

# XIAP Restricts TNF- and RIP3-Dependent Cell Death and Inflammasome Activation

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## SUMMARY

X-linked inhibitor of apoptosis protein (XIAP) has been identified as a potent regulator of innate immune responses, and loss-of-function mutations in XIAP cause the development of the X-linked lymphoproliferative syndrome type 2 (XLP-2) in humans. Using gene-targeted mice, we show that loss of XIAP or deletion of its RING domain lead to excessive cell death and IL-1 $\beta$  secretion from dendritic cells triggered by diverse Toll-like receptor stimuli. Aberrant IL-1 $\beta$  secretion is TNF dependent and requires RIP3 but is independent of cIAP1/cIAP2. The observed cell death also requires TNF and RIP3 but proceeds independently of caspase-1/caspase-11 or caspase-8 function. Loss of XIAP results in aberrantly elevated ubiquitylation of RIP1 outside of TNFR complex I. Virally infected *Xiap*<sup>-/-</sup> mice present with symptoms reminiscent of XLP-2. Our data show that XIAP controls RIP3-dependent cell death and IL-1 $\beta$  secretion in response to TNF, which might contribute to hyperinflammation in patients with XLP-2.

## INTRODUCTION

X-linked inhibitor of apoptosis protein (XIAP) is an antiapoptotic protein that inhibits induction of cell death in response to intrinsic as well as extrinsic apoptotic stimuli (Gyrd-Hansen and Meier, 2010; Jost et al., 2009). Inhibition of apoptosis by XIAP is mediated by inhibiting caspase-3 and caspase-7 via its N-terminal

baculoviral IAP repeat (BIR) domains (Deveraux et al., 1997). The first characterization of *Xiap*<sup>-/-</sup> mice showed no obvious phenotype (Olayioye et al., 2005), whereas recent studies have demonstrated a reduced capacity of *Xiap*<sup>-/-</sup> mice to clear certain infectious pathogens (Bauler et al., 2008; Prakash et al., 2010). In addition, the RING domain and the BIR2 domain of XIAP have been implicated in nuclear factor- $\kappa$ B (NF- $\kappa$ B) and mitogen-activated protein kinase (MAPK) signaling in response to activation of nucleotide-binding oligomerization domain 1 (NOD1) and NOD2 (Damgaard et al., 2012, 2013).

To date, XIAP has not been implicated in tumor necrosis factor (TNF)/TNF receptor (TNFR) signaling despite a well-defined role for its close IAP family members, cellular inhibitor of apoptosis proteins 1 and 2 (cIAP1/cIAP2), in this pathway (Gyrd-Hansen and Meier, 2010). cIAP1/cIAP2-dependent K63 ubiquitylation of receptor-interacting protein 1 (RIP1) after TNF stimulation provides a prosurvival signaling platform allowing for the induction of NF- $\kappa$ B and MAPK signaling (Bertrand et al., 2008). Loss of cIAP1/cIAP2 or deubiquitylation of RIP1 coincides with the formation of secondary TNFR signaling complexes (TNF-RSCs) that trigger cell death either through caspase-8-mediated apoptosis or through RIP3- and MLKL-dependent necroptosis (Murphy et al., 2013; O'Donnell et al., 2011; Vince et al., 2007).

There are indications that elevated necroptosis contributes to inflammation and tissue damage, at least in part, mediated by interleukin-1 $\beta$  (IL-1 $\beta$ ) (Günther et al., 2011; Kang et al., 2013). IL-1 $\beta$  is an evolutionarily conserved cytokine that potently mediates innate and adaptive immune responses, and its deregulation causes several autoinflammatory diseases (Lamkanfi and Dixit, 2012). IL-1 $\beta$  is translated as an inactive proform and requires processing into bioactive IL-1 $\beta$  by the inflammasome (Martinon et al., 2002).

Mutations within *BIRC4* (XIAP) have been identified as the genetic cause of the X-linked lymphoproliferative syndrome type 2 (XLP-2). The mutations mostly affect the C-terminal RING domain or result in complete loss of protein expression (Damgaard et al., 2013; Marsh et al., 2010; Pachlopnik Schmid et al., 2011). Clinical symptoms are mostly attributed to the aberrant activation of macrophages and dendritic cells (DCs) and the accumulation of activated T lymphocytes often in response to viral infection (Marsh et al., 2010). This hyperactivation of immune cells results in elevated systemic levels of proinflammatory cytokines, such as IL-1 $\beta$ , interferon- $\gamma$  (IFN $\gamma$ ), TNF, IL-6, and IL-18 (Marsh et al., 2010; Rigaud et al., 2006; Wada et al., 2014).

We demonstrate here an unexpected function of XIAP in curbing excessive TNF-induced inflammasome activation. This function was independent of cIAP1/cIAP2 and restricted IL-1 $\beta$  production in a RIP3-dependent manner. Viral infection of *Xiap*<sup>-/-</sup> mice resulted in a hyperinflammatory phenotype similar to that observed in patients with XLP-2, indicating that exaggerated IL-1 $\beta$  secretion might contribute to XLP-2 pathogenesis.

## RESULTS

### TLR Activation Induces Exaggerated IL-1 $\beta$ Secretion in *Xiap*<sup>-/-</sup> and *Xiap* <sup>$\Delta$ RING</sup> Dendritic Cells

XLP-2 pathology in patients is mostly ascribed to a severe hyperinflammation in response to microbial infections. We therefore hypothesized that microbial ligands trigger deregulated immune responses in *Xiap*<sup>-/-</sup> mice. To study cytokine responses, we differentiated dendritic cells (DCs) from bone marrow progenitors (BMDCs) of mice lacking XIAP or expressing a knockin allele of a RING-deleted version of XIAP termed *Xiap* <sup>$\Delta$ RING</sup> (*Xiap* <sup>$\Delta$ RING/ $\Delta$ RING</sup>) (Olayioye et al., 2005; Schile et al., 2008) (Figure S1A). After treatment with ligands for Toll-like receptor 3 (TLR3), TLR4, and TLR9, we observed a significant increase of IL-1 $\beta$  secretion from *Xiap*<sup>-/-</sup> and *Xiap* <sup>$\Delta$ RING</sup> BMDCs compared to wild-type (WT) BMDCs. Whereas TNF secretion from *Xiap*<sup>-/-</sup> and *Xiap* <sup>$\Delta$ RING</sup> cells was comparable to WT, the exaggerated IL-1 $\beta$  secretion correlated with increased cell death (Figure 1A). A similar phenotype was also observed in bone marrow-derived macrophages (BMDMs) (Figure S1B). IL-1 $\beta$  secretion and cell death, measured by a decrease of intracellular ATP levels or lactate dehydrogenase (LDH) release, occurred between 4 and 8 hr of lipopolysaccharide (LPS) exposure only in *Xiap*<sup>-/-</sup> BMDCs (Figure 1B). However, NF- $\kappa$ B and MAPK signaling and subsequent gene induction were largely identical in both genotypes (*Xiap*<sup>-/-</sup> and WT) in response to LPS, despite a minor elevation of phosphorylated p38 in *Xiap*<sup>-/-</sup> cells (Figures 1C and S1C). The elevated secretion of IL-1 $\beta$  in *Xiap*<sup>-/-</sup> BMDCs in response to LPS was remarkable because conventional inflammasome activation requires a priming and an activating signal, such as ATP, for inflammasome formation (Gross et al., 2012; Martinon et al., 2002). The hypersecretion of IL-1 $\beta$  correlated with the formation of conventional inflammasome complexes as shown by accumulation of NOD-like receptor family pyrin domain-containing 3 (NLRP3), Apoptosis-associated Speck-like protein containing a CARD (ASC), and caspase-1 within the NP-40-insoluble fraction only in *Xiap*<sup>-/-</sup> cells, but not in WT cells treated with LPS only (Figure 1D). WT BMDCs recruited

ASC and caspase-1 into the NP-40 fraction only after treatment with LPS plus ATP (Figure 1D). We also observed recruitment of caspase-8 into the NP-40 fraction in *Xiap*<sup>-/-</sup> cells but not in WT cells after LPS treatment (Figure 1D). Immunofluorescence microscopy further confirmed ASC/caspase-1 speck formation in *Xiap*<sup>-/-</sup> BMDCs treated with LPS only (Figure 1E), whereas speck formation in WT cells required the addition of ATP or nigericin (Gross et al., 2012) (Figures 1D and 1E).

