XIAP Promotes the Innate Immune Response during Cytosolic Bacterial Infection

by

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To My Parents and My Husband for their Unconditional Support

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ASC, apoptotic specklike protein with a CARD; BIR, baculoviral IAP repeat; BMDM, bone marrow derived macrophages; CARD, caspase recruitment domain; CFU, colony forming units; c-IAP, cellular inhibitor of apoptosis protein; CTL, cytotoxic T lymphocyte; DAP, diaminopimelic acid; DC, dendritic cell; dIAP, Drosophila IAP; dpi, days post infection; dsRNA, double stranded RNA; ELISA, enzyme linked immunosorbent assay; ERK, extracellular signal-regulated kinase; FADD, Fas-associated death domain; FXR, farnesoid X-activated receptor; HKLM, heat killed *Lm*; i.p., intraperitoneal; i.v. intravenous; IAP, inhibitor of apoptosis protein; IFN, interferon; IL-6, interleukin 6; Imd, Immune deficiency; IRF3, interferon regulatory factor 3; JNK, Jun N-terminal kinase; LLO, listeriolysin O; *Lm, Listeria monocytogenes*; LPS, lipopolysaccharide; LRR, leucine rich repeats; LXR, Liver X-activated receptor; MAPK, mitogen activated protein kinase; MCP, monocytes chemotactic protein; MDP, muramyl dipeptide; MEKK, MAP/ERK kinase kinase 2; MKP, MAP kinase phosphatases; MOI, multiplicity of infection; NAIP5, NLR family apoptosis inhibitory protein 5; NBS-LRR, nucleotide binding site-leucine rich repeat; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; NK, natural killer cell; NKTC, natural killer T cells; NLR, NOD-like Receptor; NOD, nucleotide oligomerization domain; PAMPs, pathogen associated molecular pattern; PRR, pattern recognition receptors; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; RICK, RIP-like interacting caspase-like apoptosis regulatory protein kinase; RING, really interesting new gene; RIP1, receptor interacting protein 1; RIP2, receptor interacting protein 2 (RICK); RLR, RIG-I like receptor; RNA, ribonucleic acid; SAP, SLAM associated protein; ssRNA, single stranded RNA; *St, Salmonella enterica Typhimurium*; TAB, TAK binding protein; TAK, TGFβ associated kinase; TipDC, TNF inos producing DC; TIR, Toll-interleukin-1 receptor domain; TLR, Toll-like Receptor; TNF, tumor necrosis factor; TRAF6, tumor necrosis associated factor 6; UBA, ubiquitin associated domain; XIAP, X-linked inhibitor of apoptosis; XLP, X-linked lymphoproliferative syndrome.

Abstract

XIAP Promotes the Innate Immune Response during Cytosolic Bacterial Infection

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The role of the innate immune system is to coordinate recognition and control of invading pathogens, and to instruct the development of adaptive immunity. Pathogens are detected by pattern recognition receptors on membranes or in the cytosol of host cells. The Toll-like receptor (TLR) family senses pathogens at the plasma membrane or within the vacuole, while surveillance of the cytosol is provided by the NOD-like receptors (NLR) and RIG-I-like receptors. The TLR family is well characterized, however the proteins involved in detection of intracellular pathogens and activation of the innate immune signaling pathways have only recently been described. We performed an affinity-based method to identify components of the cytosolic innate immune signaling pathway that associated with *Listeria monocytogenes* (*Lm*), an intracellular pathogen. We identified several classes of candidate proteins, and determined that one protein, the X-linked inhibitor of apoptosis (XIAP), is critical for *in vivo* innate immunity to *Lm* infection. Mice deficient in XIAP display a greater susceptibility to *Lm* infection. In response to cytosolic *Lm*, XIAP enhanced signaling through the NF-κB and Jun N-

terminal kinase (JNK) pathways. Additionally, XIAP promoted maximal production of pro-inflammatory cytokines upon bacterial infection *in vitro* or *in vivo*, or in response to combined treatment with Nod2 and TLR2 ligands. *In vivo,* we observed that XIAP regulates the expression of proinflammatory cytokines and is required for proper trafficking of *Lm* infected phagocytes to the white pulp of the spleen. Taken together, our results indicate that XIAP regulates the cytosolic innate immune response to *Lm* infection by promoting production of proinflammatory cytokines and coordinating TLR and NLR signaling. XIAP enhances proinflammatory cytokine production *in vivo*, promoting control of *Lm* replication and trafficking of infected phagocytes to the T cell zone of splenic follicles.

Chapter 1 : Introduction

The cells of the innate immune system, such as macrophages and neutrophils are the first responders to microbial invasion. They detect invading microbes, activate antimicrobial responses and regulate the development of adaptive immunity. The innate immune system detects pathogens using extracellular and intracellular pattern recognition receptors (PRR). The Toll-like receptor (TLR) family senses pathogens at the cell surface, by recognizing pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), peptidoglycan, lipotechoic acid and flagellin. Surveillance of PAMPs in the cytosol is provided by two families of sensors: the NOD-like receptors (NLR) and RIG-I-like receptors (RLR). The TLR family is well characterized and many of the microbial components recognized by each receptor are known. However, the proteins involved in detection of intracellular pathogens and activation of the innate immune signaling pathways have only recently been described and thus much remains unknown about regulation of cytosol specific innate immunity. The goal of my thesis work was to further define these cytosolic signaling pathways and examine how the host cell integrates extracellular (TLR) and cytosolic (NLR) signaling in response to infection.

Many pathogens can evade aspects of the immune response by growing intracellularly, however, the host can still resolve many infections, therefore, it is likely that the cytosolic

surveillance system plays an important role in host protection. Recognition of intracellular bacteria and other PAMPs could be important for several reasons: **1)** it may play a role as a failsafe detection mechanism for pathogens that limit recognition by the TLRs; **2)** cytosolic detection of pathogens may be an amplification step of the immune response, allowing cells to fine tune their response to pathogens, or may be a second signal necessary as confirmation of infection; **3)** cytosolic anti-microbial mechanisms may only be activated when cytosolic bacteria or viruses are present; **4)** the innate immune response may need intracellular detection mechanisms to aid in expression of microbial antigens in major histocompatibility complexes to alert the immune system to the presence of infected cells; **5)** additionally, cytosolic recognition of pathogens may help to instruct the immune response to develop a Th1/CTL response, which is critical resolving infection by many intracellular pathogens, rather than a TH2 (antibody) response.

