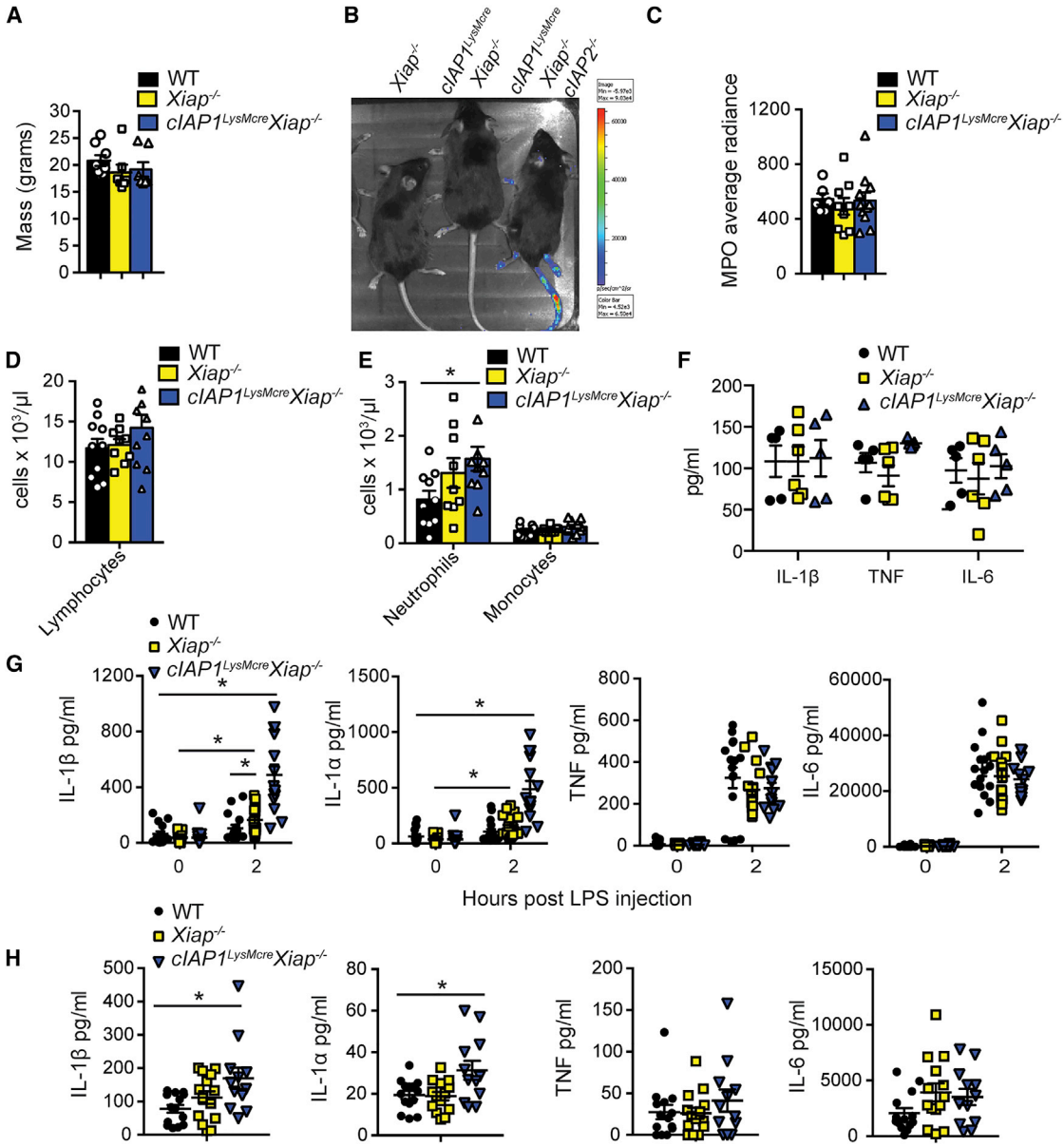

Figure 5. Mice Deficient in XIAP and cIAP1 in Myeloid Cells Are Overtly Normal but Are Hyper-Responsive to LPS-Induced IL-1β Responses In Vivo

(A) Weights of 6- to 8-week-old WT, *Xiap*$^{-/-}$ (littermates), and *cIAP1*^{LysMcre}*Xiap*$^{-/-}$ mice.

(B and C) WT, *Xiap*$^{-/-}$, *cIAP1*^{LysMcre}*Xiap*$^{-/-}$, and *cIAP1*^{LysMcre}*Xiap*$^{-/-}$*cIAP2*$^{-/-}$ mice were injected intraperitoneally with 200 mg/kg luminol, and live in vivo luminescent imaging was performed to measure MPO activity in tissues. (B) Image of MPO activity in mice and (C) average radiance of MPO activity are shown.