Of note, conventional inflammasome activation in response to ATP (Figure S1D) or nigericin (Figure 1E), and inflammasome inhibition by blocking potassium efflux or by caspase inhibition (Figure S1E), was identical in WT and *Xiap*<sup>-/-</sup> BMDCs. Together, this demonstrated that the priming signal alone induced substantial inflammasome formation and IL-1 $\beta$  secretion in *Xiap*<sup>-/-</sup> BMDCs, but not in WT BMDCs.

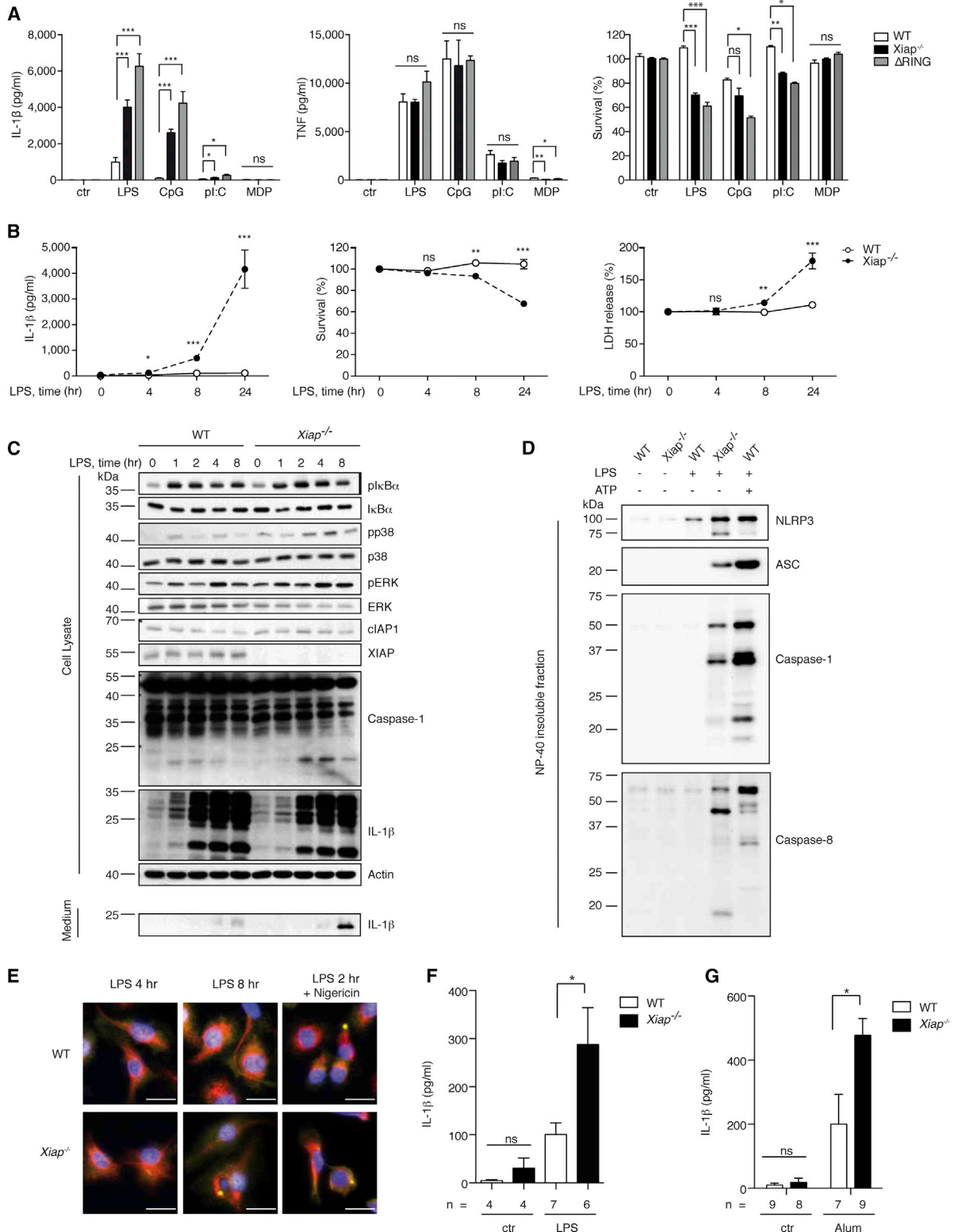
Because necrotic cell death can activate the inflammasome (Lamkanfi and Dixit, 2012), we examined the connection between IL-1 $\beta$  secretion and cell death. To characterize the contribution of damage-associated molecular pattern molecules (DAMPs) released from dying cells to the IL-1 $\beta$  secretion observed, we costimulated BMDCs with LPS plus lysates from dead cells generated by repeated freeze-thaw cycles. This treatment failed to further enhance IL-1 $\beta$  secretion after LPS (Figure S1F). In addition, we cocultured WT and *Xiap*<sup>-/-</sup> BMDCs prior to LPS stimulation. Supporting a DAMP-independent cause of inflammasome activation, we observed caspase-1 specks only in *Xiap*<sup>-/-</sup> cells, whereas directly adjacent WT cells did not contain specks (Figure S1G). Our data argue that cell-intrinsic signaling aberrations in *Xiap*<sup>-/-</sup> and *Xiap* <sup>$\Delta$ RING</sup> BMDCs resulted in increased inflammasome formation.

Similar to the systemic cytokinemia observed in patients with XLP-2, we hypothesized that activation of the innate immune system might drive an exaggerated inflammatory response. This was confirmed in a murine model of acute peritonitis elicited by intraperitoneal (i.p.) injection of LPS or the adjuvant alum, which both resulted in increased IL-1 $\beta$  levels in the peritoneal fluid of *Xiap*<sup>-/-</sup> mice when compared to WT (Figures 1F and 1G). These data show that TLR stimulation of DCs caused elevated inflammasome formation and IL-1 $\beta$  secretion in vitro and in vivo when XIAP was missing.

### IL-1 $\beta$ Secretion in Response to LPS in *Xiap*<sup>-/-</sup> BMDCs Is Mediated by TNF Signaling

We next examined what factors control the IL-1 $\beta$  secretion in *Xiap*<sup>-/-</sup> cells. IL-1 $\beta$  secretion was reduced by caspase-1 inhibitor treatment (YVAD), whereas an IL-1 $\beta$  receptor antagonist (IL-1RA; anakinra) did not substantially reduce IL-1 $\beta$  levels in *Xiap*<sup>-/-</sup> BMDCs, although some reduction was seen in *Xiap* <sup>$\Delta$ RING</sup> cells (Figure S2A). This argues against a prominent role for autocrine or paracrine IL-1 receptor signaling. In contrast, anti-TNF- $\alpha$  (Enbrel) potently inhibited IL-1 $\beta$  secretion, reduced cell death, and blocked processing of caspase-8 in *Xiap*<sup>-/-</sup> and *Xiap* <sup>$\Delta$ RING</sup> BMDCs (Figures S2A and S2B).

In line with a possible role of XIAP in regulating signaling events downstream of TNF/TNFR, we observed elevated IL-1 $\beta$  secretion and cell death in *Xiap*<sup>-/-</sup> and *Xiap* <sup>$\Delta$ RING</sup> BMDCs in response to recombinant TNF (Figure 2A). This was interesting because XIAP has not been directly implicated in TNF/TNFR signal



**Figure 1. Exaggerated IL-1 $\beta$  Secretion in *Xiap*<sup>-/-</sup> and *Xiap* <sup>$\Delta$ RING</sup> BMDCs and Mice**

(A) Secreted IL-1 $\beta$ , TNF, and survival of WT, *Xiap*<sup>-/-</sup>, and *Xiap* <sup>$\Delta$ RING</sup> BMDCs treated with LPS, CpG (250 nM), polyI:C (10  $\mu$ g/ml) or Muramyl dipeptide (MDP) (10  $\mu$ g/ml) for 24 hr. ctr, control; ns, not significant.

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transduction. Of note, we did not observe significant changes in NF- $\kappa$ B or MAPK signaling and gene induction in response to TNF in *Xiap*<sup>-/-</sup> and *Xiap* <sup>$\Delta$ RING</sup> BMDCs (Figures S2C and S2D).