Pathogen associated molecular patterns (PAMPs)

Pathogen associated molecular patterns (PAMPs) are conserved motifs that are unique to microorganisms, and are often essential for their survival thus they are highly conserved[1]. PAMPs are produced exclusively by microbes, enabling the host to differentiate self from non-self. Some of these motifs are invariant between microbes of a given class, such as LPS in Gram-negative pathogens, thus allowing the host to employ a limited number of germline encoded receptors to detect microbial infection[2].

An Evolutionary Perspective

Microorganisms affect all living organisms, therefore there is a need for self-defense in order to survive and evolve. This evolutionary pressure led to the refinement of intricate immune mechanisms, starting with the innate immune system and leading to the development of the vertebrate adaptive immune system. The fruit fly, *Drosophila melanogaster,* does not possess an adaptive immune response, and therefore must fight microbial infection solely using an innate immune response. Thus, *Drosophila* has proven to be a powerful genetic system yielding a great deal of information about conserved pathways in innate immunity. The archetypal member of the TLR signaling pathway, Toll, was first identified in *Drosophila*; flies that lack Toll are immunocompromised[3]. In *Drosophila* there are two major pathways that regulate immunity; Toll, which protects against Gram-positive and fungal pathogens, and Immune deficiency (Imd), which protects against Gram-negative pathogens. The primary response induced by microbial recognition is the production of antimicrobial peptides.

Innate immune signaling pathways are well conserved from *Drosophila* to humans [4]. The mammalian TLR pathway is primarily responsible for the detection of extracellular pathogens[5]. The Imd pathway is largely homologous to the mammalian TNF pathway (**Figure 1**). Imd is homologous to the mammalian RIP1 protein involved in TNF signaling. Many of the components of the TNF pathway including RIP, FADD and TAK play a key role in initiating immune responses to cytosolic pathogens[6,7,8]. Thus, in mammalian systems, the Toll pathway primarily detects pathogens at the cell surface, while the homologs of the Imd pathway in mammals are implicated in detecting pathogen components in the cytosol.

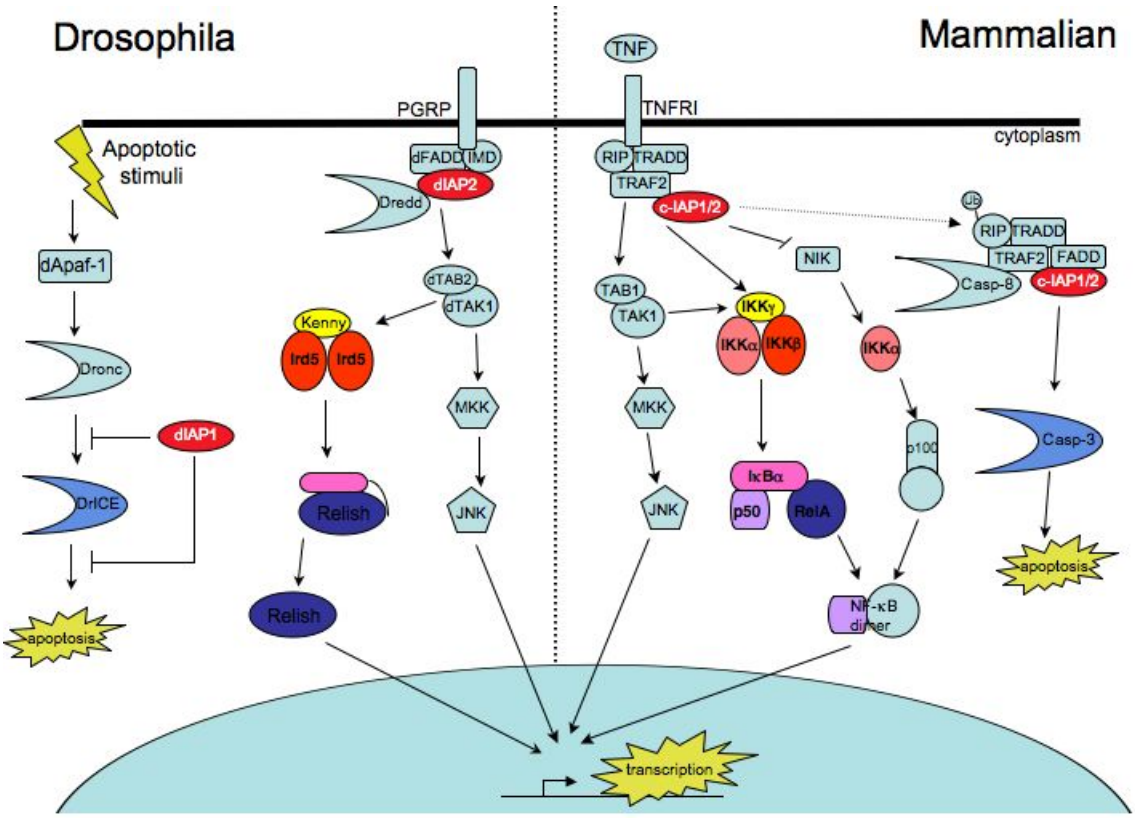


Figure 1.1 Comparison of the Drosophila Imd pathway and the mammalian TNF pathway

Inhibition of apoptosis in *Drosophila* cells by DIAP1 occurs through binding to the initiator and effector caspases, Dronc and DrICE. Similarly, direct binding and inhibition of caspase-3 in mammalian cells is mediated by XIAP. Mammalian c-IAP1 and c-IAP2 can directly bind caspases but are poor caspase inhibitors, instead acting to regulate apoptosis by indirectly modulating caspase-8 activity. Binding of TNF to its receptor results in recruitment of TRADD, RIP, and TRAF2. The c-IAPs also participate in pro-survival signaling through TNFR by associating with TRAF2. c-IAP1 and -2 ubiquitylate RIP1, which minimizes association with caspase-8, preventing apoptosis. Additionally, the association of RIP, TRAF2, and c-IAP1/2 leads to the activation of TAK and subsequent NF-┤ B and JNK activation, resulting in enhanced transcription of pro-survival genes. c-IAP1 and -2 can also inhibit NIK kinase and downstream processing of p100, thereby negatively regulating NF-┤ B activation. Thus, the effects of c-IAP1 and -2-dependent signaling on NF-┤ B are likely defined by context. A TNFR-like pathway regulates immune responses to microbial infection in *Drosophila*. Gram-negative bacteria are recognized by the peptidoglycan recognition proteins (PGRP), which activate the Imd signaling pathway. Imd is an insect homolog of mammalian RIP1. Genetic studies place Imd, dFADD, Dredd and DIAP2 upstream of or parallel to dTAK activation. D-TAK activates both the JNK and Relish pathways analogously to TAK1 in mammalian cells, promoting induction of anti-microbial peptide genes.

