Contributions to inflammation and sepsis by Inhibitor of Apoptosis proteins

by

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7-AAD – 7-amino-actinomycin D

ASK1 – apoptosis signal-regulating kinase 1

BIR – baculovirus IAP repeat

CARD – caspase activation and recruitment domain

c-IAP – cellular inhibitor of apoptosis

DD – death domain

DISC – death-inducing signaling complex

ERK – extracellular regulated kinase

FADD – Fas-associated death domain proteins

IAP – inhibitor of apoptosis

IBM – IAP binding motif

IL – interleukin

JNK – c-Jun N-terminal kinase

LPS – lipopolysaccharide

LRR – leucine-rich repeat

MAPK – mitogen-activated protein kinase

MCP-1 – monocyte chemoattractant protein-1

MIP-1α – macrophage inflammatory protein-1α

MIP-2 – macrophage inflammatory peptide-2

NEMO/IKKγ – NF-κB essential modifier/ inhibitor of κB kinase γ

NF-κB – nuclear factor-κB

NIK – NF-κB-inducing kinase

NLR – NOD-like receptor

NOD – nucleotide-binding oligomerization domain

PAMP – pathogen-associated molecular pattern

RANTES – regulated on activation, normal T cell expressed and secreted

RING – really interesting new gene

RIP1 – receptor-interacting protein kinase 1

RLH – RIG-I-like helicase

TAK1 – TGF-β activated kinase 1

TLR – Toll-like receptor

TNF – tumor necrosis factor

TNFR – tumor necrosis factor receptor

TRADD – TNFR-associated death domain protein

TRAF – TNFR associated factor

TRAMP – transgenic adenocarcinoma of the mouse prostate

UBA – ubiquitin associated domain

XIAP – X-linked inhibitor of apoptosis

Abstract

Sepsis is a significant clinical challenge that is increasing in incidence and carries a substantial risk of mortality. The syndrome is characterized by vasodilation, vascular permeability and hypoperfusion that leads to disseminated intravascular coagulation and acute organ dysfunction in severe cases. These manifestations are attributable to an overly zealous host innate immune response that causes a systemic pro-inflammatory state, and apoptosis of both immune cells and non-immune cells has been implicated in the pathogenesis of sepsis.

The primary focus of this dissertation investigates the connection between molecular regulation of apoptosis and inflammation, specifically focusing on the role of that anti-apoptotic protein c-IAP1 (cellular Inhibitor of Apotosis 1), a member of the Inhibitor of Apoptosis (IAP) family. We identified a novel role for c-IAP1 during pathologic innate immune responses that result in septic shock, using mouse models of sepsis. During sepsis c-IAP1-deficient mice demonstrated improved survival, and we identified a contribution of c-IAP1 to production of systemic cytokines during pro-inflammatory immune responses *in vivo*. While cytokine induction by the gram-negative bacterial product lipopolysaccharide (LPS) was not dependent on c-IAP1 in innate immune cells, we identified a role for c-IAP1 in the response of lung fibroblasts to pro-inflammatory stimuli, including LPS, tumor necrosis factor (TNF) and macrophage-derived cytokines. Lung fibroblasts responded to macrophage-derived TNF in a c-IAP1-

dependent manner; however, NF-κB and MAPK signaling were intact in c-IAP1-deficient fibroblasts and macrophages.

These results indicate that c-IAP1 is a critical pro-inflammatory mediator of innate immune responses during septic shock and implicate c-IAP1 in regulation of the participation of fibroblasts in cytokine networks with immune cells during inflammation. Interconnections between apoptosis and inflammatory processes mediated by IAPs are a potential mechanism to coordinate the sensitivity of the cellular suicide program and adaptation to inflammation in the extracellular environment. Characterization of the integrated regulation of inflammation and apoptosis during pathologic systemic inflammation will improve our understanding of the pathogenesis of sepsis and aid in the development of new therapeutic approaches.

Chapter 1

Introduction

Apoptosis is a controlled process of cell death in multi-cellular organisms that allows for orderly destruction of unwanted cells in the course of development or during maintenance of homeostasis. Cell death occurs following a particular, predictable pattern during normal development, and for this reason, apoptosis is also called programmed cell death. In adult organisms, tightly regulated execution of apoptosis is necessary for maintaining a physiological dynamic equilibrium between cellular proliferation and loss. The process of apoptosis can be initiated either by extracellular signaling cues (the extrinsic pathway) or by intracellular triggers (the intrinsic pathway), which are described further below. Both of these stimuli activate an intracellular cascade of endogenously expressed cysteinyl-aspartate proteases, or caspases, which are capable of cleaving essential cellular machinery to execute the cellular suicide. A cell undergoing apoptosis has a characteristic morphology that features chromatin condensation, nuclear fragmentation and blebbing from the cellular membrane. The cellular fragments that remain are cleared by phagocytic immune cells, but importantly, this process is "immunologically silent," and as such, it does not trigger an inflammatory reaction.

An illustration of the important role of apoptosis is highlighted in the adaptive immune system, where lymphocytes are primed to respond to signs of infection. Presentation of a microbial antigen with co-stimulatory molecules in the lymph node

triggers massive clonal expansion of T cells and B cells to fight off an invading pathogen. Following resolution of the infection, induction of apoptosis is responsible for culling excess lymphocytes in the peripheral tissues. Activated lymphocytes are sensitized to activation-induced cell death (AICD) by increased expression of a cell surface receptor, Fas antigen (CD95/Apo1), which detects pro-apoptotic Fas ligand (CD95L) in the environment. If mutations occur in the Fas or FasL genes that disrupt their function, which is observed in patients with autoimmume lymphoproliferative syndrome (ALPS), this clearance mechanism fails and excessive numbers of lymphocytes persist. These patients develop splenomegaly, lymphadenopathy and hepatomegaly due to increased lymphocyte numbers, and may also develop autoimmunity, anemia, thrombocytopenia and neutropenia as a consequence. Similar increases in lymphocyte numbers are also observed in mice harboring deletions in the genes for Fas (lpr/lpr mice) or FasL (gld/gld mice). Thus, in the adaptive immune system a carefully controlled process of cell death allows the organism to effectively respond to challenges in the environment (reviewed in *1*).

The process of apoptosis is also a critical safeguard during normal cellular growth and proliferation. Genome replication, reactive oxygen species generated by metabolism, and exposure to DNA-damaging toxins or irradiation are frequent sources of damage to cellular DNA. Recognition of DNA damage by protein complexes initiates the DNA repair pathways to mend the damage. These processes also induce either a halt in cell cycle progression until the damage is repaired or expression of pro-apoptotic genes that activate the intrinsic apoptosis pathway. Genomic mutations that evade recognition by the DNA repair pathways have the potential to alter the function of critical genes controlling

cellular proliferation. However, oncogenic mutations that directly unleashed uncontrolled proliferation are also caught by mechanisms that lead to cell cycle arrest or apoptosis. For cancer to develop, malignant cells do not only require uncontrolled proliferation, they must also acquire the ability to evade the intrinsic cell death program (*2*).

These examples highlight the critical roles of apoptosis in maintaining homeostatic control during normal organism growth and during adaptive responses to the environment. It is important to emphasize that apoptosis is not necessarily always protective for the organism, and perturbations in this process can lead to pathology. This dissertation will focus on understanding how regulation of apoptosis is involved in the development of pathology, specifically in the innate immune system. The innate immune system is the primary line of defense against pathogens that penetrate an organism's barriers. Innate immune cells, including macrophages, neutrophils, NK cells and dendritic cells, are professionals at recognizing pathogens, engulfing foreign entities and in many cases triggering a secondary, adaptive immune response. In the process of fighting pathogens, the innate immune response can have a number of destructive side effects on the surrounding tissue. To prevent unnecessary damage from improper activation or an excessive inflammatory response, a number of processes control the initiation and extent of inflammation. However, in some cases the innate immune system can mount an uncontrolled, overwhelming response to a microbe, resulting in a systemic inflammatory state and the manifestations of sepsis. One effect frequently associated with sepsis is widespread apoptosis of immune cells and parenchymal cells (*3*). The depletion of lymphocytes that results during sepsis can lead to a secondary immunosuppressive state and increased susceptibility to additional infections (*4-7*). We are interested in

understanding the pathology of sepsis by examining the interconnection between the molecular regulation of apoptosis and inflammation.

The cellular mechanisms that regulate apoptosis have been intensely studied since their discovery. The first understanding of the genetic components that contribute to apoptosis began in the nematode *C. elegans* with the identification of the genes *ced-3* in 1982 and *ced-4* in 1986 by Robert Horvitz, Hilary Ellis and Paul Sternberg, and the mammalian counterparts were later described in 1993 and 1997, respectively. The molecular pathways of apoptosis have now been characterized in not only *C. elegans*, but also *Drosophila,* mouse and human. Comparisons among these organisms have demonstrated evolutionary conservation of the components of apoptosis. Here, I will focus on the mammalian pathway of apoptosis, and the mechanisms that precisely regulate activation of this pathway, which are diagramed in Figure 1.1.

Molecular regulation of apoptosis

Intrinsic apoptosis

Activation of the intrinsic pathway of apoptosis occurs in response to a number of intracellular stress signals, including DNA damage, oncogene activation, growth factor deprivation or unfolded protein stress in the endoplasmic reticulum. The signals initiated in these responses influence pro- and anti-apoptotic factors that converge at the mitochondria. In healthy cells, mitochondria are the site of respiration and contain cytochrome c, a link in the oxidative phosphorylation chain, sequestered from the cytoplasm and other organelles. During apoptosis, a pore in the outer mitochondrial

membrane is formed and cytochrome c is released into the cytosol to initiate a cascade of caspase activation. The permeability of the outer mitochondrial membrane is regulated by the Bcl-2 family of proteins, which contains a number of pro-apoptotic (e.g. Bax and Bak) and anti-apoptotic (e.g. Bcl-2 and Bcl-x) members that interact to positively or negatively influence pore formation.

Release of cytochrome c into the cytosol allows its interaction with Apaf-1 (apoptotic protease-activating factor-1) in a dATP-dependent manner, and these proteins form an oligomeric complex called the apoptosome. The CARD domain within Apaf-1 recruits pro-caspase-9 to this complex, and subsequent dimerization of pro-caspase-9 within this complex unmasks its auto-proteolytic activity (*8, 9*). Activated caspase-9 is an apical caspase and initiates the cascade of activation of effector caspases, which includes caspase-3 and caspase-7. The protease activity of effector caspases is activated by cleavage, which releases an inhibitor domain from blocking the active site of the protease. Active caspase-3 cleaves essential intracellular targets, such as poly-ADP ribose polymerase (PARP), and a number of these substrates have been described (*10, 11*).

Extrinsic apoptosis

In addition to intrinsic apoptosis, apoptosis can also be triggered extrinsically by activation of death receptors on the cellular membrane. Death receptors are members of the tumor necrosis factor receptor (TNFR) superfamily that contain death domains (DD) within their intracellular tails and include TNFR1, Fas, DR4/TRAILR1 and DR5/TRAILR2. Death domains, along with the closely related death effector domains (DED) and caspase activation and recruitment domains (CARD), contain six anti-parallel α-helices and mediate homotypic protein-protein interactions. Activation of a death

receptor by its ligand promotes formation of a death-inducing signaling complex (DISC). Formation of this complex is initiated by interactions between death domains within the receptor tail and in cytosolic adapter proteins, such as FADD (Fas-associated death domain protein; *12*). The structure of the complex formed by the death domains of Fas and FADD has recently been resolved and indicates that binding of Fas ligand to Fas induces a conformational opening of the receptor tail DD (*13*). This open conformation is stabilized through receptor clustering and allows binding by the DD of FADD, which further stabilizes the oligomeric complex. Pro-caspase-8 contains a death effector domain and is recruited to the complex by binding the DED within FADD. Like caspase-9, caspase-8 is an apical caspase, and induced proximity of pro-caspase-8 proteins within the DISC promotes its activation and autolytic cleavage (*14-16*). Active caspase-8 can cleave caspase-3 and other effector caspases, as described in the intrinsic pathway above.

Signaling through the intrinsic and extrinsic pathways converges through activation of effector caspases, but there is also additional interaction between these pathways. In many cells activation of caspase-8 by the extrinsic pathway triggers a signaling feedback loop that activates pro-apoptotic factors at the mitochondrial membrane, such as the Bcl-2 family member Bid, and subsequently initiates the intrinsic apoptotic pathway as well. Cells that require this feedback loop for sufficient activation of effector caspases are called type II cells, while cells that completely activate effector caspases directly in response to caspase-8 activation are type I cells.

Inhibition of apoptosis

Apoptosis initiated by either the intrinsic and extrinsic pathway is tightly regulated through both redundant and distinct mechanisms. These mechanisms establish

the threshold of pro-apoptotic stimuli necessary to execute apoptosis, in addition to squelching spontaneous activation of apoptotic signaling components in the absence of apoptotic stimuli. As mentioned above, in the intrinsic pathway initiation of apoptosis is negatively regulated by anti-apoptotic members of the Bcl-2 family, which prevent mitochondrial membrane pore formation. Expression of these proteins is controlled by a number of factors in response to changes in the intracellular or extracellular environment, which allows conditional regulation of apoptotic sensitivity both to intrinsic apoptotic signals and to amplification of extrinsic signals in type II cells.

Activation of apoptosis through the extrinsic pathway is regulated by c-FLIP/CFLAR (CASP8 and FADD-like apoptosis regulator). Similar to pro-caspase-8, CFLAR contains two N-terminal tandem DEDs, which mediate the recruitment of CFLAR to the DISC. The C-terminal region of the CFLAR long splice variant is a caspase-like domain that lacks proteolytic active due to substitution of the active site cysteine. CFLAR blocks induction of apoptosis by competitively inhibiting access of pro-caspase-8 to the DISC. Transcriptional and post-translational regulation of CFLAR expression allows conditional control of apoptotic sensitivity to external stimuli. For example, CFLAR is expressed early during T cell activation and protects T cells against activation-induced cell death, despite increased expression of Fas. Later, decreased CFLAR expression sensitizes T cells to Fas-induces apoptosis, described in the example discussed earlier (*17*).

Regulation of apoptosis by XIAP and Smac

Apoptosis is regulated at the level of caspase activity by X-linked inhibitor of apoptosis (XIAP; *18, 19*). XIAP was isolated as a mammalian homolog of the inhibitor of

apoptosis Op-IAP from the baculovirus *Orgyia pseudotsugata* nuclear polyhedrosis virus (*20*), one member of a group of apoptosis-inhibiting baulovirus genes originally identified earlier in the *Cydia pomonella* granulosis virus (*21*). The N-terminus of XIAP contains three imperfect repeats of a conserved motif known as the baculovirus IAP repeat (BIR), which will be referred to as BIR1, BIR2 and BIR3. These ~70 amino acid domains are zinc fingers that consist of β sheets surrounded by four α helices and coordinate a zinc ion with one histidine and three cysteine residues. BIR2 and BIR3 of XIAP contain surface grooves that are recognition sites for inhibitor of apoptosis binding motifs (IBM), which are N-terminal peptide epitopes that are exposed during cleavage of pro-caspases and other proteins. The IBMs of caspases-3 and -7 bind the groove found in BIR2, and the linker region between BIR1 and BIR2 contains a binding site that specifically binds and inhibits the active sites of these effector caspases (*22*). Similarly, the surface groove of BIR3 binds the IBM of caspase-9, and a helical region at the C-terminal end of BIR3 binds and inhibits the caspase activity. Through these mechanisms, XIAP can block apoptosis induced by intrinsic or extrinsic stimuli. Furthermore, XIAP also has a role in regulation of the amplification loop between extrinsic and intrinsic apoptosis. XIAP has been demonstrated to block death receptor-induced mitochondrial permeabilization and cytochrome c release through its ability to suppress effector caspase activation (*23*).

The inhibition of caspases by XIAP can be relieved by the pro-apoptotic protein Smac/DIABLO. Freshly translated Smac contains an N-terminal mitochondrial localization sequence (MLS), which directs import of the protein through the outer mitochondrial membrane. Sequestered within this organelle, full-length Smac is cleaved

at residue 54, removing the MLS and generating the amino-terminal sequence AVPI, which is a potent IBM. Permeabilization of the outer mitochondrial membrane releases mature Smac into the cytosol, where Smac binds XIAP and displaces active caspases. Smac interacts with XIAP as a dimer, binding in the caspase-binding groove of BIR3 with high affinity (*24, 25*) and in the groove of BIR2 with lower affinity, which is strengthened through proximity-induced binding.

Future directions in further understanding apoptotic signaling

The kinetics of apoptosis have been studied using several approaches to discern the temporal relationships between apoptotic stimuli, mitochondrial membrane permeabilization, release of pro-apoptotic mitochondrial proteins, initiator caspase activation and effector caspase activation. In many cases, these experiments are complicated by the necessity of achieving precisely synchronized apoptotic stimuli in a population of cells. Most recently, imaging of individual cells undergoing apoptosis has been employed to analyze these questions (*26, 27*). Of particular interest is whether all mitochondria within the cell undergo permeabilization synchronously or discreetly. One study has reported a reported a wave of mitochondrial permeabilization through the cell (*28*). The combination of these observations with newly developed mathematical models will potentially yield further insight into the mechanisms of apoptosis (*29, 30*).

Inhibitor of Apoptosis Family

The caspase inhibitor XIAP is a member of the Inhibitor of Apoptosis (IAP) family, which is also called the BIR-containing (BIRC) family. In addition to XIAP,

seven other mammalian IAPs have also been identified, each containing one or three BIR domains, shown in Figure 1.2. BIR domains can be grouped into four types, based on conserved residues within the IBM-binding groove that influence peptide specificity (*31*). Type III BIRs contain a conserved histidine in the binding groove, which requires a proline in the third position of the IBM consensus sequence to accommodate the peptide backbone within the binding pocket. Type II BIRs contain a conserved tryptophan and do not prefer proline in the IBM. In contrast, type I and type X BIRs do not form peptide-binding grooves, but do have the potential to mediate dimerization or other protein-protein interactions.

Many IAPs have been shown to bind caspases and Smac at their BIRs, including c-IAP1, c-IAP2, ML-IAP, Survivin and BRUCE. However, the critical residues present in XIAP that permit direct inhibition of caspase activity are not conserved in other mammalian IAPs (*32-34*). The anti-apoptotic activity of these IAPs has been proposed to arise from two potential mechanisms. First, binding of Smac by these IAPs might effectively sequester cytosolic Smac and prevent it from antagonizing caspase inhibition by XIAP (*35-38*). Second, some IAPs, including c-IAP1, c-IAP2, XIAP, BRUCE and ML-IAP, have enzymatic activity that promotes the ubiquitination of caspases and Smac, targeting these pro-apoptotic proteins for proteasomal degradation (*39-43*). The ubiquitination activity of IAPs is an enormously important aspect of their function, so this aspect will be described in further detail.

Regulation of apoptosis through ubiquitination

Ubiquitination is a post-translation modification of lysine side chains in the target protein by covalent linkage to the C-terminal glycine of ubiquitin. Ubiquitin is

synthesized as a polypeptide and cleaved into individual 76 amino-acid ubiquitin moieties, which fold into a ββαββαβ structure. The process of ubiquitination involves an E1 ubiquitin-activating enzyme, an E2 ubiquitin-transferring enzyme, and an E3 ubiquitin-protein ligase that binds to the substrate. One type of E3 ubiquitin ligase is RING (really interesting new gene)-containing proteins, which have been shown to participate in ubiquitination in numerous examples (*44*). A carboxy-terminal RING domain is found in many of the IAPs, and of these E3 ubiquitin ligase activity has been definitively demonstrated for XIAP, c-IAP1 and c-IAP2. A special case among the IAPs, BRUCE contains a UBC (ubiquitin conjugating) domain that has combined E2/E3 activity.

Ubiquitin can be assembled into poly-ubiquitin chains by linking additional ubiquitin moieties onto the lysine residues within ubiquitin. The pattern of linkage influences the conformation of the poly-ubiquitin chain. Chain formation utilizing lysine 48 (K48) of ubiquitin allows interactions between hydrophobic patches on the surface the each ubiquitin moiety, promoting the association of neighboring ubiquitin into a closed cluster (*45, 46*). These ubiquitin chains are recognized by proteasome receptors to target the K48-ubiquitinated protein for degradation. In contrast, lysine 63 (K63) linkages do not allow for contact between the hydrophobic patches on neighboring ubiquitin moieties, resulting in an extended, open conformation (*47*). K63-linked ubiquitin chains are not associated with proteasomal degradation; rather, K63-mediated ubiquitination has been demonstrated to alter the function of the modified proteins in several cases, particularly influencing signaling complexes. Ubiquitin contains five additional lysine residues, but these linkages are not well studied. In some cases, ubiquitin modifications are not

extended into chains, and monoubiquitination has been implicated in membrane trafficking, endocytosis and cargo sorting. Ubiquitin modifications are reversible, and ubiquitin removal is mediated by a diverse group of deubiquitination enzymes (DUB).

The E3 ubiquitin ligase activity of XIAP, c-IAP1 and c-IAP2 has been demonstrated to promote the formation of K48-linked polyubiquitin chains that target substrates for degradation. Recent evidence also suggests that in addition to their ability to mediate K48 ubiquitination, c-IAP1 and c-IAP2 have the ability to form K63-linked ubiquitin chains on some substrates. In addition to their RING domains, XIAP, c-IAP1, c-IAP2 and ILP-2 also contain a ubiquitin associated domain (UBA), which binds polyubiquitin. The UBA domain, a three α-helix bundle containing a conserved MGF motif, is not required for the E3 ubiquitin ligase activity of these IAPs, but does contribute to their anti-apoptotic activity (*48, 49*). Of these IAPs, ILP-2 is highly unstable and has extremely limited tissue expression, so this IAP has been considered a non-functional pseudogene and will not be discussed further here (*50*).

While the ubiquitin ligase activity of IAPs can exert an anti-apoptotic effect by promoting the degradation of caspases and Smac, these are not the sole targets of ubiquitination by IAPs. XIAP, c-IAP1 and c-IAP2 have auto-ubiquitination activity and the ability to ubiquitinate each other (*51-54*), targeting the IAPs for degradation in a RING- and UBA-dependent manner. The auto-ubiquitination activity is enhanced by interaction with Smac, rapidly accelerating destabilization of these proteins.

Finally, another role for IAPs has been demonstrated during initiation of extrinsic apoptosis. c-IAP1 and c-IAP2 were originally discovered in a screen searching for members of the TNFR1 and TNFR2 signaling complexes (*55-57*). Unlike Fas, the death

receptor TNFR1 does not directly bind FADD; rather, the death domain of TNFR1 interacts with the death domains within TRADD (TNFR-associated death domain) and RIP1 (receptor interacting protein kinase 1; *58*). The intracellular tail of TNFR1 recruits a number of molecules that activate intracellular signaling pathways, forming a signaling complex called Complex I, which will be discussed extensively in the next section. Dissociation of the signaling complex from the intracellular tail of TNFR1 into the cytosol results in the recruitment of FADD by TRADD. This complex, now called Complex 2, is a DISC with the potential to activate pro-caspase-8 and initiate extrinsic apoptosis (*59, 60*). However, activation of caspase-8 is blocked in the presence of K63-linked RIP1 ubiquitination (*61*). c-IAP1 and c-IAP2 have been shown to target RIP1 for K63-linked ubiquitination within this complex (*62-64*). Thus, c-IAP1 and c-IAP2 are thought to influence sensitivity to TNF-induced apoptosis by regulating activation of caspase-8 within the TNFR1 DISC (*63-65*).

Non-apoptotic roles of IAPs in intracellular signaling pathways

c-IAP1 and c-IAP2 in signaling by the TNFR superfamily

A number of additional targets for ubiquitination have been identified for IAPs, which has led to functions for IAPs that are unrelated to apoptotic signaling (reviewed in *66*). As mentioned in the previous section, c-IAP1 and c-IAP2 are recruited to the signaling complexes activated by TNFR1 and TNFR2. c-IAP1 and c-IAP2 are recruited to these receptors through the association of their BIR1 domains with TNF receptor associated factor (TRAF) 1 and TRAF2 (*67, 68*). TRAF1 and TRAF2 are signaling adapters for several TNFR superfamily members (*69*), such as TNFR2 (*70*), CD27 (*71*),

CD30 (*72, 73*), CD40 (*67, 74*) and OX40, and they either bind to the receptor intracellular tail directly or through the intermediary TRADD.

The most widely studied signaling pathway activated by the TNF receptors is canonical NF-κB (nuclear factor-κB) signaling. Together, TRAF2 and RIP1 recruit and activate the IKK (inhibitor of κB kinase) complex (*75*). The IKK complex contains two subunits with kinase activity, IKKα and IKKβ, in addition to a regulatory subunit, nuclear factor-κB essential modulator (NEMO/IKKγ; reviewed in *76-78*). This complex phosphorylates inhibitor of κB-α (IκBα) on Ser32 and Ser36. Phosphorylated IκBα is ubiquitinated by a β-TrCP-containing complex and degraded by the proteasome. In the absence of IκBα, nuclear localization sequences within NK-κB subunits are unmasked. These sequences are recognized by nuclear import machinery to translocate the subunits, such as p65/RelA, Rel-B, c-Rel and p50, to the nucleus, where they bind as heterodimers or homodimers to their DNA consensus sequences and regulate transcriptional activation of the target genes. Activation of an NF-κB transcriptional program affects a diverse array of processes within the cell, including developmental programs, cell growth, apoptosis and immune responses. NF-κB is activated by a wide range of signals, such as pro-inflammatory cytokines, pathogens, antigen receptors or cellular stress from external or internal factors.

Extensive ubiquitination modifications occur within the TNFR signaling complex that influence the activation of signaling pathways (reviewed in *79*). Many of the modifications have been attributed to the E3 ubiquitin ligase activity of c-IAP1, c-IAP2 and TRAF2, which is also a RING-containing ubiquitin ligase. As mentioned previously, c-IAP1 and c-IAP2 have been shown to target RIP1 for K63-linked ubiquitination. K63

ubiquitination of RIP1 is detected by a polyubiquitin-binding domain within NEMO (*80, 81*), which is thought to recruit the IKK complex to the TNFR signaling complex. NEMO is also a target of ubiquitination by c-IAP1 and c-IAP2, which promotes activation of canonical NF-κB complexes (*82*). Ubiquitin chains within the signaling complex are removed by a number of DUBs, allowing for replacement of K63-linked polyubiquitin with K48-linked chains. RIP1, for example, is initially modified with K63-linked polyubiquitin during TNFR signaling and is later modified with K48-linked polyubiquitin, which targets RIP1 for proteasomal degradation (*83*). Editing of RIP1 ubiquitination can be performed by A20, which is NF-κB inducible and not only selectively removes K63-linked polyubiquitin, but also attaches K48-linked polyubiquitin (*84*).

Many members of the TNFR superfamily, including CD40, CD30, BAFFR, LTβR and TNFR2, also trigger non-canonical activation of NF-κB. Through TRAF-mediated activation of NF-κB-inducing kinase (NIK/MAP3K14) and IKKα homodimers, the non-canonical NF-κB member p100/NF-κB2 is phosphorylated. Phospho-p100 is ubiquitinated by β-TrCP and degraded by the proteasome into active p52 subunits. c-IAP1, c-IAP2, TRAF2 and TRAF3 affect non-canonical NF-κB signaling by promoting the K48-ubiqutination of NIK, targeting for it degradation and thus reducing p52 activation (*85-88*). The importance of non-canonical NF-κB activation is particularly evident during B cell development. Mutations that disrupt NFκB2 result in reduced B cell populations and disrupted splenic and lymph node architecture (*89*); moreover, mutations that disrupt NIK (aly/aly) result in alymphoplasia characterized by immunodeficiency,

disorganized splenic and thymic structures and the absence of lymph nodes and Peyer's patches (*90*).

In addition to NF-κB signaling pathways, TNF also activates MAPK (mitogen-activated protein kinase) signaling. MAPK signaling is characterized by activation of an apical kinase, a member of the MAP3K (MAP2K kinase) superfamily, which initiates a cascade of phosphorylation and activation of MAP2Ks (MAPK kinase) and subsequently MAPKs. Activation of these kinases ultimately results in the phosphorylation of transcription factor subunits, such as c-Jun, of the AP-1 transcription factor complex. TNF has been demonstrated to activate multiple MAPK signaling pathways, specifically JNK/SAPK (c-Jun N-terminal kinase/ stress activated protein kinase), ERK (extracellular regulated kinase) and p38 MAPK (*91, 92*) pathways. Although TRAF2 has been identified as an important signaling component during activation of MAPK pathways by TNF, the identity and contributions of specific MAP3Ks has not been completely characterized. One important apical kinase is ASK1 (apoptosis signal-regulating kinase 1), which is required for JNK and p38 activation by TRAF2 (*93-95*). Intriguingly, c-IAP1 has been demonstrated to ubiquitinate ASK1, which results in its degradation and termination of JNK and p38 signals. (*96*).

The spatial and temporal coordination of signaling is an important aspect of TNFR activation. For one TNFR family member, CD40, activation of the JNK signaling cascade requires dissociation of the signaling complex into the cytosol. This transition has been demonstrated to require c-IAP1/2-mediated degradation of TRAF3 (*97*). In a number of cases, activation of TNFR family members is associated with membrane translocation into detergent-insoluble lipid rafts (*98-100*), and recent studies indicate

these rafts might be essential for activation of intracellular signaling (*101*). Receptor activation subsequently leads to ubiquitination and degradation of TRAF2 (*102, 103*), TRAF1 (*104*) and c-IAP1 (*105*), and lipid rafts have been implicated as the site of ubiquitination (*100, 106*). The role of c-IAP1 and c-IAP2 in TNF-activated signaling pathways will be discussed in further detail in Chapter 4.

Additional regulation of intracellular signaling by IAPs through ubiquitination

Further functional roles for c-IAP1 have also been recently uncovered, which appear unrelated to its function in TNFR-activated signaling. Another target of K48-linked ubiquitination by c-IAP1 is Mad1. Mad1, Myc and Max are basic helix-loop-helix leucine zipper transcription factors that bind as heterodimers to the promoters of target genes. Myc/Max heterodimers activate transcription of genes involved in regulation of proliferation, differentiation and apoptosis. Mad1 antagonizes Myc by dimerizing with Max and recruiting co-repressors to Myc-responsive elements at target genes to block transcription. Deregulation of Myc activity is prevalent in numerous cancers, and by de-repressing Myc activity through its actions on Mad1, c-IAP1 potentially cooperates in promotion of proliferation, transformation and tumorigenesis (*107*).

In addition to its function as a *bona fide* caspase inhibitor, XIAP has additional critical roles in copper metabolism and NF-κB signaling. Intracellular copper is closely regulated by chaperones, which transfer copper from membrane transporters to the intracellular proteins that require copper as a co-factor. XIAP tightly binds copper, but copper-bound XIAP undergoes a conformational change that destabilizes the protein (*108*). This decrease in XIAP stability not only affects the apoptotic sensitivity of the cell,

but also copper metabolism within the cell. COMMD1 (Copper metabolism MURR1 domain-containing 1) is an essential component of the copper excretion pathway (*109*) that is targeted for degradation by the ubiquitin ligase activity of XIAP (*110*). COMMD1 was initially discovered because inherited mutations are prevalent in Bedlington terrier dogs and lead to copper toxicosis (*111*). Under conditions of elevated intracellular copper, there is degradation of XIAP and subsequently stabilized COMMD1 and enhanced copper excretion. COMMD1 has an additional effect beyond copper metabolism, because it is a component of an E3 ubiquitin ligase that controls ubiquitination and degradation of chromatin-bound NF-κB subunits, resulting in termination of transcription of NF-κB dependent genes (*112-114*). Through COMMD1, XIAP has the potential to regulate NF-κB signaling and copper homeostasis.