(D and E) Peripheral blood was collected at sacrifice of 6- to 8-week-old WT and IAP mutant mice for analysis of leucocyte counts by Advia and flow cytometry; (D) lymphocyte and (E) neutrophil and monocyte numbers are shown.

(F) Serum was collected from 6- to 8-week-old WT and IAP mutant mice and analyzed by ELISA for the indicated cytokines.

(G and H) WT, *Xiap*$^{-/-}$, and *cIAP1*^{LysMcre}*Xiap*$^{-/-}$ mice were pre-bled and then intraperitoneally injected with 100 μg LPS. Following sacrifice 2 hr later, (G) serum and (H) peritoneal lavage fluid were collected and levels of IL-1β, IL-1α, TNF, and IL-6 were measured by ELISA (n ≥ 12/genotype).

In (A) and (C)–(H), each symbol represents an individual mouse. Data are the mean ± SEM (Mann-Whitney test, *p < 0.05). See also Figure S5.

hand, MyD88-mediated TRAF2 degradation still occurred regardless of the status of TRIF (Figure 6C).

To determine whether TRIF-induced type I IFN production countered TLR-induced cIAP1 loss, we next examined BMDMs from IFN receptor 1 (IFNAR1)-deficient mice. LPS-induced cIAP1 degradation occurred more efficiently in *Ifnar1*$^{-/-}$ BMDMs compared to WT cells (Figure 6D). Moreover, TRIF-mediated priming of IFNAR1-deficient BMDMs was unable to prevent

TLR-MyD88-induced cIAP1 degradation (Figure 6D). In contrast, TLR-induced TRAF2 degradation in WT and *Ifnar1*$^{-/-}$ BMDMs was similar with all stimuli (Figure 6D).

The activation of IFNAR1 with recombinant IFNβ not only protected WT BMDMs against P_3Cys- and CpG-dependent cIAP1 degradation but also prevented efficient TLR-mediated precursor IL-1β and NLRP3 expression in both WT and XIAP-deficient cells (Figures 6E and 6F). Consequently, IFNβ treatment abrogated LPS- and P_3Cys-induced caspase-1 and IL-1β processing and secretion in *Xiap*$^{-/-}$ BMDMs, but it did not markedly alter TNF secretion (Figures 6G–6K). Remarkably, IFNβ-mediated stabilization of cIAP1 also correlated with reduced LPS and P_3Cys killing of XIAP-deficient macrophages (Figures 6L and 6M). Collectively, these data show that type 1 IFN signaling acts in an inhibitory loop to block TLR-Myd88-induced cIAP1 degradation, cell death, and IL-1β activation in the absence of XIAP.

To define if IFNβ acts transcriptionally to induce cIAP1 expression, we examined cIAP1 protein and mRNA levels following stimulation of BMDMs with IFNβ alone. IFNβ stimulation resulted in a very modest increase in cIAP1 levels at both the protein and mRNA levels (Figures S6A and S6B), unlike the potent induction of IRF7 following either LPS or IFNβ treatment (Figures S6C and S6D). Therefore, IFNβ protection against TLR-induced loss of cIAP1 is unlikely to be solely a result of increased gene transcription.

TLR-Induced TNFR2 Expression Is Required for the cIAP1-TRAF2 Degradation that Allows Efficient IL-1β Activation and Cell Death in XIAP-Deficient Macrophages

LPS-induced TNF production is required for IL-1β activation and cell death in XIAP-deficient cells (Yabal et al., 2014), but the roles of the two TNFRs, TNFR1 and TNFR2, in this process have not been clarified. Given that TLR-mediated cIAP1-TRAF2 degradation is reminiscent of TNFRSF signaling, we asked if TNFR2 contributed to the XIAP-deficient phenotype. Notably, both LPS and P_3Cys stimulation of macrophages induced the production of TNF, TNFR2 mRNA, and increased cell surface TNFR2 protein levels (Figures S6F–S6J). These events correlated with TLR-induced cIAP1/TRAF2 degradation, and they occurred immediately prior to caspase-8 processing and the detection of secreted IL-1β (Figures S6E and S2B). Remarkably, TNFR2-deficient, but not TNFR1-deficient, BMDMs were completely protected from LPS-, P_3Cys-, CpG-, and R837-induced cIAP1 and TRAF2 degradation (Figure 7A). Therefore, TLR-induced TNF signaling through TNFR2 drives cIAP1-TRAF2 degradation upon stimulation with TLR ligands, and this correlates with caspase-8-mediated activation of IL-1β.