Extracellular Detection of Pathogens (TLRs)

The Toll-like receptors (TLRs) play an essential role in recognizing extracellular and vacuolar bound foreign particles and initiating the innate immune response. The TLR family has expanded throughout evolution: *Drosophila* only has one membrane receptor for the Toll pathway, while humans have 10 different TLRs and mice have 12 TLR family members. Mammalian TLR- 3, 7, 8 and 9 are expressed on vacuolar membranes, while TLR- 1, 2, 4, 5, 6 and 10 are expressed on the cell surface[9]. The vacuolar TLRs recognize nucleic acids including dsRNA, ssRNA, and CpG DNA motifs. The cell surface TLRs recognize many bacterial ligands, including lipopeptides, LPS and flagellin, as well as some endogenous ligands[2]. The TLR proteins are transmembrane receptors containing 19-25 extracellular leucine rich repeats (LRR) and an intracellular Toll/interleukin-1 receptor (TIR) domain[10]. The LRR domain forms a horseshoe shaped structure that provides binding sites for PAMPs. The ability of the TLRs to recognize a wide variety of PAMPS lies in their marked deviation from the LRR consensus sequence, the most crucial deviation being insertions, which are commonly the sites of PAMP recognition[9]. Ligand binding by the LRR triggers dimerization of several TLR proteins and induces conformational changes, which allows the cytosolic TIR domains to recruit adaptor molecules inducing a phosphorylation cascade (**Figure 2**). Phosphorylation of NF-κB and the MAP kinase family leads to activation and translocation of transcription factors to the nucleus, where they induce transcription of costimulatory molecules and proinflammatory cytokine genes, including TNF and IL-6[10].

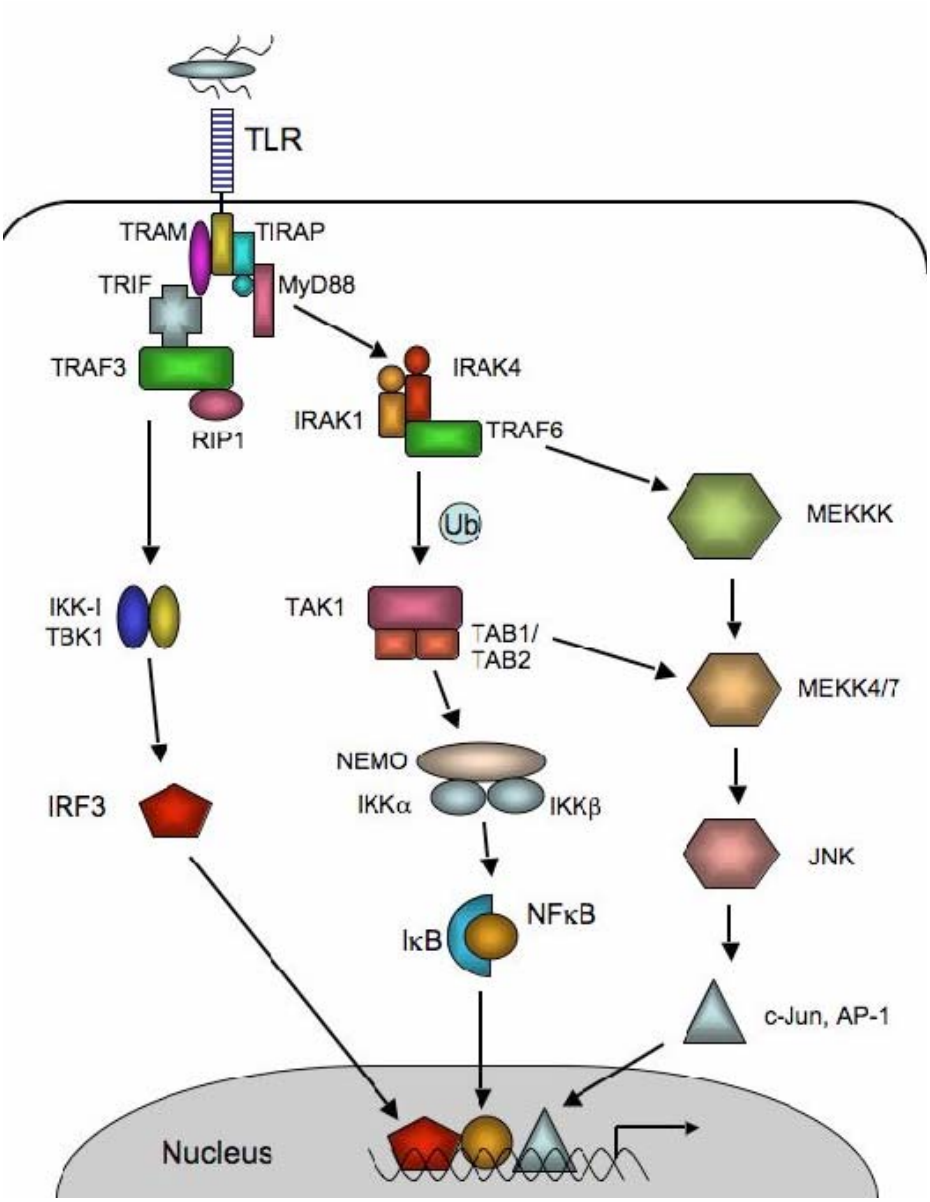


Figure 1.2 TLR signaling pathway

Ligand-binding to TLR recruits the intracellular adaptor molecules Toll-interleukin 1 receptor (TIR) domain-containing adapter protein (TIRAP) and MyD88 to the cytosolic TIR domain of the receptor. IRAK4 and IRAK1 then associate with the complex activating TRAF6. TRAF6 recruits and activates TGF-B activated Kinase 1 (TAK1). TAK1 forms a complex with the TAK binding proteins TAB1, TAB2 and TAB3, and then activates the IKK complex. IKK phosphorylates the IκB family and leads to the activation of NF-κB. TAK1 also activates members of the MAP kinase family including JNK, p38 and ERK. TLR4 can also signal through a complex of TRAF3, TRIF, TRAM and RIP1 to activate TANK binding kinase 1 (TBK1) and subsequently the transcription factor IRF3. Activation of NF-κB, IRF3 and the MAP kinase family leads to activation and translocation of transcription factors to the nucleus.