To better define the role of XIAP in the control of TNF signaling, we intercrossed *Xiap*<sup>-/-</sup> mice with mice deficient for TNF (*Tnf*<sup>*tm1Gkh*</sup>) (Pasparakis et al., 1996). BMDCs from *Tnf*<sup>-/-</sup>*Xiap*<sup>-/-</sup> double-deficient mice differentiated normally in culture (Figure S2E). Supporting a critical role of TNF/TNFR signaling for the observed phenotype, we found that genetic deletion of TNF reduced IL-1 $\beta$  secretion to WT levels after LPS (Figure 2B). Exogenous addition of recombinant TNF to *Tnf*<sup>-/-</sup>*Xiap*<sup>-/-</sup> BMDCs fully recapitulated the excessive IL-1 $\beta$  production observed in *Xiap*<sup>-/-</sup> cells (Figure 2B). Deletion of TNF also rescued *Xiap*<sup>-/-</sup> BMDCs from cell death, implicating TNF-induced signaling events in both inflammasome activation and cell death (Figure 2B). Importantly, canonical inflammasome activation with ATP after a short priming stimulus with LPS caused normal inflammasome activation in *Tnf*<sup>-/-</sup> and *Tnf*<sup>-/-</sup>*Xiap*<sup>-/-</sup> BMDCs (Figure S2F). This is in agreement with a previous report that showed that TNFR signaling was not required for canonical IL-1 $\beta$  maturation in response to a priming and activating signal when all IAPs were antagonized (Vince et al., 2012).

To understand the contribution of XIAP to IL-1 $\beta$  secretion and lymphoproliferation in vivo, we analyzed mice immunized with antigen-coated alum after 12 days. Alum induces a sustained inflammatory response primarily driven by NLRP3 inflammasome activation in inflammatory CD11c<sup>+</sup> DCs (Eisenbarth et al., 2008; Kool et al., 2008). We observed an elevated splenic infiltration of inflammatory cells, such as neutrophils, macrophages, and eosinophils, in *Xiap*<sup>-/-</sup> mice when compared to WT mice (Jordan et al., 2004) (Figure 2C), which is reminiscent of the splenomegaly observed in many patients with XLP-2. In support of a critical role for TNF/TNFR signaling, we observed that the exaggerated splenic infiltration in *Xiap*<sup>-/-</sup> mice was absent in *Tnf*<sup>-/-</sup>*Xiap*<sup>-/-</sup> mice (Figure 2C).

These data demonstrated that XIAP controls signaling events downstream of TNF/TNFR and thereby protects from aberrant inflammasome formation and cell death. The largely identical phenotype observed in both *Xiap* <sup>$\Delta$ RING</sup> and *Xiap*<sup>-/-</sup> BMDCs implicated that XIAP functions as a ubiquitin ligase in this context.

### Protection from Excessive Inflammasome Activation and Cell Death Is Predominantly Mediated by XIAP

Cell death triggered by TNF is often a consequence of deregulated ubiquitylation of RIP1 (Vandenabeele et al., 2010). cIAP1/cIAP2 are ubiquitin ligases of RIP1 and have been shown to protect from aberrant IL-1 $\beta$  secretion after TLR4 activation (Vince et al., 2012). To delineate the roles of cIAP1/cIAP2, from those

of XIAP, we utilized the small molecule monovalent IAP antagonist (SMAC mimetic) LCL161. This compound has a higher affinity for cIAP1/cIAP2 than for XIAP and causes rapid degradation only of cIAP1/cIAP2, but not of XIAP (Figure 2D) (Weisberg et al., 2010).

cIAP1/cIAP2 depletion by LCL161 at 50 nM readily induced IL-1 $\beta$  secretion and cell death only in *Xiap*<sup>-/-</sup> cells, but not in WT cells (Figure 2E). Upon stimulation with LPS, WT cells depleted of cIAP1/cIAP2 failed to increase IL-1 $\beta$  secretion up to doses of 500 nM LCL161 (Figure 2F). In contrast, depletion of cIAP1/cIAP2 potently increased IL-1 $\beta$  secretion in *Xiap*<sup>-/-</sup> cells (Figure 2F). Interestingly, the IL-1 $\beta$  production was mostly TNF/TNFR dependent because *Tnf*<sup>-/-</sup> and *Tnf*<sup>-/-</sup>*Xiap*<sup>-/-</sup> cells secreted significantly less IL-1 $\beta$  compared to WT and *Xiap*<sup>-/-</sup> cells, respectively (Figure 2F). A TNF-independent contribution to IL-1 $\beta$  secretion and cell death was observed only at LCL161 doses higher than 50 nM (Figure 2F).

To exclude the possibility that depletion of cIAP1 by LCL161 in *Xiap*<sup>-/-</sup> cells was not sufficient to deplete cIAP2 (Varfolomeev et al., 2007), we probed for NF- $\kappa$ B-inducing kinase (NIK) stabilization. We observed a similar elevation of NIK in both WT and *Xiap*<sup>-/-</sup> cells (Figures S2G and S2H), indicating that activation of the noncanonical NF- $\kappa$ B pathway did not contribute to the differential levels of IL-1 $\beta$  secretion and cell death.

In summary, depletion of cIAP1/cIAP2 was insufficient for IL-1 $\beta$  secretion and cell death to occur when XIAP was present. Depletion of cIAP1/cIAP2 only further promoted both outcomes when XIAP was deleted. We conclude that protection from excessive inflammasome activation and cell death in DCs after TNFR or TLR4 activation is predominantly mediated by XIAP.

### RIP3 Is Required for Cell Death and Inflammasome Activation in *Xiap*<sup>-/-</sup> BMDCs after TNF/TNFR or TLR4 Stimulation

RIP3 is a mediator of TNF-induced cell death and inflammation (Murphy and Silke, 2014; Vandenabeele et al., 2010). We therefore determined the requirement for RIP3 for both outcomes in XIAP-deficient cells by crossing *Xiap*<sup>-/-</sup> mice with *Rip3*<sup>-/-</sup> mice (Newton et al., 2004). *Rip3*<sup>-/-</sup>*Xiap*<sup>-/-</sup> BMDCs differentiated normally in culture (Figure S3A). After stimulation with TNF (Figure 3A) or LPS (Figures S3B and S3C), the secretion of processed IL-1 $\beta$  into the medium observed in *Xiap*<sup>-/-</sup> cells was completely abolished by loss of RIP3. The reduction in IL-1 $\beta$  levels correlated with normal cell survival in response to TNF or LPS, implying that inflammasome formation and cell death signaling are both consequences of RIP3 activity (Figures 3A and S3C). Surprisingly, processing of caspase-8 into the p17 subunit was absent in *Rip3*<sup>-/-</sup>*Xiap*<sup>-/-</sup> BMDCs after TNF or LPS treatment (Figures 3B and S3B). In addition, deletion of

(B) Secreted IL-1 $\beta$ , cell viability, and LDH release of WT and *Xiap*<sup>-/-</sup> BMDCs treated with LPS (5 ng/ml).