To determine if TNFR2-induced cIAP1 degradation impacts cell death and IL-1β responses in XIAP-deficient macrophages, we first utilized a neutralizing anti-TNFR2 antibody. Similar to the genetic loss of TNFR2 alone, TNFR2 neutralization prevented LPS- and P_3Cys-dependent cIAP1 and TRAF2 degradation (Figures 7B and S7A). Significantly, the treatment of *Xiap*$^{-/-}$ BMDMs with anti-TNFR2 to prevent cIAP1 degradation limited LPS- or P_3Cys-mediated IL-1β and caspase-1 processing and secretion, as well as caspase-8 activation and cell death (Figures 7C–7F, S7C, and S7D). Conversely, TNFR2 neutralization did not blunt LPS- or P_3Cys-induced TNF production (Figures S7B, S7E, and S7F).

If TNFR2-induced cIAP1 degradation is essential for TLR-induced IL-1β activation following XIAP loss, then direct targeting of cIAP1 by IAP antagonist treatment should bypass the requirement for TNFR2 signaling. Consistent with this, the neutralization of TNFR2 failed to inhibit TLR-induced IL-1β and caspase-1 activation and secretion in XIAP-deficient cells that were also treated with the cIAP-targeting IAP antagonist 711 (birinapant) (Figures 7E, 7F, and S7F). Similarly, treatment of TNFR2-deficient BMDMs with LPS or P_3Cys, combined with the IAP antagonist Cp. A to inhibit XIAP and cIAPs (Condon et al., 2014; Vince et al., 2007), resulted in robust IL-1β secretion and cell death akin to WT cells (Figures S7G and S7H).

To demonstrate the absolute requirement for TNFR2 in promoting IL-1β and cell death responses resulting from the loss of XIAP, we generated *Xiap*$^{-/-}$*Tnfr2*$^{-/-}$ mice. Analysis of these mice recapitulated results using anti-TNFR2. Specifically, LPS- and P_3Cys-induced cIAP1-TRAF2 degradation and subsequent IL-1β secretion and cell death, but not TNF secretion, were blocked in *Xiap*$^{-/-}$*Tnfr2*$^{-/-}$ cells, compared to the loss of XIAP alone (Figures 7G, 7H, S7I, and S7J). Notably, akin to TNFR2 neutralization studies, IAP antagonist targeting of cIAPs eliminated the requirement for TNFR2 signaling for IL-1β activation and cell death (Figures 7G and 7H).

IAP antagonist targeting of XIAP, cIAP1, and cIAP2 can promote an LPS-TLR4-TRIF axis to drive caspase-8 and IL-1β activation in the absence of TNF-TNFR1 signaling (Lawlor et al., 2015). In line with these results, neutralizing TNFR1 failed to reduce IL-1β secretion in LPS-stimulated *Xiap*$^{-/-}$ BMDMs, although, as expected, it did block TNF- and Cp. A-induced killing (Figures S7K–S7M). On the other hand, P_3Cys-induced TLR1/2 does not utilize TRIF to activate caspase-8, and presumably it activates caspase-8 via autocrine TNF-TNFR1 activation. Consistent with this idea, TNFR1 blockade reduced P_3Cys-induced IL-1β, but not TNF, secretion (Figures S7L and S7M). Intriguingly however, neither TNFR1 nor TNFR2 were essential for P_3Cys-induced IL-1β secretion or cell death when XIAP and cIAP1/2 were inhibited with the IAP antagonist Cp. A (Figures S7G and S7H). This finding indicates that the induction of alternative death ligands may eventually substitute for TNFR1-induced caspase-8 activation.

DISCUSSION

XIAP deficiency can result in a hyperinflammatory disease, X-linked HLH, that is often triggered by pathogen infections. Why this is the case has remained unclear, as XIAP has typically been implicated in the inhibition of apoptosis, but not the repression of inflammatory responses. Intriguingly, X-linked HLH can present with clinical features reminiscent of diseases caused by autoactivating NLRP3 inflammasome mutations that result in pathological IL-1β activation (Christiansen et al., 2016; Speckmann et al., 2013; Standing et al., 2012; Wada et al., 2014). Our findings show that XIAP deficiency sensitizes myeloid cells to TLR- or TNFR1-mediated cell death and NLRP3 signaling via