Cytosolic Detection of Pathogens (NLRs and RLRs)

A primary function of the NLR family, which includes more than 23 proteins, is to sense cytosolic PAMPs. Nod1 and Nod2 were the first described NLR proteins, identified based upon their homology to plant NBS-LRR proteins. The NBS-LRR proteins are thought to act as guard proteins in plants that protect against pathogens[11]. The NLR proteins contain a LRR domain which mediates ligand sensing, a central nucleotide oligomerization domain (NOD) responsible for protein-protein interactions with other NOD domain containing proteins, and a domain for the initiation for signaling such as caspase recruitment domain (CARD), PYRIN or baculoviral inhibitor of apoptosis repeats (BIR) domain[12]. Similar to the TLR proteins, the LRR domain of the NLR proteins is thought to recognize microbial products, triggering oligomerization via the NOD domain to activate a cellular response governed by the signaling domains.

The two most well characterized NLR proteins, Nod1 and Nod2, participate in innate immune sensing of diaminopimelic acid (DAP) and muramyl dipeptide (MDP) containing motifs respectively, which are components of peptidoglycan found in the bacterial cell wall[13,14]. DAP is primarily found in Gram-negative organisms, while MDP is the minimal unit of peptidoglycan in both Gram-positive and Gram-negative organisms. Nod1 and Nod2 signal through a common downstream mediator, RIP2 (RICK), a protein that interacts with IKKγ to activate the NF-κB signaling pathway[15]. Additionally, the Nod1 and Nod2 proteins activate MAPK signaling through CARD6 and CARD9 respectively[16,17]. At the start of this thesis, the other NLR family members

were not well characterized; their ligands and the subsequent signaling pathways that were activated had not yet been identified.

During my thesis research, a more detailed model of cytosolic innate immune signaling has emerged. There are two different models of innate immune activation by NLRs in response to intracellular bacteria; Nod1 and Nod2 primarily activate NF-κB and MAPK signaling, whereas many other NLRs activate the inflammasome and NF-κB (**Figure 3**) [18]. The inflammasome is a cytosolic complex of proteins that is assembled in response to cytosolic bacterial PAMPs and membrane perturbation[19]. The pivotal enzyme in the inflammasome is caspase-1, which is activated by NLR proteins[20]. Cleavage activates caspase-1 allowing it to process the immature pro-inflammatory cytokines, pro IL-1β and pro IL-18, to their mature and active forms; additionally, caspase-1 can induce an inflammatory cell death. TLR signaling induces the transcription and translation of pro-IL-1β, however a second activation event must activate caspase-1 in order to get cleavage and secretion of mature IL-1β. This observation indicates that the intracellular pathogen detection systems synergize with the TLRs to amplify cytokine and chemokine production, increasing the stimulatory environment that instructs the cells of the adaptive immune response[21].

The RIG-I like family of receptors (RLR) include RIG-I and Mda5, two RNA helicases that have recently been identified as essential components for the innate antiviral response[22,23]. Upon recognition of ssDNA or dsDNA in the cytosol, these viral sensors trigger activation NF-κB and IRF transcription factors resulting in antiviral