Through a distinct mechanism, XIAP also influences NF-κB and JNK1 activation by the TGF-β (transforming growth factor-β)/BMP (bone morphogenetic protein) receptor superfamily. XIAP binds to TGF-βRI (TGF-β receptor type I; *115, 116*) and the signaling intermediate TAB1 (TAK-1 binding protein; *117*) to mediate activation of TAK1 (TGF-β activated kinase 1) by the receptor (*118-120*) and subsequently activate NF-κB and JNK signaling. However, one dissenting paper has suggested XIAP causes degradation of TAK1 and inhibits signaling instead (*121*). Limited evidence has been observed *in vivo* for contribution of XIAP to NF-κB signaling. XIAP-deficient mice are generally healthy without profound defects in apoptosis (*122*), but altered kinetics of NF-κB activation were observed in association with delayed mammary gland development in pregnant mice (*123*).

XIAP and to a lesser extent c-IAP1 and c-IAP2 also regulate the stability of the signaling protein C-Raf indirectly. C-Raf is a Ser/Thr protein kinase that participates in the Ras-stimulated cascade of MAPK signaling that activates ERK and contributes to cell growth, migration, differentiation, survival and proliferation. C-Raf is a binding partner of IAPs (*124*), which results in its degradation by the proteasome. However, this degradation is independent of IAP ubiquitin ligase activity and is instead mediated by the Hsp90 chaperone protein. Through this activity, XIAP has been shown to regulate ERK signaling and cell motility (*125*). Thus, the extensive functions of XIAP affect a broad array of cellular processes. The broad range of substrates targeted for ubiquitination by XIAP and other IAPs suggests the possibility that additional substrates will continue to be identified, leading to additional, novel functions of IAPs.

Cell proliferation and division – Survivin and BRUCE

The small protein Survivin, which consists of a single BIR and coiled-coil domain, does not have ubiquitin ligase activity but does play a non-apoptotic role in cellular proliferation. Expression of Survivin is extremely cell-cycle specific, and Survivin binds to centromeres during cell division in a complex called the chromosomal passenger complex (*126, 127*). The proper function of this complex is necessary for microtubule-mediated chromosomal segregation to opposite spindle poles (*128*). Failure of this complex disrupts polarized microtubule attachment and results in multinucleated, polyploid cells (*129-131*).

Later during cytokinesis, BRUCE, the largest IAP by far at 528kD, is necessary to cut the final channel between the dividing cells (*132*). Survivin and BRUCE are the only IAPs essential for viable mouse embryo development, and each has been highly

conserved through evolution relative to the rest of the family (*133*). A wide variety of cancers demonstrate dramatic changes in Survivin expression and localization, which may represent significant changes in Survivin function that promote tumorigenesis (reviewed in *134*).

Clinical significance of IAPs

Tumorigenesis

The IAPs have received enormous interest for a possible role in tumorigenesis, since their anti-apoptotic function is a potential mechanism of malignant cell survival (reviewed in *135*). Indeed, increased expression of IAPs, including XIAP, c-IAP1, c-IAP2, ML-IAP and Survivin, has been observed in a number of tumor specimens. Evidence for an apoptotic role of these IAPs has come from a number of studies using cancer cell lines that are depleted in expression of IAPs through targeted genetic, RNA interference, antisense oligonucleotide, dominant negative or pharmacological approaches. In a number of cancer cell lines, reduction of IAP expression sensitizes cells to apoptosis *in vitro* (*136*) and reduces tumor growth *in vivo* (*137-148*).

Unfortunately, the approaches widely used to dissect the contributions of a gene or pathway to tumorigenesis have a number of limitations. In many cases, cancer cell lines are utilized that are cultured from aggressive cancers, which have presumably acquired a large number of mutations or polyploidy during the progression of the malignancy in the original host and later in culture. For this reason, these cells are an imperfect model of initial tumor development, since a number of factors that potentially participated in cancer development might no longer be relevant in cells at this advanced

stage. Furthermore, *in vivo* studies often rely on xenograft models of tumor growth, in which human cancer cell lines are injected into immunodeficient mice. This approach is limited in its ability to model the initial emergence of malignant cells within the context of an intact immune and stromal environment.

We have attempted to address these limitations by examining tumor development in an immunocompetent mouse using the transgenic adenocarcinoma of the mouse prostate (TRAMP) model. Prostate-specific expression of SV40 T-antigen in TRAMP mice interferes with the function of retinoblastoma and p53 tumor suppressors and results in 100% penetrance of prostate tumors, with a substantial proportion developing metastatic disease. In an evaluation of prostate cancer development in the presence and absence of XIAP, we found no evidence for a protective effect of XIAP deficiency in tumor onset or overall survival. Furthermore, tumor histology revealed similar patterns of differentiation and frequencies of apoptosis and proliferation in tumors (*149*). This is a stark contrast to pre-clinical and early phase clinical trials of therapeutics that target XIAP or other IAPs, which have generated promising preliminary responses. These results highlight the need for improved models of cancer development. A number of innovative approaches are currently being developed that will vastly advance our evaluation of therapeutic targets.

In general, altered expression or function of IAPs in cancer is not associated with genomic mutations, but c-IAP1 and c-IAP2 are notable exceptions. Microamplifications of the human 11q22 region, which includes c-IAP1 and c-IAP2, have been identified in pancreatic cancer (*150*), lung cancer (*151*), medulloblastoma (*152*), squamous cell carcinoma (*153-155*), glioblastoma multiforme (*156*), hepatocellular carcinoma (*157*) and

osteosarcoma (*158*). In mouse models of hepatocellular carcinoma and osteosarcoma, focal amplicons of the syntenic murine chromosome 9A1 region have been observed. c-IAP1 and c-IAP2 have been identified as critical genes within this amplicon that contribute to tumorigenesis, in cooperation with p53 or Myc mutations (*157, 158*). We have observed a moderately protective effect in c-IAP1-deficient mice using the TRAMP model (please refer to Appendix), which has abrogated function of p53. Since expression of both XIAP and c-IAP1 are increased in TRAMP tumors (*159*), the differential contributions of these IAPs to TRAMP tumor growth implicate non-apoptotic activities of c-IAP1 in tumor development.

In multiple myeloma, a plasma cell-derived malignancy, bi-allelic deletions of the region encoding c-IAP1 and c-IAP2 have been detected. These mutations are associated with constitutive activation of non-canonical NF-κB signaling, and mutations in a number of other components in this pathway (e.g. NIK, TRAF2, TRAF3, NFKB2, CD40 and LTBR) have also been detected (*160*). Patients with these mutations demonstrated relatively improved response to proteasome inhibitors, which can inhibit activation of canonical and non-canonical NF-κB signaling.

A final instance of oncogenic mutations involving IAPs is the t(11;18) (q21,q21) translocation of c-IAP2 and MALT1 in MALT lymphoma, a non-Hodgkins lymphoma that develops from B-cells in mucosal-associated lymphoid tissue (MALT). The expressed protein contains the BIR and UBA domains of c-IAP2 (*49*) fused with MALT1, which is a caspase-like protein normally involved in activation of NF-κB by antigen receptors on lymphocytes (*161*). This fusion constitutively activates NF-κB by BIR-induced oligomerization (*162-164*). These examples also provide further evidence,

in addition to the roles of c-IAP1/Mad1/Myc and Survivin previously described, for non-apoptotic contributions of IAPs to tumor development.

Immune function

Additional clinical consequences of XIAP relate to the development and activation of the immune system. XIAP loss-of-function mutations have been identified in patients with X-linked lymphoproliferative syndrome (XLP), an extremely rare and fatal immunodeficiency that manifests as lymphohistiocytosis, splenomegaly, hypogammaglobulinema, fulminant infectious mononucleosis from Epstein-Barr γ-herpesvirus and lymphoma. These patients have a deficiency in natural killer T-lymphocytes (NKT cells), and apoptosis of lymphocytes from patients is enhanced in response to Fas, TRAIL or T cell antigen receptor stimulation (*165*).

The pathogenesis of XLP is unclear, but XLP can also result from other mutations at the Xq25 locus that affect expression of the neighboring gene SAP (SLAM-associated protein). Patients with loss of SAP function have similar manifestations of XLP as patients with mutations in XIAP, including a deficit in NKT cells. However, the functional similarities end there between XIAP and SAP, which is an SH2-containing adapter for the SLAM (signaling lymphocytic activation molecule) family of CD2 immunoglobulin receptors. A number of functions for SAP have been identified by analyzing SAP-deficient mice, which are defective in immune responses to the murine γ-herpesvirus MHV-68, NKT cell development, NK and $CD8^+$ T cell cytotoxicity and $CD4^+$ T cell-dependent cytokine production and germinal center formation (*166*). In contrast, XIAP-deficient mice have normal NKT cell populations, intact germinal center formation, normal cytokine responses to MHV-68 and normal SAP expression (*167*).

Thus, XIAP might have a role in the pathogenesis of XLP distinct from the role of SAP. In addition to enhanced sensitivity of patient lymphocytes to apoptosis, cells from XIAP-deficient mice are more sensitive to MHV-68-induced apoptosis, which is associated with increased viral production (*167*). Also, overexpression of XIAP has been shown to be associated with resistance to apoptosis in the experimental autoimmune encephalitis (EAE) model, which correlates with exacerbation of disease (*168*). In other models, sensitization of lymphocytes to antigen-induced cell death has been shown to deplete virus-specific $CD8^+$ T cells and lead to impaired cytotoxicity and reduced viral clearance (*169*). Further study is necessary, but these findings potentially suggest an apoptotic role for XIAP in XLP.

A non-apoptotic role of XIAP has also been implicated in contributions to immune responses through the role of XIAP in NF-κB and MAPK signaling described in the previous section. During *Listeria monocytogenes* infection, XIAP has been shown to enhance activation of both canonical NF-κB and JNK signaling in activated macrophages. Activation of the JNK pathway through XIAP contributes to enhanced cytokine production, specifically IL-6, by *L. monocytogenes*-infected activated macrophages *in vitro*. IL-6 expression is also augmented *in vivo* by XIAP, and the contributions of XIAP to the innate immune response protect mice from listeriosis-induced lethality (*170*). Thus, XIAP has the potential to modulate immune responses through apoptotic and non-apoptotic roles in different settings.

Another example of the non-apoptotic role of IAPs in immune responses is NAIP, which contains a nucleotide-binding oligomerization domain (NOD) and leucine-rich repeats (LRR) characteristic of the NOD-like receptors (NLR). This family of pattern

recognition receptors is a critical component of the innate immune response to intracellular PAMPs. Mice encode six Naip genes, and mice that do not express functional Naip5 demonstrate profound susceptibility to *L. pneumophila.* Indeed, Naip5 has been shown to recognize intracellular *Legionella pneumophila* flagella and activate IL-1β cytokine production (*171*), (*172*). The BIR domains of NAIP have low affinity for caspases or Smac; thus, the role for NAIP is thought to be non-apoptotic but possibly influences cell death by other processes discussed in the next section.

Inflammation and cell death

Inflammation is triggered as a component of the innate immune system's response against invading pathogens. Cells of the innate immune system communicate through a large number of cytokines, chemokines and other pro-inflammatory mediators to target leukocytes to the site of insult. Activation of innate immune cells is initially triggered by recognition of pathogen-associated molecular patterns (PAMP) by three main classes of pattern recognition receptors: NLRs (mentioned in the previous section), TLRs (Toll-like receptors) and RLH (RIG-like helicases). Signaling through each of these groups involves a number of DD, DED and CARD-containing proteins, similar to apoptotic signaling cascades. Of particular note, a subset of NLRs activate intracellular signaling through Ipaf-1, which is related to Apaf-1 in the intrinsic apoptotic pathway. Upon activation, Ipaf-1 oligomerizes to form an "inflammasome." This complex recruits a special subset of caspases that includes human caspases-1, -4, -5 and -12 and murine caspases-1, -11 and -12, which do not have a demonstrated role in apoptosis. Rather, the pro-inflammatory caspases have ICE (interleukin converting enzyme) activity, which

cleaves pro-interleukin (IL)-1β and pro-IL-18 into mature cytokines for secretion. In addition, activation of caspase-1 can result in a non-apoptotic cell death characterized by plasma membrane pore formation and rupture, called pyroptosis (reviewed in *173*). Release of intracellular contents during pyroptosis stimulates an inflammatory response, distinctly different from the non-inflammatory cell death that occurs as a result of apoptosis.

Death of innate immune cells might represent an important mechanism for limiting the extent or severity of inflammation, similar to the importance of antigen-induced cell death in regulation of the adaptive immune system. On the other hand, during severe, uncontrolled inflammation observed in patients with sepsis, excessive death of immune cells has been thought to lead to subsequent immune suppression with detrimental consequences (*6, 7*). In a number of mouse models of sepsis, improved survival was observed by blocking apoptosis through overexpression of anti-apoptotic Bcl-2 (*174-178*), suppression of pro-apoptotic Bcl-2 family members (*179, 180*), suppression of Fas or caspase-8 (*181*), knock-out of caspase-7 (*182*) or administration of caspase inhibitors (*183, 184*); furthermore, increased severity of illness and more apoptotic leukocytes were observed in Bcl-2-knockout mice (*185*). Thus, targeting apoptosis has been identified as an attractive therapeutic option in patients with sepsis, who have substantially increased risk for acute and chronic morbidity and mortality otherwise (*186*).

In addition to modulating cell death, another outcome of pattern recognition receptor activation by PAMPs is signaling by the NF-κB and MAPK pathways. The transcription factors activated by these pathways induce an enormous array of target

genes, many of which are pro-inflammatory or pro-survival. The IL cytokine and TNF ligand families are included in this group, both of which can further activate NF-κB and MAPK signaling within receptor-expressing cells. Activation of NF-κB cascades by pattern recognition receptors, as well as TNFR and IL-R families, is significantly affected by K63- and K48-linked ubiquitination of signaling proteins. For some pattern recognition receptors, components of the TNFR signaling pathway, such as TRAF2 and RIP1, mediate activation of NF-κB signaling. Related to RIP1, RIP2 also plays a role in NF-κB activation by certain pattern recognition receptors. Interestingly, RIP2 is a target of ubiquitination by c-IAP1. The notable similarities between inflammatory signaling pathways activated by pattern recognition receptors and TNFR family members alludes to a number of possible roles for c-IAP1 and c-IAP2 during PAMP recognition. The implications of signaling by pattern recognition receptors and potential contributions of IAPs will be discussed further in Chapters 4 and 5.

An intriguing functional role for c-IAP2 in inflammation was recently uncovered by Conte and colleagues (*187*). Mice with genetically targeted deletion of c-IAP2 were observed to have improved survival during endotoxemia. This observation was associated with reduced systemic pro-inflammatory cytokines and increased sensitivity of immune cells from these mice to apoptosis. The authors concluded that c-IAP2 is a critical pro-survival factor within innate immune cells that drive systemic inflammation. In the absence of c-IAP2, the authors suggested that this population of immune cells is depleted, resulting in decreased systemic cytokines and improved disease outcomes. These results are an interesting contrast to other models of sepsis discussed above, which indicated that anti-apoptotic mechanisms resulted in improved survival. It is compelling to conjecture

that the non-apoptotic functions of c-IAP2 also modulate inflammatory responses, leading to a disparate role for this anti-apoptotic molecule during sepsis. The interconnections between IAPs in apoptosis and intracellular signaling pathways are a potential mechanism to coordinate the sensitivity of the cellular suicide program and adaptation to inflammation in extracellular environment. Certainly, this process must be better understood before apoptotic pathways can be therapeutically targeted, either specifically with IAP antagonists or broadly, in sepsis or in other diseases with components of inflammation, such as cancer.

Overview of dissertation

This dissertation will investigate the connection between molecular regulation of apoptosis and inflammation, specifically focusing on the role of c-IAP1. A potential functional role for c-IAP1 was explored in sepsis, based on previous observations of the contributions of c-IAP2 in this model. These studies were also extended to a model of viral infection to determine whether c-IAP1 contributes to a broad pro-inflammatory response. Results from these *in vivo* studies are described in Chapter 2. The contribution of c-IAP1 to regulation of pro-inflammatory cytokine production is examined in both immune cells and non-immune cells in Chapter 3. In Chapter 4, intracellular signaling pathways that regulate cytokine production in response to PAMPs or TNF are examined in the context of c-IAP1. The implications of these results in the pathology of inflammatory processes and the molecular signaling that regulates them will be discussed in Chapter 5.

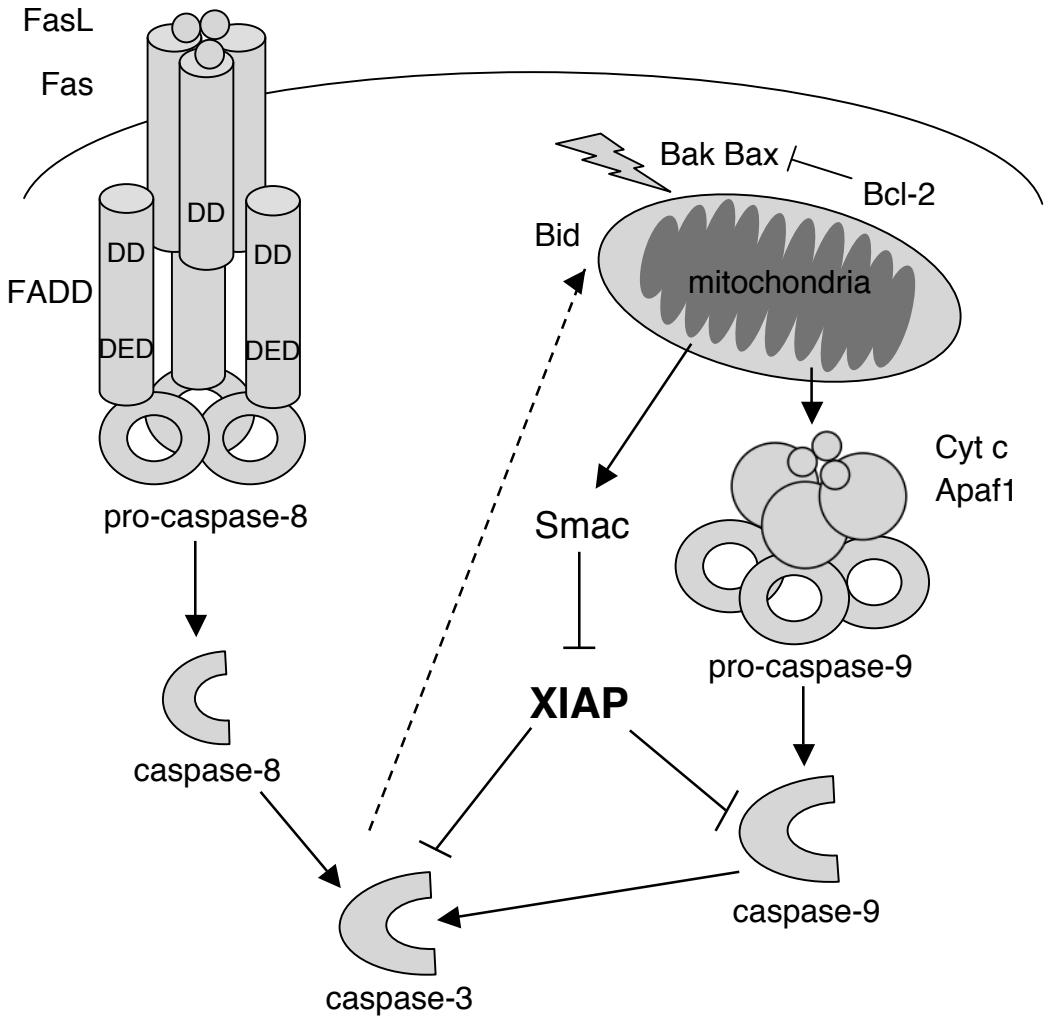

Figure 1.1 Apoptotic signaling by the extrinsic and intrinsic pathways.

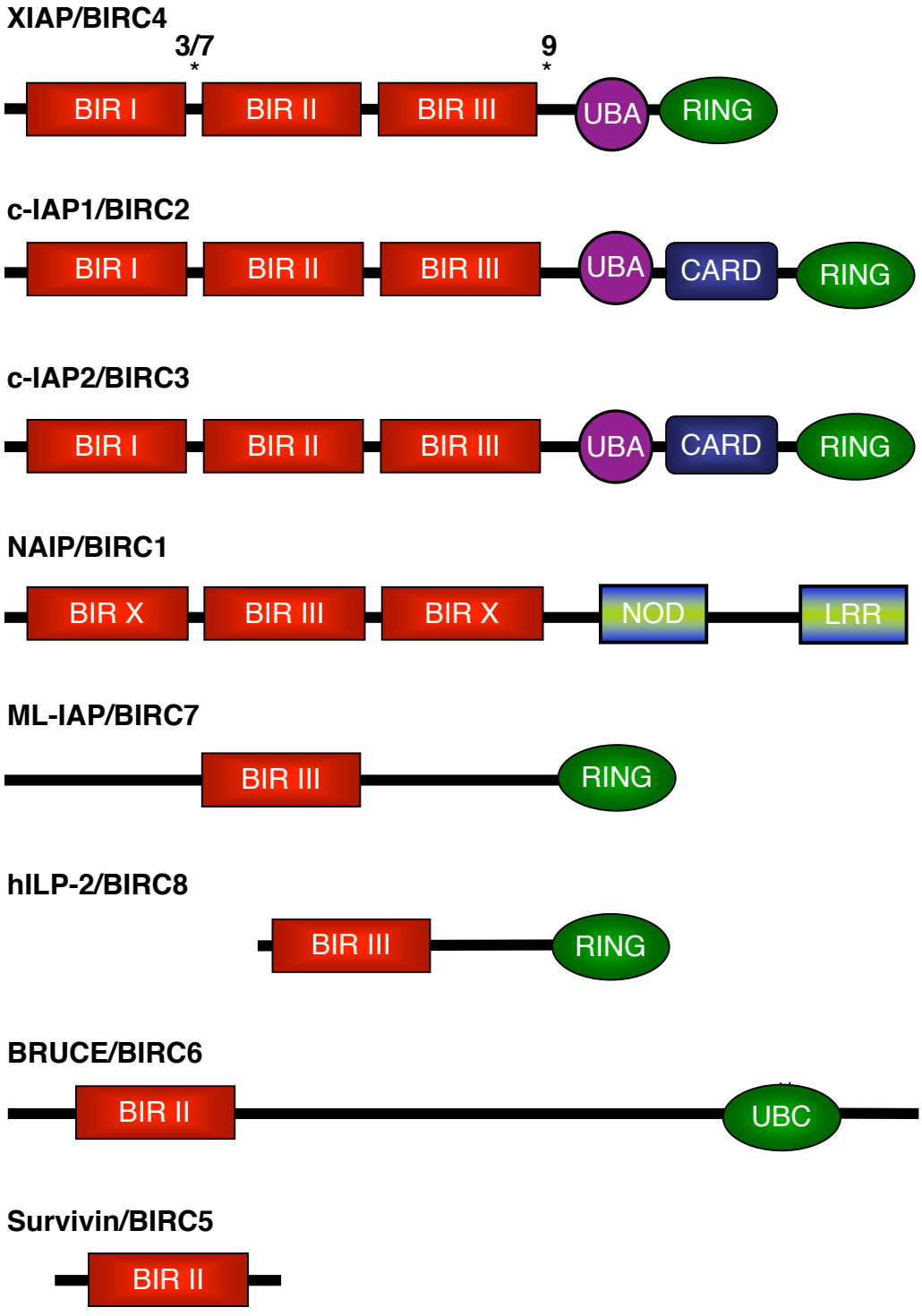

Figure 1.2 Members of the mammalian IAP family. BIR: Baculovirus IAP repeats; type I, II, III and X described in the text. UBA: ubiquitin associated domain. UBC: ubiquitin conjugating domain. RING: really interesting new gene. NOD: nucleotide-binding oligomerization domain. LRR: leucine-rich repeat. CARD: caspase activation and recruitment domain.

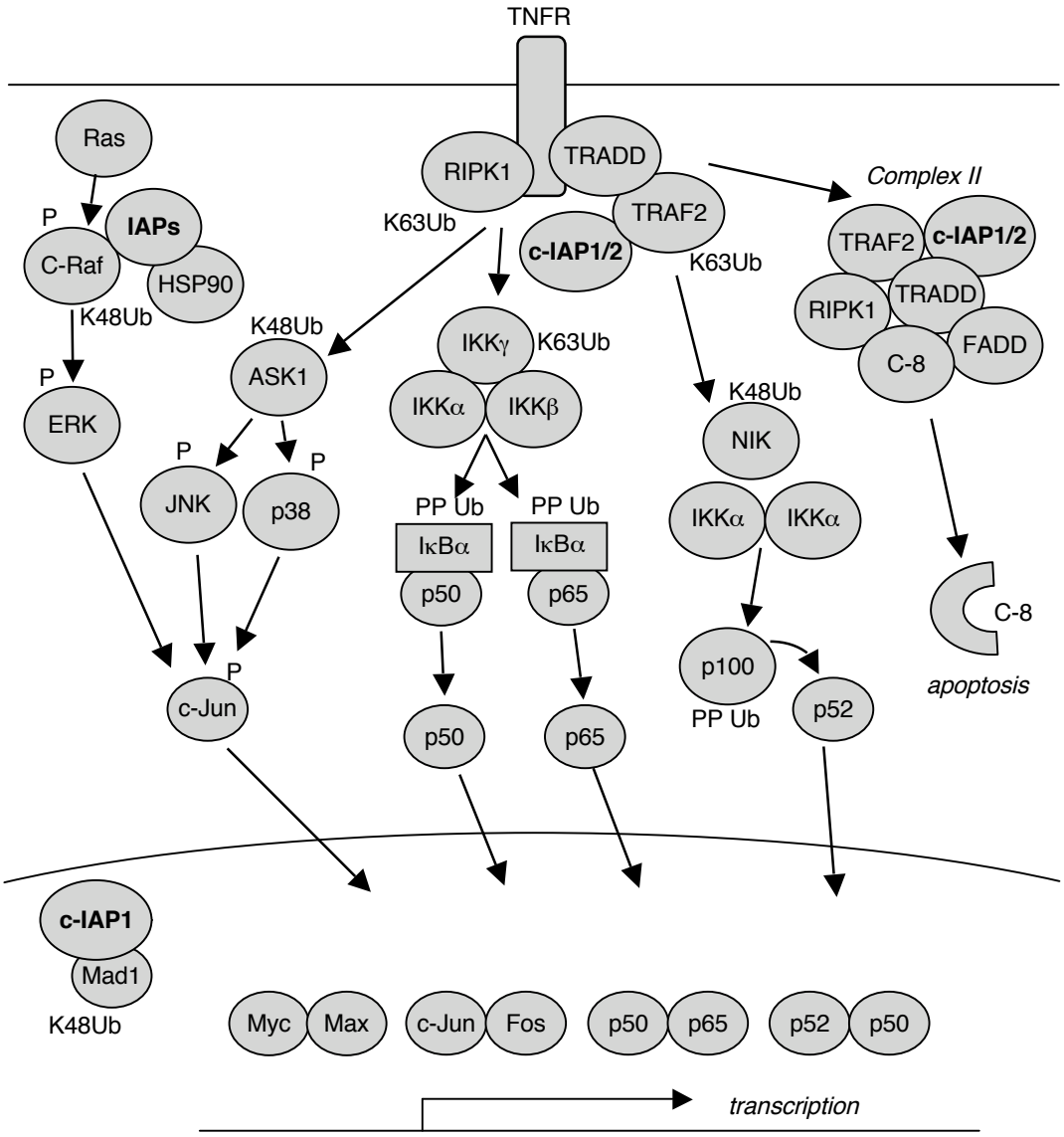

Figure 1.3 Non-apoptotic signaling influenced by c-IAP1.

Chapter 2

The effect of c-IAP1 on inflammation *in vivo*

Abstract

Cellular inhibitor of apoptosis 1 (c-IAP1) is a component of the TNFR signaling complex that has been implicated in apoptosis, cell proliferation and cancer development. Here we identify a pro-inflammatory role for c-IAP1 during endotoxic shock. *c-IAP1*$^{-/-}$ mice demonstrated significantly improved overall survival and decreased serum concentrations of pro-inflammatory cytokines IL-1β, IL-6, IL-12, RANTES, MCP-1 and TNF following challenge with LPS. Disruptions in cytokine production were not a result of altered cytokine production at the site of endotoxin challenge or increased apoptosis of cytokine-producing cells. Interestingly, decreased concentrations of IL-1β, IL-12 and MCP-1 were observed in the lungs of *c-IAP1*$^{-/-}$ mice following intraperitoneal challenge with LPS. These findings identify a contribution of c-IAP1 to production of systemic cytokines during pathologic pro-inflammatory immune responses.

Introduction

Sepsis is a significant clinical challenge that is increasing in incidence and carries a substantial risk of mortality (*188, 189*). The syndrome is characterized by vasodilation, vascular permeability and hypoperfusion that leads to disseminated intravascular

coagulation and acute organ dysfunction in severe cases. These manifestations are attributable to an overly zealous host innate immune system response to a pathogen (*190*), which results in excessive pro-inflammatory cytokines that are not limited to the local site of microbial infection (*191*). This systemic inflammatory response is characterized by an initial surge in serum TNF (tumor necrosis factor), IL-6, IL-1β and MIP-2 (macrophage inflammatory peptide-2 or CXCL2), followed later by the chemokines MCP-1 (monocyte chemoattractant protein-1 or CCL2), RANTES (regulated on activation, normal T cell expressed and secreted or CCL5) and additional pro-inflammatory mediators. The pro-inflammatory cytokine cascade is a central component of the uncontrolled, disproportionate inflammation that results in the pathological sequelae of sepsis.

The pathogenesis of sepsis involves an early, central role for the pro-inflammatory cytokine TNF. The protective effects of blocking TNF during sepsis have been demonstrated using TNF receptor-null mice or TNF-neutralizing proteins (*192-195*). While attempts to block the actions of TNF have failed to improve the clinical response of patients with sepsis (*196, 197*), it remains clear that the TNF-mediated activation events are critical for the subsequent cytokine cascades. Production of pro-inflammatory mediators is triggered by TNF through the activation of intracellular signaling cascades, including NF-κB and MAPK signaling.

The importance of TNF as a pro-inflammatory mediator suggests a potentially critical role for c-IAP1 and c-IAP2 during inflammation and septic shock. c-IAP1 and c-IAP2 are signaling molecules that have the ability to influence these TNF-induced pro-inflammatory cellular responses. c-IAP1 and c-IAP2 have previously been shown to be

components of the TNF receptor signaling complex leading to activation of NF-κB (*55, 56*). In addition to modulating the pro-survival affects of NF-κB activation, c-IAP1 and c-IAP2 have been shown to regulate initiation of caspase activation in response to the TNF (*63-65*).