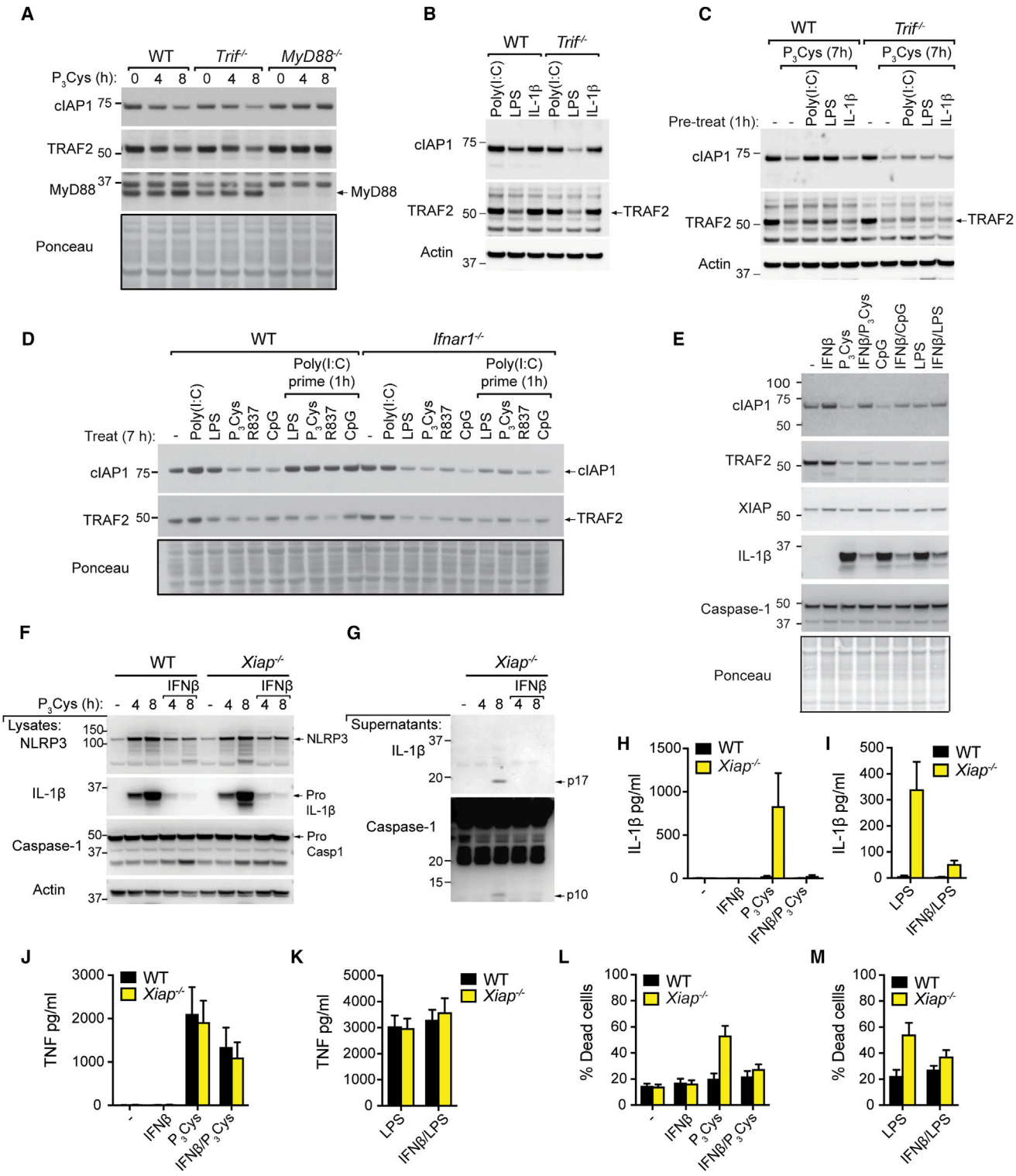

Figure 6. A TRIF-Type I IFN Axis Acts to Block TLR-MyD88-Induced cIAP1 Degradation and Cell Death

(A and B) WT, *MyD88*$^{-/-}$, and *Trif*$^{-/-}$ BMDMs were treated with P_3Cys (500 ng/mL), Poly(I:C) (4 μg/mL), LPS (100 ng/mL), and recombinant murine IL-1β (200 ng/mL) for 7 hr, and total cell lysates were analyzed by immunoblot.

(C) WT and *Trif*$^{-/-}$ BMDMs were pre-treated with or without Poly(I:C) (4 μg/mL), LPS (100 ng/mL), or recombinant murine IL-1β for 1 hr prior to stimulation with P_3Cys (500 ng/mL) for a further 7 hr. Total cell lysates were analyzed by immunoblot.