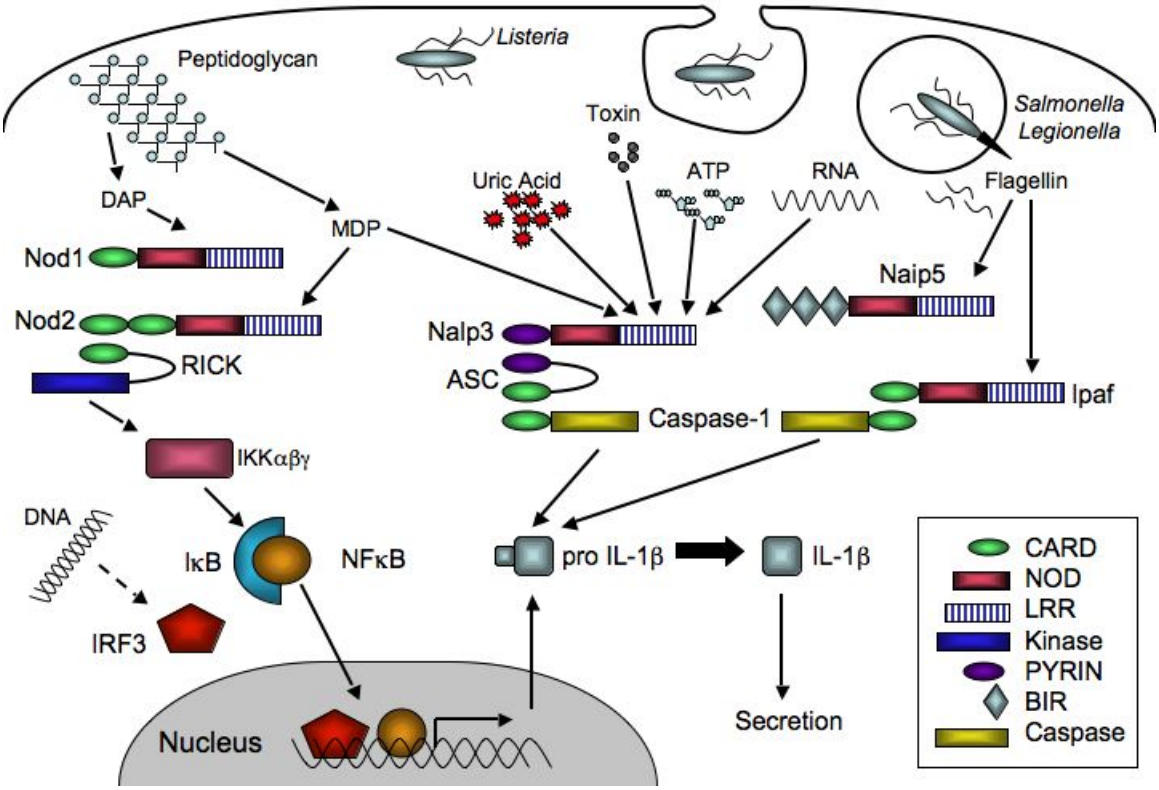


Figure 1.3 The NLR signaling pathway

Intracellular pattern recognition receptors detect the presence of bacterial components in the cytosol, resulting in the activation of a pro-inflammatory response. Nod1 and Nod2 sense peptidoglycan-derived muropeptides in the cytosol and form a complex with RICK (RIP-like interacting caspase-like apoptosis regulatory protein kinase; also known as RIP2). Activation of RICK leads to NF-κB translocation to the nucleus to induce transcription of cytokine genes. Nalp3 recognizes MDP and bacterial RNA, as well as endogenous uric acid crystals and high concentrations of ATP in the cytosol (Nalp1b, which responds to anthrax lethal toxin, is not shown). Activation of Nalp3 results in the formation of the inflammasome (which includes Nalp3, apoptosis-associated speck-like protein containing a caspase-recruitment domain, and caspase-1), inducing cleavage and activation of caspase-1. Active caspase-1 cleaves pro-IL-1β into its mature form, IL-1β, which is then secreted. Naip5 and Ipaf sense cytosolic flagellin and activate caspase-1. Cytosolic DNA is sensed by an unknown receptor, activating the transcription factor interferon regulatory factor 3 (IRF3).

immune responses, including expression of type-1 interferons[24]. Several components of the TNF signaling pathway including FADD and RIP1 are required for induction of type 1 interferon by intracellular dsRNA stimulation[6]. The involvement of FADD and RIP1 in mammalian cytosolic immunity is evidence that the Imd signaling pathway of *Drosophila* has likely evolved in mammals to protect cells against intracellular pathogens.

Immunity to *Listeria monocytogenes*

Immunologists and microbiologists alike have employed *Listeria monocytogenes (Lm)* to study the interaction between the host and pathogen. *Lm* is amenable to genetic manipulation; since it can infect the mouse, the immune response triggered by *Lm* in the murine model of infection is very well characterized. *Listerial* species are commonly found in the environment and can be carried by a number of mammalian and avian species, including in the gastrointestinal tract of 5-10% of the human population[25]. Humans are exposed to *Lm* by ingesting contaminated food products such as unpasteurized dairy products and incompletely cooked meat. Immunocompromised individuals and infants are susceptible to infection by *Lm*, where it most commonly causes septicemia and meningitis, but can also lead to septic abortion[25].

The majority of *Lm* infection studies in the mouse do not occur via the normal gastrointestinal route of infection but rather by intraperitoneal (i.p.) or intravenous (i.v.) injection of the bacteria. Thus, the majority of our knowledge about the immune response to *Lm* is from systemic infections, where *Lm* is initially taken up by phagocytes.

Two main sites of *Lm* replication in the mouse are in the liver and spleen. Due to the availability of well defined mutants and the wealth of information know about the immune response to *Lm*, we chose to use it as a model system to study the cytosolic innate immune response.

Lm can be taken up by phagocytosis or can induce its own uptake by secreting an invasin protein, internalin A. Binding of internalin A to E-cadherin on epithelial cells initiates phagocytic uptake[26]. After bacterial entry, *Lm* is then able to escape from the vacuole by secreting listeriolysin O (LLO) and the phospholipases C, which induce vacuolar membrane damage (**Figure 4**) [27,28]. Upon rupture of the vacuole, *Lm* gains access to the cytosol, which upregulates production of the surface protein, ActA, which nucleates host actin polymerization[29]. Actin-based motility propels the bacteria through the cytosol. When bacteria encounter the cell membrane, they can protrude from the cell and be ingested by neighboring cells, allowing *Lm* to spread from cell to cell without being exposed to the extracellular environment.

The innate immune response is critical for controlling *Lm* replication during the early phase of infection[30]. The adaptive immune response, specifically CD8+ T cells, is essential to achieve clearance of *Lm*[31,32,33]. *Lm* has several PAMPs, including lipotechoic acid, flagellin and MDP, which trigger innate immune recognition of the pathogen. TLR and NLR stimulation by *Lm* induces activation of the NF-κB and MAP kinase signaling pathways, resulting in the induction of a proinflammatory response[34]. NLR activation also leads to assembly of the inflammasome[35]. Upon cytosolic