Cell death is a widespread, harmful consequence of sepsis that significantly affects both the acute and the chronic course of the disease. Profound apoptotic defects are not present in naïve *c-IAP1*$^{-/-}$ mice or *c-IAP2*$^{-/-}$ mice, and these mice develop normally. Deletion of c-IAP2 in mice has been reported to sensitize macrophages to apoptosis in response to apoptotic stimuli *in vitro* or in a model of sepsis *in vivo* (*187*). Challenging these mice with the gram-negative bacterial endotoxin component lipopolysaccharide (LPS) resulted in decreased serum cytokine production and protection from lethality (*187*). One notable difference between c-IAP1 and c-IAP2 is that expression of c-IAP2 is inducible, for example in response to LPS, while c-IAP1 is constitutive and ubiquitous (*187*). From these observations, c-IAP2 has been suggested to contribute to systemic inflammation during sepsis through its role as a critically induced pro-survival gene that protects macrophages from apoptosis.

In the studies described here, we sought to characterize the role of c-IAP1 during innate immune responses using genetically targeted mice. We show that, like c-IAP2, c-IAP1 is also a critical mediator of innate immune responses during septic shock. c-IAP1-deficient mice demonstrated improved survival during endotoxic shock and significantly decreased production of systemic cytokines. However, unlike c-IAP2-deficient mice, which have been described to exhibit enhanced sensitivity to apoptosis of macrophages, c-IAP1 deficiency did not appear to affect macrophage viability. The absence of c-IAP1

did not result in global deficiencies in immune responses, because cytokine production and leukocyte infiltration in response to viral infection were normal in *c-IAP1*$^{-/-}$ mice. These findings suggest that c-IAP1 alters activation of the cytokine cascades that lead to catastrophic septic responses.

Materials and Methods

Mouse models

c-IAP1$^{-/-}$ mice have been described previously (*53*) and were obtained from Dr. Tak Mak (University of Toronto, Ontario, Canada). Age-matched wild-type mice were obtained from Jackson Laboratories (Bar Harbor, ME), unless littermates are specifically stated. All mice are on C57BL/6 background, backcrossed at least six generations, and maintained under specific pathogen-free conditions at the University of Michigan Medical School Animal Facility. The University of Michigan Committee on Use and Care of Animals approved all animal studies.

Cecal ligation and puncture procedures were performed as previously described (*198*). *c-IAP1*$^{-/-}$ (*n=4*) and *c-IAP1*$^{+/-}$ (*n=5*) littermates were used at 9-weeks old. Briefly, mice were anesthetized by intraperitoneal injection of Ketamine HCL (112.5 mg/kg; Abbott Laboratories, N. Chicago, IL) and Anased (7.5 mg/kg; Lloyd Laboratories, Shenandoah, IA). Under sterile surgical conditions, a 1- to 2-cm midline incision was made to the ventral surface of the abdomen, and the cecum was fully exposed through this incision. The cecum was ligated at its base with a 4-0 silk suture without causing bowel obstruction. The ligated cecum was punctured twice with a 21-gauge needle. The abdominal incision was closed using a surgical staple, and 1 ml of sterile normal saline

was administered subcutaneously for fluid resuscitation. Survival was monitored for one week after these procedures. To control for the surgical procedure, sham-operated mice underwent an identical laparotomy but did not undergo cecal ligation or puncture, and all mice receiving the sham operation survived.

Endotoxic shock was induced by intraperitoneal injection of LPS (*E. coli* O111:B4, #L2630, Sigma-Aldrich, St. Louis, MO) in groups of age- and sex-matched mice. Age-matched wild-type mice were obtained from Jackson Laboratories (Bar Harbor, ME). For survival experiments, mice (n=7) were injected with doses of 40 μg LPS/g body weight or 100 μg LPS/g body weight in 500 μL volume, and health was monitored for seven days. For sub-lethal endotoxemia, mice were injected with 2.5 μg LPS/g body weight in 200 μL volume into the peritoneum. Blood, liver, lung and peritoneal lavage were collected after 1 hour (*c-IAP1*$^{-/-}$ n=5, *c-IAP1*$^{+/+}$ n=3), 4 hours (*c-IAP1*$^{-/-}$ n=6, *c-IAP1*$^{+/+}$ n=6), or 24 hours (*c-IAP1*$^{-/-}$ n=4, *c-IAP1*$^{+/+}$ n=5). Serum was obtained by clotting for at least one hour at room temperature, followed by centrifugation. Peritoneal lavage cells were counted, and cytospin slides were stained with DiffQuik (Dade Behring, Newark, DE). Lung and liver samples were homogenized with a Tissue Tearor in 1mL 0.1% Triton X-100 PBS with protease inhibitor cocktail.

RSV was derived from a clinical isolate at the University of Michigan (*199-201*). The virus was propagated in Hep2 cells (American Type Culture Collection) and titered using Vero cells (American Type Culture Collection), as previously described (*202*). Mice were anesthetized and infected intratracheally with RSV, as previously described (*199, 200, 203*). Lungs were harvested one day (*c-IAP1*$^{-/-}$ n=6; *c-IAP1*$^{+/+}$ n=4) or eight days (*c-IAP1*$^{-/-}$ n=5; *c-IAP1*$^{+/+}$ n=4) following infection for cytokine analysis. Lung

histology specimens and lymph nodes were also harvested on day eight. Histology slides were stained with hematoxylin and eosin (H&E) or periodic acid Schiff (PAS). Custom primers for qRT-PCR of RSV G protein have been previously described (*204*).

Quantification of apoptosis

To analyze apoptosis, peritoneal lavage cells from *c-IAP1*$^{-/-}$ mice and wild-type littermates were harvested in cold saline 2.5 hours following intraperitoneal injection with 10 μg LPS/g body weight (n=5). Total cell number was obtained by counting using a hemacytometer, and cells were stained using Annexin V-phycoerythrin (PE) and 7-amino-actinomycin D (7-AAD) in Annexin V binding buffer (BD Pharmingen, San Jose, CA) in the dark for 15 min. Cells were analyzed by flow cytometry on a Beckman Coulter FC500 instrument (Fullerton, CA). Mononuclear cells were identified by forward scatter/side scatter profile, and apoptotic cells were identified as Annexin V and 7-AAD positive using FlowJo software (Tree Star, Inc., Ashland, OR).

Quantification of cyotokines

Cytokine protein concentrations were quantified using a bead-based cytokine assay analyzed by the Bio-plex Suspension Array System following manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA).

To quantify expression of cytokine genes of interest, RNA was harvested in Trizol (Invitrogen, Carlsbad, CA) and isolated following manufacturer's instructions. cDNA was generated using Moloney Murine Leukemia Virus (MMLV) Reverse Transcriptase (Invitrogen). Levels of mRNA were analyzed by qPCR using Taqman gene expression assays (Applied Biosystems, Foster City, CA). Gene expression was

normalized to GAPDH mRNA for each sample and expressed as fold increase compared to the negative control within the experiment.

Statistical Analyses

Mouse survival data were analyzed using Kaplan-Meier analysis and log-rank statistical tests with the survival package in the R statistical program (*205*).

For analysis of cytokines, ANOVA was used with a Student Newman-Keuls post-test to determine the statistical significance of differences. A p-value <0.05 was considered to be statistically significant.

Results

c-IAP1$^{-/-}$ mice have improved survival during polymicrobial sepsis and endotoxic shock

Recent evidence that supports a role for c-IAP2 in the pathogenesis of sepsis prompted us to investigate a potential role for the highly homologous protein, c-IAP1. We utilized two mouse models of sepsis, which arise from peritonitis induced through either a polymicrobial insult or gram-negative bacterial endotoxin. We first examined *c-IAP1*$^{-/-}$ mice in a model of polymicrobial sepsis induced by cecal ligation and puncture. During this procedure, the cecum was exposed through an incision in the abdominal wall under general anesthetic and sterile surgical conditions. Without causing bowel obstruction, the cecum was ligated using silk surgical suture and then punctured twice with a needle. The abdominal incision was closed, and mice were monitored for health for one week. Sham surgery was performed without cecal ligation and puncture on

additional mice, and all mice receiving a sham operation survived for the duration of the study. Kaplan-Meier analysis of survival over the seven days following surgery for mice that underwent cecal ligation and puncture are displayed in Figure 2.1. In wild-type controls, peritonitis induced by cecal ligation and puncture resulted in 60% survival. In contrast, survival of *c-IAP1*$^{-/-}$ mice was 100% over the following week. Statistical analysis was performed using a log-rank test, which yielded a p-value of 0.18, which did not meet the criteria for statistical significance.

Although the comparison between *c-IAP1*$^{+/+}$ and *c-IAP1*$^{-/-}$ mice receiving cecal ligation and puncture was not statistically significant, we felt that the complete protection of *c-IAP1*$^{-/-}$ mice suggested a potential area of further study. We investigated the role of c-IAP1 in sepsis further by utilizing a second model, endotoxic shock induced by intraperitoneal injection of LPS. Shown in Figure 2.2A, seven days following administration of 40 μg LPS/g body weight, survival of *c-IAP1*$^{-/-}$ mice was 86%, compared to 14% survival of wild-type mice. This was a statistically significant improvement in survival, p=0.008, using a log-rank test. At a higher dose of 100 μg LPS/g body weight shown in Figure 2.2B, survival of *c-IAP1*$^{-/-}$ mice was 63%, while 25% of wild-type controls survived. This result suggests that at higher doses of LPS, c-IAP1 deficiency is not completely protective. At this higher dose, the difference between *c-IAP1*$^{-/-}$ and *c-IAP1*$^{+/+}$ mice was not statistically significant, p=0.059. The survival of wild-type mice was not worsened at the 100 μg/g dose compared to the 40 μg/g dose, indicating that the maximal lethality of endotoxic shock had been reached, and might explain why the difference between *c-IAP1*$^{-/-}$ and *c-IAP1*$^{+/+}$ mice was not statistically

significant. Overall, these data indicate that c-IAP1 participates in the pathogenesis of endotoxic shock.

c-IAP1$^{-/-}$ mice demonstrate reduced serum cytokines during endotoxic shock

The activation of TNF and subsequent cytokine cascades during sepsis contributes to organ pathology and eventual lethality. The finding that c-IAP1 influences survival during endotoxic shock prompted us to examine the effect of c-IAP1 on the cytokine cascades that lead to acute organ dysfunction. We analyzed cytokine protein concentrations of TNF and IL-1β, two critical primary response cytokines, in addition to the pro-inflammatory cytokines IL-6, IL-12(p70), MCP-1 and RANTES and the anti-inflammatory cytokine IL-10 utilizing a multiplex bead array system. Shown in Figure 2.3, serum levels of early systemic cytokines, such as TNF and IL-1β, were elevated at one hour following challenge with LPS. Cytokine concentrations did not significantly differ between *c-IAP1*$^{-/-}$ and *c-IAP1*$^{+/+}$ mice at this time. Later, at four hours following challenge with LPS, systemic production of the pro-inflammatory cytokines IL-6, IL-12, RANTES and MCP-1 was dramatically increased in *c-IAP1*$^{+/+}$ mice compared to uninjected controls. At this time, serum concentrations of IL-6, IL-12, RANTES, MCP-1, IL-1β, TNF were significantly decreased in *c-IAP1*$^{-/-}$ mice compared to *c-IAP1*$^{+/+}$ mice. These data suggest that c-IAP1 contributes to systemic pro-inflammatory cytokine production during sepsis.

In contrast, the serum concentration of IL-10 was unchanged in *c-IAP1*$^{-/-}$ mice. This cytokine is induced during sepsis and plays an anti-inflammatory role that counters pro-inflammatory cytokines. Reduced pro-inflammatory cytokines in the presence of normal IL-10 concentrations during endotoxemia in *c-IAP1*$^{-/-}$ mice might lead to an

unabated protective effect of anti-inflammatory cytokines. The dose of LPS used in this experiment (2.5 μg LPS/g body weight) was sub-lethal, and systemic inflammation, as indicated by serum cytokines, was resolved by 24 hours in both *c-IAP1*$^{-/-}$ and *c-IAP1*$^{+/+}$ mice. In all, these striking results suggest a clear contribution of c-IAP1 related to the pro-inflammatory cytokine cascade, associated with a pathology that leads to lethality in sepsis.

Local inflammation and apoptosis are unaffected in *c-IAP1*$^{-/-}$ mice

Systemic cytokine production during endotoxic shock was disrupted in *c-IAP1*$^{-/-}$ mice, but the critical early pro-inflammatory cytokines TNF and IL-1β were unaffected during the earliest phase of the systemic response. The ability to generate early systemic cytokines implies the presence of a robust local innate immune response to LPS. Thus, we sought to characterize the inflammatory response at the site of LPS challenge. In wild-type mice, chemokine-induced neutrophil infiltration was evident in peritoneal lavage samples four hours following intraperitoneal injection. c-IAP1 deficiency did not interfere with infiltration of neutrophils into the peritoneum (Fig. 2.4A), indicating that the local inflammatory response persists despite the loss of c-IAP1.

IAP family members have been implicated in TNF-induced apoptosis (*63, 87, 88, 206, 207*), which suggests the possibility that c-IAP1 might protect against apoptosis during innate immune responses. A reduction in the population of cytokine-producing cells could subsequently contribute to diminished systemic cytokine production. Indeed, excessive apoptosis of LPS-activated macrophages and decreased serum cytokines have been previously reported in *c-IAP2*$^{-/-}$ mice (*187*). Therefore, to explore the possibility that the resistance to LPS might be due to the apoptotic death of macrophages or

lymphocytes, we examined peritoneal mononuclear populations by histological examination of peritoneal lavage samples 24 hours following challenge with LPS. Surprisingly, the number of peritoneal mononuclear cells recovered from *c-IAP1*$^{-/-}$ mice was similar to wild-type mice, shown in Figure 2.4B. These results were further confirmed by flow cytometric quantification of mononuclear cells in peritoneal lavage samples four hours post-injection of LPS, shown in Figure 2.4C. To specifically identify cells that have undergone apoptosis, these samples were stained using the apoptotic marker Annexin V and the viability dye 7-AAD. As shown in Figure 2.4D, the percentage of apoptotic mononuclear cells (Annexin V^{+} 7-AAD^{+}) was not significantly different between *c-IAP1*$^{-/-}$ mice (25.1%) and wild-type mice (23.2%). Thus, a local direct effect on apoptosis did not appear to be causative of the altered systemic cytokine levels.

To complete our analysis of LPS-induced inflammation within the peritoneum, we quantified cytokine concentrations in peritoneal lavage fluid from mice challenged with LPS. Shown in Figure 2.5, concentrations of IL-1β, IL-6, RANTES and MCP-1 were significantly induced by LPS injection, however, only marginal changes were observed in the concentrations of TNF, IL-12 and IL-10. For all of the cytokines analyzed, there were no significant differences between *c-IAP1*$^{-/-}$ and *c-IAP1*$^{+/+}$ mice at any time point. From our analysis of peritoneal inflammation following LPS exposure, we conclude that the local response to LPS is intact in c-IAP1-deficient mice.

The peritoneum is intimately related with the liver, which receives circulation through the hepatic portal venous system. Kupffer cells, resident macrophages within the liver, might be exposed to to endotoxin administered through intraperitoneal route, and

are a potential source of systemic cytokine production. To investigate this as a possible reason for altered systemic inflammation in c-IAP1-deficient mice, we analyzed cytokine concentrations within the liver during endotoxemia. Depicted in Figure 2.6, liver concentrations of IL-1β, MCP-1, RANTES, MIP-1α and IL-10 were elevated in response to LPS challenge, but IL-6, IL-12 and TNF in the liver were not responsive to LPS. For IL-1β and MIP-1α, concentrations did not differ between *c-IAP1*$^{-/-}$ and *c-IAP1*$^{+/+}$ mice at any time point tested. *c-IAP1*$^{-/-}$ mice did demonstrate decreased MCP-1 compared to wild-type mice at four hours following challenge but not at one hour, when MCP-1 concentrations in the liver were at peak values. In contrast, RANTES and IL-10 concentrations were elevated in *c-IAP1*$^{-/-}$ mice compared to wild-type mice. Based on these results, cytokine production within the liver did not correlate with the observed changes in systemic cytokine levels in *c-IAP1*$^{-/-}$ mice.

Distant effects of c-IAP1 during endotoxemia

The observations that c-IAP1 did not affect local inflammation or early systemic cytokine induction led us to the hypothesis that c-IAP1 might modulate cytokine production during subsequent amplification of systemic pro-inflammatory cytokines. We explored this possibility by characterizing cytokines in tissues distant from the site of LPS challenge. During sepsis, patients develop pathology in the lungs that can result in significant morbidity. Using the mouse model of endotoxemia, we observed elevated cytokine production in the lungs as early as one hour following LPS challenge, shown in Figure 2.7. Compared to wild-type mice, *c-IAP1*$^{-/-}$ mice had significantly lower concentrations of IL-1β, IL-12 and MCP-1 at four hours following LPS challenge. Concentrations of TNF and Il-6 were also depressed in *c-IAP1*$^{-/-}$ mice, but these

differences were not statistically significant. While analysis of cytokine expression by qRT-PCR for IL-1β, IL-6, MIP-1α, RANTES and MCP-1 demonstrated greatly increased mRNA in response to LPS, differences in expression between *c-IAP1*$^{-/-}$ and *cIAP1*$^{+/+}$ were not evident (data not shown). The observed changes in cytokine protein concentrations in the lungs suggest that the effect of c-IAP1 on serum cytokines is associated with cytokine production in distant tissues, but not with the local innate immune response at the site of exposure.

TNF is an early response cytokine that promotes additional production of pro-inflammatory mediators; thus, TNF is a potential mediator for amplification of systemic cytokine cascades. The role of c-IAP1 in intracellular signaling initiated by TNF is a possible mechanism through which c-IAP1 might affect systemic cytokine cascades without disrupting local inflammation. We challenged mice with recombinant mouse TNF to determine whether subsequent cytokine amplification was affected in c-IAP1-deficient mice. In mice exposed to TNF through intraperitoneal injection, shown in Figure 2.8A, production of IL-12, MCP-1 and RANTES was induced, but only RANTES was significantly different in *c-IAP1*$^{-/-}$ mice. Similarly, for mice injected with TNF intravenously, shown in Figure 2.8B, induction of IL-1β, IL-6, IL-12, MCP-1, MIP-1α and RANTES was observed. However, in *c-IAP1*$^{-/-}$ mice, intravenous TNF resulted in significantly increased cytokine concentrations of IL-1β, IL-6, MIP-1α and MCP-1 compared to wild-type mice. Despite the role of c-IAP1 in TNFR-mediated intracellular signaling, diminished responsiveness to TNF was not observed in c-IAP1-deficient mice. These observations imply that abrogation of TNF-induced cytokine cascades is not

responsible for the decreased systemic cytokine production that occurs as a result of c-IAP1 deficiency.

Alternative model of innate immune responses

To expand our characterization of alternative potential roles of c-IAP1 in innate immune responses, we examined a mouse model of viral infection. In part, this study will address whether the effect of c-IAP1 on innate immune responses that contribute to sepsis represents either a universal or narrow deficiency in cytokine responses. Our model of viral infection was intratracheal administration of respiratory syncytial virus (RSV), which results in a lower respiratory tract infection accompanied by immune cell infiltration to the airways and mucus production. *c-IAP1*$^{-/-}$ mice infected with RSV were first assessed for lung inflammation by histology, shown in Figure 2.9 (A-D). Immune cell infiltration was grossly similar to infected wild-type mice. Mucus production, which was assessed by PAS staining, shown in Figure 2.9 (E-F), was also similar.

In addition to lung pathology, cytokine responses to RSV infection were assessed at time points that corresponded with early innate immune responses to RSV (day 1; *208*) and later development of pathology (day 8). Although there was a slight decrease in cytokine mRNA expression in *c-IAP1*$^{-/-}$ mice on day 8 for all cytokines analyzed, there were no significant differences in lung cytokine expression between c-IAP1-deficient and wild-type mice on day 1 or day 8 (Fig. 2.10). Examination of cytokine mRNA expression in draining lymph nodes harvested from RSV-infected mice yielded similar results (Figure 2.10C).

Finally, infected mice were analyzed for the ability to control and clear RSV. Expression of RSV G protein transcripts, assessed using qRT-PCR, was used as a proxy

for the extent of RSV infection. On day 8 following infection, the quantity of RSV transcripts in the lung was similar between c-IAP1-deficient and wild-type mice. In the draining lymph nodes, RSV was not detectable in either *c-IAP1*$^{-/-}$ or *c-IAP1*$^{+/+}$ mice (data not shown). Since RSV infection of c-IAP1-deficient mice resembled wild-type mice in each of the measures we assessed, we concluded that the protective effect observed in c-IAP1-deficient mice during sepsis does not extend to a viral model of inflammation.

Discussion

In cases of severe sepsis, pro-inflammatory cytokine production is uncontrolled and results in systemic production of cytokines, such as TNF, IL-6 and IL-1β, that contribute to the clinically significant manifestations of septic shock. Using two mouse models of sepsis, we observed improved survival in c-IAP1-deficient mice, and we have identified a role for c-IAP1 in systemic pro-inflammatory cytokine production during endotoxemia. Decreased serum concentrations of IL-1β, IL-6, IL-12, RANTES, MCP-1 and TNF were observed in *c-IAP1*$^{-/-}$ mice, but serum concentrations of IL-10 were unaltered, suggesting that the balance of inflammatory mediators is shifted to a protective, anti-inflammatory response (*209*). We were particularly interested in whether c-IAP1 played a role in primary, local responses to LPS, and we determined that initial cytokine production and local inflammation were not affected. We hypothesized c-IAP1 modulates cytokine production during subsequent amplification of systemic pro-inflammatory cytokines based on the results *in vivo*, and this question will be addressed further *in vitro* in the next chapter. Surprisingly, we did not see evidence *in vivo* that c-IAP1 influences cytokine cascades by affecting responses to TNF. The role of c-IAP1

was not due to a broad disruption in innate immune cell function, because c-IAP1-deficient mice responded appropriately to RSV infection. Interestingly, TNF production is involved in RSV-induced inflammation (*210*). The absence of significant differences in c-IAP1-deficient mice following RSV infection indicates that the role of c-IAP1 does not extend to all inflammatory processes mediated by TNF.

These results highlight critical functional similarities and differences between the highly homologous proteins c-IAP1 and c-IAP2. The reported outcomes of endotoxic shock in c-IAP2-deficient mice resemble the observations of c-IAP1-deficient mice we report here, specifically, improved survival and reduced serum cytokine concentrations (*187*). In contrast, *c-IAP2*$^{-/-}$ macrophages demonstrated dramatic sensitivity to apoptosis during endotoxic shock, which we did not observe in *c-IAP1*$^{-/-}$ mice. A number of studies have examined the contributions of c-IAP1 and c-IAP2 to apoptotic sensitivity *in vitro*, often using small molecules that target both c-IAP1 and c-IAP2 for degradation. Enhanced sensitivity to apoptosis was observed in these cells, which was dependent on autocrine production of TNF (*87, 88, 206, 207*). Individual contributions of c-IAP1 and c-IAP2 to apoptotic sensitization have recently been characterized in murine embryonic fibroblasts, but the published findings describing the role for c-IAP1 are conflicting. In two instances, loss of c-IAP1 sensitized to TNF-induced apoptosis (*49, 88*); however, another study found no alterations in sensitivity to TNF-induced apoptosis in *c-IAP1*$^{-/-}$ fibroblasts (*211*). This latter observation was reflected in our data demonstrating that apoptosis of macrophages during endotoxemia was unaffected in *c-IAP1*$^{-/-}$ mice.

The dispensable role of c-IAP1 in apoptosis might be the result of compensation by c-IAP2, which is post-translationally upregulated in *c-IAP1*$^{-/-}$ cells (*53*). Compensation

between c-IAP1 and c-IAP2 has been observed in signaling pathways, including both canonical NF-κB (*64, 212*) and non-canonical NF-κB (*85, 86*) activation. Loss of either c-IAP1 or c-IAP2 alone did not abrogate signaling, which instead occurred following loss of both c-IAP1 and c-IAP2. The deficiency in cytokine production observed in our experiments might result from a failure of c-IAP2 to compensate for loss of c-IAP1 during excessive inflammatory stress, when upregulation of c-IAP2 expression is critical. On the other hand, functions of c-IAP1 distinct from those of c-IAP2 could also be responsible for the defects in cytokine networking in *c-IAP1*$^{-/-}$ cells. In either case, there is now evidence that both c-IAP1 and c-IAP2 contribute to inflammation, despite differing patterns of expression.

The results of these studies have led to interesting new directions for the role of IAPs during inflammation, which will be investigated further in the next chapters. In Chapter 3, the effect of c-IAP1 on pro-inflammatory cytokine expression will be examined in immune and non-immune cells *in vitro*. These studies will also continue to address whether the role of c-IAP1 in inflammation is linked to possible effects on TNF responsiveness. In Chapter 4, these results will be put into the context of molecular functions for c-IAP1, by studying the effect of c-IAP1 on signaling cascades relevant to inflammation.

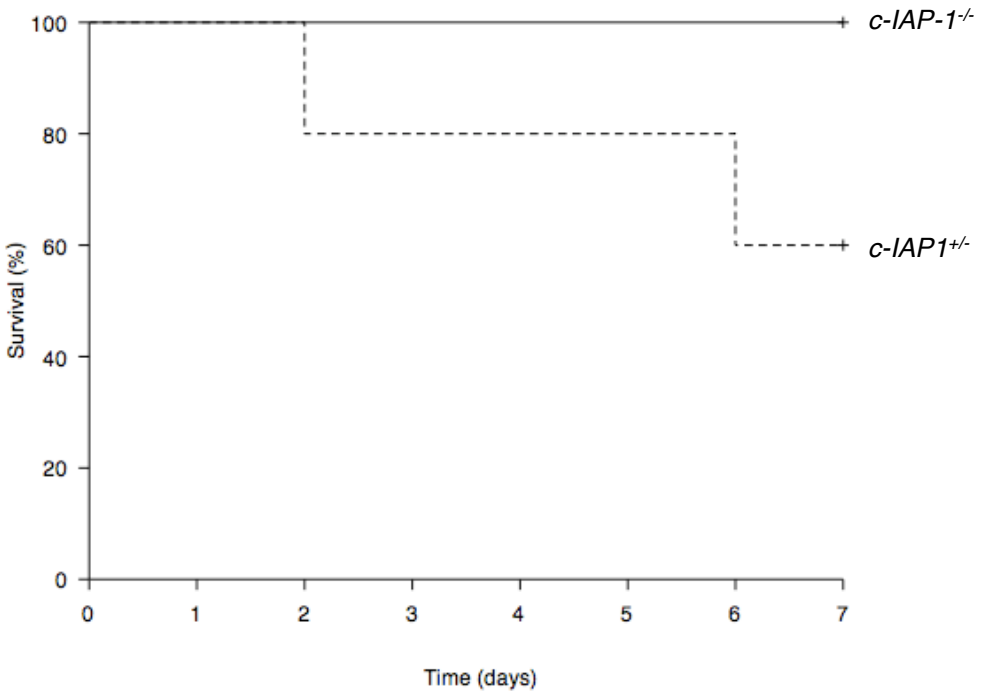

Figure 2.1 *c-IAP1*$^{-/-}$ mice have improved survival in polymicrobial sepsis. Cecal ligation and puncture was performed on *c-IAP1*$^{-/-}$ mice (solid line) and wild-type controls (dashed line) to induce polymicrobial sepsis. Survival was monitored for seven days following surgery, and Kaplan-Meier analysis of the data is displayed. Log-rank test was utilized for statistical analysis, p= 0.18.

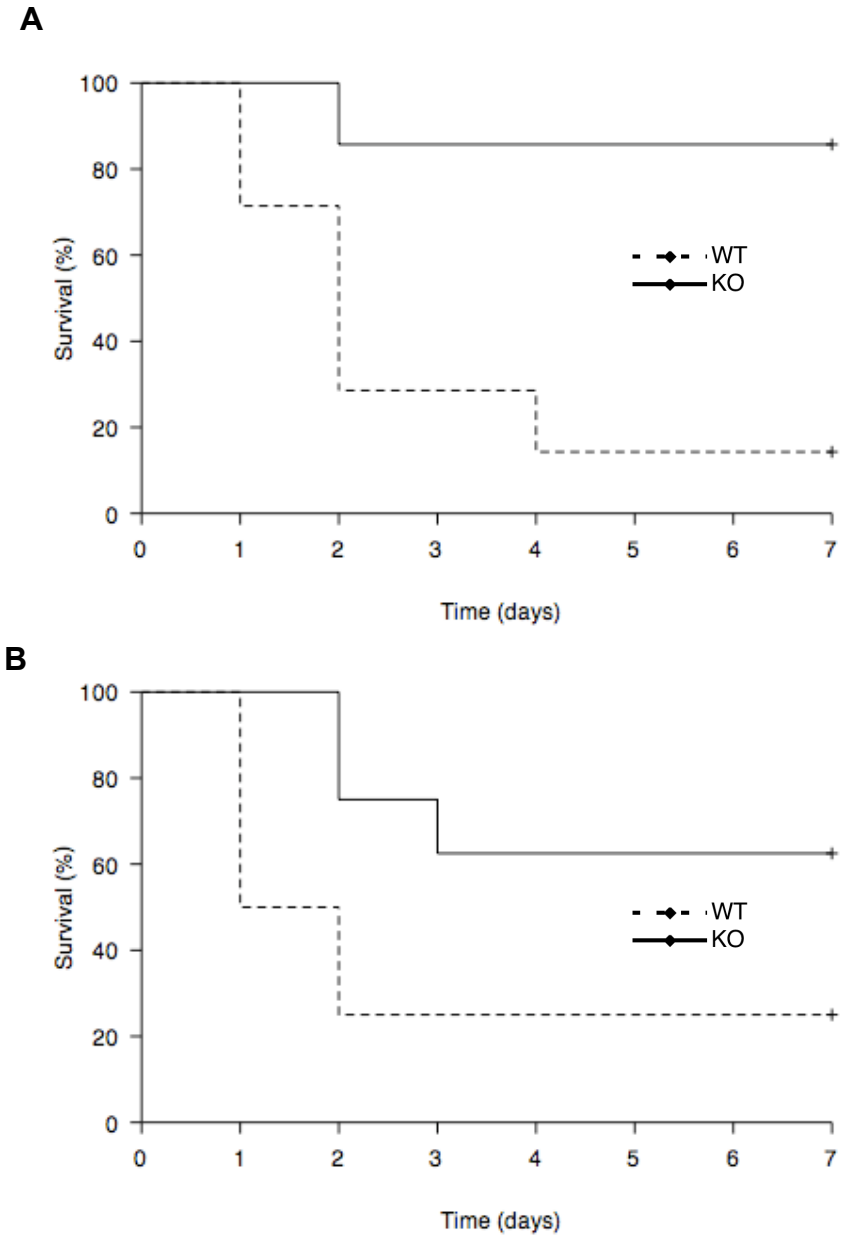

Figure 2.2 *c-IAP1*$^{-/-}$ mice have improved survival in endotoxemia. *c-IAP1*$^{-/-}$ mice (solid line) and wild-type controls (dashed line) were challenged with intraperitoneal injection of LPS. Survival was monitored for seven days following LPS, and Kaplan-Meier analysis of the data is displayed. Statistical significance was determined by log-rank test. (A) 40 μg/g body weight LPS, p= 0.008. (B) 100 μg/g body weight LPS, p= 0.059.