(legend continued on next page)

TLR-induced upregulation of TNFR2 and TNFR2-driven degradation of the related IAP, cIAP1. In support of the central role for cIAP1 degradation in triggering these pathways in the absence of XIAP, the requirement for TNFR2 is bypassed if cIAP1 is independently removed from cells through IAP antagonist treatment. Therefore, TLR co-operation with TNFR2 signaling in innate immune cells defines XIAP and cIAP1 as central suppressors of inappropriate caspase-8-driven NLRP3, IL-1β, and cell death responses.

Infectious or genetic hyperinflammatory disease in susceptible mice and humans can be driven through excessive TLR signaling, and it is often referred to as HLH or macrophage activation syndrome (Janka and Lehmberg, 2014). Previous research demonstrated that XIAP deficiency allowed for spontaneous TLR-induced IL-1β activation in a range of innate immune cells by engaging caspase-8 to both activate the NLRP3 inflammasome and process IL-1β directly (Lawlor et al., 2015; Vince et al., 2012; Wicki et al., 2016; Yabal et al., 2014). Unlike WT murine BMDMs, TLR signaling in human monocytes and murine dendritic cells has been reported to engage caspase-8 to activate NLRP3 and cause spontaneous activation and secretion of IL-1β (Gaidt et al., 2016; Moriwaki et al., 2015). It will, therefore, be of interest to determine whether TLR- and TNFR2-induced cIAP1-TRAF2 degradation or XIAP expression levels contribute to caspase-8 activation and NLRP3 responses in these cell types.

Chemical depletion of cIAPs using IAP antagonist compounds sensitizes macrophages to TLR-induced IL-1β activation and secretion upon XIAP deletion. This agrees with the spontaneous inflammatory phenotype that presents in mice with myeloid cell-restricted deletion of cIAP1/2 and XIAP and that is absent in single-IAP gene-targeted mice (Lawlor et al., 2015; Wong et al., 2014). Triple-IAP-deficient mice exhibit systemic inflammation, including prominent arthritis and elevated IL-1β and TNF serum levels, which resemble the IL-1-driven pathology of NLRP3-autoactivating mice (Bonar et al., 2012). This work therefore alluded to the potential that the inflammatory XIAP-deficient phenotype, revealed upon microbial infection or TLR stimulation, is likely to be modified by changes in cIAP expression levels. In this regard, our results demonstrate a significant reduction in total cellular cIAP1 and TRAF2 levels upon TLR-MyD88-driven TNFR2 signaling. This decrease in cIAP1 is required for the efficient activation of IL-1β observed upon XIAP loss, because (1) myeloid cell genetic deletion of cIAP1 alone on an XIAP-deficient background significantly enhances IL-1β activation in vitro and in vivo; (2) blocking cIAP1 degradation by ablating TNFR2 signaling prevents caspase-8, NLRP3, and IL-1β activation in XIAP-knockout mice; and (3) inducing cIAP1 degradation by IAP antagonist treatment allows efficient TLR-induced IL-1β activation in XIAP-targeted macrophages, even in the absence of TNFR2.

Mice with myeloid cell-specific loss of cIAP1 and XIAP were viable and healthy. This contrasts to the embryonic lethality following germline loss of XIAP and cIAP1 (Moulin et al., 2012), indicating that the deletion of myeloid cell IAPs does not drive this lethal phenotype. However, when mice with healthy myeloid cells deficient in both cIAP1 and XIAP were challenged with LPS, serum IL-1β levels were elevated above the already exacerbated levels observed in $Xiap^{-/-}$ animals, proving that in vivo caspase-8/NLRP3-induced IL-1β responses are dictated by both myeloid cell XIAP and cIAP1 expression levels. The increased LPS-induced IL-1β serum levels in $cIAP1^{LysMcre}Xiap^{-/-}$ mice when compared to $Xiap^{-/-}$ mice is likely to reflect the complete loss of cIAP1 achieved in $cIAP1^{LysMcre}Xiap^{-/-}$ animals versus the partial cIAP1 degradation (~60%) achieved in XIAP-deficient mice.