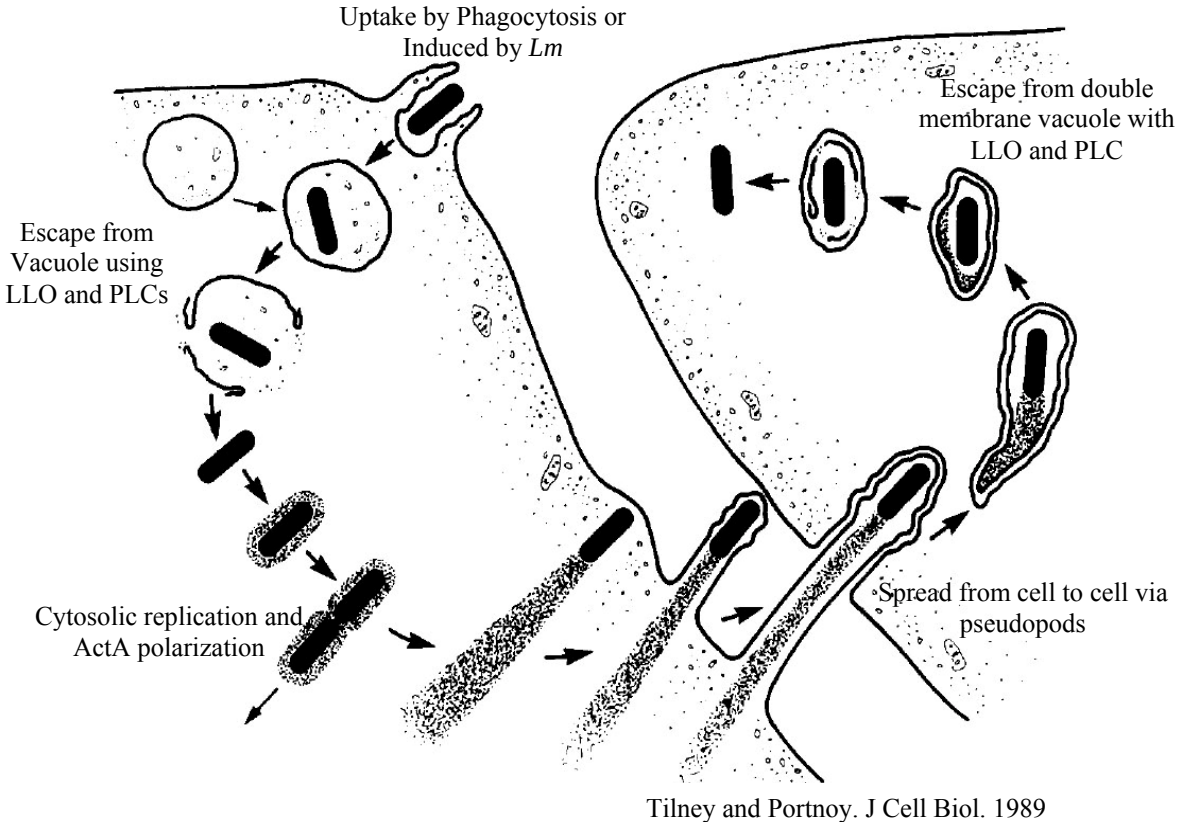


Figure 1.4 *Listeria monocytogenes* Lifecycle

localization of *Lm*, a distinct pattern of gene expression is rapidly induced, which includes a number of proinflammatory cytokine genes. For example, the interferon beta (*ifnb*) gene is induced by cytosolic bacteria but not by vacuole bound bacteria[34]. Additionally, TLR activation leads to the upregulation of *il1b* gene expression; however, cleavage and secretion of IL-1β only occur after cytosolic receptor induced inflammasome activation. Mice deficient in the adaptor proteins Myd88 or RICK, critical adaptors for the TLR and NLR pathways, are very susceptible to *Lm* infection, indicating the importance of intracellular and extracellular recognition of this pathogen for control and clearance by the immune system[7,36].

Upon pathogen detection, the host sets up a state of inflammation, recruiting innate immune cells to the site of infection. Recognition of *Lm* by the TLRs and NLRs induces the production and secretion of several proinflammatory cytokines including Interferon- γ (IFNγ), Interluekin-6 (IL-6), Interleukin-12 (IL-12), Interleukin-1β (IL-1 β) and Tumor Necrosis Factor-α (TNF) and several adhesion molecules[25,37]. These proinflammatory cytokines promote recruitment and activation of innate immune cells including macrophages, dendritic cells (DC), natural killer cells (NK cells) and neutrophils[38]. The adhesion molecules direct phagocyte trafficking to the spleen and lymph nodes where they can present antigens to the T cells and B cells that make up the adaptive arm of the immune response[39]. CD8α+ DC are required for early *Lm* entry into the spleen, suggesting that they are the major population of cells trafficking *Lm* from sites of infection to the spleen[40]. Once in the spleen, *Lm* is observed in the T cell zone of lymphoid follicles, presumably in phagocytes, where other immune cells including

dendritic cells and neutrophils are recruited to fight the infection and aid in presentation of antigen to T cells[41].

In response to infection by *Lm*, a series of coordinated interactions occur between the cells of the innate immune system resulting in proinflammatory cytokine production and subsequent immune cell activation. Cells infected directly by *Lm*, such as macrophages and epithelial cells, respond by producing several proinflammatory cytokines including IL-6, TNF and IL-12[36,42,43]. TNF, IL-12 and IL-18 activate NK cells to produce IFNγ, which synergizes with TNF to enhance the microbicidal activity of macrophages[38]. Activation of macrophages upregulates expression and activity of inducible nitric oxide synthase and NADPH oxidase, the enzymes responsible for producing nitric oxide and superoxide. respectively. Both the oxidative burst of macrophages and the production of nitric oxide are critical to controlling *Lm* infection, specifically through their microbicidal effects on *Lm* in the vacuole, which prevents vacuolar escape[44,45,46,47]. IL-6 enhances the production of IFNγ as well as promoting recruitment of neutrophils to sites of infection[48,49]. Crosstalk between innate immune cells is critical for proper function and pathogen control.

One of the common themes that is emerging is the importance of spatial positioning in the developing immune response. One potential role of cytosolic immunity is to enhance signaling in cooperation with the TLR signaling pathway, as seen for IL-1β signaling; however, the proteins involved in this synergy and regulation are unknown. In the cytosol, *Lm* triggers a specific pattern of genes that is responsible for control of infection.

LLO-deficient *Lm,* which are trapped in the vacuole and therefore do not trigger cytosol specific genes, also do not promote protective immunity, suggesting that the cytosol specific response to infection is critical to protect the host from future infection[50]. Some of the cytosol specific genes triggered, such as *ifnb*, are not induced by any of the NLR proteins known to respond to *Lm*. Therefore, there are additional uncharacterized pathways triggered by cytosolic *Lm*[51]. It has been hypothesized that the defect in protective immunity during an LLO-deficient *Lm* infection is due to a mislocalization of the infected phagocytes. Cell harboring vacuolar *Lm* are not recruited to the T cell zone of the splenic follicles but are instead found in the marginal zone. To clarify some of these questions, my thesis research focused on defining the molecular requirements for the cytosolic immune response to *Lm* infection.