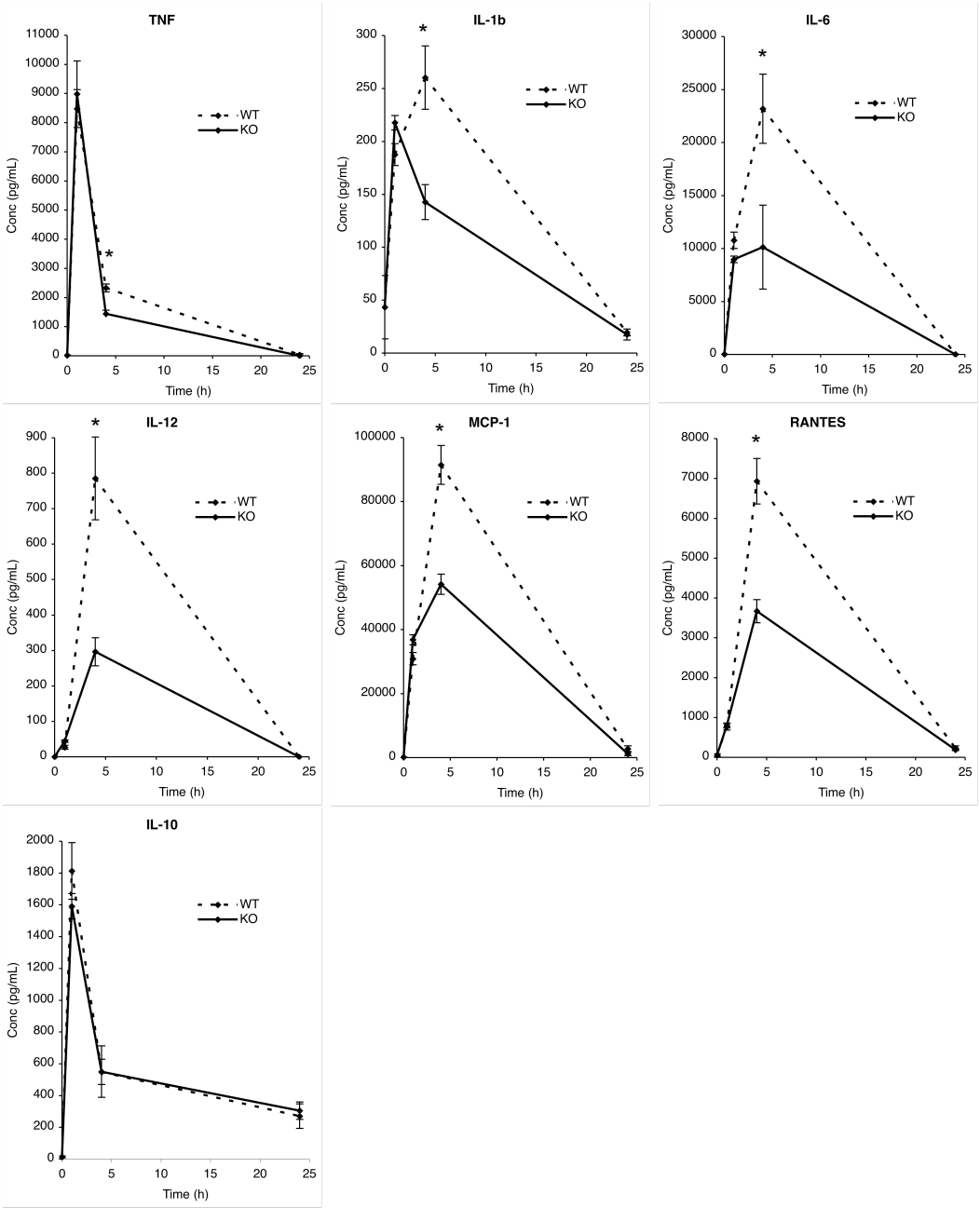

Figure 2.3 Serum cytokine levels are decreased during endotoxic shock in *c-IAP1*$^{-/-}$ mice. *c-IAP1*$^{-/-}$ mice and wild-type controls were challenged with intraperitoneal LPS (2.5 μg/g body weight). Serum was harvested 1, 4 or 24 hours post-injection and analyzed for cytokine concentrations using Bio-plex cytokine assays. Error bars indicate SEM, and * indicates $p < 0.05$.

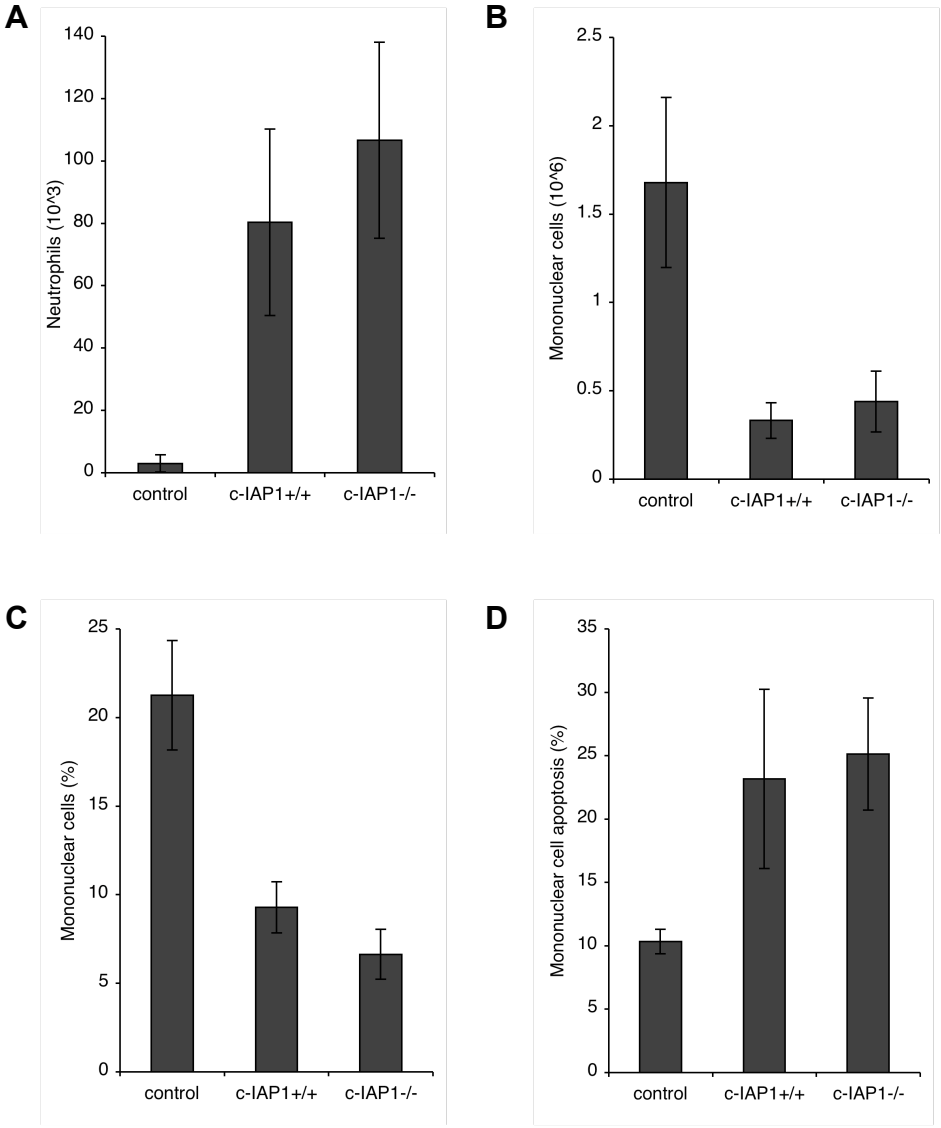

Figure 2.4 Peritoneal immune cells from c-IAP1-deficient mice do not exhibit changes in infiltration or apoptosis in response to LPS (A) Peritoneal lavage was harvested four hours following intraperitoneal challenge with LPS (2.5 μg/g body weight). Neutrophils were assessed by histological examination of DiffQuik stained peritoneal lavage cells. (B) Macrophages were assessed by DiffQuik stain of peritoneal lavage cells 24 hours following intraperitoneal injection with LPS (2.5 μg/g body weight). (C) *c-IAP1*$^{-/-}$ mice and wild-type littermates were challenged with LPS for 2.5 hours (i.p. 10ug/g body weight). Mononuclear cells were identified in peritoneal lavage samples by flow cytometry forward scatter/side scatter profile. (D) Within the population of mononuclear cells (shown in C), Annexin V-positive 7-AAD-positive apoptotic cells were analyzed by flow cytometry. Error bars in all graphs represent SEM.

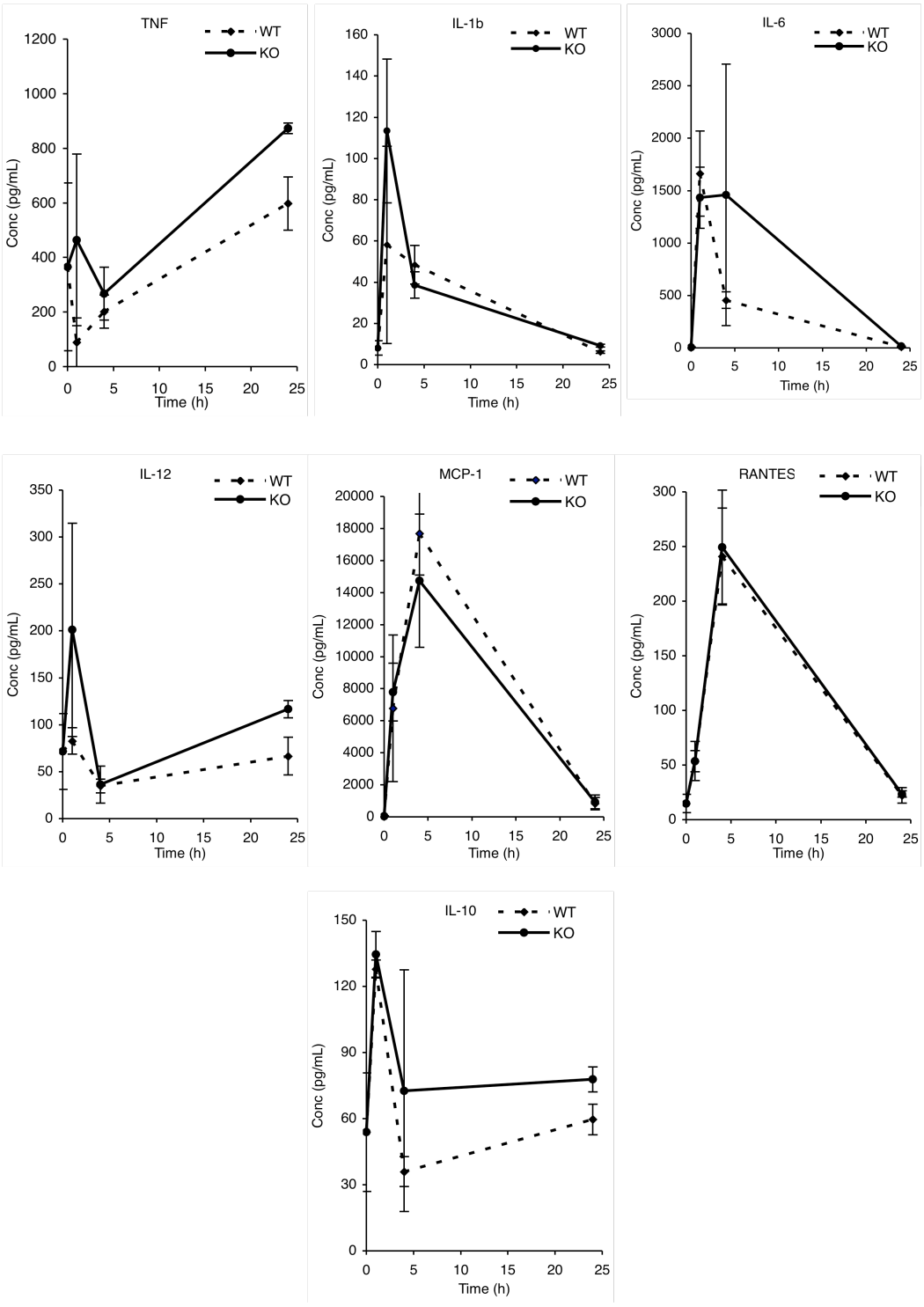

Figure 2.5 Peritoneal lavage cytokine concentrations are unaffected during endotoxic shock in *c-IAP1*$^{-/-}$ mice. *c-IAP1*$^{-/-}$ mice and wild-type controls were challenged with intraperitoneal LPS (2.5 μg/g body weight). Peritoneal lavage fluid was harvested 1, 4 or 24 hours post-injection and analyzed for cytokine concentrations using Bio-plex cytokine assays. Error bars indicate SEM.

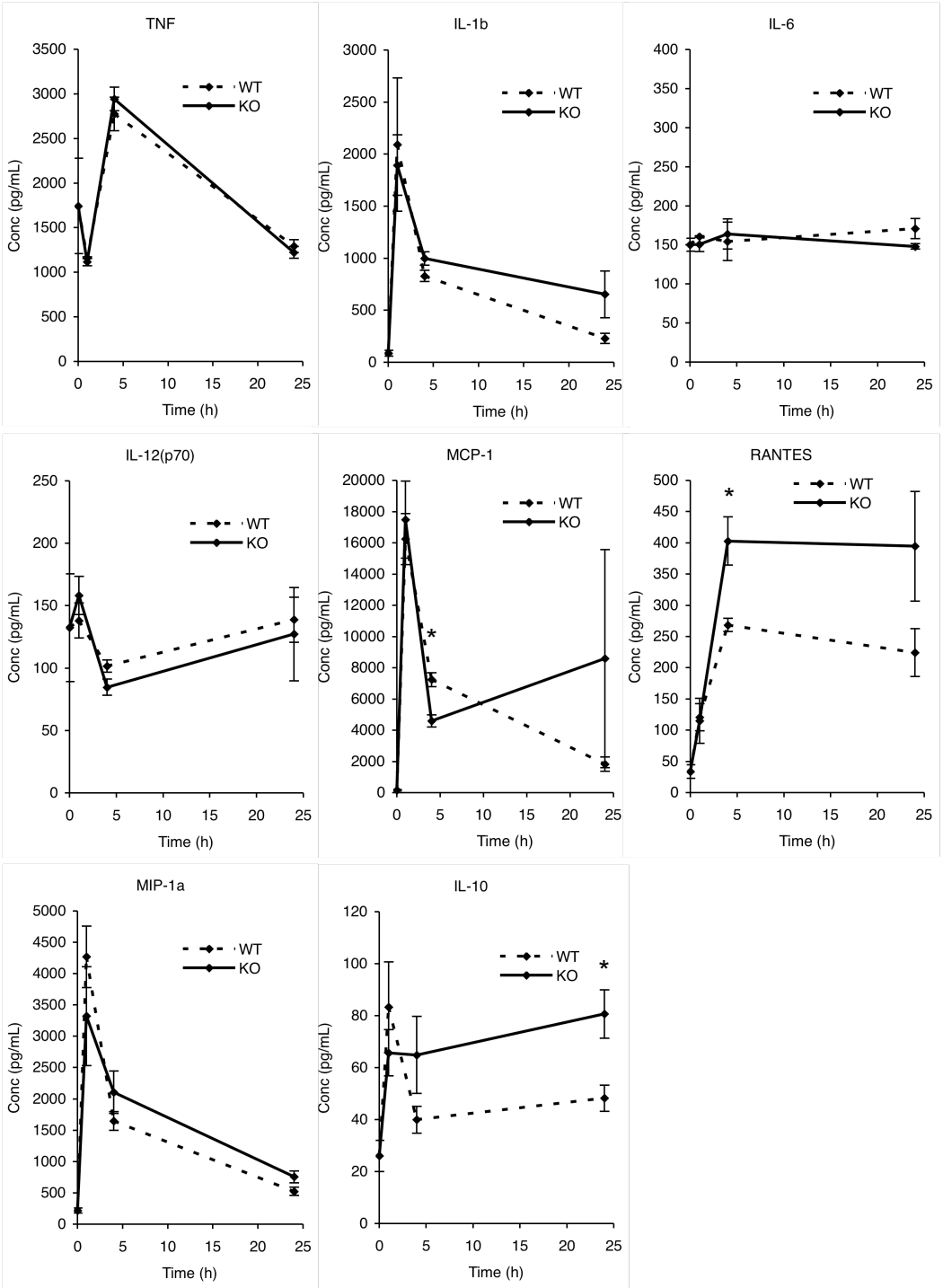

Figure 2.6 Liver cytokines during endotoxic shock do not correlate with systemic cytokines in *c-IAP1*$^{-/-}$ mice. *c-IAP1*$^{-/-}$ mice and wild-type controls were challenged with intraperitoneal LPS (2.5 μg/g body weight). Liver samples were harvested 1, 4 or 24 hours post-injection and analyzed for cytokine concentrations using Bio-plex cytokine assays. Error bars indicate SEM, and * indicates $p < 0.05$.

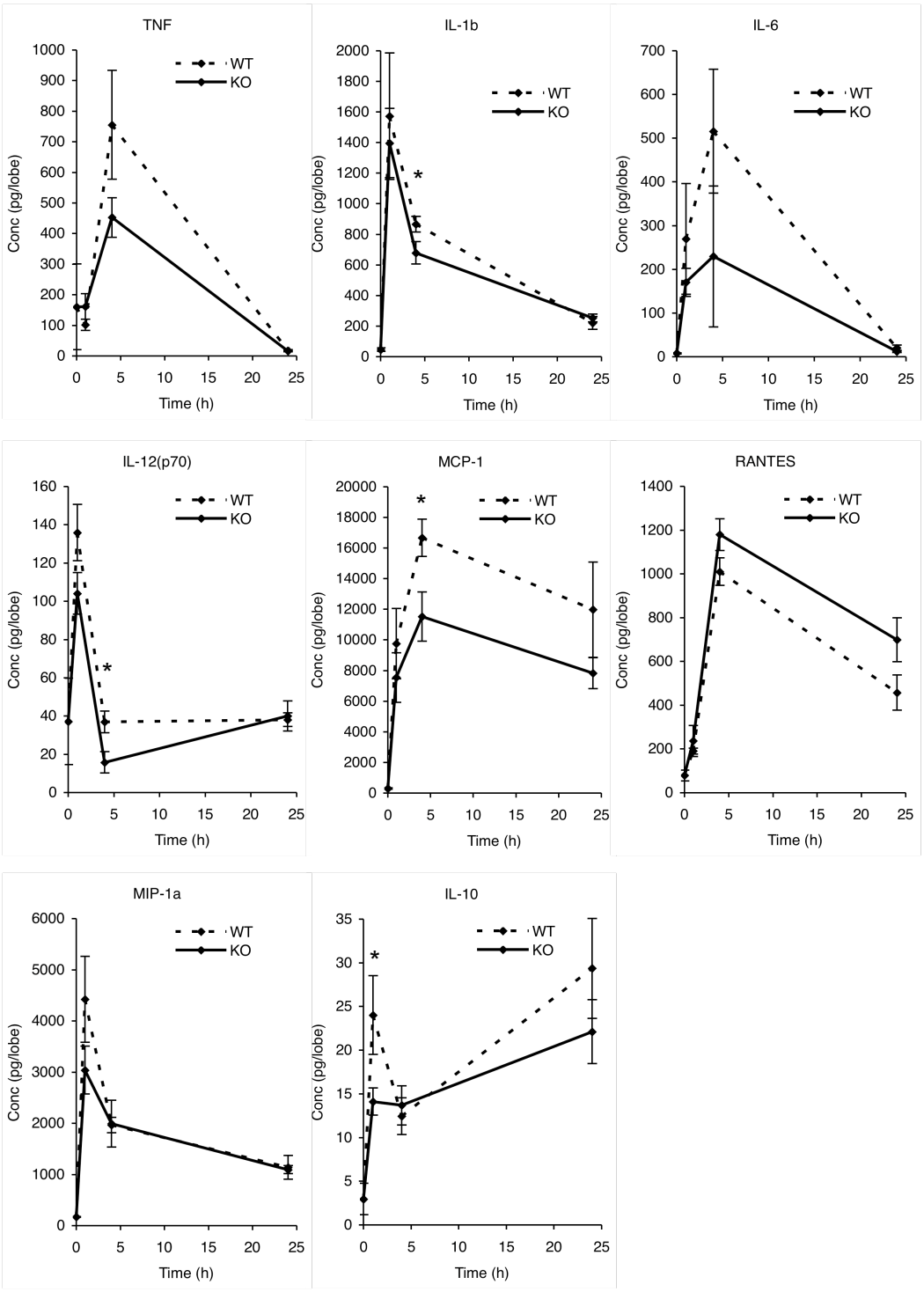

Figure 2.7 Lung concentrations of MCP-1, IL-1b and IL-12 are decreased during endotoxic shock in c-IAP1-/- mice. *c-IAP1*$^{-/-}$ mice and wild-type controls were challenged with intraperitoneal LPS (2.5 μg/g body weight). Lungs were harvested 1, 4 or 24 hours post-injection and analyzed for cytokine concentrations using Bio-plex cytokine assays. Error bars indicate SEM, and * indicates $p < 0.05$.

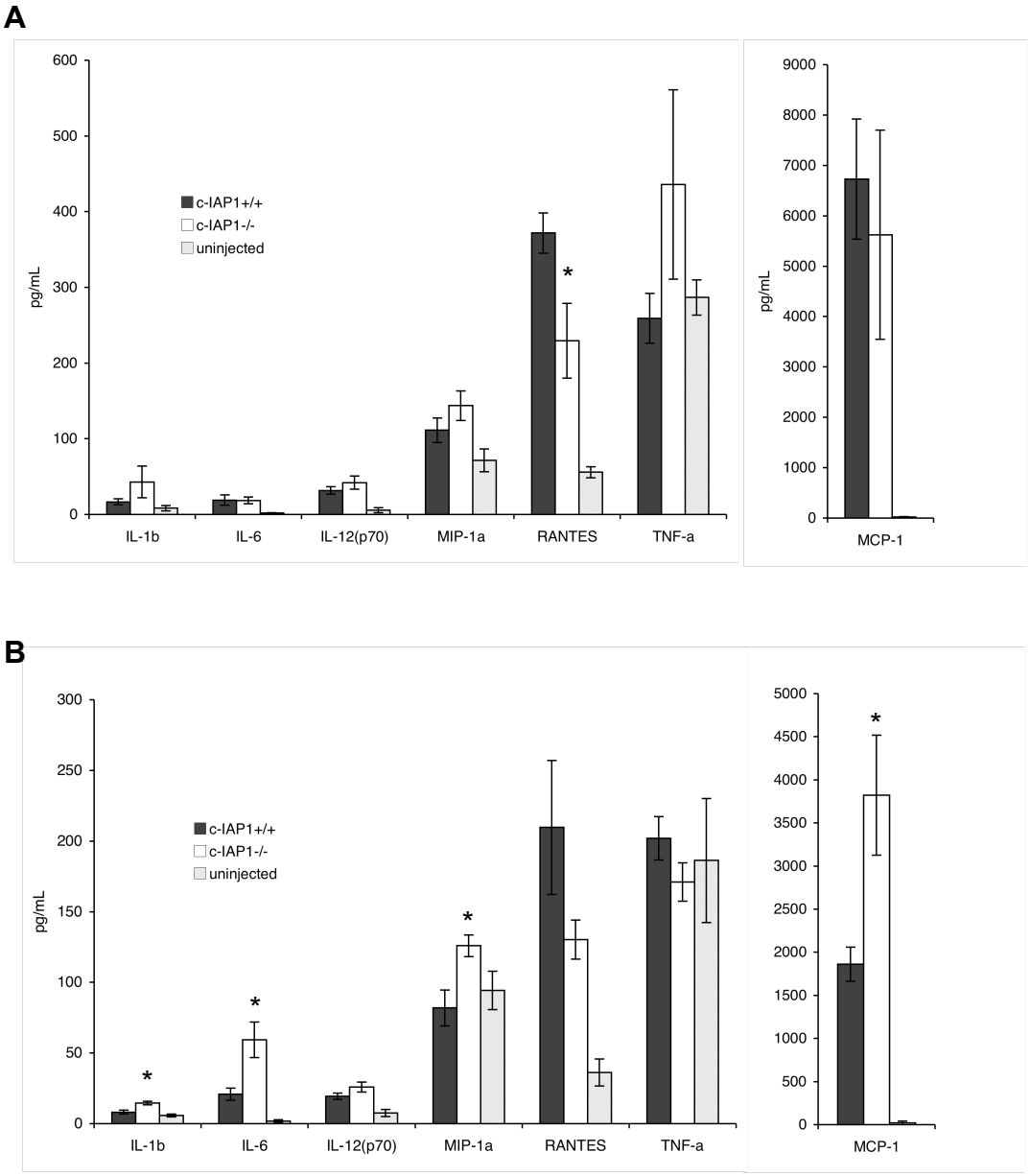

Figure 2.8 Serum cytokine concentrations following challenge with TNF. *c-IAP1*$^{-/-}$ and *c-IAP*$^{+/+}$ mice were challenged with recombinant mouse TNF (Roche) for four hours. Serum was harvested and analyzed by Bio-plex. Error bars indicate SEM, and * indicates $p<0.05$. (A) A dose of 420 ng TNF was administered by intraperitoneal injection in a volume of 100 μL (n=5). (B) A dose of 350 ng TNF was administered intravenously in a volume of 100 μL (n=4).

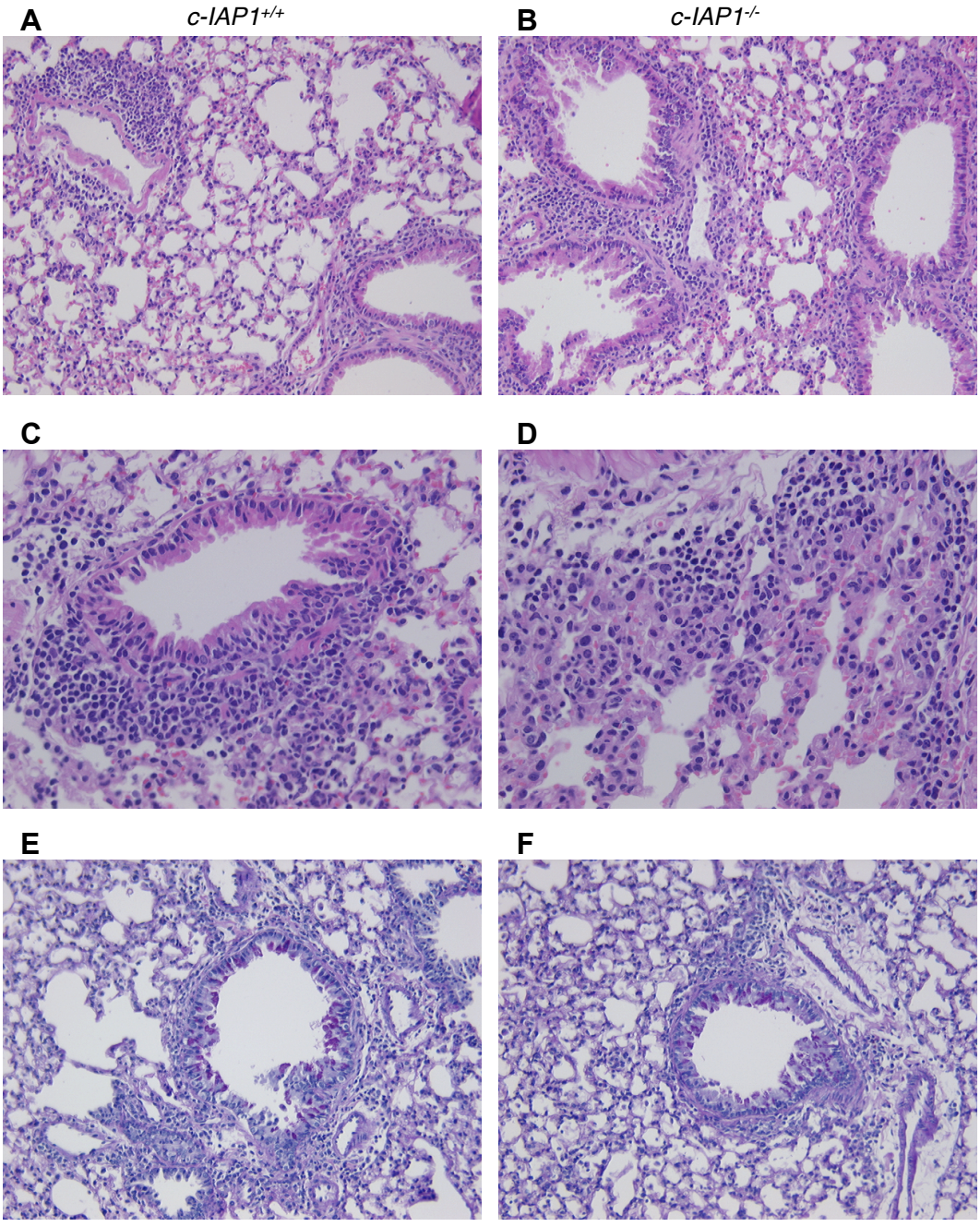

Figure 2.9 RSV-induced lung inflammation in *c-IAP1*$^{-/-}$ mice. *c-IAP1*$^{+/+}$ mice (A,C,E) and *c-IAP1*$^{-/-}$ mice (B,D,F) were administered intratracheal RSV. Lungs were harvested for histology on day eight. (A-B) H&E, 20x magnification (C-D) H&E, 40x magnification (E-F) PAS, 20x magnification

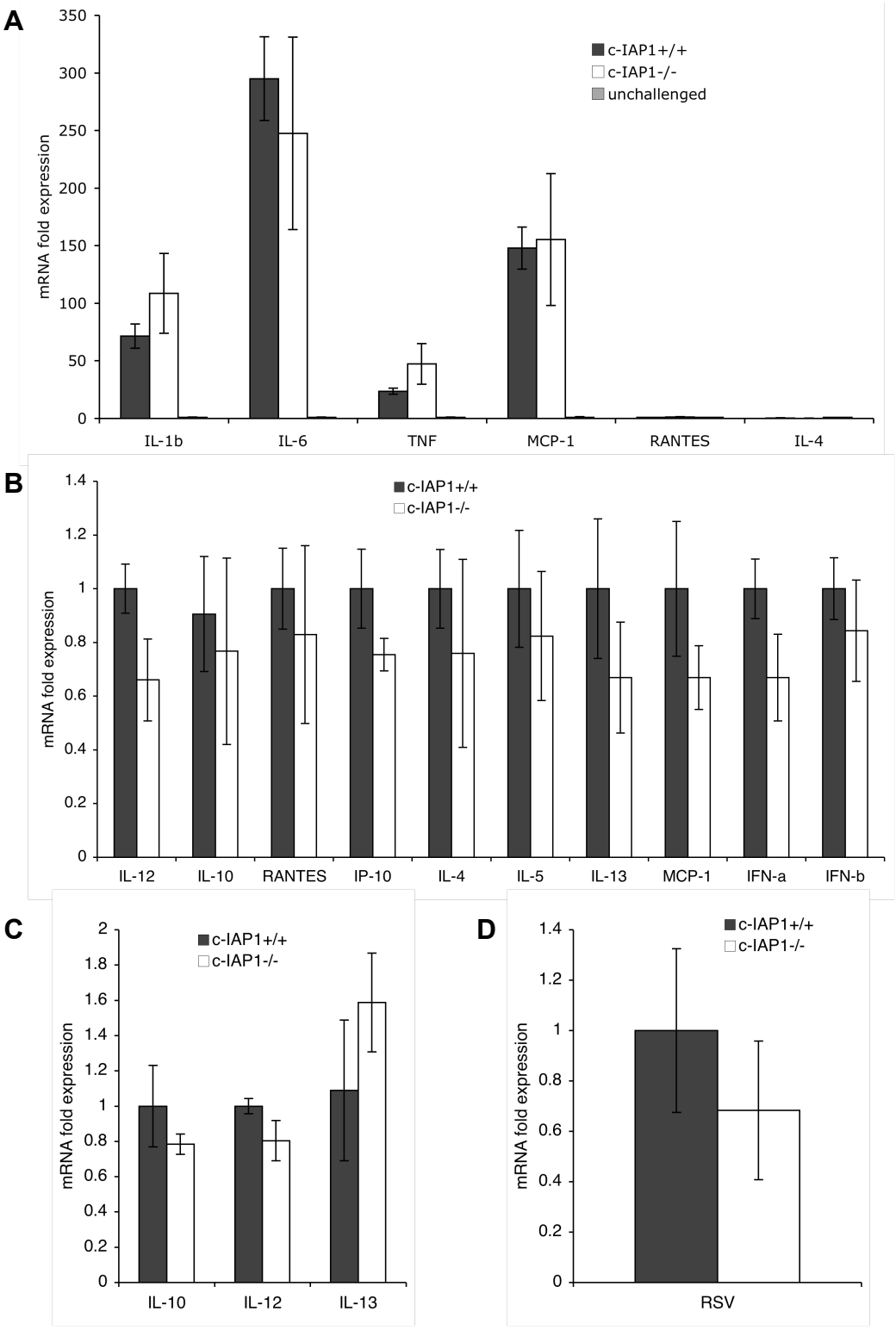

Figure 2.10 Cytokine induction in *c-IAP1*$^{-/-}$ mice following RSV infection. (A) RNA harvested from lungs one day following RSV infection was analyzed by qRT-PCR. (B) qRT-PCR analysis of lung RNA on day 8 of RSV infection. (C) RNA harvested from mediastinal lymph nodes on day 8 of RSV infection, analyzed by qRT-PCR. (D) qRT-PCR for RSV g protein in lung RNA harvested on day 8. Error bars indicate SEM in all panels.