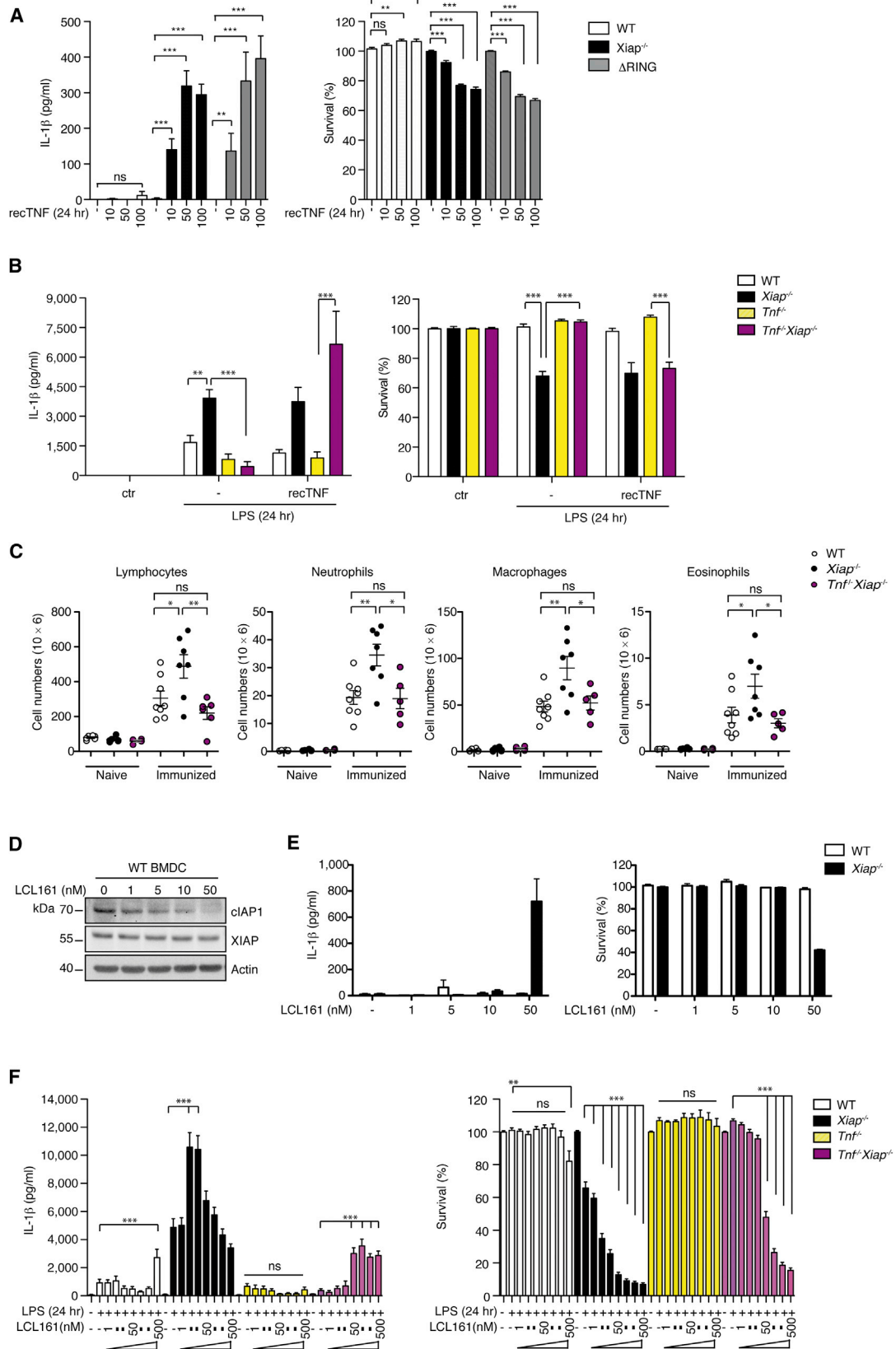
(C) Immunoblotting of cell lysates and culture media from WT and *Xiap*<sup>-/-</sup> BMDCs treated with LPS (5 ng/ml).

(D) Immunoblotting of NP-40 insoluble fractions isolated from BMDCs after treatment with LPS (5 ng/ml) for 8 hr, or treatment with LPS for 2 hr plus ATP (5 mM) for 1 hr.

(E) Fluorescence microscopy of ASC/caspase-1 specks in WT and *Xiap*<sup>-/-</sup> BMDCs treated as indicated. Cells were stained for caspase-1 (yellow), nuclei (blue), and vimentin (red). Scale bars represent 20  $\mu$ m.

(F and G) IL-1 $\beta$  from peritoneal fluid of WT and *Xiap*<sup>-/-</sup> mice injected with (F) LPS (1  $\mu$ g) or (G) alum crystals (700  $\mu$ g) i.p. for 2 hr. n, number of mice.

Error bars represent mean  $\pm$  SEM. Other data represent mean  $\pm$  SEM of at least three independent experiments performed in triplicates. See also Figure S1.



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*Rip3* blocked inflammasome formation as measured by assembly of ASC and caspase-1 within the NP-40 fraction (Figure 3C). The critical role of RIP3 was also confirmed in vivo, where the amount of IL-1 $\beta$  induced by LPS in *Xiap*<sup>-/-</sup> mice was reduced in *Rip3*<sup>-/-</sup>*Xiap*<sup>-/-</sup> mice (Figure 3D). These findings identify XIAP as a negative regulator of RIP3-dependent signaling in response to TNF or LPS and detect a critical role for RIP3 in the full activation of caspase-8 in this process.

### XIAP Regulates Ubiquitylation of RIP1 outside the TNF-RSC

The finding that XIAP restricted inflammasome activation and cell death downstream of TNF/TNFR signaling prompted us to investigate the impact of XIAP loss on the formation of the TNF-RSC. We performed immunoprecipitations of the TNF-RSC using FLAG-tagged TNF in WT or *Xiap*<sup>-/-</sup> HCT116 human colorectal carcinoma cells, which have previously been shown to fully form the TNF-RSC and propagate XIAP-dependent signaling (Damgaard et al., 2012; Krieg et al., 2009). We observed no differences in the composition or the ubiquitylation of proteins within the TNF-RSC when XIAP was absent (Figure S3D). This is in line with our data that showed no significant changes in NF- $\kappa$ B and MAPK activation, and gene induction, in *Xiap*<sup>-/-</sup> and *Xiap* <sup>$\Delta$ RING</sup> BMDCs after TNF treatment (Figures S2C and S2D).

We next tested whether XIAP alters RIP1 expression by probing for the mRNA and protein levels of RIP1 in BMDCs. No significant changes were observed for RIP1 mRNA in response to either LPS or TNF (Figure S3E). However, compared to WT BMDCs, we observed elevated RIP1 protein levels in *Xiap*<sup>-/-</sup> cells at baseline and after 8 hr of LPS treatment (Figure 3E). The baseline elevation of RIP1 and the levels after stimulation were dependent on TNF signaling because RIP1 levels were lower in *Tnf*<sup>-/-</sup>*Xiap*<sup>-/-</sup> cells (Figure 3E). The differences in protein levels despite normal mRNA expression suggest that XIAP affects RIP1 in a posttranslational manner.

We therefore examined whether changes in the ubiquitylation of RIP1 account for its increase. To pull down polyubiquitin chains from BMDCs, we utilized tandem ubiquitin binding entities (TUBEs) fused to glutathione S-transferase (GST). Intriguingly, we found that RIP1 ubiquitylation was substantially elevated after 30 and 60 min of TNF treatment in *Xiap*<sup>-/-</sup> BMDCs (Figure 3F). This finding was surprising because our data did not show an increase of ubiquitin on RIP1 within the TNF-RSC (Figure S3D) and because depletion of cIAP1/cIAP2 by SMAC mimetics reduces RIP1 ubiquitylation (Bertrand et al., 2008). These data therefore suggest that XIAP regulates RIP1 ubiquitylation independently from cIAP1/cIAP2 and outside the TNF-RSC.

We next investigated whether RIP3 contributes to the ubiquitylation of RIP1, possibly in a RIP1-RIP3-containing protein complex (Cho et al., 2009; Feoktistova et al., 2011; Tenev et al., 2011). We found that genetic deletion of RIP3 completely blocked the aberrant RIP1 ubiquitylation observed in *Xiap*<sup>-/-</sup> cells (Figure 3G). Collectively, these data show that XIAP contributes to the regulation of the ubiquitylation status of RIP1 in a RIP1-RIP3-containing complex outside of the TNF-RSC.