Chapter 2 : Identification of host cytosolic proteins that associate with the surface of *Listeria monocytogenes*

ABSTRACT

The innate immune system is responsible for early detection of pathogens and controlling the infection, while instructing the development of the adaptive immune system. Pathogens are detected by pattern recognition receptors on membranes or in the cytosol of host cells. Many pathogens limit extracellular detection by the immune system by growing intracellularly, however pathogens or pathogen associated molecular patterns (PAMPs) can be recognized by cytosolic Nod-like receptors (NLR) or Rig-I like receptors (RLR). Here we describe an approach designed to identify components of the cytosolic innate immune signaling pathway that associate with cytosolic *Listeria monocytogenes* (*Lm*). We identified several classes of candidate proteins, and determined that one protein, the X-linked inhibitor of apoptosis (XIAP), is critical for *in vivo* innate immunity to *Lm* infection.

INTRODUCTION

The innate immune response has two main functions: to detect and control initial levels of bacterial infection, and to instruct the adaptive immune response by presenting antigen in an activating context. The immune system recognizes extracellular pathogens via the

Toll-like Receptors (TLRs) and intracellular pathogens via the Nod-like Receptors (NLRs) or Rig-I like Receptors (RLRs). These families of sensors recognize pathogen-associated molecular patterns (PAMPs) that are characteristic of microbial organisms, such as lipopolysaccharide or peptidoglycan[19]. Recognition of these components by the host cell initiates signaling cascades that result in the induction of MAP kinase and NF-κB pathways, leading to production of proinflammatory cytokines and other costimulatory molecules[12]. The TLR signaling cascades have been extensively characterized, but the cytosolic NLR and RLR pathways are less well understood[52]. Additionally, when both TLR and NLR ligands are introduced simultaneously, some reports suggest that the TLR and NLR pathways synergize to enhance the immune response, but the mechanisms that govern synergy between these two pathways are poorly understood[53].

Listeria monocytogenes (Lm) is commonly used as a tool to study the immune response because *Lm* is amenable to genetic manipulation and the mouse model of infection is very well characterized[25]. The experimental results of these infections can be more easily interpreted as they can be fit into a larger context of knowledge already gained from similar experiments. Since *Lm* is intracellular, the bacterium is exposed to the extracellular space upon initial infection; thereafter, it is protected from humoral immunity by spreading from cell to cell. Therefore, the host must develop a cell-mediated, cytotoxic T cell immune response to clear the infection[33].

The immune response to infection is initiated by innate immune recognition of PAMPs. *Lm* has several PAMPs that may induce the innate immune response, including flagellin, peptidoglycan, lipotechoic acid, and lipoproteins[54,55,56]. PAMP recognition is critical to controlling the infection, as TLR2-deficient mice are more susceptible to infection, displaying reduced cytokine and costimulatory molecule production[54]. MyD88, a TLR adaptor protein, is crucial for initiating the immune response to *Lm*[36]. Additional TLRs likely also play a role in detecting *Lm,* as Myd88-deficient mice are more susceptible to infection than TLR2-deficient mice[54]. Activation of NLR proteins can trigger three main pathways, either NF-κB and MAP kinase signaling or activation of the inflammasome. *Lm* infection can induce all three pathways. Two NLR proteins, Nod1 and Nod2, are activated by cytosolic *Lm* inducing the NF-κB and MAP kinase pathway[57,58]. However mice deficient in both Nod1 and Nod2 do not display enhanced susceptibility to systemic infection, indicating that neither of these NLRs is solely responsible for the cytosolic sensing of *Lm*[59]. Additionally, several NLRs are responsible for inflammasome activation upon *Lm* infection including NALP3, Ipaf and another unknown receptor that requires ASC[60]. While much is known about detection of *Lm* by the innate immune system, it is clear that there are still sensors important for recognizing *Lm* that are not yet identified.

The immune response to *Lm* infection is complex, both innate and adaptive immunity are required for clearance. Upon infection, *Lm* is initially taken up into a vacuole, which it escapes by secreting listeriolysin O (LLO), a pore forming protein. The wildtype strain of *Lm* stimulates both the extracellular TLR and cytosolic NLR signaling pathways upon

infection[54,57]. However, a strain deficient in LLO is unable to gain access to the cytosol and thus will only stimulate the TLR immune response[25]. Studies comparing the immune response induced by these two different stains indicate that specific patterns of gene expression are induced upon cytosolic localization of wildtype *Lm* that differ from the patterns seen after infection with the vacuolar, LLO-deficient strain of *Lm*[34,61]. To identify some of the host cell components that contributed to the cytosolic immune response to *Lm* infection, we developed an assay to isolate proteins from the host cell cytosol that associated with the surface of *Lm*. We identified a number of proteins that associated with *Lm* that could be categorized into three main groups: those associated with TGFβ signaling, with TNF signaling, or those involved in ubiquitin modification pathways.

RESULTS

Identification of host cytosolic proteins that associate with *Listeria monocytogenes*

Since differential immune responses are induced by vacuole-bound bacteria and cytosolic bacteria, and bacteria that gain access to the cytosol are largely intact, I hypothesized that the host could recognize components of the bacterial surface. Since cytosolic *Lm* rapidly replicate, it is unlikely the bacteria were damaged in the vacuole. Therefore, non-surface exposed bacterial components would likely be shielded from the immune system and unable to trigger the initial cytosolic immune response. Bacterial components degraded in the vacuole can be released into the cytosol by an unidentified mechanism and recognized by the NLR proteins; however, immune detection by this mechanism is delayed compared to the immediate response observed upon bacterial release into the

cytosol[55]. Preliminary studies suggested that the cytosolic immune response did not respond to *Lm* proteins, as macrophages scrape loaded with bacterial lysates that were digested with proteinase K were still able to induce cytosol-specific gene expression (M. O'Riordan, unpublished observations). Since the immune response to cytosolic *Lm* has been shown in several different cell types, we isolated cytosolic proteins from primary macrophages and two different epithelial cell lines, Caco2 and HeLa cells. Our affinity-based assay was developed in a manner that promoted the identification of cytosolic innate immune components and minimized the identification of proteins that non-specifically associated with *Lm* (**Fig. 2.1**). We observed several different protein bands on Coomassie stained gels that consistently associated with *Lm* in our affinity-based assay (**Fig 2.2, 2.3**). After determining the identity of these bands by mass spectroscopy, we had a number of candidate proteins that might regulate the innate immune response to cytosolic *Lm* (**Fig 2.4, 2.5, 2.6**).