Chapter 3

The effect of c-IAP1 on inflammatory cytokine regulation

Abstract

In our studies on the role of c-IAP1 during sepsis, c-IAP1 contributed to excessive, systemic pro-inflammatory cytokine production. In this chapter, we investigate the role of c-IAP1 in the regulation of pro-inflammatory cytokine induction. We determined that cytokine production by LPS-stimulated immune cells is not dependent on c-IAP1. Intriguingly, we identified a role for c-IAP1 in the response of lung fibroblasts to pro-inflammatory stimuli such as LPS and TNF. These results are in agreement with our observations *in vivo* of disruptions in cytokine production in serum and lungs after the initial phase of cytokine production, which suggested to us that c-IAP1 contributes to the processes that amplify cytokine production. We analyzed the effect of macrophage-derived cytokines on lung fibroblast cytokine induction using conditioned media, and in these experiments we observed that lung fibroblasts responded to macrophage-derived TNF in a c-IAP1-dependent manner. Consequently, c-IAP1 might regulate the participation of fibroblasts in cytokine networks with immune cells during inflammation.

Introduction

Microbial infections are recognized by innate immune cells, chiefly macrophages at the site of infection, using specialized pattern recognition receptors. In particular, gram-negative bacteria trigger activation of the pattern recognition receptor TLR4 by shedding endotoxin from the outer cell wall. LPS, the biologically active component of endotoxin, binds TLR4 and initiates an intracellular signaling cascade that includes NF-κB and MAPK signaling. These signaling programs activate transcription of pro-inflammatory genes, which includes cytokines responsible for recruiting and activating additional innate immune cells at the site of infection. Cytokines also effect changes in stromal and endothelial tissues that facilitate the function of responding innate immune cells. Vasodilation, vascular permeability and expression of adhesion molecules, for example, are dramatically increased by the actions of cytokines on endothelial cells and smooth muscle cells. As a result, blood flow increases and circulating leukocytes roll along the capillary wall and easily extravasate into inflamed tissues. Cytokine networks are established in which immune cells and non-immune cells both produce pro-inflammatory mediators (*213-216*). These networks enhance the pro-inflammatory microenvironment by reinforcing the activation of resident immune cells and recruiting infiltrating immune cells (*217, 218*). In severe, systemic inflammatory responses during sepsis, the initial source of cytokine production is likely macrophages and dendritic cells that are activated by pathogen-derived molecules. However, the resulting pathology of systemic inflammation is not limited to the immune system and disrupts homeostasis in a number of organ systems. Consequently, the processes that lead to multiple organ failure

in severe sepsis involve the interactions of a diverse group of immune and non-immune cells.

Our investigations into the role of c-IAP1 during inflammation *in vivo* using models of sepsis identified a contribution of c-IAP1 to excessive, systemic pro-inflammatory cytokine production. These results prompted us to further investigate the role of c-IAP1 in the regulation of pro-inflammatory cytokine induction. First we were interested in whether c-IAP1 is necessary for immune cells to recognize LPS through the TLR4 pathway. Decreased cytokine production in c-IAP1-deficient mice could be a consequence of a defect in TLR pathway activation. Our *in vivo* observations of early cytokine responses to endotoxemia, both within the peritoneum and systemically, indicated that c-IAP1 was not required during initiation of cytokine production. From these findings, we hypothesized that recognition of LPS was intact in c-IAP1-deficient innate immune cells. Disruptions in cytokine production were observed at a later time in serum and lungs, which suggested to us that c-IAP1 contributes to the processes that amplify cytokine production, possibly by influencing sensitivity to the potent pro-inflammatory cytokine TNF. The hypothesis that c-IAP1 contributes to inflammation by increasing responsiveness to TNF is strengthened by molecular experiments that have implicated c-IAP1 as a signaling molecule in the pathways triggered by TNF. In the next studies, we examined the effect of c-IAP1 on induction of pro-inflammatory cytokines during responses to TNF. These studies were extended to non-immune cells because of the importance of these cells during both healthy, physiologic immune responses and during severe, systemic inflammation. We focused on lung fibroblasts based on the observations *in vivo* of disrupted cytokines in the lung during endotoxemia. Fibroblasts in

the lung parenchyma are active participants during inflammation (reviewed in *219*). They have been reported to produce cytokines, including IL-1β, TNF, IL-6, MCP-1 and MIP-1α, extracellular matrix proteins and matrix-degrading enzymes, adhesion molecules and angiogenesis factors. We integrated the roles of macrophages and lung fibroblasts *in vitro* by analyzing the effect of macrophage-derived cytokines on lung fibroblast cytokine induction using conditioned media (*220*).

Intriguingly, here we report a role for c-IAP1 in cytokine induction in lung fibroblasts. However, c-IAP1 was not required for recognition of LPS by macrophages and dendritic cells. c-IAP1 in lung fibroblasts enhanced responsiveness to pro-inflammatory stimuli, and consequently might influence the participation of fibroblasts in cytokine networks with immune cells.

Materials and Methods

Cell culture and reagents

Bone marrow was flushed from tibia and femur bones of naïve mice using cold RPMI 1640. Bone marrow-derived macrophages were cultured from the recovered bone marrow cells as described previously (*221*) in polystrene Petri dishes (Nunc Lab-Tek, Thermo Fisher Scientific, Rochester, NY) in RPMI containing 20% fetal calf serum (FCS), 30% L929 cell-conditioned medium, glutamine, penicillin and streptomycin, with media replenished on day 3 of culture. After 6 days of culture, adherent cells were collected with Accutase (eBioscience, San Diego, CA) and plated in RPMI containing 10% FCS, glutamine, streptomycin and penicillin. To culture bone marrow-derived

dendritic cells, bone marrow was treated with red blood cell lysis buffer and cultured in the presence of 10 ng/mL GM-CSF (R&D Systems, Minneapolis, MN) in RPMI containing 10% FCS, penicillin, streptomycin and glutamine.

Peritoneal macrophages were harvested from naïve mice by peritoneal lavage with cold phosphate-buffered saline (PBS). Cells were treated with RBC lysis buffer and plated in RPMI supplemented with 10% FCS, glutamine, penicillin and streptomycin. After overnight culture, non-adherent cells were removed.

Lung fibroblasts were harvested from lungs of naïve mice and digested with 1 mg/mL Collagenase A (Roche Applied Sciences, Indianapolis, IN) and 20 U/mL DNase (Sigma-Aldrich) in RPMI with 5% FCS for 30 min, followed by dispersion and RBC lysis. Cells were plated in DMEM containing 10% FCS, glutamine, penicillin, streptomycin and amphotericin B for two passages. For stimulation, lung fibroblasts were cultured in DMEM containing 0.5% FCS, glutamine, penicillin, streptomycin and amphotericin B.

Mouse embryonic fibroblasts were isolated from individual embryos on day 14.5 from timed matings of two c-IAP1 heterozygous mice following standard procedures. Experiments were performed with female *c-IAP1*$^{+/+}$ and male *c-IAP1*$^{-/-}$ cells from littermate embryos (*211*). After two passages, *c-IAP1*$^{+/+}$ and *c-IAP1*$^{-/-}$ cells were transformed by serial infection with lentiviruses expressing Ras and E1A (early region 1A; *222*). Cells were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine and 1% penicillin/streptomycin. For stimulation, fibroblasts were cultured in DMEM containing 0.5% FCS, penicillin, glutamine, streptomycin and amphotericin B.

For each experiment, wild-type and c-IAP1-deficient cells were obtained from littermates. All cells were maintained at 37°C with 5% CO2. Cells were stimulated *in vitro* with LPS (*E. coli* O128:B8, #L3129, Sigma-Aldrich) or recombinant murine TNF (Roche Applied Sciences). For cytokine networking experiments, wild-type macrophages were stimulated with 2 ng/mL LPS for 24 hours, without washing the cells following LPS exposure. Macrophage-conditioned media was diluted 1:2.5 in lung fibroblast media and transferred to lung fibroblast cultures. After four hours, lung fibroblasts were harvested for RNA isolation. For neutralization of TNF in cell culture supernatants, 7 μg/mL anti-TNF (monoclonal rat IgG_1 clone MPG-XT3; Upstate, Lake Placid, NY) was added.

Flow cytometry

Cells were incubated with Fc block and subsequently stained with CD40-FITC and CD80-PE antibodies (BD Pharmingen, San Diego, CA) at a dilution of 1:200 in PBS containing 1% FCS. Cells were analyzed by flow cytometry using a Beckman Coulter FC500 instrument (Fullerton, CA) and FlowJo software (Tree Star, Inc.).

Quantification of cytokines

Cytokine protein concentrations were quantified using a bead-based cytokine assay analyzed by the Bio-plex Suspension Array System following manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA).

To quantify expression of cytokine genes of interest, RNA was harvested in Trizol (Invitrogen, Carlsbad, CA) and isolated following manufacturer's instructions. cDNA was generated using Moloney Murine Leukemia Virus (MMLV) Reverse Transcriptase (Invitrogen). Levels of mRNA were analyzed by qPCR using Taqman gene

expression assays (Applied Biosystems, Foster City, CA). Gene expression was normalized to GAPDH mRNA for each sample and expressed as fold increase compared to the negative control within the experiment.

Statistical analyses

For analysis of cytokines, ANOVA was used with a Student Newman-Keuls post-test to determine the statistical significance of differences. A p-value <0.05 was considered to be statistically significant.

Results

LPS-induced cytokine production is unaltered in *c-IAP1*$^{-/-}$ macrophages and dendritic cells

Our studies of c-IAP1 in a mouse model of endotoxic shock identified an effect of c-IAP1 on the severity of pro-inflammatory cytokine production, which was not associated with an alteration in the population of macrophages. We explored the possibility that induction of cytokine production by LPS was impaired in c-IAP1-deficient innate immune cells. We first examined the response of bone marrow-derived macrophages to LPS exposure *in vitro* and observed that production of cytokines TNF, IL-1β, IL-6, IL-12, MCP-1 and RANTES were all unaffected in c-IAP1-deficient cells (Fig. 3.1). We confirmed these results in peritoneal macrophages by culturing adherent cells harvested by peritoneal lavage. c-IAP1-deficient cells again demonstrated intact induction of TNF, IL-1β, IL-6, IL-12, MCP-1 and RANTES in response to LPS (Fig. 3.2).

In addition to macrophages, dendritic cells are also exquisitely sensitive to pathogen-derived molecules and contribute to cytokine production during innate immune responses (*223, 224*). Bone marrow-derived dendritic cells from *c-IAP1*$^{-/-}$ mice also responded normally to LPS *in vitro*, inducing production of TNF, IL-1β, IL-6, IL-12, MCP-1 and MIP-1α at 4 hours (Fig. 3.3) and 24 hours (data not shown) following stimulation. Upon activation, dendritic cells up-regulate expression of co-stimulatory molecules, including CD40 and CD80. We assessed expression of these cell surface receptors on of dendritic cells using flow cytometry. Bone marrow-derived dendritic cells expressed CD40 and CD80 in response to LPS and TNF, and it was not altered in dendritic cells from *c-IAP1*$^{-/-}$ mice (Fig. 3.4). Baseline expression of these markers in untreated cells was similar between c-IAP1-deficient and wild-type cells (data not shown). Expression of these molecules requires autocrine cytokine production and stimulation (*224*); hence, proper expression of maturation markers is one indication that this aspect of cytokine production is functionally intact.

These results indicate that c-IAP1-deficient innate immune cells were capable of recognition of LPS and production of the pro-inflammatory cytokines TNF and IL-1β, which agrees with observations *in vivo* that early pro-inflammatory cytokine induction was not affected by c-IAP1. The observation that c-IAP1-deficient innate immune cells also produced appropriate quantities of IL-6, IL-12, MCP-1, MIP-1α and RANTES indicates that the disruption these cytokines observed *in vivo* did not result from a defect in production of these cytokines by macrophages or dendritic cells.

Role of c-IAP1 in contributions of non-immune cells to inflammation

Since innate immune cells did not require c-IAP1 for LPS-induced cytokine production, we examined other cytokine-producing cells to understand the mechanism of diminished cytokine production in c-IAP1-deficient mice. While innate immune cells are an initial source of cytokines following recognition of a pathogen *in vivo*, further inflammatory cytokine production can result from contributions to cytokine networks by both innate immune cells and non-immune structural cells. We hypothesized that c-IAP1 modulates subsequent amplification of systemic inflammatory cytokine production. Since we observed altered cytokine production in the lungs, we chose to examine lung fibroblasts, a structural cell that responds to the pro-inflammatory environment during endotoxic shock and contributes to the resulting lung pathology.

Unlike macrophages and dendritic cells, lung fibroblasts from *c-IAP1*$^{-/-}$ mice were defective in LPS-induced induction of IL-1β, IL-6, MCP-1 and MIP-2, shown in Figure 3.5A. We confirmed a role for c-IAP1 in fibroblasts using an independent source of fibroblasts, mouse embryonic fibroblasts (MEFs) derived from c-IAP1-deficient or wild-type mice. c-IAP1-deficient MEFs were also impaired in responses to LPS, failing to fully activate expression of TNF or IFNβ (Fig. 3.6). In addition to gene expression, we could also measure increased protein concentrations of cytokines in supernatants from LPS-stimulated wild-type fibroblasts. Cytokine concentrations from c-IAP1-deficient lung fibroblasts stimulated with LPS were inconsistently affected, resulting in decreased cytokine production in 50% (2/4) of c-IAP1-deficient fibroblast cell cultures and no change in 50% (2/4; data not shown).

We concluded that c-IAP1 has a role in inflammatory responses of fibroblasts to LPS; however, c-IAP1 is not absolutely required for LPS responsiveness because c-IAP1-deficient fibroblasts are not completely defective in cytokine induction following LPS exposure. Since fibroblasts are not the primary cells to respond to LPS in tissues, we hypothesized that the effect of c-IAP1 on cytokine production in response to LPS might be mediated indirectly through other cytokines, instead of directly disrupting LPS responses activated by TLR4.

Cytokine networking is disrupted in *c-IAP1*$^{-/-}$ cells

The pro-inflammatory environment of the lung during endotoxic shock includes not only LPS but also a number of cytokines that stimulate lung fibroblasts, including TNF. Using recombinant mouse TNF, we observed increased concentrations of cytokines, such as MCP-1, RANTES and IL-12, in stimulated wild-type fibroblasts (Fig. 3.7). We first tested whether TNF produced by lung fibroblasts contributed to LPS induction of pro-inflammatory cytokines in an autocrine manner. We blocked the activity of TNF using a neutralizing antibody and stimulated lung fibroblasts with 10ng/mL LPS for 24 hours. Compared to lung fibroblasts stimulated with LPS alone, we observed three distinct results in cytokine production, shown in Figure 3.7. For MCP-1, autocrine TNF signaling contributed to cytokine production, since neutralization of TNF significantly decreased LPS-induced MCP-1 concentrations. RANTES also followed a similar trend, although the decrease in concentrations was not statistically significant. For IL-6 and IL-12, there was no apparent dependence on TNF for cytokine production, but this cannot be concluded with certainty because LPS did not significantly induce these cytokines in the lung fibroblasts. Finally, IL-1β and MIP-1α were also dependent on autocrine TNF

stimulation for full cytokine production, but paradoxically, TNF alone was not able to induce cytokine production. In the case of IL-1β, stimulation with TNF activated gene transcription (Fig. 3.5B) but additional post-translational cleavage of pro-IL-1β is required that is not activated by TNF alone. On the other hand, LPS was sufficient for secretion of IL-1β, but autocrine TNF signaling induces additional transcriptional activation and enhances IL-1β production. To examine the role of c-IAP1 during cytokine induction by TNF, we analyzed cytokine mRNA harvested from lung fibroblasts of c-IAP1-deficient mice and wild-type littermates. Following four-hour stimulation with TNF, RNA expression of IL-1β, IL-6, MCP-1 and MIP-2 were all significantly decreased in c-IAP1-deficient lung fibroblasts (Fig. 3.5B). Together, these data indicate that c-IAP1 contributes to the response of structural cells in a pro-inflammatory environment.

During lung inflammation, the predominant source of TNF in the microenvironment of the fibroblasts is likely macrophages, rather than autocrine production. In this context, TNF would be accompanied by a number of other cytokines and inflammatory mediators produced by the rapid response of innate immune cells to detection of LPS. To simulate this environment *in vitro*, we characterized the response of c-IAP1-deficient lung fibroblasts to cytokines produced by LPS-stimulated macrophages. Bone marrow-derived macrophages from naïve wild-type mice were stimulated with 2 ng/mL LPS for 24 hours *in vitro* to elicit cytokine production. Conditioned media containing macrophage-produced cytokines and residual LPS was harvested and diluted in lung fibroblast media. Subsequently, lung fibroblasts from wild-type or *c-IAP1*$^{-/-}$ mice were incubated with macrophage-conditioned media for four hours. Cytokine gene induction in lung fibroblasts was assessed by qRT-PCR, and the results are shown in

Figure 3.8. Induction of IL-1β, IL-6, MCP-1 and MIP-2 were significantly decreased in *c-IAP1*$^{-/-}$ lung fibroblasts compared to wild-type littermates.

Finally, we attempted to confirm that the contributions of c-IAP1 observed during responses to LPS and TNF in lung fibroblasts are relevant and significant in an environment with multiple pro-inflammatory stimuli. We neutralized TNF in macrophage-conditioned media to determine whether TNF production by macrophages contributed to cytokine induction in lung fibroblasts. Compared to fibroblasts stimulated with conditioned media alone, neutralization of TNF significantly diminished cytokine induction in wild-type lung fibroblasts for IL-1β, IL-6, MCP-1 and MIP-2 (Fig. 3.8). These findings confirm that a significant component of cytokine activation was stimulated by macrophage-derived inflammatory mediators and not solely by residual LPS. Intriguingly, neutralization of TNF did not affect cytokine induction in c-IAP1-deficient fibroblasts, compared to macrophage-conditioned media alone. This interesting result implies that TNF does not have a role in cytokine induction in c-IAP1-deficient cells, likely due to signaling defects through TNFR in fibroblasts from *c-IAP1*$^{-/-}$ mice.

Discussion

Control of cytokine production is critical to regulate the extent of the immune response during inflammation. In our studies on the role of c-IAP1 during inflammation *in vivo*, c-IAP1 contributed to pro-inflammatory cytokine production, and we investigated the mechanism of c-IAP1 in the regulation of pro-inflammatory cytokine induction further in this chapter. Interestingly, we identified a role for c-IAP1 in the response of lung fibroblasts to pro-inflammatory stimuli such as LPS and TNF. This effect was cell-

specific, since cytokine production by macrophages or dendritic cells stimulated with LPS was not dependent on c-IAP1. These results suggest initial recognition of LPS and early cytokine production should be intact in c-IAP1-deficient mice, which was in agreement with our observations *in vivo*.

In lung fibroblasts, activation of cytokines by LPS was amplified by an autocrine TNF signaling loop, and our results implied a role for c-IAP1 in TNF-mediated activation of cytokines. In this light, it is perhaps not surprising that c-IAP1 was not involved in cytokine induction by macrophages or dendritic cells, since these cells rapidly produce cytokines following detection of LPS without a requirement for TNF (*225*). Lung fibroblasts and other non-immune cells participate in networks with immune cells to further enhance the pro-inflammatory environment (*214, 219, 226, 227*). The role of c-IAP1 in lung fibroblasts potentially contributes to these processes that amplify cytokine production. The ability of macrophage-derived cytokines to induce cytokine expression in lung fibroblasts was impaired in c-IAP1-deficient cells, and the response of lung fibroblasts to macrophage-derived TNF in particular was dependent on c-IAP1. Consequently, c-IAP1 might regulate the participation of fibroblasts in cytokine networks with immune cells during inflammation. Disruption of the cytokine networks that coordinate innate immune cells and structural cells during an innate immune response would be predicted to impair further amplification of cytokine cascades. During an excessive innate immune response, such as sepsis, the amplification of cytokine cascades involves immune and non-immune cells in distant tissues as a result of the systemic pro-inflammatory environment. In our investigations of sepsis, we observed a contribution of c-IAP1 to serum cytokine concentrations after the initial wave of early response

cytokines, and to lung cytokine concentrations. Taken together, these results support the hypothesis that c-IAP1 contributes to TNF-responsiveness *in vivo*, resulting in a cell-specific contribution to pro-inflammatory cytokine production that enhances pathogenic systemic cytokine cascades during sepsis.

While excessive inflammation is detrimental to the host, as seen during sepsis, the innate immune system is necessary to fight bacterial pathogens. Local cytokine cascades that activate and recruit leukocytes are critical for eliminating invading pathogens (*226*). Disruptions in cytokines, such TNF, during this response result in uncontrolled microbial proliferation and worsened outcomes (*228, 229*). It would be therapeutically desirable to uncouple the harmful effects of inflammation from the essential role of innate immune response in pathogen clearance. One report has suggested cell- and pathway-specific contributions during inflammation that differentially lead to harmful or beneficial effects. In this example, the role of NF-κB signaling in vascular endothelial cells during sepsis was studied using a transgenic mouse model with inducible and endothelial cell-specific expression of mutant IκBα, which has substitutions at Ser32 and Ser36 that prevent its phosphorylation and degradation (*230*). Endothelial cells influence leukocyte recruitment by increasing adhesion molecule expression and vascular permeability, but these responses can also lead to hypotension and multiple organ injury if they are uncontrolled. Blockade of NF-κB activation by mutant IκBα in the endothelial cells of these mice abrogated adhesion molecule expression, permeability, neutrophil infiltration, hypotension, intravascular coagulation and multiple organ injury in models of sepsis. As a result, survival was improved, and importantly, these mice retained the ability to clear bacterial pathogens. Thus, targeting NF-κB in an endothelial cell-specific manner might

be a potential therapeutic approach for sepsis, but cell specificity for this pathway would be difficult to achieve in patients.

The finding that endothelial cell activation of NF-κB has specific effects on innate immune processes raises the intriguing question of the general role of non-immune cells during innate immune responses. Would disrupting contributions of non-immune cells to inflammation reduce the harmful effects of inflammation, while still preserving sufficient innate immune cell activation for the beneficial effects of pathogen clearance? If this is the case, then decreasing the responsiveness of non-immune cells might be an alternative therapeutic approach to sepsis. If our proposed mechanism of the cell-specific activity of c-IAP1 is accurate, then c-IAP1 would be a candidate target for this approach. Further studies of c-IAP1 might lead to a new generation of sepsis therapeutics with the potential to successfully ameliorate excessive inflammation without diminishing clearance of pathogens, if administered sufficiently early during the course of inflammation to patients at risk of developing sepsis following trauma or burns, for example.

The next step would be to characterize c-IAP1 in a broader range of non-immune cells involved in inflammation, in particular endothelial cells. Indeed, a role for c-IAP1/2 in endothelial cells has already been identified in zebrafish. Loss-of-function mutation in the sole c-IAP ortholog resulted in severe disruptions in vascular integrity due to a failure of death receptor-mediated signaling in endothelial cells (*65*). In mice, disruption of c-IAP1 alone does not result in the catastrophic phenotype observed in zebrafish. We predict that interference with both c-IAP1 and c-IAP2 might be tolerable in adult animals, and that the catastrophic results that occur during development are due to increased apoptotic sensitivity that occurs as a part of certain stages of the developmental program.

While *in vitro* studies of the role of c-IAP1 in endothelial cells and other non-immune cells might be informative, we anxiously await the development of conditional c-IAP1 knockout mice to rigorously examine cell-specific contributions of c-IAP1 to inflammation *in vivo.*

In addition to the potential implications for development of therapeutics, further examination of the cell-specific role of c-IAP1 on inflammation and pathogen clearance *in vivo* would provide insight into the physiologic functions of TNF. We have proposed that c-IAP1 acts to modulate signaling activated by TNF, which is essential for clearance of certain bacterial infections, especially intracellular pathogens such as *Mycobacterium tuberculosis* and *Listeria monocytogenes,* Further studies are needed to understand whether c-IAP1 is necessary for clearance of these organisms, and if so, whether this function is cell-type specific.

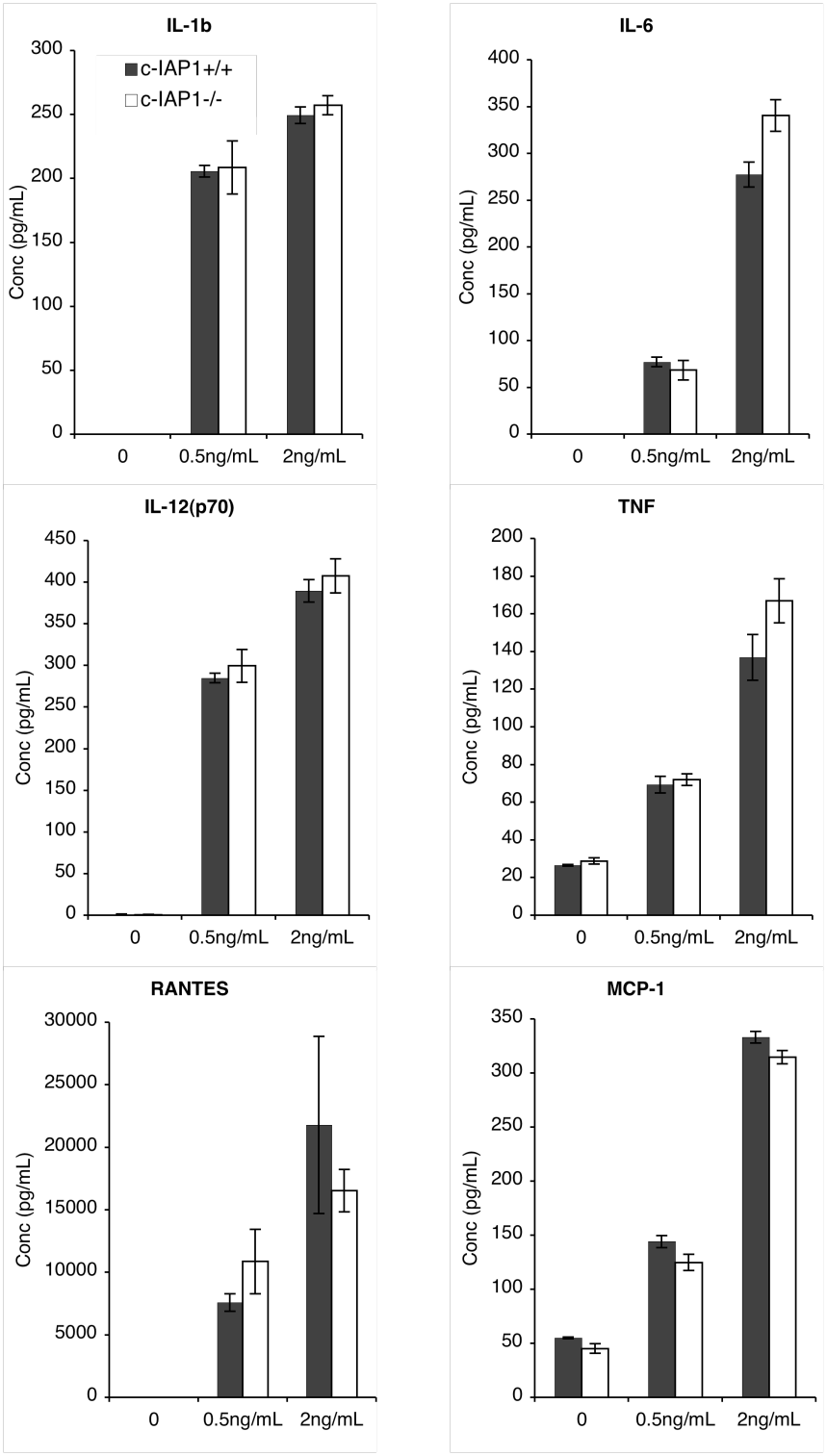

Figure 3.1 Cytokine production is unaffected in c-IAP1-deficient macrophages. Bone marrow-derived macrophages were cultured from *c-IAP1*$^{-/-}$ mice and wild-type littermates in the presence of L929 cell-conditioned medium and 20% FCS for 6 days. Macrophages were then plated in 10% FCS and stimulated with LPS at a dose of 0.5 ng/mL or 2 ng/mL for 20 hours, and supernatant was analyzed for cytokine concentrations using Bio-plex beads. Error bars represent SEM for triplicates. Results are representative of two independent experiments.