TRAF2 and cIAPs were first identified through the analysis of TNFR2-interacting components (Rothe et al., 1994, 1995), and their degradation through TNF superfamily receptors, such as TNFR2 and FN14, triggers non-canonical NF-κB and sensitizes cell lines to TNFR1-mediated killing (Fotin-Mleczek et al., 2002; Rauert et al., 2010; Siegmund et al., 2016; Varfolomeev et al., 2007; Vince et al., 2008). Our findings now document that TLRs also require non-canonical NF-κB for optimal cytokine production and that this is promoted by the induction of TNF-TNFR2 signaling and cIAP1-TRAF2 degradation. Unlike experiments performed in immortalized cell lines (Moulin et al., 2012; Vince et al., 2007), our work demonstrates that, in macrophages, cIAP loss alone does not suffice for efficient LPS or TNF killing or IL-1β activation and that the removal of XIAP is essential (Lawlor et al., 2015). This is particularly intriguing, as unlike cIAP1, XIAP does not associate with the intracellular portion of TNFR1 (Yabal et al., 2014), nor has it been described within TLR or TNFR2 complexes. Perhaps XIAP, redundantly with cIAP1, inhibits another component important for causing TLR-induced cell death and IL-1β responses. Regardless, it is notable that LPS-induced cIAP1 degradation was enhanced in XIAP-deficient cells, highlighting the possibility that XIAP interactions with cIAPs may influence IAP stability under specific conditions (Silke et al., 2005).

We have demonstrated how TLRs and TNFR2 can cooperate to drive inflammatory responses through non-canonical NF-κB activity and, upon XIAP deficiency, NLRP3 inflammasome and IL-1β activation. Other than XIAP, the loss or mutation of several components involved in the regulation of TNF and TLR signaling, including A20, LUBAC, and RIPK1, can precipitate in autoinflammatory disease in mice or humans and sensitize to

(D) WT and $Ifnar1^{-/-}$ BMDMs were pre-treated with or without Poly(I:C) (4 μg/mL) for 1 hr prior to stimulation with Poly(I:C) (4 μg/mL), LPS (100 ng/mL), P_3Cys (500 ng/mL), R837 (4 μg/mL), or CpG (2 μg/mL) for a further 7 hr. Cellular lysates were analyzed by immunoblot for the indicated proteins.
(E) WT BMDMs were pre-treated with or without recombinant murine IFNβ (1,000 units/mL) for 1 hr prior to stimulation with P_3Cys (500 ng/mL), CpG (2 μg/mL), or LPS (100 ng/mL) for a further 7 hr, as indicated. Immunoblots were then performed for the indicated proteins.
(F–M) WT and $Xiap^{-/-}$ BMDMs were pre-treated with or without recombinant murine IFNβ (1,000 units/mL) for 0.5–1 hr prior to stimulation with P_3Cys (500 ng/mL) or LPS (100 ng/mL) for up to 8 hr. (F and G) Total cell lysates were examined by immunoblot as indicated. (H–K) Cell supernatants were analyzed by ELISA for levels of IL-1β and TNF, as indicated. (L and M) Cell death was measured by PI uptake and flow cytometry.
For (A)–(G), experiments were performed at least two times (H, J, and L, five pooled experiments each containing n = 2–3 mice; I, K, and M, three pooled independent experiments each containing n = 3 mice per group). Data are the mean + SEM. See also Figure S6.