### Cell Death Is Induced Independently of Inflammasome Activation in *Xiap*<sup>-/-</sup> BMDCs

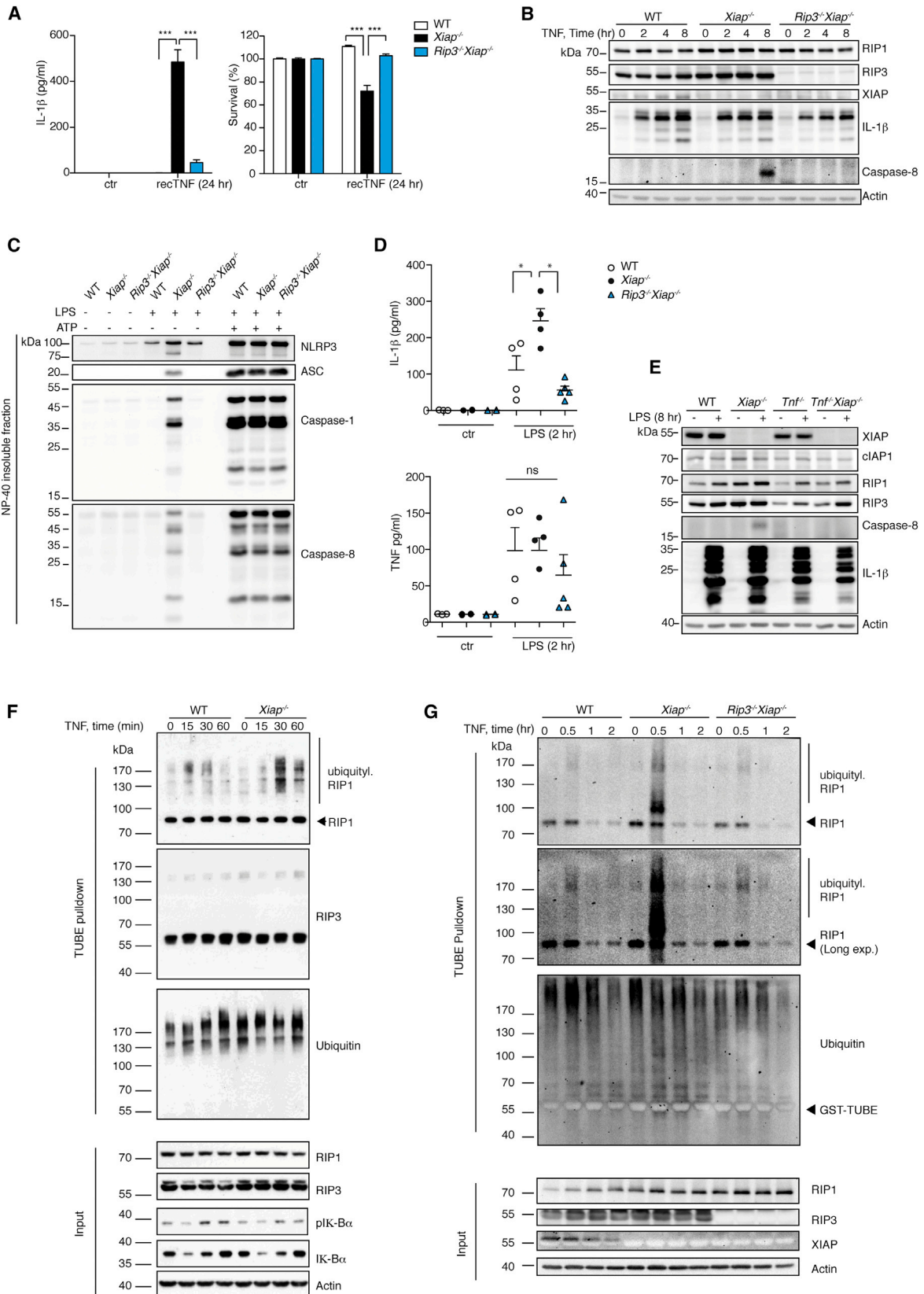
The critical role of RIP3 for the inflammasome activation and cell death induction in *Xiap*<sup>-/-</sup> cells prompted us to examine the relationship between both outcomes. We therefore generated *Caspase 1/11*<sup>-/-</sup>*Xiap*<sup>-/-</sup> mice (Kuida et al., 1995). In line with previous literature, canonical inflammasome activation induced by ATP was completely blocked in BMDCs from such mice (Figures S4A and S4B).

After LPS treatment, we found a substantial reduction in IL-1 $\beta$  levels in *Caspase 1/11*<sup>-/-</sup>*Xiap*<sup>-/-</sup> mice compared to *Xiap*<sup>-/-</sup> mice, implicating conventional inflammasome formation in this process (Figures 4A–4C). Interestingly, IL-1 $\beta$  levels in *Caspase 1/11*<sup>-/-</sup>*Xiap*<sup>-/-</sup> mice at 24 hr were still substantially elevated compared to WT cells (Figure 4C). This suggested that processing of pro-IL-1 $\beta$  by caspase-8 might cooperate with caspase-1 for IL-1 $\beta$  maturation. Indeed, *Caspase 1/11*<sup>-/-</sup>*Xiap*<sup>-/-</sup> BMDCs retained the elevated caspase-8 activity also observed in *Xiap*<sup>-/-</sup> cells (Figures 4A and 4B). Moreover, inhibition of caspase-8 with the inhibitor Z-IETD-FMK (IETD) further reduced IL-1 $\beta$  levels at 8 and 24 hr (Figures 4B and 4C), implying that caspase-8 contributes to IL-1 $\beta$  maturation in *Xiap*<sup>-/-</sup> BMDCs. We therefore tested the recruitment of both caspases into the NP-40 fraction in response to LPS. We observed that LPS treatment was sufficient to cause recruitment of caspase-8 and caspase-1 into this fraction in *Xiap*<sup>-/-</sup> cells, whereas WT cells required canonical inflammasome activation with ATP for this to occur (Figures 1D, 3C, and 4D). Of note, recruitment of caspase-8 to the NP-40 fraction was unperturbed in mice lacking caspase-1/caspase-11 (Figure 4D). In addition, inhibition of caspase-8 with IETD did not prevent caspase-1 recruitment to the NP-40 fraction in *Xiap*<sup>-/-</sup> cells (Figure 4E). Collectively, these data show that recruitment and activation of caspase-1 and caspase-8 occur independently of each other but that both cooperate in the processing of pro-IL-1 $\beta$ .

Importantly, we observed no effect of deletion of caspase-1/caspase-11 on the cell death of *Xiap*<sup>-/-</sup> cells (Figures 4B and 4C). Additional inhibition of caspase-8 (Figures 4B and 4C) and complete inhibition of all caspase activity using the caspase

### Figure 2. XIAP Protects against Excessive IL-1 $\beta$ Secretion and Cell Death in a TNF-Dependent Manner

- (A) IL-1 $\beta$  and cell survival of WT, *Xiap*<sup>-/-</sup>, and *Xiap* <sup>$\Delta$ RING</sup> BMDCs treated with recombinant TNF (recTNF) at 10, 50, and 100 ng/ml.  
 (B) IL-1 $\beta$  and cell viability of WT, *Xiap*<sup>-/-</sup>, *Tnf*<sup>-/-</sup>, and *Tnf*<sup>-/-</sup>*Xiap*<sup>-/-</sup> BMDCs after LPS (5 ng/ml) with or without recTNF (100 ng/ml).  
 (C) Splenic lymphocytes, neutrophils, macrophages, and eosinophils from WT (n = 8), *Xiap*<sup>-/-</sup> (n = 7), and *Tnf*<sup>-/-</sup>*Xiap*<sup>-/-</sup> (n = 5) mice 12 days after injection i.p. with coated alum crystals. Each dot represents a mouse, and error bars represent mean  $\pm$  SEM.  
 (D) Immunoblots of WT BMDCs treated with IAP antagonist LCL161 for 2 hr.  
 (E) IL-1 $\beta$  and cell survival of WT and *Xiap*<sup>-/-</sup> BMDCs after LCL161 for 24 hr.  
 (F) IL-1 $\beta$  and cell viability of WT, *Xiap*<sup>-/-</sup>, *Tnf*<sup>-/-</sup>, and *Tnf*<sup>-/-</sup>*Xiap*<sup>-/-</sup> BMDCs after LPS and LCL161 at 1, 5, 10, 50, 100, 200, and 500 nM for 24 hr. Error bars represent mean  $\pm$  SEM of at least three independent experiments performed in triplicates. See also Figure S2.



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inhibitor ZVAD also failed to rescue the viability of *Xiap*<sup>-/-</sup> cells (Figure S4C). Whereas the precise function of caspase-8 in this context remains unresolved, our data clearly showed that cell death induction was nonapoptotic and independent from inflammasome activation.

### Loss of XIAP Drives Excessive Inflammation in a Murine Model of EBV Mononucleosis

XLP-2 pathology in patients is often triggered by an Epstein-Barr virus (EBV) infection. As a murine model for EBV mononucleosis, we studied the immune response of *Xiap*<sup>-/-</sup> mice to murine  $\gamma$ -herpesvirus 68 (MHV-68) (Barton et al., 2011). In this model, intranasal (i.n.) infection with MHV-68 results in a productive infection of the respiratory tract (Stevenson and Efstathiou, 2005) that is followed by the transfer of the infection to lymphoid tissues. Latent infection is established in the spleen within 2–3 weeks postinfection (p.i.), after which the number of latently infected splenocytes returns to basal levels (Barton et al., 2011) (Figure 5A).