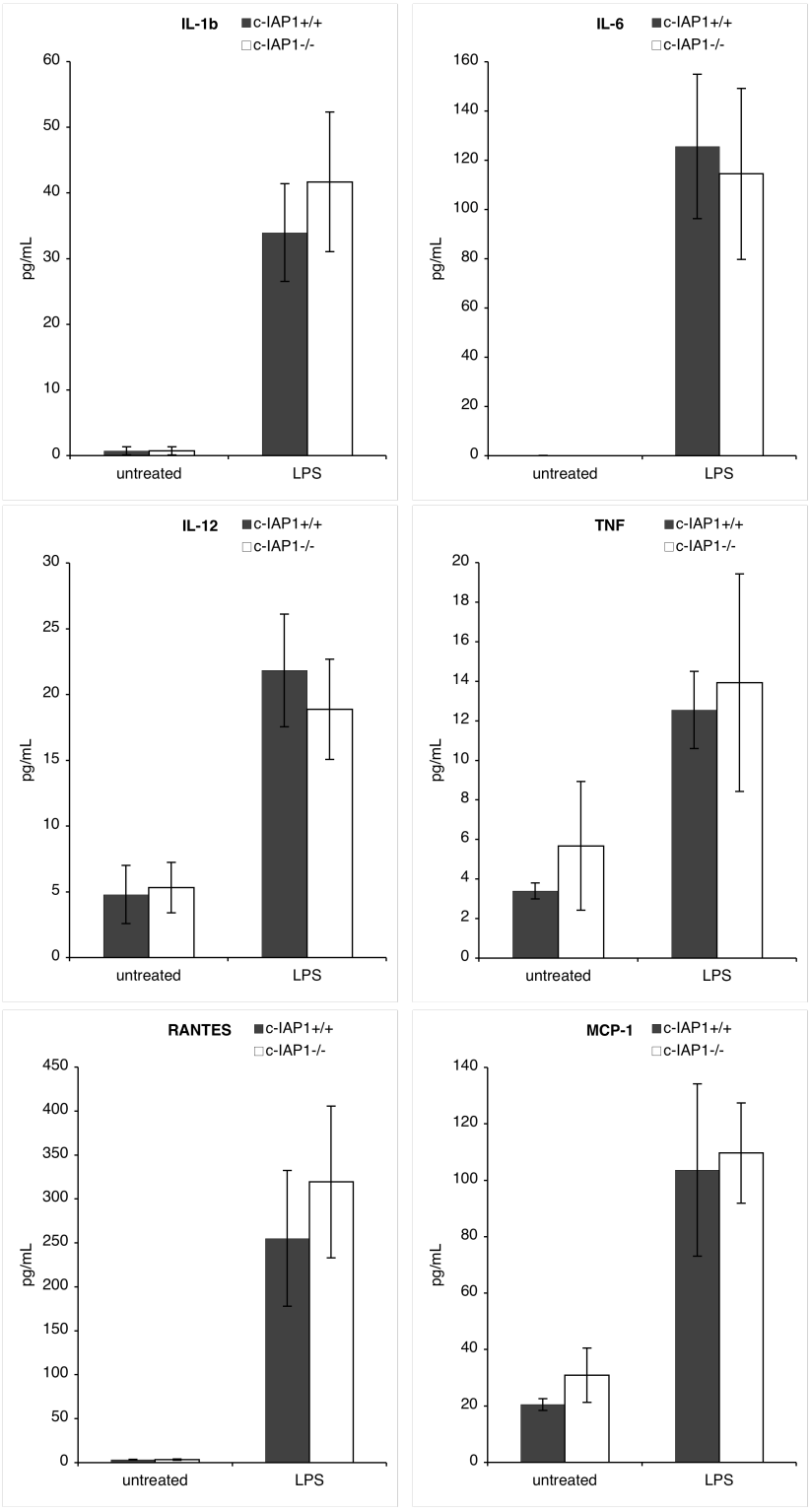

Figure 3.2 Cytokine production by *c-IAP1*$^{-/-}$ peritoneal macrophages is normal. Peritoneal macrophages were harvested by lavage from c-IAP1-deficient mice or littermate controls. Cells were cultured in the presence of 0.5 ng/mL LPS for 30 hours, then supernatant was analyzed for cytokine concentrations using Bio-plex beads. Error bars represent SEM for triplicates.

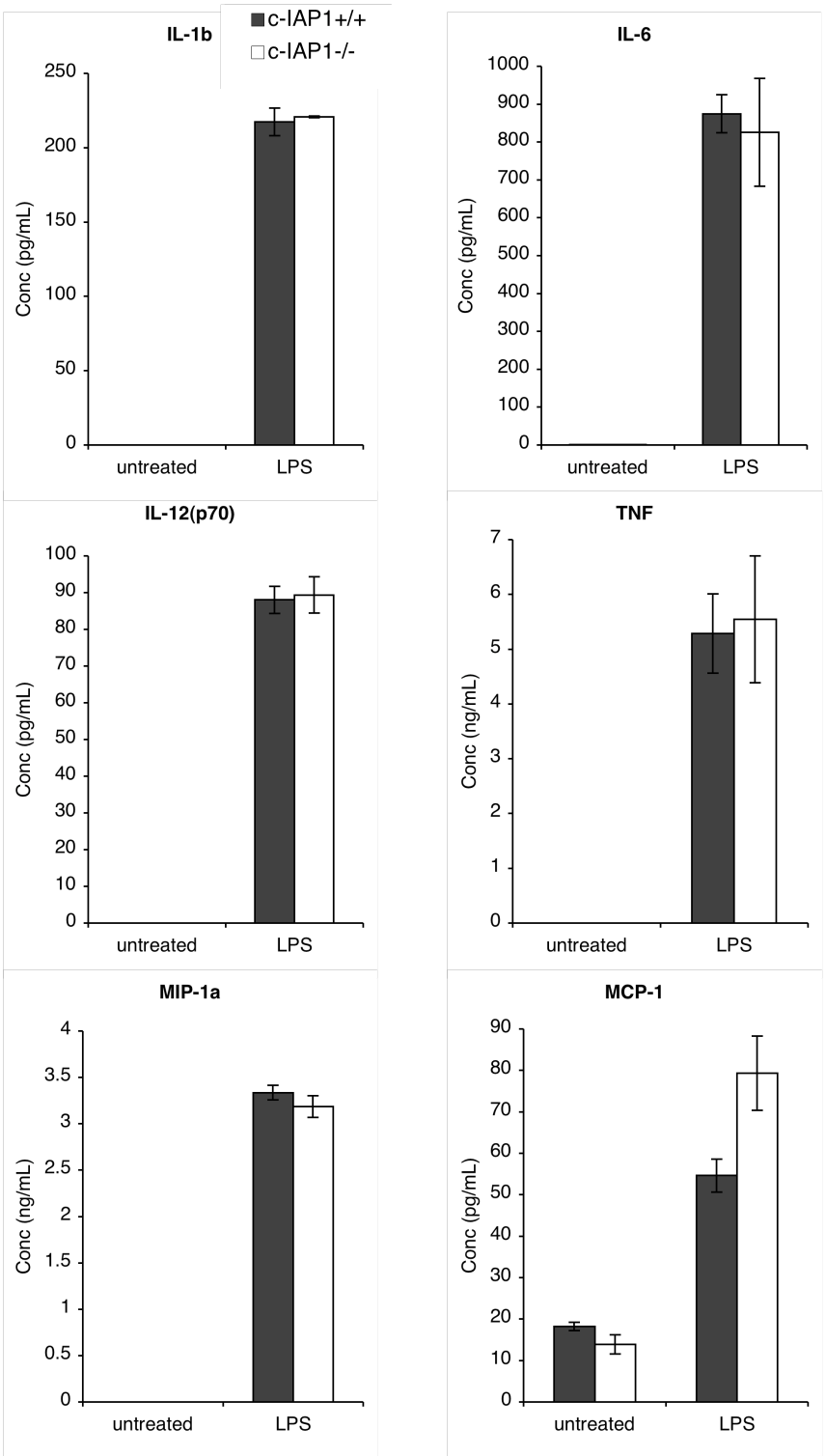

Figure 3.3 Cytokine production by c-IAP1-deficient dendritic cells is unaffected. Dendritic cells were cultured from bone marrow cells of c-IAP1-deficient mice or wild-type littermates using 10ng/ml GM-CSF. Bone marrow-derived dendritic cells were stimulated with 10ng/mL LPS for four hours. Supernatants from stimulated cells were analyzed for cytokine concentrations using Bio-plex beads. Error bars represent SEM for triplicates.

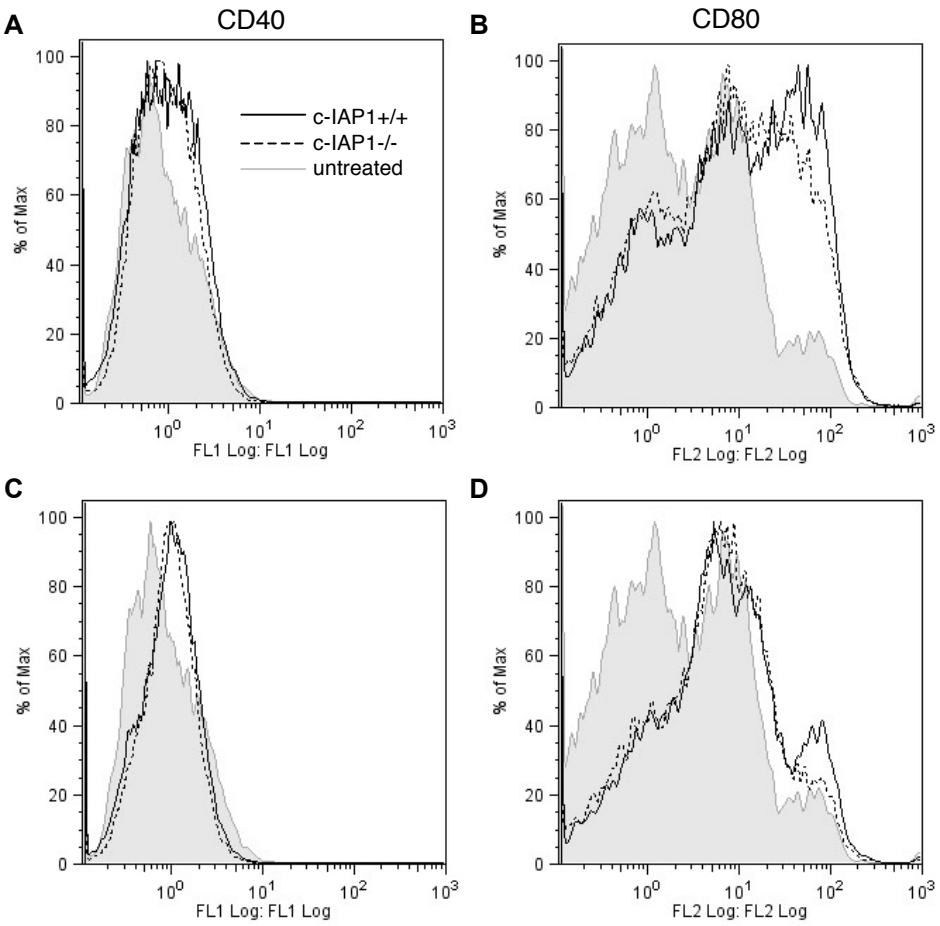

Figure 3.4 Maturation of c-IAP1-deficient dendritic cells is normal. (A,B) Bone marrow-derived dendritic cells were stimulated with 500 ng/mL LPS for 20 hours. c-IAP1-deficient cells (solid line), wild-type cells (dashed line) and untreated cells (filled) were analyzed for expression of maturation markers CD40 (A) and CD80 (B) by flow cytometry. (C,D) Bone marrow-derived dendritic cells were stimulated for 20 hours with 20 ng/mL TNF. c-IAP1-deficient cells (solid line), wild-type cells (dashed line) and untreated cells (filled) were analyzed for expression of CD40 (C) and CD80 (D).

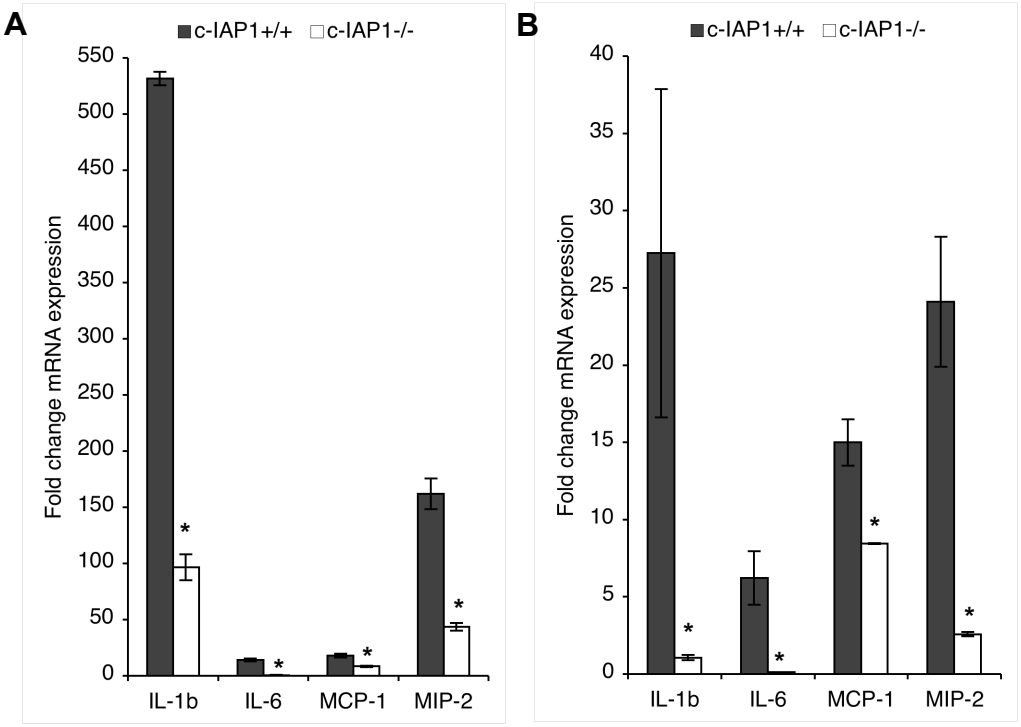

Figure 3.5 Cytokine induction is impaired in c-IAP1-deficient lung fibroblasts. Lung fibroblasts from *c-IAP1*$^{-/-}$ mice and littermate controls were stimulated with (A) 10 ng/mL LPS or (B) 5 ng/mL murine TNF for four hours. Isolated RNA was analyzed by qPCR for expression of IL-1β, IL-6, MCP-1 and MIP-2. Error bars represent SEM for triplicates, and * indicates $p < 0.05$. Results are representative of two independent experiments.

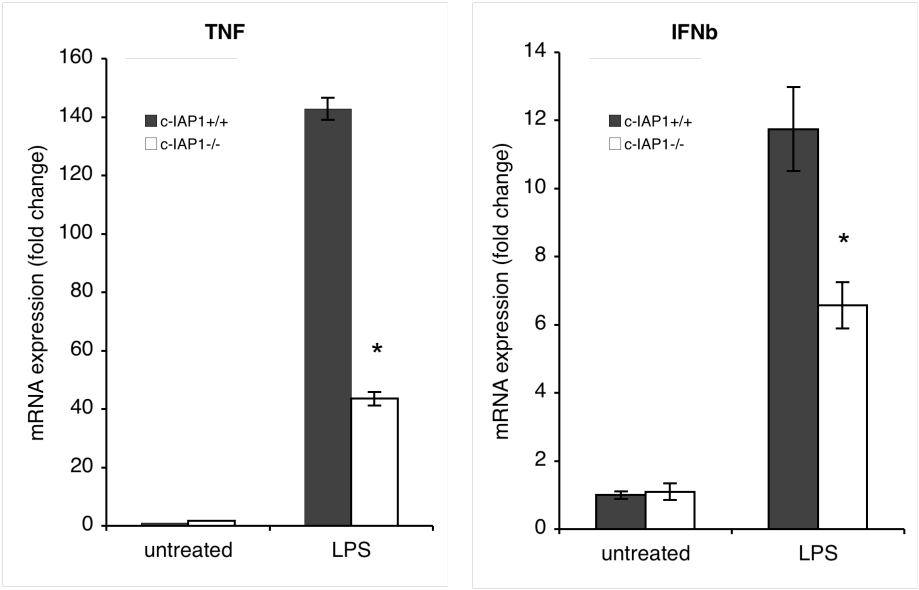

Figure 3.6 Cytokine induction is impaired in c-IAP1-deficient mouse embryonic fibroblasts. MEFs from *c-IAP1*$^{-/-}$ and *c-IAP1*$^{+/+}$ littermates were stimulated with 50 ng/mL LPS for four hours, and isolated RNA was analyzed by qPCR for expression of TNF and IFNβ. Error bars represent SEM for triplicates, and * indicates $p < 0.05$.

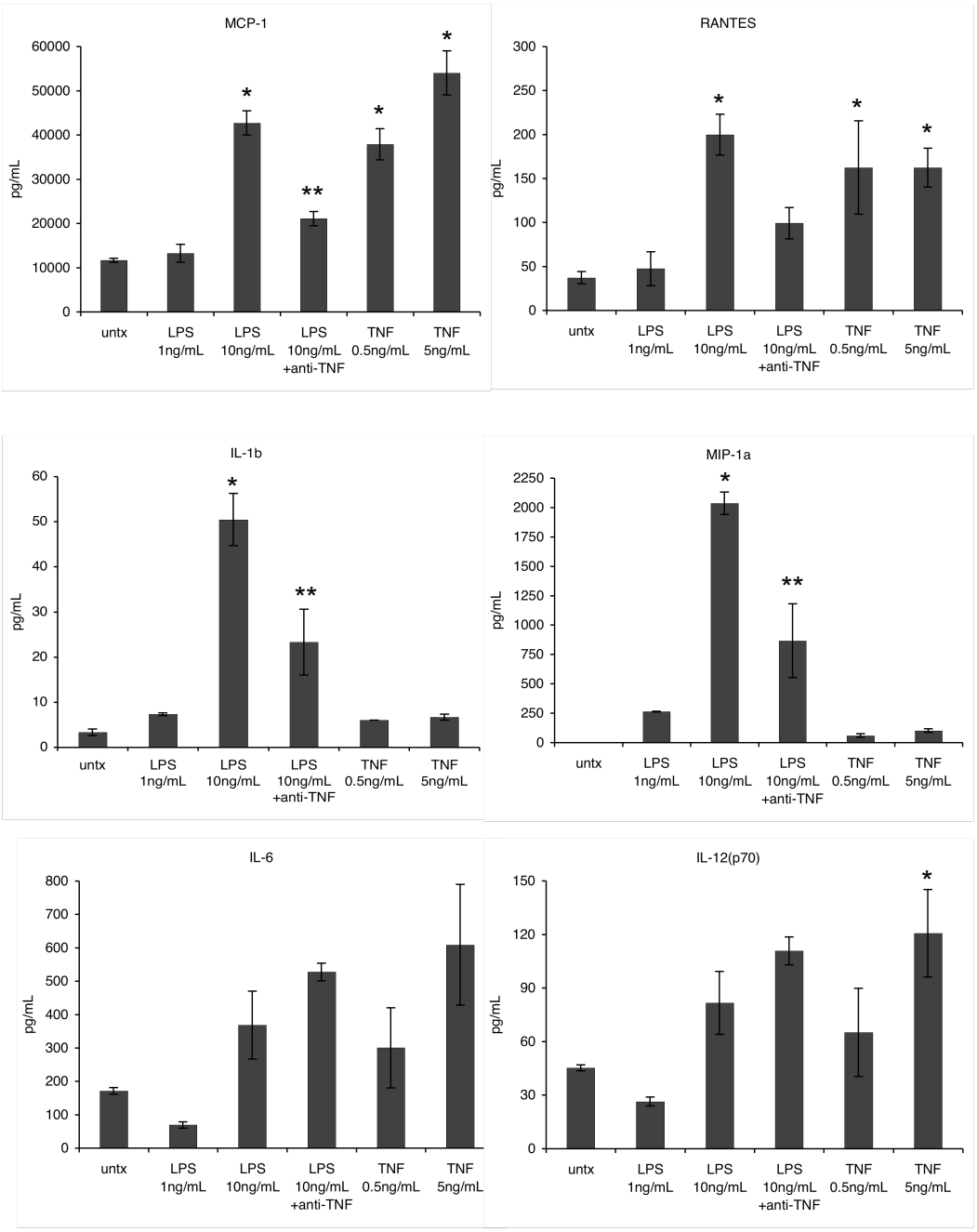

Figure 3.7 Autocrine TNF contributes to cytokine induction by LPS in lung fibroblasts. Wild-type lung fibroblasts were stimulated for 24 hours, and supernatant was harvested for cytokine quantification using Bio-plex beads. Error bars are SEM for triplicates. * indicates $p<0.05$ in comparison to untreated, and ** indicates $p<0.001$ in comparison to 10 ng/mL LPS alone.

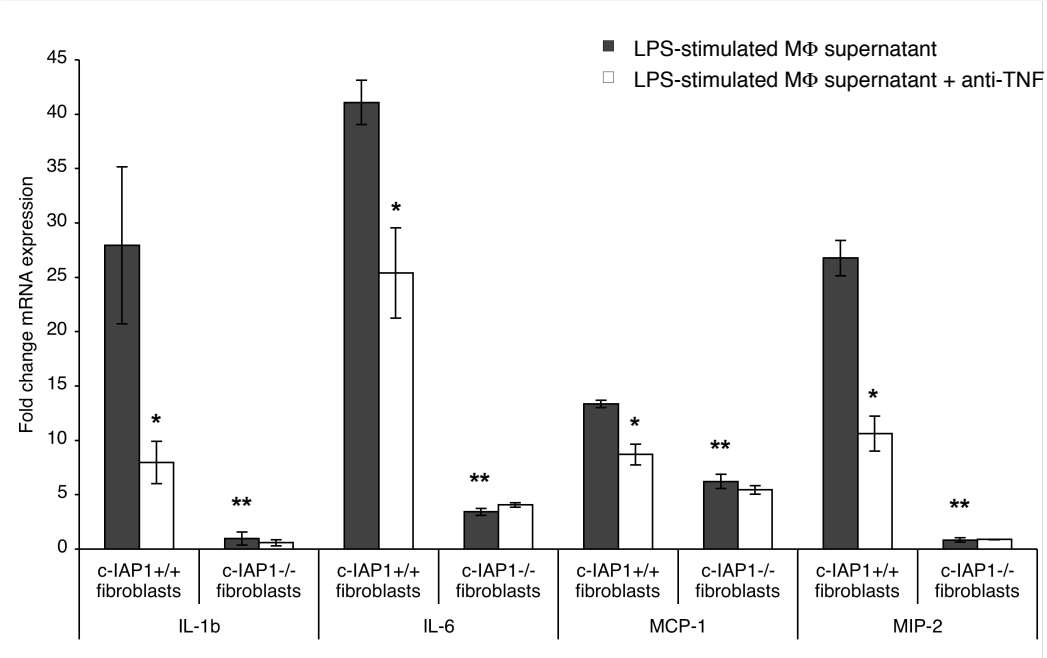

Figure 3.8 Cytokine networking between macrophages and c-IAP1-deficient lung fibroblasts is impaired. Wild-type bone marrow-derived macrophages were stimulated with 2 ng/mL LPS for 24 hours. Lung fibroblasts were then stimulated four hours with macrophage-conditioned media, and isolated RNA was analyzed by qPCR for expression of IL-1β, IL-6, MCP-1 and MIP-2. Lung fibroblasts were also stimulated with macrophage-conditioned media treated with TNF neutralizing antibody. Error bars represent SEM for triplicates. ** indicates $p<0.05$ for comparison between *c-IAP1*$^{-/-}$ and *c-IAP1*$^{+/+}$ lung fibroblasts stimulated with macrophage-conditioned media. * indicates $p < 0.05$ for comparison of macrophage-conditioned media in the absence or presence of TNF neutralizing antibody.

Chapter 4

The effect of c-IAP1 on intracellular signaling pathways that lead to pro-inflammatory cytokine production

Abstract

Since alterations in pro-inflammatory cytokine induction were observed in *c-IAP1*$^{-/-}$ mice *in vivo* and c-IAP1-deficient cells *in vitro*, we explored potential mechanisms for the effect of c-IAP1 by investigating two central signaling pathways that contribute to inflammatory responses, NF-κB and MAPK signaling. Although pro-inflammatory cytokine activation was disrupted, c-IAP1-deficient fibroblasts demonstrated intact NF-κB signaling. We further explored components of the MAPK signaling pathways, including p38 MAPK, JNK, MEK1, ERK1/2 and c-Jun, which are activated by phosphorylation. Phosphorylation of MAPK signaling components was not impaired in c-IAP1-deficient lung fibroblasts and macrophages. Examination of RIP1, a TNFR-associated signaling molecule, revealed differential ubiquitination in c-IAP1-deficient cells. While we have observed an effect of c-IAP1 in this upstream role, these changes did not correlate with differential activation of the two primary signaling pathways associated with TNFR signaling. Further exploration of roles for c-IAP1 in additional pathways will be necessary to determine the mechanism by which transcriptional activation of cytokines is affected.

Introduction

Intracellular processes that lead to inflammation are largely coordinated by two central signaling pathways, NF-κB and MAPK signaling. The NF-κB pathway is essential for a wide variety of developmental and homeostatic programs, including a central role in the development and function of cells in both the innate and adaptive immune systems. This pathway regulates a large, diverse set of genes with pro-inflammatory functions. A number of these target genes and several components of this signaling pathway have well-established contributions to the pathogenesis of sepsis, as previously reviewed (*231*). While it has not been as exhaustively studied as NF-κB activation, MAPK signaling also has been demonstrated to have an important role in inflammation and sepsis (*232-235*). Each of these pathways controls transcription of hundreds of genes with pleotropic effects, and their activation and regulation have been described in Chapter 1.

There is evidence for evolutionary conservation of the involvement of IAPs in signaling pathways activated during innate immune responses. A *Drosophila* IAP ortholog, DIAP2, is critical for activation of innate immune responses in response to gram-negative bacteria. Recognition of gram-negative bacteria by cell surface receptors in *Drosophila* triggers signaling pathways that share remarkable resemblance to components of mammalian TNFR signaling, including central roles for NF-κB and MAPK pathways. Genetic studies in *Drosophila* indicated that during innate immune responses, DIAP2 was required for signaling mediated by *imd* (*immune deficiency*), the *Drosophila* ortholog of mammalian RIP1 (*236-240*). Innate immune responses in

mammals have diversified to include important components of cytokine networking and adaptive immune responses, and mammalian IAPs have diversified to include CARD domains. In the midst of these changes, many components of these signaling pathways have been conserved, which compelled us to specifically examine the effect of c-IAP1 on RIP1 during TNFR-mediated signaling.

Further evidence in mammalian models has implicated c-IAP1 as a component of the NF-κB and MAPK signaling pathways. For example, the ubiquitin ligase activity of c-IAP1 putatively targets signaling molecules RIP1, TRAF2, NEMO, NIK and ASK1 for K63-linked or K48-linked ubiquitination, which are critical modifications during TNFR-mediated activation of canonical NF-κB, non-canonical NF-κB and MAPK signaling, as detailed in Chapter 1. For activation of JNK signaling in particular, TRAF2 has been demonstrated to be an essential component of the TNFR signaling complex (*241, 242*). The consequences of the effect of c-IAP1 on JNK signaling has been demonstrated in response to activation of CD40, a member of the TNFR family. c-IAP1 influenced the duration of CD40-induced JNK signaling (*97*), and has also been shown to affect CD40-induced cytokine production in macrophages (*243*).

Our studies of c-IAP1 during inflammation *in vivo* and *in vitro* led us to conclude that c-IAP1 contributed to inflammation largely by affecting the responsiveness of non-immune cells, such as fibroblasts, to TNF. Through this role, c-IAP1 potentially influenced the participation of non-immune cells in networks with immune cells that amplify inflammation. Since TNF is a potent stimulus for both NF-κB and MAPK signaling, in this chapter we will examine mechanisms by which c-IAP1 potentially contributes to activation of these signaling pathways during inflammation. We analyzed

activation of the NF-κB and MAPK signaling pathways in c-IAP1 deficient cells. We did not observe defects in NF-κB activation in c-IAP1-deficient lung fibroblasts or macrophages. Alterations observed in MAPK signaling in c-IAP1-deficient cells did not correlate with the pattern of pro-inflammatory cytokine expression from previous results. Based on these findings, we explored a potential role for the highly homologous protein, c-IAP2, as a compensatory mechanism in c-IAP1-deficient cells. However, we did not observe changes in c-IAP2 mRNA expression in c-IAP1-deficient cells. There is increasing evidence for additional apoptotic and non-apoptotic roles for c-IAP1, described in Chapter 1, and the potential involvement of these functions in inflammation will be addressed in the discussion.

Materials and Methods

Cell culture and reagents

c-IAP1-deficient lung fibroblasts and mouse embryonic fibroblasts were isolated and cultured in DMEM containing 10% FCS, glutamine, penicillin, streptomycin and amphotericin B as described in Chapter 3. Culture conditions for macrophages and dendritic cells derived from bone marrow were also described in Chapter 3. For each experiment, wild-type and c-IAP1-deficient cells were obtained from littermates. c-IAP2-deficient mouse embryonic fibroblasts were kindly provided by Dr Robert Korneluk (Department of Pediatrics, University of Ottawa, ON, Canada). For stimulation, fibroblasts were cultured in DMEM containing 0.5% FCS, glutamine, penicillin, streptomycin and amphotericin B.

Cells were stimulated *in vitro* with LPS (*E. coli* O128:B8, #L3129, Sigma-Aldrich) or recombinant murine TNF (Roche Applied Sciences).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed with all steps done on ice, as described in (*244*). Nuclear extracts were prepared from cells washed with buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM PMSF, and 0.5 mM DTT) and pelleted by centrifugation at 200 x g for 5 min. Buffer A was aspirated, and the pellet was resuspended by gentle pipetting in 30 µl of buffer A supplemented with 0.1% Nonidet P-40 and incubated on ice for 10 min. The nuclear pellet was isolated by centrifugation at 20,800 x g in a refrigerated microcentrifuge at 4 °C for 10 min, resuspended in 20 µl of cold buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1 mM PMSF, 0.5 mM DTT, and 25% glycerol), and rotated at 4 °C for 15 min. The nuclear extract was clarified by centrifugation at 20,800 x g for 15 min, and 10 µl was transferred to a fresh tube and diluted with 60 µl of modified buffer D (20 mM HEPES, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 0.1 mM PMSF, 0.5 mM DTT, and 20% glycerol), flash frozen, and stored at –80 °C.

Two complementary oligonucleotides containing NF-κB consensus binding sites, 5'-TGCAAGGGACTTTCCGCTGGGGACTTTCC-3' and 5'-TGCAGGAAAGTCCCCAGCGGAAAGTCCCT-3', were annealed, and 50 ng was radiolabeled in a Klenow reaction in the presence of [α-^{32}P]dCTP. The radiolabeled probe was purified over a Sephadex column in TE containing 50 mM NaCl. To test for the presence of NF-κB in the nuclear extracts, 2 µl of nuclear extracts were incubated for 20 min at room temperature with 1 µg of poly(dI-dC)·poly(dI-dC) in modified buffer D

(minus glycerol) in a total volume of 20 µl. 0.2 µl of ^{32}P-radiolabeled probe was added, and the entire reaction was separated on a nondenaturing 4% polyacrylamide gel. Autoradiography was performed overnight at –20°C.

Immunoprecipitation and immunoblotting

Whole cell lysates were prepared using Triton lysis buffer (1% Triton-X 100, 25 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM $NaVO_4$ and 1 mM PMSF) supplemented with protease inhibitors (Roche). For immunoprecipitations, lysates were incubated with 1 µg RIP1 antibody (mouse monoclonal, BD Transduction Laboratories, San Diego, CA) on a rotator for four hours at 4°C. Protein G agarose beads were added for 1 hour on a rotator at 4°C, then beads were washed four times with lysis buffer. Proteins were eluted in 30 µL lysis buffer containing 50 mM DTT and 1x NuPAGE LDS sample buffer (Invitrogen) and boiled at 95°C for 5 min.