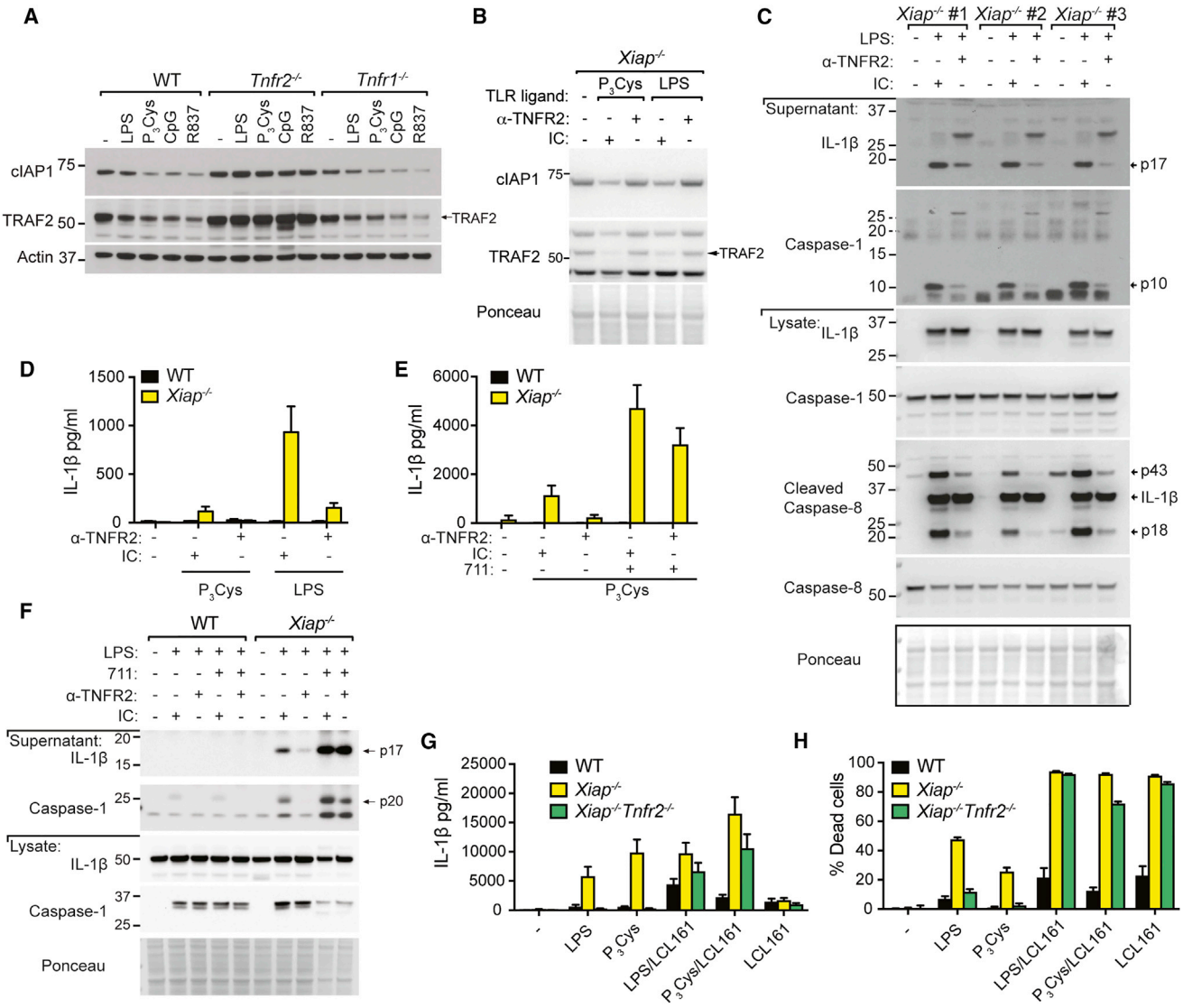

Figure 7. TLR-MyD88 Induce TNFR2 to Drive cIAP1 and TRAF2 Degradation that Is Required for IL-1β Activation in the Absence of XIAP

(A) WT, *Tnfr1*$^{-/-}$, and *Tnfr2*$^{-/-}$ BMDMs were treated with LPS (100 ng/mL), P_3Cys (500 ng/mL), CpG (2 μg/mL), and R837 (4 μg/mL) for 7 hr, and cellular lysates were immunoblotted for the indicated proteins (one of three experiments).

(B–D) WT and *Xiap*$^{-/-}$ BMDMs were pre-treated with anti-TNFR2 or isotype control (IC) hamster IgG (20 μg/mL) for 30 min prior to treatment with P_3Cys (500 ng/mL) or LPS (100 ng/mL) for 7 hr. (B and C) Cell lysates and supernatants were analyzed by immunoblot as indicated (one of two experiments). (D) Supernatants were analyzed for IL-1β by ELISA. Data are the mean + SEM (n = 3 mice per group, one of three experiments).

(E and F) WT and *Xiap*$^{-/-}$ BMDMs were pre-treated with anti-TNFR2 or IC hamster IgG (20–40 μg/mL) for 30 min prior to treatment with P_3Cys (500 ng/mL) for 2 hr and IAP antagonist 711 (1 μM, targets cIAP1 and cIAP2 preferentially), as indicated, for a further 6 hr. (E) Supernatants were analyzed for IL-1β by ELISA. Data are the mean + SEM (n = 3 mice per group, two pooled independent experiments). (F) Immunoblots were performed on total cell lysates and supernatants (one of two experiments).

(G and H) WT, *Xiap*$^{-/-}$, and *Xiap*$^{-/-}$ *Tnfr2*$^{-/-}$ BMDCs were pre-treated with LPS (20 ng/mL) or P_3Cys (500 ng/mL) for 2–3 hr and then treated, as indicated, with IAP antagonist LCL161 (1 μM) and harvested after 24 hr. (G) Supernatants were analyzed for IL-1β and (H) cell viability was measured (expressed as percentage dead cells).

Data are the mean + SEM (three to four pooled independent experiments). See also Figures S7 and S8.