Despite normal viral clearance during early infection (days 6 and 16) (Figure 5B; data not shown), we found increased viral genomic loads in the spleens of infected *Xiap*<sup>-/-</sup> mice during late (day 43) and very late (day 84) latency (Figure 5B). On day 16, the cellularity of *Xiap*<sup>-/-</sup> and *Xiap* <sup>$\Delta$ RING</sup> spleens was increased compared to those of WT mice, which was reflected by the increased numbers of myeloid and lymphoid populations in the spleen (Figure 5C). Because CD4<sup>+</sup> T lymphocytes are important for the regulation of the viral loads of MHV-68, we measured T cell populations during active infection and early latency (Figure 5D). Despite equivalent values on day 6 p.i., the levels of CD4<sup>+</sup> effector T cells, regulatory T cells (Treg), and IFN $\gamma$ <sup>+</sup> T cells were substantially elevated in *Xiap*<sup>-/-</sup> and *Xiap* <sup>$\Delta$ RING</sup> mice at day 16 p.i., a phenotype also observed in patients with XLP-2 (Figure 5D).

In line with our *in vitro* data, we found substantially elevated IL-1 $\beta$  levels in the peripheral blood of *Xiap*<sup>-/-</sup> mice at day 16 p.i., whereas TNF was comparable between both genotypes (Figure 5E). Together, this shows that  $\gamma$ -herpesvirus infection drives hyperinflammation in *Xiap*<sup>-/-</sup> mice, similar to what is seen in EBV-infected patients with XLP-2.

Based on our findings showing the TNF-dependent nature of the excessive IL-1 $\beta$  secretion in *Xiap*<sup>-/-</sup> BMDCs and mice, we hypothesized that deletion of TNF would ameliorate some of the phenotypes observed. Indeed, during early infection at day 6 p.i., *Tnf*<sup>-/-</sup>*Xiap*<sup>-/-</sup> mice failed to induce IL-1 $\beta$  induction, whereas at day 16 p.i., stimuli other than TNF induced IL-1 $\beta$

production comparable to the levels observed in *Xiap*<sup>-/-</sup> mice (Figure 5E). Consistently, we observed a reduction in myeloid and lymphoid subpopulations infiltrating the spleen in *Tnf*<sup>-/-</sup>*Xiap*<sup>-/-</sup> mice at day 16 p.i. (Figures 5F and 5G). Finally, in agreement with our previous data, deletion of RIP3 also reduced the cellular infiltrations observed in spleens of *Xiap*<sup>-/-</sup> mice after viral infection (Figure S5A), further supporting a role for RIP3-dependent cell death and inflammation observed in *Xiap*<sup>-/-</sup> mice.

In summary, the phenotype observed in *Xiap*<sup>-/-</sup> and *Xiap* <sup>$\Delta$ RING</sup> mice mimicked XLP-2 pathology, which is characterized by the effort of the immune system to clear an infectious pathogen, such as EBV, causing severe hyperinflammation. Importantly, genetic deletion of TNF ameliorated the symptoms, supporting the causative nature of TNF in this process and indicating a potential therapeutic benefit of TNF inhibition for patients with XLP-2 during early infection.

### DISCUSSION

Excessive RIP3-dependent cell death has been shown to cause elevated cytokine production and inflammation collectively resulting in severe tissue damage (Murphy and Silke, 2014). This is exemplified, for example, by the development of severe terminal ileitis in Crohn's disease due to necroptosis of Paneth cells (Günther et al., 2011). Necroptosis-inhibiting proteins are therefore critical regulators of tissue homeostasis (Günther et al., 2011; Kang et al., 2013; Welz et al., 2011). Our data identify XIAP as an inhibitor of RIP3-dependent cell death and inflammation and show that loss of XIAP expression in mice causes hyperinflammation that mimics human XLP-2 pathology (Marsh et al., 2010).

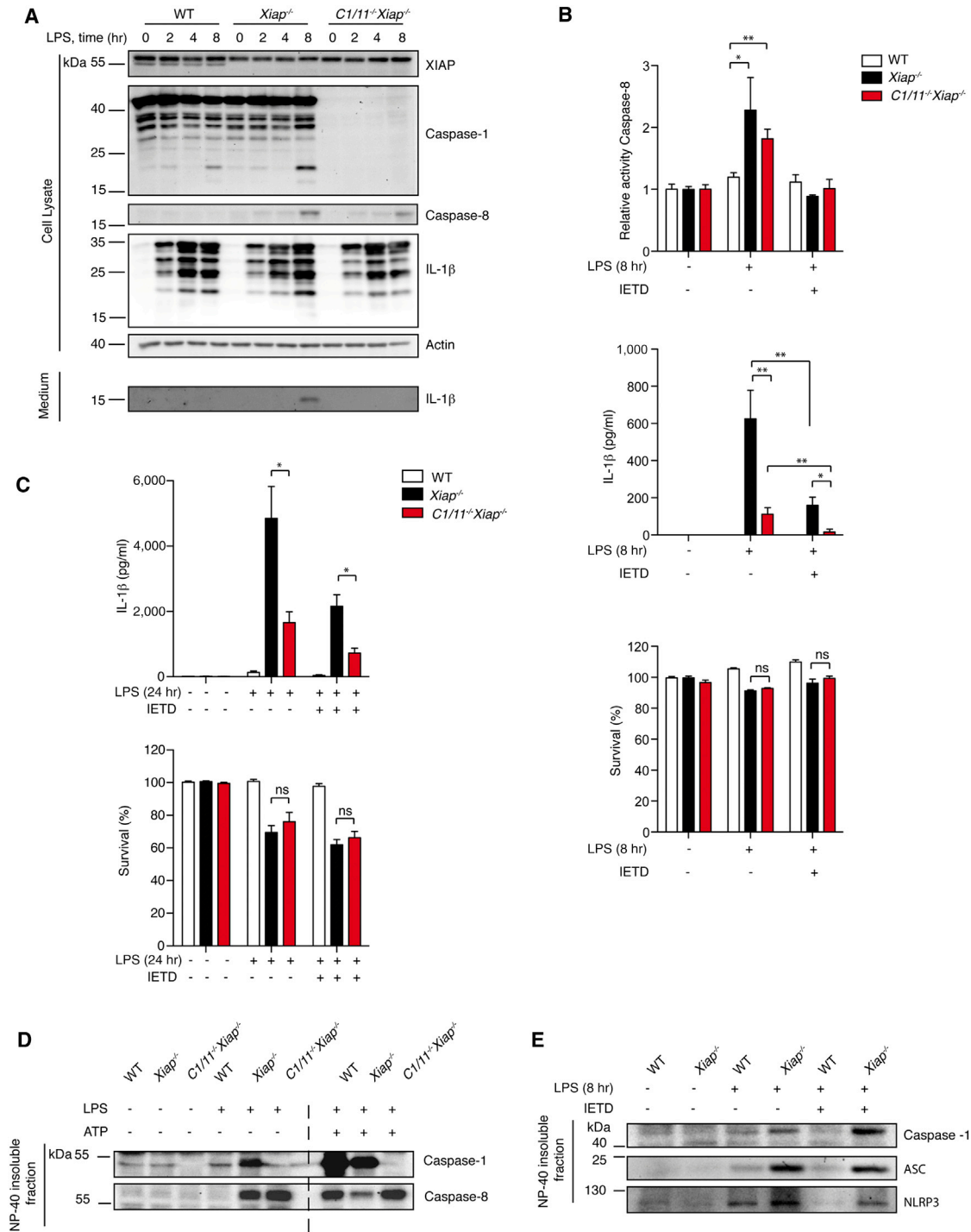
Unexpectedly, our data place XIAP downstream of TNF signaling, despite the fact that it is not directly involved in the formation of the TNF-RSC I. The role of XIAP in TNFR signaling has mainly been characterized in mouse embryonic fibroblasts (MEFs). Contrary to cIAP1, XIAP is not involved in propagating canonical NF- $\kappa$ B activation in response to TNF in MEFs (Moulin et al., 2012) and fails to increase retention of RIP1 within the TNF signaling complex I (Vince et al., 2007). In contrast, our data place XIAP's function outside of the TNF-RSC I, where it controls the cellular fate of DCs by regulating RIP1 ubiquitylation together with RIP3 within a TNF/TNFR-induced signaling complex IIb (also termed ripoptosome or necrosome) (Murphy and Silke, 2014; Tenev et al., 2011). Whether XIAP directly interacts with RIP1 and/or RIP3 or whether the aberrant ubiquitylation of