Samples were resolved by SDS/PAGE (4–12% gradient gels; Invitrogen), transferred on to nitrocellulose membranes (Invitrogen) and blocked in 5% (w/v) non-fat dried skimmed milk powder in Tris-buffered saline containing 0.1% Tween 20. Membranes were incubated with the specified primary antibody, followed by secondary horseradish-peroxidase-conjugated antibody (1:2000; GE Healthcare). ECL (enhanced chemiluminescence; GE Healthcare) and Kodak XAR film were used for visualization. Mouse monoclonal multiubiquitin antibody clone FK2 (Stressgen/Assay Designs, Ann Arbor, MI) was used at 1:2000 dilution. Rat monoclonal anti-c-IAP1 was obtained from John Silke (Department of Biochemistry, La Trobe University, Melbourne, Australia) and used at 1:500 dilution (*88*). Mouse monoclonal phospho-JNK/SAPK (T183/W185) was obtained from Cell Signaling Technologies and used at 1:2000 dilution for immunoblot.

Quantification of protein phosphorylation

Cells were lysed using the Bio-Plex Cell Lysis Kit (Bio-Rad, Hercules, CA) Phosphoprotein concentrations were quantified using bead-based phosphoprotein and total protein assays analyzed by the Bio-plex Suspension Array System following manufacturer's instructions (Bio-Rad). For data analysis, fluorescent units of for each sample were normalized to the untreated samples, and the extent of phosphorylated target protein was determined relative to the total target protein.

Quantification of mRNA expression

To quantify expression of cytokine genes of interest or c-IAP2, RNA was harvested in Trizol (Invitrogen, Carlsbad, CA) and isolated following manufacturer's instructions. cDNA was generated using Moloney Murine Leukemia Virus (MMLV) Reverse Transcriptase (Invitrogen). Levels of mRNA were analyzed by qPCR using Taqman gene expression assays (Applied Biosystems, Foster City, CA). Gene expression was normalized to GAPDH mRNA for each sample and expressed as fold increase compared to the negative control within the experiment.

Statistical analyses

For analysis of gene expression and phosphoprotein results, ANOVA was used with a Student Newman-Keuls post-test to determine the statistical significance of differences. A p-value <0.05 was considered to be statistically significant.

Results

Activation of NF-κB is normal in c-IAP1-deficient cells

Based on our investigations of the role of c-IAP1 during inflammation *in vivo* and *in vitro,* we hypothesized that c-IAP1 contributes to inflammation by affecting the responsiveness of non-immune cells, such as fibroblasts, to TNF produced in the pro-inflammatory environment. TNF potently activates signaling through the NF-κB pathway, and we examined activation of this pathway by TNF and by LPS in c-IAP1-defiicent lung fibroblasts. Transcription of NF-κB target genes is activated by translocation of NF-κB subunits into the nucleus, which is dependent on phosphorylation and degradation of the inhibitory protein IκBα. We quantitatively assessed phosphorylated IκBα and total IκBα using a bead-based immunoassay. In lung fibroblasts, IκBα phosphorylation and degradation were not significantly induced by LPS at the time points evaluated, but treatment with TNF for 10 minutes resulted in significant IκBα phosphorylation (Fig. 4.1 A-C). c-IAP1-deficient and wild-type lung fibroblasts demonstrated equivalent induction of IκBα phosphorylation in response to TNF, which was rapidly extinguished within 20 minutes of stimulation in both *c-IAP1*$^{-/-}$ and *c-IAP1*$^{+/+}$ cells.

We next confirmed that translocation of NF-κB subunits into the nucleus was intact following IκBα phosphorylation. Nuclear extracts were incubated with radiolabeled probes, and bound NF-κB subunits were resolved by non-denaturing gel electrophoresis. Both LPS and TNF stimulation resulted in nuclear translocation of NF-

κB subunits in MEFs, which was comparable in c-IAP1-deficient and wild-type cells (Fig. 4.1D).

Our experiments examining cytokine gene regulation *in vitro* suggest a cell-specific role for c-IAP1 during responses to LPS. We hypothesized that the mechanism for the cell-specific effect was the requirement of an autocrine TNF loop in lung fibroblasts for stimulation of cytokine induction by LPS. In support of this hypothesis, we observed that the effect of TNF on lung fibroblasts was c-IAP1-dependent. On the other hand, macrophages are rapidly activated and induce cytokine production upon stimulation with LPS in a TNF-independent manner. By assessing NF-κB activation by LPS and TNF in macrophages, we could examine the contribution of c-IAP1 in cells that are more responsive to LPS than lung fibroblasts. LPS and TNF each triggered phosphorylation and degradation of IκBα in macrophages, and the kinetics were similar in *c-IAP*$^{-/-}$ and *c-IAP1*$^{+/+}$ cells (Fig. 4.2). Expression of c-IAP1 protein in wild-type but not *c-IAP1*$^{-/-}$ mice was confirmed by immunoblot (Fig. 4.2D).

Additional characterization of signaling in c-IAP1-deficient cells

Since NF-κB activation was intact in c-IAP1-deficient lung fibroblasts and macrophages, we proceeded to analyze activation of the MAPK pro-inflammatory signaling pathways. JNK, ERK1/2, p38 MAPK, the upstream kinase MEK1 and the AP-1 transcription factor subunit c-Jun were analyzed for phosphorylation in lung fibroblasts following stimulation with LPS or TNF (Fig. 4.3A). Overall, the MAPK cascades were robustly activated in c-IAP1-deficient lung fibroblasts by TNF. A small but significant decrease in ERK1/2 activation was observed after 20 minutes of TNF stimulation. Conversely, a significant increase in ERK1/2 phosphorylation was following 35 minutes

of stimulation of LPS, and it is unclear what effect these conflicting changes would have on pro-inflammatory cytokine induction. Results for JNK signaling were verified by immunoblot in MEFs (Fig4.3B), which confirmed phosphorylation of JNK in both c-IAP1-deficient and wild-type MEFs following stimulation with TNF, but not following LPS exposure. Importantly, at longer time points phosphorylation of JNK decayed, and kinetics were similar in wild-type and c-IAP1-deficient cells.

We again compared signaling in lung fibroblasts to macrophages and observed dramatic enhancement of MAPK activation by LPS in macrophages (Fig. 4.4). Activation of the p38 MAPK, ERK1/2 and MEK1 kinases was rapidly induced by LPS and TNF in both c-IAP1-deficient and wild-type macrophages, and the signals appeared to decay with similar kinetics as well. Activation of JNK phosphorylation by LPS was greater in wild-type macrophages than c-IAP1-deficient macrophages at 30 minutes following stimulation, but the relevance is unclear since macrophages appear to induce cytokine production normally in response to LPS. JNK phosphorylation induced by TNF in macrophages was not significantly different between c-IAP1-deficient and wild-type cells.

Given our findings that NF-κB and MAPK signaling were generally intact in both lung fibroblasts and macrophages following stimulation with TNF or LPS, we next examined whether ubiquitination of RIP1, a critical component of the TNFR signaling complex, is altered in c-IAP1-deficient cells. A putative target of the ubiquitin ligase activity of c-IAP1, RIP1 ubiquitination was determined by immunoprecipitation from stimulated cells, separation by gel electrophoresis and immunoblot with an antibody that recognizes multi-ubiquitin. RIP1 was ubiquitinated in wild-type cells stimulated with

TNF, shown in Figure 4.5, but not in response to LPS (data not shown). The patterns of ubiquitination were altered in c-IAP1-deficient lung fibroblasts, resulting in reduced ubiquitination of RIP1. We attempted to correlate changes in RIP1 ubiquitination with alterations in NEMO ubiquitination, but ubiquitination of NEMO was not detectable in response to TNF using this approach (data not shown). Thus, while disruptions in the TNF signaling complex are evident in c-IAP1-deficient lung fibroblasts, we have not observed an effect on the primary signaling pathways that lead to subsequent pro-inflammatory responses.

Normal expression of c-IAP2 in c-IAP1-deficient cells

Functional similarities of c-IAP1 and c-IAP2 have been described for their roles in intracellular signaling pathways and apoptosis. We examined whether c-IAP2 had a similar effect to c-IAP1 on cytokine induction in response to pro-inflammatory stimuli. An effect of c-IAP1 on cytokine induction in MEFs was demonstrated in the previous chapter (Fig. 3.6). Interestingly, induction of IL-6 expression in c-IAP2-deficient MEFs was impaired compared to wild-type controls during responses to LPS (Fig. 4.6C), implicating c-IAP2 in regulation of cytokine induction as well. The potential for overlapping or redundant roles of c-IAP1 and c-IAP2 during responses to pro-inflammatory stimuli suggested that c-IAP2 might compensate for the absence of c-IAP1during inflammatory responses. We first examined expression of c-IAP2 in macrophages following treatment with TNF of LPS. c-IAP1-deficient macrophages do not demonstrate alterations in cytokine production (Fig. 3.1), and we observed comparable induction of c-IAP2 in wild-type and c-IAP1-deficient macrophages (Fig. 4.6B). This result indicates that increased expression of c-IAP2 in c-IAP1-deficient cells

following stimulation is not a mechanism of compensation in macrophages to preserve cytokine expression despite a lack of c-IAP1. We next examined c-IAP1-deficient lung fibroblasts, which demonstrated impaired induction of cytokines in response to pro-inflammatory stimuli (Fig. 3.5). We tested whether decreased cytokine induction in these cells resulted from a failure to properly induce expression of c-IAP2 following pro-inflammatory stimuli in the absence of c-IAP1. However, we again observed comparable expression of c-IAP2 in c-IAP1-deficient and wild-type lung fibroblasts (Fig. 4.6A), indicating that c-IAP1 deficiency does not result in impaired transcriptional activation of c-IAP2 following pro-inflammatory stimuli. Thus, the observed reduction in cytokine induction in c-IAP1-deficient lung fibroblasts is a result of decreased induction of c-IAP2.

Discussion

c-IAP1 and c-IAP2 have both been demonstrated to influence innate immune responses, but it is unclear whether the individual contributions of these ubiquitin ligases are redundant or distinct during signaling pathways that are relevant to innate immune responses. We did not observe transcriptional compensation by c-IAP2 in c-IAP1-deficient cells, but post-translational effects on the stability of c-IAP2 have been shown to have a dramatic effect on c-IAP2 protein expression in c-IAP1-deficient cells, even in the absence of alterations in c-IAP2 mRNA expression (*53*). As a result, the responses of cells deficient in only c-IAP1 or c-IAP2 are difficult to predict. In fact, the roles reported in the literature for c-IAP1 and c-IAP2 during activation of signaling pathways by TNF are conflicting. While our experiments in this study do not resolve the multitude of

findings in the literature, we will describe these findings and discuss possible mechanisms that might influence the contributions of c-IAP1 to intracellular signaling.

In our studies of canonical NF-κB signaling, TNF-induced activation of the canonical NF-κB signaling pathway was observed to be intact in c-IAP1-deficient lung fibroblasts, MEFs and macrophages. These findings confirm studies during the initial characterization of *c-IAP1*$^{-/-}$ mice, which demonstrated appropriate nuclear translocation of NF-κB subunits in MEFs and activation of NF-κB reporter genes in splenocytes and thymocytes by TNF (*53*). Similarly, another study also demonstrated that loss of c-IAP1 alone did not disrupt TNF activation of NF-κB, as measured by IκBα degradation, in hepatocytes or MEFs (*212*). Rather, this study identified redundant contributions of c-IAP1 and c-IAP2 to NF-κB signaling, which were disrupted when expression of both c-IAP1 and c-IAP2 was suppressed. In contrast, two studies have reported effects on NF-κB activation when only c-IAP1 is deleted. One report has demonstrated a disruption in IκBα degradation by TNF in *c-IAP1*$^{-/-}$ MEFs (*64*), while in the opposite direction, another study of *c-IAP1*$^{-/-}$ MEFs demonstrated activation of NF-κB reporter assays (*88*). Conflicting findings have also been reported in studies of RIP1 ubiquitination by c-IAP1. Whereas one study demonstrated that c-IAP1 and c-IAP2 have redundant functions in TNF-induced RIP ubiquitination using MEFs and skeletal muscle fibroblasts (*212*), other studies in *c-IAP1*$^{-/-}$ MEFs have reported reduced ubiquitination of RIP1 (*63, 64*) and prolonged RIP1 binding to TNFR1 upon stimulation (*88*). Despite using similar cells, stimuli and detection methods, the contributions of c-IAP1 and c-IAP2 appear to be extremely context-dependent.

In addition to these discrepancies in the role of c-IAP1 in TNF-stimulated signaling, there are also inconsistent effects on the basal activation of canonical NF-κB signaling when c-IAP1 expression is lost. In our studies and others, unstimulated c-IAP1-deficient cells did not demonstrate alterations in IκBα phosphorylation or degradation, NF-κB nuclear translocation or production of NF-κB-regulated cytokines (*53, 212*). However, in one study mentioned above, activity in an NF-κB reporter assay was spontaneously induced in c-IAP1-deficient MEFs, which was suppressed by re-expression of c-IAP1 (*88*). Similar effects on NF-κB activation were found following treatment of wild-type cells with IAP antagonists, including increased recruitment and prolonged binding of RIP1 to TNFR1, phosphorylation and degradation of IκBα, phosphorylation of the p65 NF-κB subunit, activation in an NF-κB reporter assay, induction of TNF and MCP-1 expression and TNF-dependent induction of apoptosis (*87, 88*).

In addition to regulation of canonical NF-κB signaling activation, c-IAP1 has also been implicated in regulation of non-canonical NF-κB signaling, characterized by processing of p100 to p52. Activation of this pathway is controlled by the regulatory kinase NIK, which is a target for K48-linked ubiquitination by c-IAP1. Degradation of both c-IAP1 and c-IAP2 using small molecule antagonists results in stimulus-independent stabilization of NIK and processing of p100 (*87, 88*). Characterization of the individual roles of c-IAP1 and c-IAP2 during non-canonical signaling are unresolved. While c-IAP1 and c-IAP2 have been reported to have redundant roles in promoting the degradation of NIK (*85, 86*), another report has demonstrated NIK stabilization and p100 processing in *c-IAP1*$^{-/-}$ MEFs (*88*).

The kinetics of disrupting c-IAP1 and/or c-IAP2 is likely an important determinant of the outcome of intracellular signaling or apoptotic signaling. As an example, stable expression of mature pro-apoptotic Smac in the cytosol does not result in apoptosis (unpublished data), while acute introduction of Smac or Smac-based small molecules can rapidly induce cell death. Cells harvested from mice that have developed fully in the absence of c-IAP1 might have adapted different signaling and apoptotic thresholds, which are not representative of acute alterations in IAP protein stability during apoptosis or IAP antagonist treatment. For this reason, future studies using mice or cells with disruptions in expression of IAPs will require careful design to accurately model perturbations in intracellular or apoptotic signaling.

In light of the inconclusive or conflicting effects of c-IAP1 on TNFR signaling described in the chapter, a constructive comparison for the cell type-specific role for c-IAP1 might be drawn to recently published findings in TRADD-deficient mice. TRADD binds to TNFR1 and is required to recruit TRAF2, and subsequently c-IAP1 and c-IAP2 into the TNFR1 signaling complex. Although RIP1 recruitment to the receptor tail does not require TRADD, ubiquitination of RIP1 requires TRADD-mediated recruitment of these ubiquitin ligases. Interestingly, TRADD is required for activation of NF-κB and MAPK signaling in MEFs; however, TRADD-deficient macrophages were not defective in NF-κB or MAPK signaling or production of TNF (*245-247*). The authors concluded that macrophages possess redundant mechanisms either for RIP1 ubiquitination or for activation of NF-κB and MAPK independent of RIP1 ubiquitination. If there are redundant pathways that ensure activation of NF-κB and MAPK in macrophages despite a failure to recruit of TRAF2, c-IAP1 or c-IAP2 to the signaling complex in the absence

of TRADD, then similar pathways are likely responsible for the cell-specific effects observed in c-IAP1-deficient mice.

These contributions of TRADD to TNFR1 signaling place the findings for *c-IAP1*$^{-/-}$ mice in perspective, but the conclusions are complicated by the TRADD-independent role of c-IAP1 in TNFR signaling, through direct recruitment of TRAF2 to other TNFR family members. In particular, there is evidence that c-IAP1, c-IAP2 and their binding partners TRAF2 and TRAF1 are critically regulated by TNFR2, which contains a TRAF-binding sequence to directly bind TRAF2 and does not contain a death domain to bind TRADD. TNF stimulation of TNFR2 induces translocation of the receptor, along with TRAF2 and c-IAP1, into a detergent-insoluble fraction where the c-IAP1 and TRAF2 complex is targeted for degradation by the proteasome. As a result of TNFR2 signaling, TNFR1-stimulated apoptosis is enhanced and activation of JNK and NF-κB is inhibited, summarized in (*248*). The contrasting contributions of these receptors to innate immune responses have been demonstrated during bacterial infections *in vivo,* using TNFR1-deficient mice and TNFR2-deficient mice (*229*). Variations or inconsistencies in the observed role of c-IAP1 using isolated cells *in vitro* would be expected to depend not only on the general sensitivity of the cell to TNF, but also on the relative contributions of TNFR1 and TNFR2 sensitivity in each particular cell culture. This subtle mechanism, which can be influenced by factors such as TRAF1 expression (*248*), might result in large discrepancies between the results in different laboratories with otherwise similar cells and conditions.

Intriguingly, c-IAP1 and TRAF2 have recently been implicated in the process of monocyte differentiation into macrophages, through an initial phase of TRAF2-mediated

NF-κB activation, followed by c-IAP1-mediated TRAF2 degradation that sensitizes cells to CD40-initiated JNK activation leading to cytokine production (*243, 249*). A mechanism for c-IAP1- and c-IAP2-dependent activation of JNK by CD40 has also been recently proposed (*97*). These findings suggest that the contributions of c-IAP1 might not be evident in bone marrow-derived macrophages if these cells have not yet matured to express CD40. Rather, the contribution of c-IAP1 might only be expected to affect cytokine production by mature macrophages that express CD40.

MAPK activation by the TNFR family, while less extensively studied than NF-κB signaling, appears to involve complex regulation through phosphorylation and ubiquitination (reviewed in *250*). In several studies, transient activation of JNK signaling by TNF has been proposed to cooperate with NF-κB signaling to induce cytokine expression. Our observations of MAPK signaling in c-IAP1-deficient cells during this time frame generally indicated that MAPK signaling was intact. The consequences of sustained JNK activation are less clear, having been associated with apoptosis during inhibition of NF-κB in some cases (*251*) and with increased cytokine production dependent on XIAP in another case (*170*). The mechanism of the effect of XIAP on JNK activation is an interesting question for further study to determine whether the effect of XIAP is mediated by inhibition of caspases, by activation of intracellular signaling through TAK1 or by another non-apoptotic function. Modulation of sustained JNK signaling pathways by c-IAP1 during inflammatory processes warrants further study, to examine whether pro-inflammatory cytokines are regulated in this manner *in vitro* and *in vivo*. These studies would have potential mechanistic consequences for the role of monoubiquitination of receptors during trafficking into detergent-insoluble fractions,

regulation of TRAF2 stability by c-IAP1 and the role of c-IAP1, c-IAP2 and TRAFs in dissociation of signaling complexes from the receptor tail. While this chapter in its entirety focuses on the role of c-IAP1 in response to TNF stimulation though the NF-κB and MAPK pathways, numerous additional functions for c-IAP1 might also contribute to the role of c-IAP1 in innate immune responses, which will be discussed further in Chapter 5.

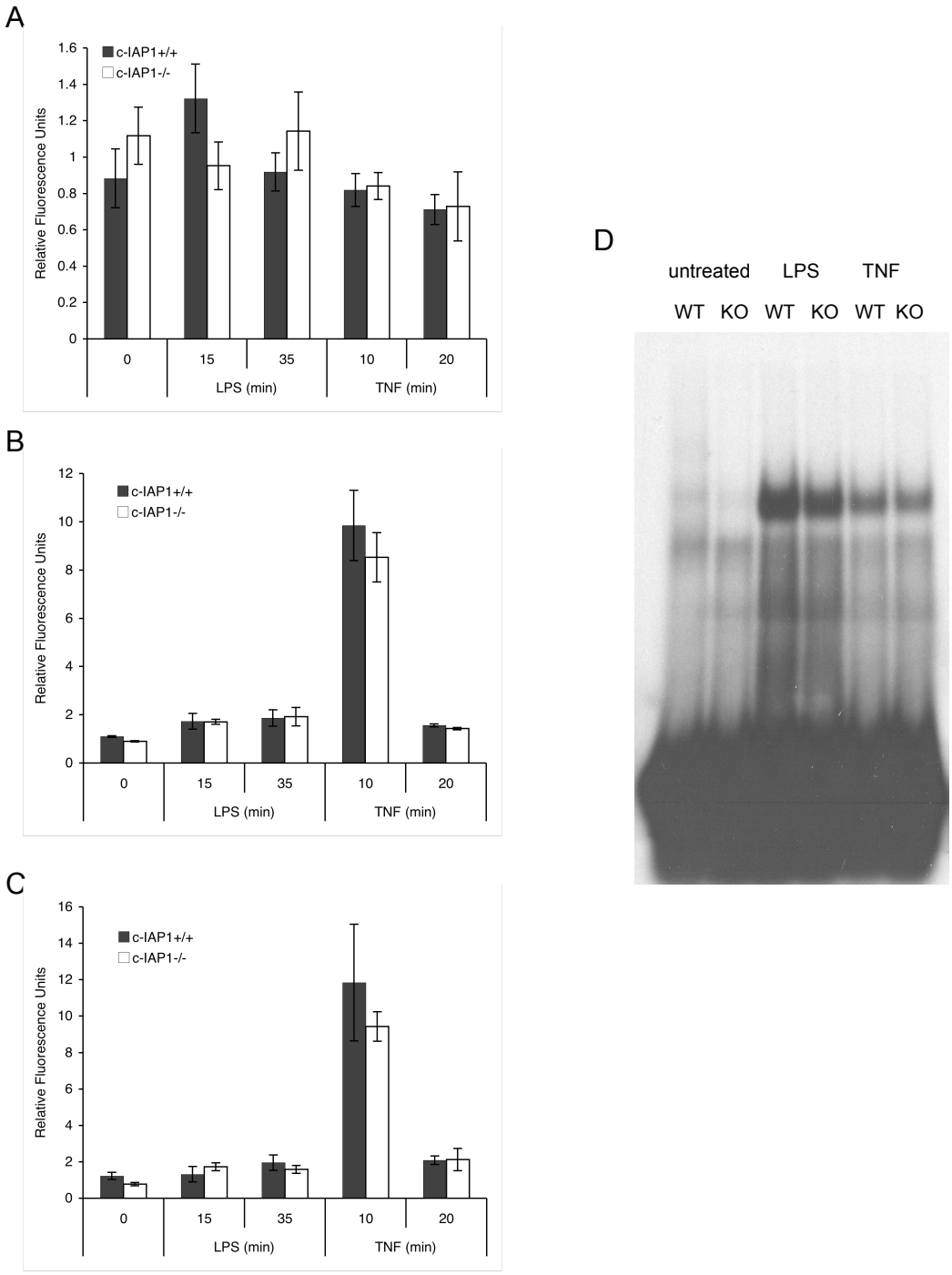

Figure 4.1 Activation of NF-κB is intact in c-IAP1-deficient fibroblasts. (A-C) Lung fibroblasts were stimulated with 100 ng/mL LPS or 10 ng/mL TNF for the indicated times, and protein concentrations were quantified using Bio-plex beads for (A) total IκBα and (B) phosphorylated IκBα. (C) The proportion of phosphorylated IκBα relative to total IκBα was calculated. Error bars are SEM for triplicates. (D) *c-IAP1*$^{-/-}$ and *c-IAP1*$^{+/+}$ MEFs were stimulated with 500 ng/mL LPS or 500 U/mL TNF for 45 min before harvesting nuclear extracts. The presence of NF-κB in nuclear extracts was probed with radiolabeled oligonucleotides and separated on a non-denaturing gel.

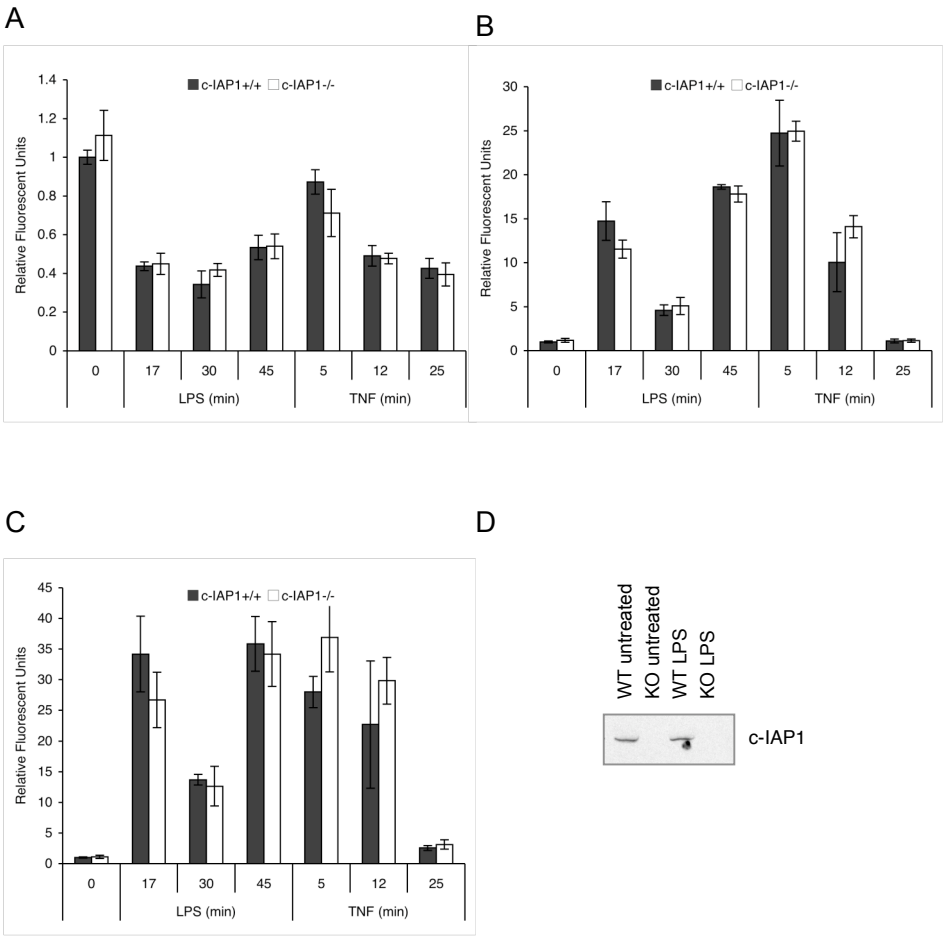

Figure 4.2 NF-κB signaling is intact in macrophages. Quantification of protein in bone-marrow derived macrophages stimulated with 100 ng/mL LPS or 10 ng/mL TNF using Bio-plex beads for (A) total IκBα and (B) phosphorylated IκBα. (C) The proportion of phosphorylated IκBα relative to total IκBα is displayed. Error bars are SEM for triplicates. (D) c-IAP1 expression was analyzed in wild-type and c-IAP1-deficient bone marrow-derived dendritic cells by immunoblot. 23 μg protein was loaded in each lane, and the membrane was probed with monoclonal anti-c-IAP1 antibody.

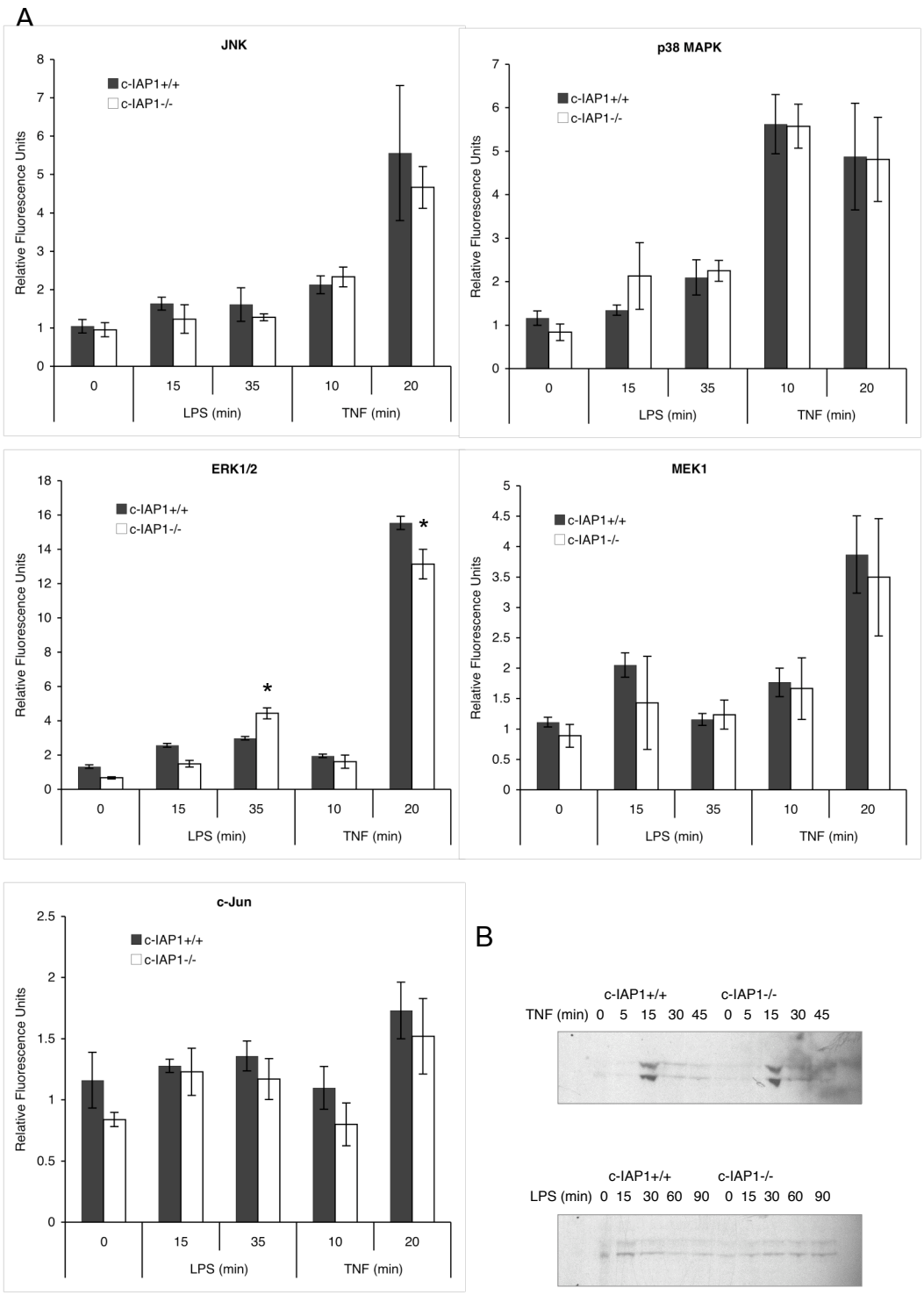

Figure 4.3 MAPK signaling in fibroblasts. (A) Lung fibroblasts were stimulated with 100 ng/mL LPS or 10 ng/mL TNF, and target protein concentration was quantified using Bio-plex beads. Results are displayed as the ratio of phosphorylated target protein to total target protein. Error bars are SEM for triplicates. (B) MEFs were stimulated with 5 ng/mL TNF or 500 ng/mL LPS for the indicated times, and whole cell lysates were probed for phospho-JNK.