TNF- or TLR-induced NLRP3 inflammasome signaling (Vince and Silke, 2016). Notably, delineating the mechanisms by which their loss triggers pathological IL-1β activity upon TLR or TNF engagement is likely to reveal novel pathways and therapeutic targets applicable to treating both rare and common inflammatory conditions. In this regard, it will be interesting to determine if our insights into TLR-induced IL-1β activation resulting from XIAP deficiency may contribute to other hereditary autoinflammatory conditions or be required for beneficial immune responses to microbial infections.

EXPERIMENTAL PROCEDURES

Mice

All mice were housed under standard conditions at WEHI or at the Technische Universität München. All procedures were approved by the WEHI or the District Government of Upper Bavaria. Female and male mice were at least 6 weeks old at the time of experimentation, and all animals were age- (6–8 weeks) and sex- (male) matched for in vivo experiments. cIAP1 (*Birc2*$^{flox/flox}$) and cIAP2 (*Birc3*$^{FRT/FRT}$) mice were generated on a C57BL/6 background (Moulin et al., 2012) and backcrossed to the XIAP-deficient mouse, which had been backcrossed to C57BL/6 mice (n > 10 generations). These mice were utilized to generate conditional deletion of cIAP1 in myeloid cells (Lysozyme M Cre transgenic), and they are referred to as *cIAP1*^{LysMcre}*Xiap*$^{-/-}$ mice. Other mouse strains are detailed in the the Supplemental Experimental Procedures.

Cell Culture

To generate BMDMs and bone marrow-derived dendritic cells (BMDCs), bone marrow was harvested from the femoral and tibial bones. For BMDMs, bone marrow was cultured in DMEM containing 10% fetal bovine serum (FBS, Sigma), 50 U/mL penicillin, and 50 μg/mL streptomycin (complete media) and supplemented with 20% L929 cell-conditioned medium for 6 days (37°C and 10% CO_2). To derive BMDCs, red blood cell lysis was performed prior to seeding in VLE-RPMI (Biochrome) supplemented with 10% FBS (SeraPlus, Pan Biotech), Glutamine, penicillin, streptomycin, β-mercapto-ethanol (Gibco), and 20 nM GM-CSF (PeproTech). Culture medium was supplemented on days 3 and 6 of culture. Unless otherwise indicated, BMDMs and BMDCs were routinely plated at 4 × 10^5 cells/well in 24-well tissue culture plates (BD Falcon) or 2 × 10^5 cells/well in 96-well flat-bottom tissue culture plates. Alternatively, BMDMs were plated at 3 × 10^5 or 1 × 10^5 cells in 24-well or 96-well non-tissue culture-treated plates for flow cytometric analysis. Treatment conditions and reagents are detailed in the Supplemental Experimental Procedures.

Cytokine Analysis

IL-1β (R&D Systems), IL-1α (BioLegend), TNF, IL-6, IL-10, and MCP-1 (eBioscience) ELISA kits or IL-1β, IL-6, and TNF ProcartaPlex assays (Thermo Fisher Scientific) were routinely used to assay supernatants, serum, or peritoneal fluid, according to the respective manufacturer's instructions. In some cases, serum IL-1β was measured using an enhanced Cytokine Bead Array (BD Biosciences). In the indicated experiments, supernatants were analyzed utilizing a Bio-plex (Bio-plex Pro Cytokine 23-plex assay, Bio-Rad).

Endotoxin Model

Mice were bled retro-orbitally at least 4 days prior to LPS challenge to obtain baseline serum for analysis. Mice were injected with 100 μg LPS (Ultra-pure, Invivogen) and 2 hr later sacrificed, and cardiac bleeds and peritoneal lavages were performed to collect serum and peritoneal fluid. Samples were stored at −80°C until analysis.

Statistical Analyses

The Mann-Whitney test was used to analyze the level of significance between MPO measurements and to compare in vivo cytokine levels, and an unpaired, two-tailed Student's t test was used to analyze differences in cell populations. For each test, p values < 0.05 were considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.06.073.

AUTHOR CONTRIBUTIONS

The project was conceived by J.E.V. and K.E.L.; the experiments were designed by J.E.V., K.E.L., R.F., M.Y., and P.J.J.; and the manuscript was written by J.E.V., K.E.L., R.F., M.Y., and P.J.J. The experiments were performed by K.E.L., R.F., M.Y., J.E.V., S.A.C., K.W.C., S.Z., C.G., Y.Z., T.A.N., C.H., D.B.D., S.M.C., and A.J.V. Expert advice and essential reagents were provided by K.C.P., K.S., J.S., and D.L.V. All authors assisted with data interpretation and manuscript editing.

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