### Figure 3. TNF Induces Elevated Ubiquitylation of RIP1 and Drives RIP3-Dependent Inflammasome Formation and Cell Death in *Xiap*<sup>-/-</sup> Mice

- (A) IL-1 $\beta$  secretion and survival of WT, *Xiap*<sup>-/-</sup>, and *Rip3*<sup>-/-</sup>*Xiap*<sup>-/-</sup> BMDCs treated with TNF (100 ng/ml). Data represent mean  $\pm$  SEM of at least three independent experiments performed in triplicates.
- (B) Immunoblots of WT, *Xiap*<sup>-/-</sup>, and *Rip3*<sup>-/-</sup>*Xiap*<sup>-/-</sup> BMDCs treated with TNF (100 ng/ml).
- (C) Immunoblots of NP-40 insoluble fractions of WT, *Xiap*<sup>-/-</sup>, and *Rip3*<sup>-/-</sup>*Xiap*<sup>-/-</sup> BMDCs after LPS (5 ng/ml) for 8 hr, or LPS (10 ng/ml) primed for 2 hr plus ATP (5 mM) for 1 hr.
- (D) Cytokines from peritoneal fluid of WT (n = 4), *Xiap*<sup>-/-</sup> (n = 4), and *Rip3*<sup>-/-</sup>*Xiap*<sup>-/-</sup> (n = 5) mice after 2 hr of LPS (1  $\mu$ g) i.p. Each data point represents one mouse. Error bars represent mean  $\pm$  SEM.
- (E) Immunoblots of extracts from WT, *Xiap*<sup>-/-</sup>, *Tnf*<sup>-/-</sup>, and *Tnf*<sup>-/-</sup>*Xiap*<sup>-/-</sup> BMDCs after treatment with LPS (5 ng/ml).
- (F) Immunoblots of GST-TUBE ubiquitin pull-down from WT and *Xiap*<sup>-/-</sup> BMDCs treated with TNF (100 ng/ml).
- (G) Immunoblots of GST-TUBE ubiquitin pull-down from WT, *Xiap*<sup>-/-</sup>, and *Rip3*<sup>-/-</sup>*Xiap*<sup>-/-</sup> BMDCs treated with TNF (100 ng/ml).

See also Figure S3.





**Figure 4. Caspase-1/Caspase-11 and Partially Caspase-8 Drive IL-1 $\beta$  Processing in *Xiap*<sup>-/-</sup> Mice, but Both Are Dispensable for Cell Death**

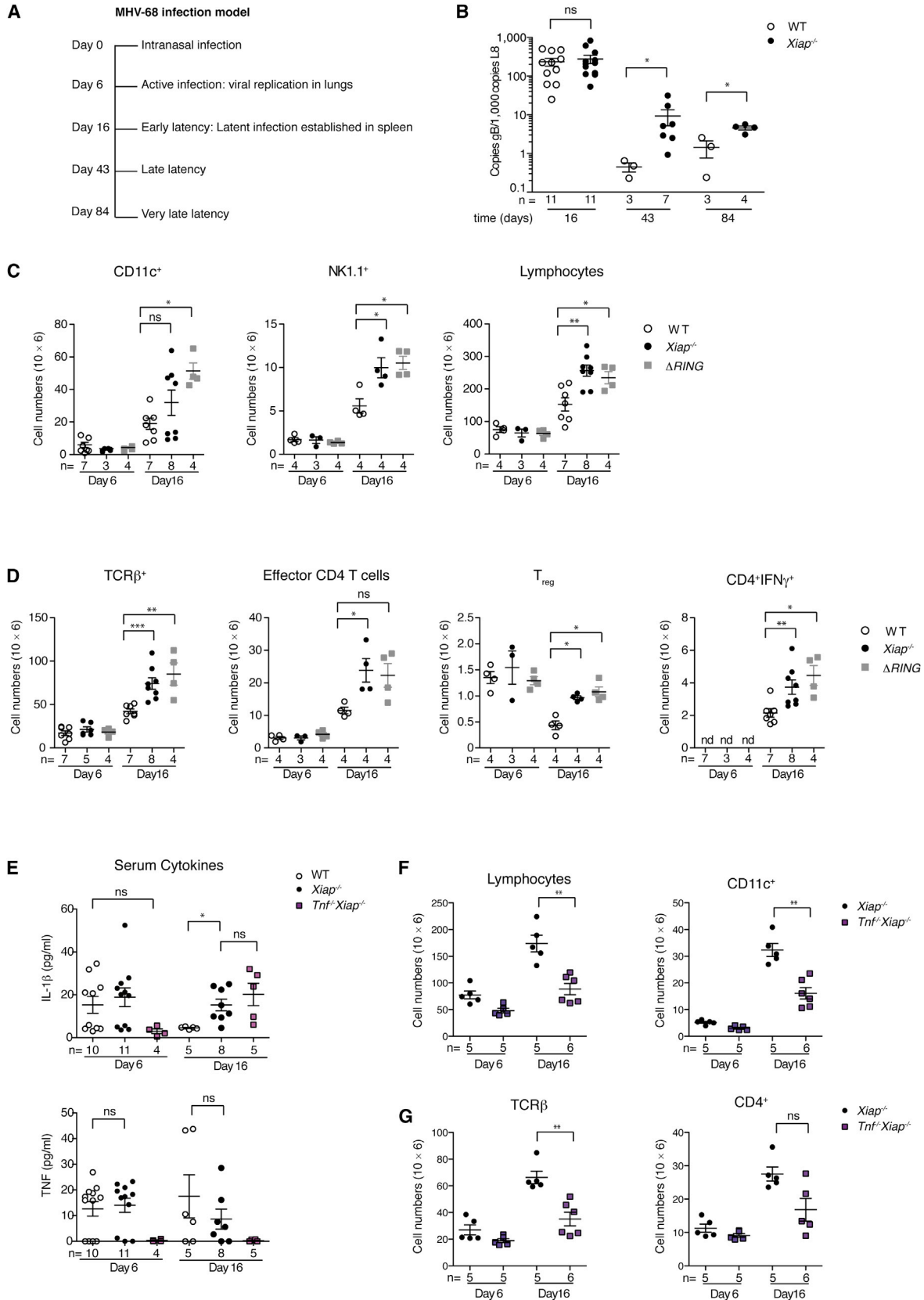
(A) Immunoblots of cell extracts and media from WT, *Xiap*<sup>-/-</sup>, and *Caspase 1/11*<sup>+/-</sup>*Xiap*<sup>-/-</sup> BMDCs treated with LPS (5 ng/ml).

(B and C) Relative caspase-8 activity, IL-1 $\beta$  secretion, and survival of WT, *Xiap*<sup>-/-</sup>, and *Caspase 1/11*<sup>+/-</sup>*Xiap*<sup>-/-</sup> BMDCs after LPS (5 ng/ml) with or without the caspase-8 inhibitor IETD-fmk (IETD) (10  $\mu$ M) for (B) 8 hr or (C) 24 hr.

(D) NP-40 insoluble fractions of WT, *Xiap*<sup>-/-</sup>, and *Caspase 1/11*<sup>+/-</sup>*Xiap*<sup>-/-</sup> BMDCs after LPS (5 ng/ml) for 8 hr, or LPS for 2 hr (10 ng/ml) plus ATP (5 mM) for 1 hr.

(E) NP-40 fractions of WT and *Xiap*<sup>-/-</sup> BMDCs with LPS (5 ng/ml) for 8 hr with or without IETD.

Error bars represent mean  $\pm$  SEM of at least three independent experiments performed in triplicates. See also Figure S4.



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RIP1 favors binding to RIP3 remains unresolved, and these questions will be important to further investigate in the future.