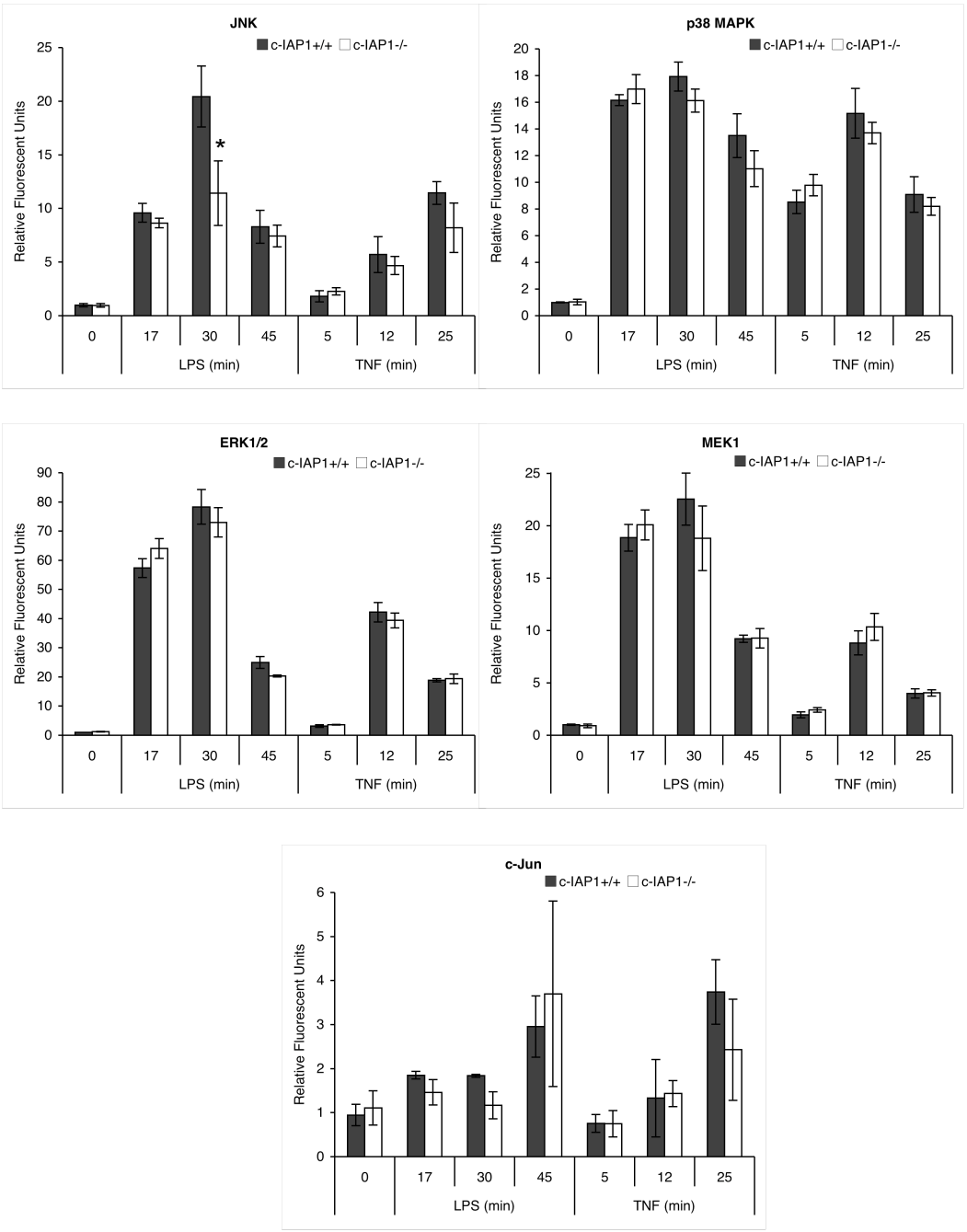

Figure 4.4 MAPK signaling in macrophages. Bone marrow-derived macrophages were stimulated with 100 ng/mL LPS or 10 ng/mL TNF, and target protein concentration was quantified using Bio-plex beads. Results are displayed as the ratio of phosphorylated target protein to total target protein. Error bars are SEM for triplicates.

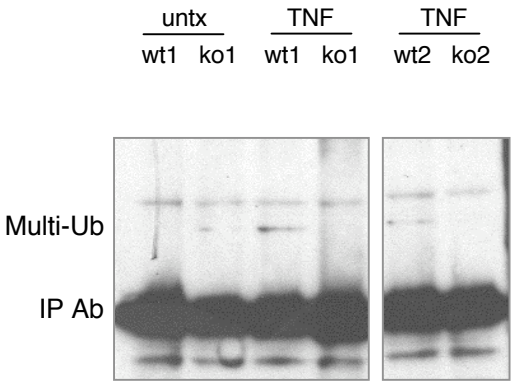

Figure 4.5 RIP1 ubiquitination during TNFR signaling. Lung fibroblasts were stimulated with 20 ng/mL TNF for 10 minutes (*n*=2). Whole cell lysates were immunoprecipitated using RIP1 antibody and separated by gel electrophoresis. Blots were probed using multi-ubiquitin antibody, which recognizes poly-ubiquitination of RIP1. Anti-mouse secondary antibody binds to the monoclonal primary antibody, as well as the mouse immunoprecipitation RIP1 antibody.

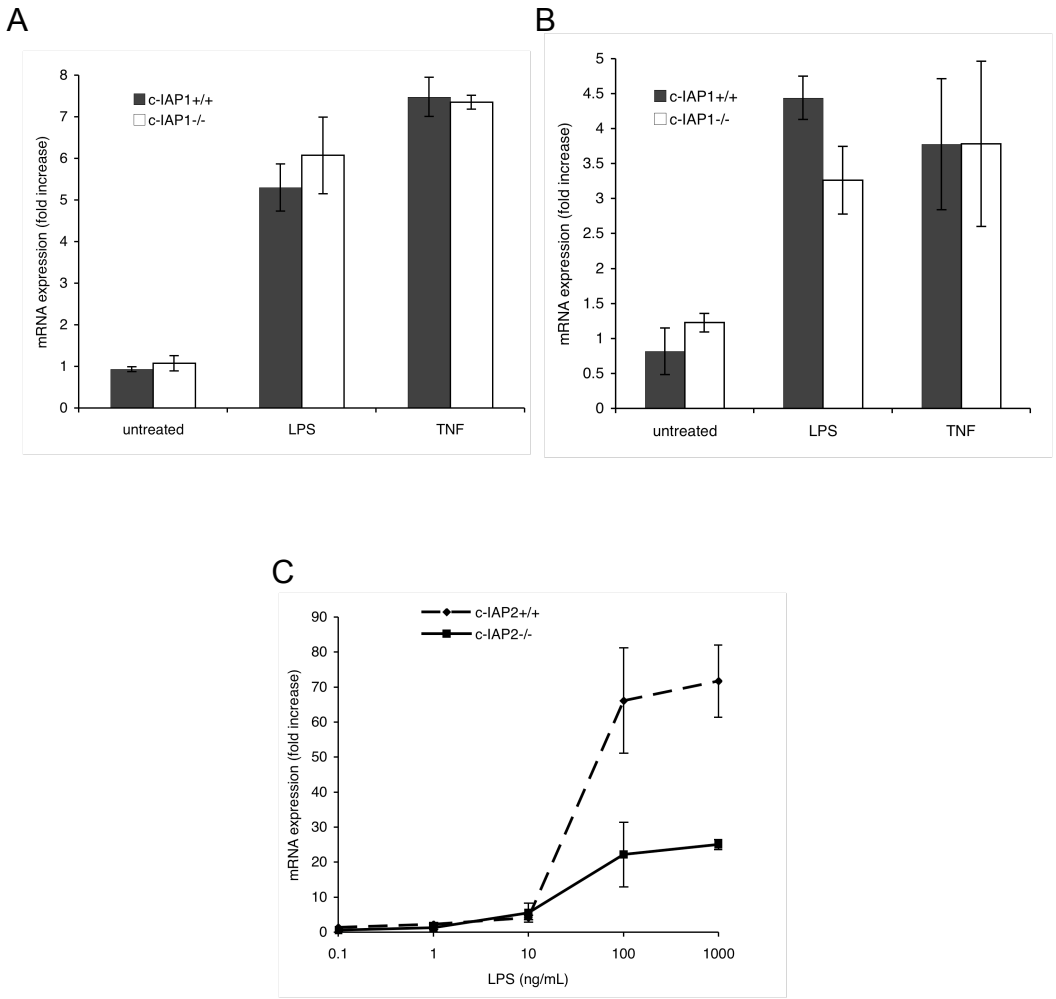

Figure 4.6 Induction of c-IAP2 is not altered in c-IAP1-deficient lung fibroblasts or macrophages. (A) Lung fibroblasts were stimulated with 10 ng/mL LPS or 5 ng/mL TNF for four hours. RNA was isolated and analyzed for expression of c-IAP2 by qRT-PCR. (B) Bone-marrow derived macrophages were stimulated with 2 ng/mL LPS or 5 ng/mL TNF for four hours. RNA was harvested and analyzed for c-IAP2 expression by qRT-PCR. (C) Expression of c-IAP2 is required for induction of cytokine transcription. c-IAP2-deficient and wild-type MEFs were stimulated with increasing doses of LPS for four hours, and induction of IL-6 mRNA was assessed by qRT-PCR. In all panels, error bars are SEM for triplicates.

Chapter 5

Discussion

In the studies described in this dissertation, we have attempted to elucidate one aspect of the interconnections between apoptosis and inflammation by focusing on the role of the anti-apoptotic protein c-IAP1 during inflammation and sepsis. In cases of sepsis, pro-inflammatory cytokine production is uncontrolled and results in systemic production of cytokines, such as TNF, IL-6 and IL-1β, which potentially contribute to hypotension and hypoperfusion associated with septic shock. Using two mouse models of sepsis, we showed that c-IAP1 was a critical pro-inflammatory mediator of innate immune responses during septic shock, described in Chapter 2. We observed improved survival in c-IAP1-deficient mice, and we identified a role for c-IAP1 in pro-inflammatory systemic cytokine production during endotoxemia.

To understand the contributions of c-IAP1 to excessive inflammation during sepsis, we characterized the extent of inflammation and cytokine production at the site of endotoxin challenge and in distant tissues. During the primary, local responses to LPS, initial cytokine production and local inflammation were not affected in c-IAP1-deficient mice, and apoptosis of cytokine-producing cells was also unaltered. Interestingly, decreased concentrations of IL-1β, IL-12 and MCP-1 were observed in the lungs of *c-IAP1*$^{-/-}$ mice following intraperitoneal challenge with LPS. These findings identify a contribution of c-IAP1 to production of systemic cytokines during pathologic pro-

inflammatory immune responses. We hypothesized that c-IAP1 did not affect initial inflammatory responses, but instead modulated cytokine production during subsequent amplification of systemic pro-inflammatory cytokines. Investigating the role of c-IAP1 in the regulation of pro-inflammatory cytokine induction *in vitro*, we determined that cytokine production by LPS in innate immune cells, such as macrophages and dendritic cells, was not dependent on c-IAP1. Intriguingly, we identified a role for c-IAP1 in the response of lung fibroblasts to pro-inflammatory stimuli, such as LPS, TNF and macrophage-derived cytokines. Using macrophage-conditioned media, we observed that lung fibroblasts responded to macrophage-derived TNF in a c-IAP1-dependent manner. Consequently, c-IAP1 might regulate the participation of fibroblasts in cytokine networks with immune cells during inflammation, as discussed in Chapter 3. We explored potential mechanisms for the effect of c-IAP1 by investigating signaling pathways that contribute to inflammatory responses in response to LPS or TNF. NF-κB and MAPK signaling were intact in c-IAP1-deficient fibroblasts and macrophages. We did observe changes in ubiquitination of RIP1 in c-IAP1-deficient lung fibroblasts, and later in this chapter we will outline additional signaling pathways of interest to further explore the contribution of c-IAP1 to intracellular signaling that leads to inflammatory responses.

Similar to c-IAP1-deficient mice, *c-IAP2*$^{-/-}$ mice have been reported to demonstrate improved survival and reduced systemic pro-inflammatory cytokines during endotoxemia (*187*). It is interesting that c-IAP1-deficient mice and c-IAP2-deficient mice both demonstrate protection during models of sepsis, because these proteins have different patterns of expression during inflammation. The expression of c-IAP1 is ubiquitous in many tissues and is not altered during inflammation. On the other hand, c-

IAP2 typically has very low expression and is induced in response to NF-κB activation during inflammation, which we observed in LPS-stimulated lung fibroblasts (Fig. 4.6). However, there is also the possibility of compensation between c-IAP1 and c-IAP2 as a result of overlapping or redundant functions of these highly homologous proteins, discussed previously in Chapter 4. Although we did not observe additional transcription of c-IAP2 in c-IAP1-deficient cells, we cannot rule out post-translational effects on c-IAP2 stability that would allow increased expression of c-IAP2 to compensate in the absence of c-IAP1, as reported previously (*53*). Importantly, the improved survival of c-IAP1-deficient mice and c-IAP2-deficient mice implies a failure of complete compensation between c-IAP1 and c-IAP2 during excessive inflammation. c-IAP2-deficient mice are defective in induction of c-IAP2 during inflammation, which is critical for maximal cytokine production. In c-IAP1-deficient mice, impaired induction of cytokines in response to inflammatory stimuli could result from two possible mechanisms: (i) insufficient compensation for the absence of c-IAP1 during the immediate response to an inflammatory stimuli, prior to c-IAP2 induction by NF-κB, or (ii) failure to induce increased expression of c-IAP2 due to upregulation of c-IAP2 in naïve c-IAP1-deficient cells.

Unlike c-IAP1-deficient mice, c-IAP2-deficient mice demonstrated increased sensitivity of macrophages to apoptosis, which potentially depletes cytokine-producing cells and results in impaired systemic cytokine production. This represents an unusual role for an anti-apoptotic protein during sepsis-induced apoptosis; in contrast, other studies have supported blocking apoptosis during sepsis to prevent excessive lymphocyte death and improve survival, as described in Chapter 1. The role of c-IAP2 during sepsis

might result from an indirect effect on apoptosis, potentially by altering TNFR-mediated apoptosis and intracellular signaling (*63-65*). Thus, we speculate that non-apoptotic functions of c-IAP1 and c-IAP2 contribute to inflammatory responses during sepsis, resulting in distinct roles for c-IAP1 and c-IAP2 in the pathogenesis of sepsis compared to other anti-apoptotic molecules. Since dissecting the roles of c-IAP1 and c-IAP2 is complicated by redundancy and compensation, we have attempted to investigate the effect on inflammation in the absence of both c-IAP1 and c-IAP2 by using a small molecule inhibitor. We utilized a bivalent compound based on the tetrapeptide IBM of Smac, which has been shown to rapidly induce proteasomal degradation of c-IAP1 and c-IAP2 (*63, 136*). However, the impact of this compound on cytokine induction and intracellular signaling pathways in response to pro-inflammatory stimuli has been inconclusive.

While our experiments using TNF-neutralizing antibody on lung fibroblasts *in vitro* suggested that TNF-mediated signaling was the primary defect in c-IAP1-deficient lung fibroblasts, our subsequent studies of TNF-induced NF-κB and MAPK failed to correlate changes in intracellular signaling pathways with the observed alterations in cytokine gene induction. It is tempting to speculate that c-IAP1 contributes to inflammatory processes through additional or alternative roles. Recruitment of c-IAP1 and c-IAP2 into signaling complexes is mediated by direct association with the well-established binding partners TRAF2 and TRAF1. Recent studies have identified novel roles for TRAF2 in inflammatory processes, in addition to its function in TNFR-mediated signaling. Based on contributions of TRAF2 and other putative ubiquitination targets of c-IAP1, potential roles for c-IAP1 during signaling by pattern recognition receptors will

be briefly discussed here. Innate immune cells recognize PAMPs using three types of pattern recognition receptors, NLRs, TLRs and RLHs, which were introduced in Chapter 1, and examples of potential contributions of c-IAP1 will be described for each.

The best-characterized of the RIG-like helicases is RIG-I, a receptor that recognizes cytosolic viral RNA. The primary signaling adaptor for RIG-I is MAVS, which is anchored in outer mitochondrial membrane, and both of these molecules are CARD-containing proteins. Activation of signaling pathways and subsequently anti-viral cytokines by RIG-I through MAVS requires K63-ubiquitination of RIG-I, and two ubiquitin ligases have been identified that ubiquitinate RIG-I at different sites (*252, 253*). This example highlights the critical role of ubiquitination during signaling pathways that coordinate inflammation. Intriguingly, a signaling complex containing MAVS has been identified, which also contains TRAF2 (*254*), TRADD, FADD and RIP1 (*255*). Furthermore, MAVS undergoes caspase-mediated cleavage during apoptosis and also possibly during activation (*256, 257*). These interactions are highly suggestive of a potential role for c-IAP1, similar to its recruitment to TNFR signaling complexes. Infection with RSV *in vivo* is one model to study RIG-I-mediated signaling, since RSV stimulates RIG-I activation during infection (*258*). However, our analysis of c-IAP1-deficent mice infected with RSV did not demonstrate alterations in cytokine production, as detailed in Chapter 2. In comparison, MAVS-deficient mice were impaired in TNF, IL-6 and IL-1β production one day following RSV infection (*208*). While the molecular signaling complex suggests a role for c-IAP1 in RIG-I signaling, evidence for a function role of c-IAP1 in this process was elusive.

A TRADD and RIP1 signaling complex has also been identified in one signaling pathway activated by the TLR family of receptors. TLRs recognize extracellular or endosomal PAMPs, such as recognition of LPS by TLR4. Some members of the TLR family, such as TLR4 and TLR3, activate signaling pathways through the adapter TRIF. TRADD, RIP1 and TRAF1 have been identified as components of the TRIF signaling complex (*246, 247, 259, 260*). Furthermore, stimulation of these receptors results in ubiquitination of RIP1. However, we were limited in our investigation of the effect of c-IAP1 on RIP1 ubiquitination in this process, because we were unable to detect RIP1 ubiquitination in response to LPS stimulation using the methods described in Chapter 4.

Similar to RIG-I, NOD-like receptors also detect cytosolic PAMPs. NOD1 and NOD2 have the ability to activate the NF-κB pathway through the signaling molecule RIP2 (*261*), which is a CARD-containing protein that interacts with c-IAP1 (*262*). Ubiquitination of RIP2 by K63-linked chains is critical for NF-κB activation and cytokine induction by promoting recruitment of TAK1, and it will be interesting to see in future experiments whether RIP2 is a substrate for ubiquitination by c-IAP1, similar to the related protein RIP1. We attempted to stimulate cells with muramyl dipeptide (MDP), a NOD ligand, in combination with low doses of LPS, but curiously we did not observe enhanced cytokine induction compared to LPS alone. Thus, the potential for NOD activation by contaminating lipopeptides in LPS cannot be excluded, despite <1% protein contamination specified by the LPS purification method. Further experiments will be necessary to delineate the regulation of RIP2 and RIP1 ubiquitination by c-IAP1 during responses to TLR and NOD agonists.

During an inflammatory response to PAMPs, signaling cascades activated by pattern recognition receptors are integrated within the cell. Activation of NF-κB signaling can be initiated by each of the pathways described, and synergistic activation occurs following stimulation of multiple pathways. One point of integration is the IKK regulatory subunit, NEMO. K63-linked ubiquitination of NEMO has been implicated in activation of NF-κB following activation of TNFRs, TLRs and NLRs (*263*). Moreover, NEMO is also an adapter in RIG-I signaling pathway that influences not only NF-κB activation, but also IRF3/7 activation (*264*). One group generated a transgenic mouse model to specifically investigate the role of K63-linked ubiquitination of NEMO by mutating the site of ubiquitination (NEMO K392R). Strikingly, NEMO K392R mice were resistant to endotoxic shock and demonstrated impaired cytokine production, but NF-κB and MAPK signaling were intact in macrophages, thymocytes and MEFs (*265*). The remarkable similarity of mice expressing ubiquitin-defective NEMO and c-IAP1-deficient mice implicates the function of c-IAP1 as a ubiquitin ligase of NEMO as a critical component during inflammation. We were unable to detect NEMO ubiquitination in response to TNF or LPS using the method described in Chapter 4, but further experiments in this area are certainly warranted.

Evidence for a role of IAPs during integration of pattern recognition receptor signaling has been established for XIAP. During stimulation with TLR and NLR ligands, XIAP contributed to synergistic induction of cytokine production by macrophages (*170*). Further understanding of the contributions of XIAP, c-IAP1 and c-IAP2 to inflammatory responses, and the corresponding effects on apoptosis, has the potential to uncover a critical link in regulation of these two important processes. Elucidating the mechanism of

integration of apoptosis and inflammatory responses will clarify the response of innate immune cells, adaptive immune cells and non-immune cells during successful, physiologic clearance of an invading pathogen, and the pathologic response of these cells during sepsis. The effects of IAPs on inflammatory responses appears to be extremely dependent on cell-type and stimulus; consequently, extensive characterization of a variety of cells is necessary in both physiologic and pathologic contexts. The responses under these conditions would help model the factors that influence apoptotic and inflammatory responses. In addition to apoptosis, there is increasing evidence for integration of inflammation with other processes that can lead to cell death, such as pyroptosis (*173, 266, 267*), autophagy (*268-270*), reactive oxygen species (*271*) and genotoxic stress (*272*). Further investigation in these areas will also be informative for understanding inflammation in the context of cell death.

The development of therapeutics that target apoptosis for sepsis or non-infectious causes of systemic inflammatory responses will require further investigation into the regulatory links between inflammation and apoptosis. One therapeutic approach that has been pursued is broadly blocking apoptosis using caspase inhibitory small molecules. Based on the findings in the studies presented here, narrowing this approach to specifically target with IAP antagonists might be an additional strategy to prevent the harmful consequences of sepsis. However, we hypothesize that these molecules affect the non-apoptotic functions of IAPs since loss of c-IAP1 or c-IAP2 has the opposite effect of other anti-apoptotic targets. As discussed in Chapter 2, these compounds might have the exciting potential to potentially block the harmful effects of inflammation without sacrificing bacterial clearance. Small molecule IAP antagonists that trigger degradation

of c-IAP1 and c-IAP2 are already in development. In large part, these molecules were designed as cancer therapeutics by sensitizing cancer cells to apoptosis. The important contributions of inflammation during cancer development and progression (*273, 274*) are another possible target for therapeutics, and the use of small molecules to target c-IAP1 in cancer might have additional non-apoptotic mechanisms of action that modulate the inflammatory microenvironment of the tumor. As such, these compounds represent an intriguing therapeutic approach that could potentially induce tumor regression both through a direct sensitization to apoptosis in malignant cells and indirectly by mitigating pro-inflammatory cytokines that support tumor growth. Pre-clinical testing of these effects will require mouse models that preserve host-tumor interactions intact, including a competent immune system.

In summary, this dissertation has examined the interconnection of apoptosis and inflammation by investigating a novel role for c-IAP1 during responses to pro-inflammatory stimuli. During sepsis, c-IAP1-deficient mice demonstrated improved survival, and we identified a contribution of c-IAP1 to production of systemic cytokines during pathologic pro-inflammatory immune responses *in vivo*. Although cytokine production by LPS in innate immune cells was not dependent on c-IAP1, we identified a role for c-IAP1 in the response of lung fibroblasts to pro-inflammatory stimuli, such as LPS, TNF and macrophage-derived cytokines. Lung fibroblasts responded to macrophage-derived TNF in a c-IAP1-dependent manner; however, NF-κB and MAPK signaling appeared to be intact in c-IAP1-deficient fibroblasts and macrophages. These results indicate that c-IAP1 is a critical pro-inflammatory mediator of innate immune responses during septic shock and that c-IAP1 might regulate the participation of

fibroblasts in cytokine networks with immune cells during inflammation. Characterization of the regulation of inflammation and apoptosis during pathologic systemic inflammation will improve our understanding of the pathogenesis of sepsis and aid in the development of new therapeutics.

Appendix

c-IAP1 deficiency in a mouse model of prostate cancer

Introduction

The transgenic adenocarcinoma of the mouse prostate (TRAMP) model is a genetically engineered mouse model of cancer that allows for the study of autochonous tumor development, which importantly, occurs in the context of interactions with stromal tissue in an immunocompetent host. Expression of the oncogenic SV40 T-antigens is under control of the probasin promoter, which drives prostate-specific expression. SV40 T-antigens interfere with the function of retinoblastoma (Rb) and p53 tumor suppressors, resulting in uncontrolled cellular proliferation. Tumors progress through stages of prostate intraepithelial neoplasia (PIN) and adenocarcinoma, and mice develop prostate tumors with 100% penetrance with a substantial proportion developing metastatic disease.

The TRAMP model has been previously utilized to demonstrate that blocking apoptosis by simultaneously over-expressing Bcl-2 in prostate tissue resulted in reduced time to tumor formation (*275*). Since expression of both XIAP and c-IAP1 are increased in TRAMP tumors compared to normal prostate epithelium (*159*), we utilized the TRAMP model to examine the role of IAPs in tumor development and progression. We used XIAP-deficient TRAMP mice to assess the requirement of XIAP during prostate

cancer development and growth (*149*). In an evaluation of tumor development in XIAP-deficient TRAMP mice and control TRAMP mice, we found no evidence for a protective effect of XIAP deficiency in tumor onset or overall survival, and tumor histology revealed similar patterns of differentiation, apoptosis and proliferation in prostate tumors. In the study described here, we generated c-IAP1 deficient TRAMP mice to further assess the requirement for IAPs in prostate cancer development.

Methods

Generation and genotyping of TRAMP and c-IAP1-deficient mice have been previously described. All mice were bred and maintained on a C57BL/6 background. Research was conducted on a UCUCA-approved protocol in a manner consistent with the NIH Guidelines for the Care and Use of Laboratory Animals. Beginning at 12 weeks of age, weekly abdominal palpation by two independent observers was used to determine tumor onset. Mice were euthanized when moribund. Statistical analyses for Kaplan–Meier plots were performed using a two-sided log rank test with the survival package in the R statistical program (*205*).

Results and Discussion

c-IAP1-deficient and control TRAMP mice were monitored for tumor onset and overall health. Kaplan–Meier curves for tumor onset are shown in Figure A.1A. Latency to tumor onset was increased in c-IAP1-deficient TRAMP mice (median 33.3 weeks) compared to wild-type TRAMP controls (median 30.3 weeks). The difference was

statistically significant as determined by a two-sided log-rank analysis, p-value = 0.045. Kaplan-Meier curves for overall survival are shown in Figure A.1B. Overall survival appeared to be extended in c-IAP1-deficient mice (median 46.0 weeks) compared to TRAMP controls (median 42.6 weeks). However, this effect was not statistically significant (p=0.213). Thus, we have observed a moderately protective effect in c-IAP1-deficient mice using the TRAMP model. The differential requirement for c-IAP1 and XIAP during TRAMP tumor growth potentially implicates non-apoptotic activities of c-IAP1 in tumor development. These results also suggest that the activity of IAP antagonists currently in clinical trials might result from their effect on c-IAP1 rather than XIAP.

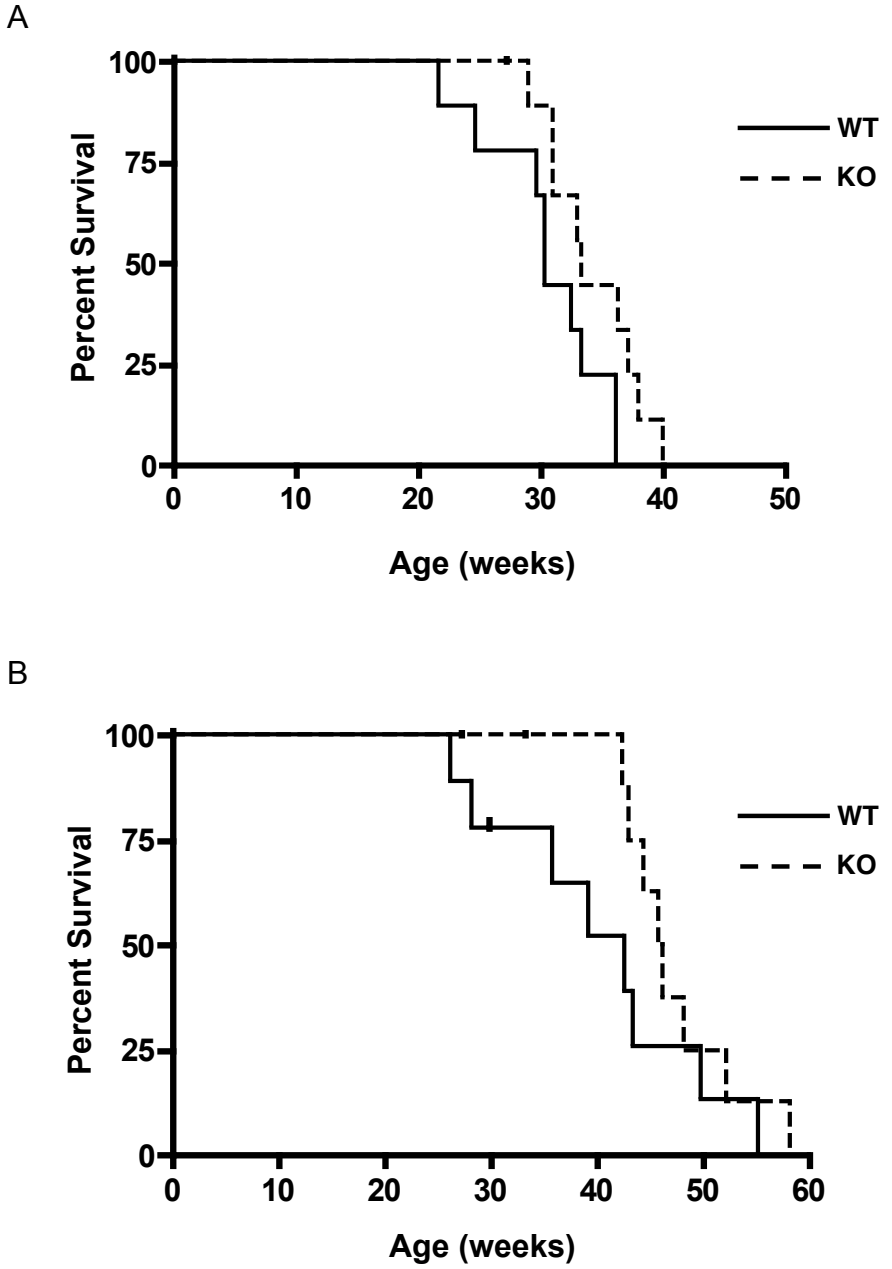

Figure A.1 Survival of c-IAP1-deficient TRAMP mice. Kaplan–Meier curves for tumor onset and overall survival were plotted for c-IAP1-deficient (dotted line) and wild-type (solid line) TRAMP mice. (A) The probability of remaining without tumor is plotted against age in weeks. A two-sided log-rank analysis yielded p-value of 0.045. (B) The probability of survival is plotted against age in weeks, p=0.213.

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