The ubiquitylation status of RIP1 is a key factor that influences the switch between cell survival, apoptosis and RIP3-dependent cell death downstream of the TNFR. RIP1 has been shown to be decorated with M1, K11-, K48-, and K63-linked ubiquitin chains (Bertrand et al., 2011; Gerlach et al., 2011; Haas et al., 2009). cIAP1/cIAP2 have been implicated as mediators of the K48 and K63 linkages (Bertrand et al., 2011). Loss of these K48/K63 linkages, either via the action of specific deubiquitinases (DUBs) or by IAP antagonists, leads to cell death (Feoktistova et al., 2011; O'Donnell et al., 2011), whereas loss of the linear M1 linkage attenuates the NK- $\kappa$ B transcriptional response also resulting in elevated cell death (Gerlach et al., 2011; Haas et al., 2009; Ikeda et al., 2011). *Xiap* <sup>$\Delta$ RING</sup> mutant mice, which express a truncated XIAP missing only the ligase function, presented with an almost identical phenotype compared to *Xiap*<sup>-/-</sup> mice. This supports the notion that XIAP functions as a ubiquitin ligase in TNFR/TNF signaling and that coordinated ubiquitylation events downstream of TNFR are tightly controlled by IAPs, including XIAP.

We observed an aberrant and prolonged ubiquitylation of RIP1 after TNF stimulation in cells lacking XIAP. Our data argue that XIAP functions as a ubiquitin ligase controlling the ubiquitylation of RIP1 either directly or indirectly, e.g., by affecting another ubiquitin ligase or DUB, which is important for ensuring correct RIP1 ubiquitylation. Indeed, there is emerging evidence that RIP1 ubiquitylation is tightly balanced by multiple ubiquitin ligases and DUBs and that altering this balance results in aberrant signaling and cell death (Gerlach et al., 2011; Keusekotten et al., 2013; O'Donnell et al., 2011; Wertz et al., 2004).

We show that XIAP cooperates with cIAP1/cIAP2 in diverging TNFR/TNF signaling away from cell death. However, XIAP clearly functions independently of cIAP1/cIAP2. This is exemplified by the fact that deletion of XIAP alone is sufficient to drive the observed phenotype and by the observation that deletion of XIAP causes aberrantly elevated ubiquitylation of RIP1 instead of the reduced RIP1 ubiquitylation observed when cIAP1/cIAP2 are inhibited (Bertrand et al., 2008)

XLP-2 is currently considered a familial hemophagocytic lymphohistiocytosis (HLH) disease despite the lack of the characteristic defects of CD8<sup>+</sup> T cell and natural killer cell cytotoxicity usually present in familial patients with HLH (Marsh et al., 2010). The protection of DCs and macrophages against RIP3-dependent cell death and inflammation afforded by XIAP argues that loss-of-function mutations constitute a major underlying cause of XLP-2 pathology. TNF was identified as the prime causative cytokine in our study, and genetic inhibition ameliorated some

disease-specific symptoms. Despite the likely contribution of alternative inflammatory pathways, such as IFN signaling, we suggest that patients with XLP-2 might profit from therapeutic TNF inhibition when used early during an inflammatory episode (Mischler et al., 2007).

## EXPERIMENTAL PROCEDURES

### Mice

*Xiap*<sup>-/-</sup> (Olayoye et al., 2005) and *Xiap* <sup>$\Delta$ RING</sup> (Schile et al., 2008) mice have been previously described. *Tnf* *tm1Gkl* (*Tnf*<sup>-/-</sup>) (Pasparakis et al., 1996) and *Casp1*<sup>*tm1Flv/J*</sup> (*Caspase-1/Caspase-11*<sup>-/-</sup>) (Kuida et al., 1995) mice were purchased from Jackson Laboratories. *Rip3*<sup>-/-</sup> mice were obtained under a material transfer agreement from Genentech and have been previously described by Newton et al. (2004). Both male and female mice deficient for *BIRC4* were denoted *Xiap*<sup>-/-</sup>. All animal experiments were performed in compliance with protocols approved by the local animal ethics committee guidelines.

### Inflammasome Formation in BMDCs

To monitor inflammasome formation by fluorescence microscopy, cells treated with or without nigericin (5  $\mu$ M) (Sigma-Aldrich) for 20–30 min after LPS priming for 2 hr were seeded on chamber slides and probed with antibodies against caspase-1 (Casper-2 clone; Adipogen) and vimentin (D21H3; Cell Signaling Technology). Images were acquired with a Leica DMRBE fluorescence microscope. For inflammasome-enrichment studies, cells were treated with LPS or TNF for the indicated times, and the insoluble fraction of the NP-40-lysed cells was collected by centrifugation and analyzed by SDS-PAGE and immunoblotting for ASC, caspase-1 (Casper-1 clone), as previously described (Gross et al., 2012).

### Alum-Induced Peritonitis and Immunization

Eight to 12-week-old age- and sex-matched mice were injected i.p. with 700  $\mu$ g Inject Alum Adjuvant (Thermo Scientific) in 200  $\mu$ l PBS. Peritoneal fluid was collected 2 hr postinjection and concentrated to 100  $\mu$ l with 10 kDa MWCO Vivaspin filters (Vivascience), and cytokines were measured by Cytokine Bead Array (BD Biosciences). For immunization, ovalbumin (100  $\mu$ g) (Sigma-Aldrich; A2512) and LPS (10  $\mu$ g) (Sigma-Aldrich; L2880) were precipitated with alum (4.4  $\mu$ g) (Roth P724.1). Each mouse was injected with the precipitate that was washed once with PBS. Spleens of mice were analyzed by flow cytometry 12 days later.

### Intranasal MHV-68 Infection

Intranasal (i.n) infection of mice was performed as previously described by El-Gogo et al. (2007) and Stevenson and Efstathiou (2005). In brief, mice were infected i.n. with  $5 \times 10^4$  plaque-forming units. Lytic virus titers of infected lungs were determined on day 6, and splenic latent viral load was performed by real-time PCR. At days 6 and 16 p.i., spleens of infected mice were analyzed by flow cytometry analysis, and cytokines were measured from blood sera.

### Statistical Analyses

The Wilcoxon-Mann-Whitney test was used to compare levels of serum cytokines and differences in cellular populations. All values are expressed as the mean  $\pm$  SEM, and  $p < 0.05$  (\*),  $p < 0.005$  (\*\*), and  $p < 0.0005$  (\*\*\*) were

## Figure 5. *Xiap*<sup>-/-</sup> and *Xiap* <sup>$\Delta$ RING</sup> Mice Infected with MHV-68 Develop Excessive Inflammation

(A) Schematic overview of the MHV-68 infection model.

(B) MHV-68 viral genomic load of spleens from infected WT and *Xiap*<sup>-/-</sup> mice determined on days 16, 43, and 84 p.i.

(C) Flow cytometric analyses of spleens of infected WT, *Xiap*<sup>-/-</sup>, and *Xiap* <sup>$\Delta$ RING</sup> mice were analyzed at days 6 and 16 p.i.

(D) T cell subsets of infected mice were analyzed as in (C). Effector T cells were defined as TCR $\beta$ <sup>+</sup>, CD4<sup>+</sup>, CD44<sup>high</sup>, CD62L<sup>low</sup>, and T-regs as CD4<sup>+</sup>, CD25<sup>+</sup>, and FOXP3<sup>+</sup>.

(E) IL-1 $\beta$  and TNF from sera of infected WT, *Xiap*<sup>-/-</sup>, and *Tnf*<sup>-/-</sup> *Xiap*<sup>-/-</sup> mice at days 6 and 16 p.i.

(F) Flow cytometric analysis of spleens of infected *Xiap*<sup>-/-</sup> and *Tnf*<sup>-/-</sup> *Xiap*<sup>-/-</sup> mice at days 6 and 16 p.i.

(G) T cell numbers from mice in (F).

Each dot represents a mouse, and error bars represent mean  $\pm$  SEM. See also Figure S5.

considered statistically significant. Statistical analyses and graphing were performed with GraphPad Prism software.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.05.008>.

## AUTHOR CONTRIBUTIONS

P.J.J. conceived and supervised the project, analyzed the data, and wrote the manuscript. M.Y. conceived and performed the experiments, analyzed the data, and wrote the manuscript. N.M., H.A., N.K., C.J.G., R.B.D., and O.G. performed experiments. H.K., M.R., and M.G.-H. provided critical reagents and samples. T.K., M.H., A.S., J.R., O.G., C.P., and M.G.-H. gave conceptual advice and wrote the paper.

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