Interestingly, many of the proteins identified could be classified into one of three groups: components of the TGFβ signaling pathway, the TNF signaling pathway or proteins involved in ubiquitin modification, such as ABIN, Smad9, and SNX6. TGFβ is an immunosuppressive molecule that aids in resolving innate immune responses[63]. Two of the other proteins identified from the macrophage assay were members of the IAP protein family that regulate TNF signaling, c-IAP1 and c-IAP2[62]. TNF is an immunostimulatory molecule that is proinflammatory and functions to enhance the innate immune response[64]. Additionally, the TNF pathway is homologous to the *Drosophila* Imd pathway, which regulates the immune response to Gram-negative pathogens. Many

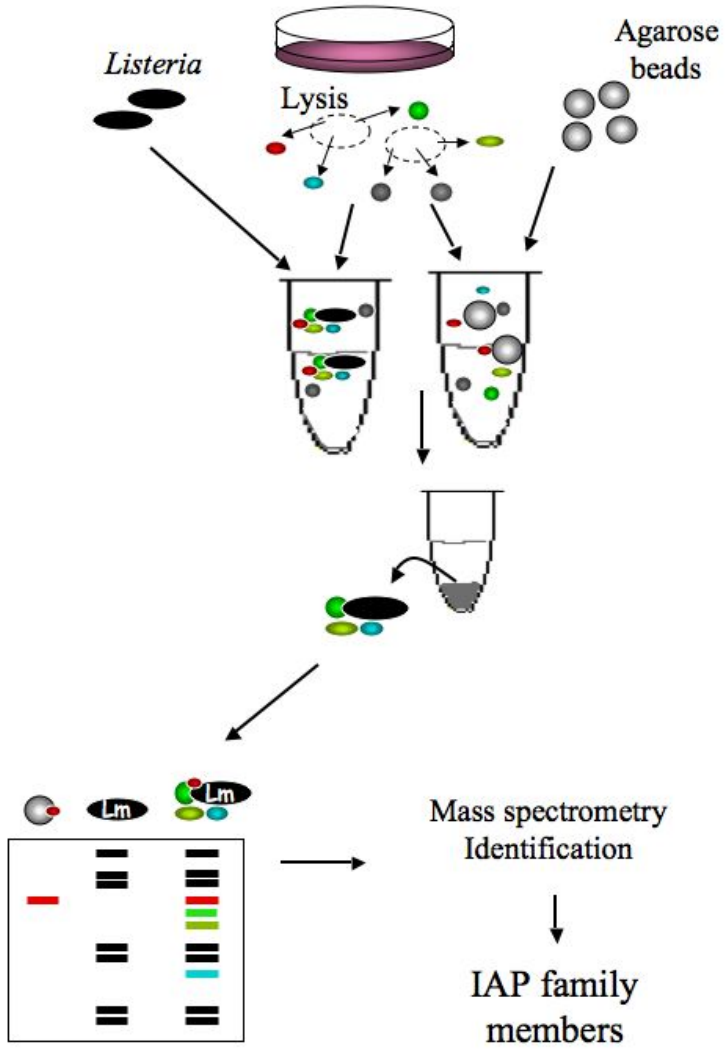


Figure 2.1 Affinity Association Assay.

Host cells were lysed, nuclei and membranes were removed by centrifugation. Cytosolic protein lysate was incubated with agarose beads for 1h prior to remove any proteins that bound non-specifically. *Lm* was boiled in SDS-PAGE running buffer to remove bacterial surface proteins. The cytosolic protein lysate was then incubated with boiled *Lm* or protein A/G beads overnight. Beads or *Lm* were pelleted and washed to remove any unbound proteins. The resulting proteins were separated by SDS-PAGE gel electrophoresis and visualized by Coomassie blue gel stain. Proteins were identified by Mass Spectrometry.

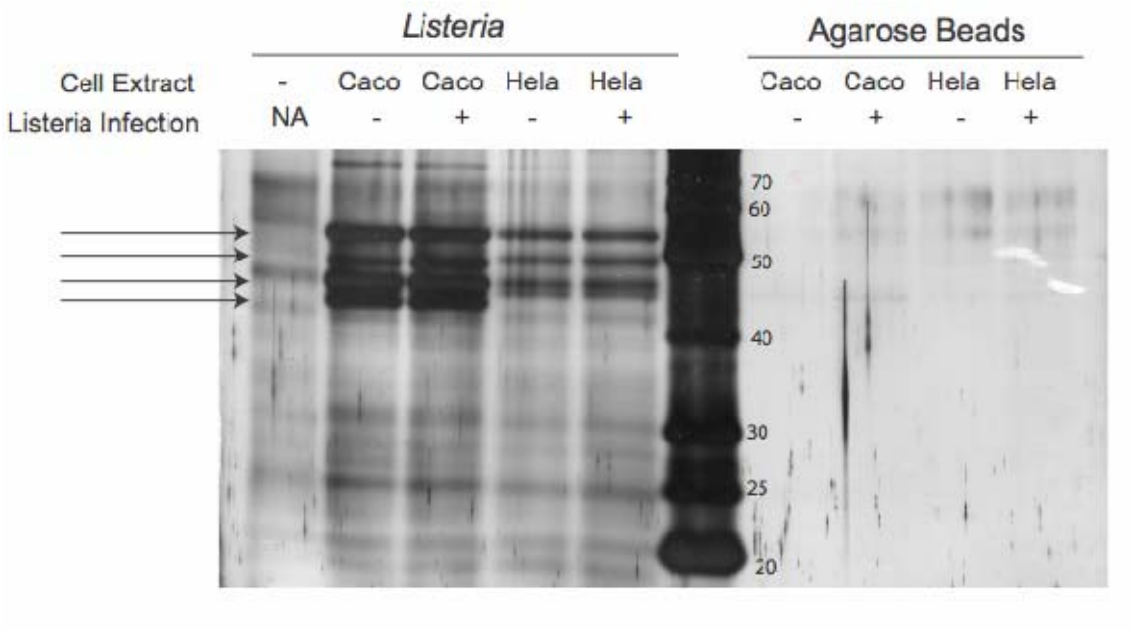


Figure 2.2 HeLa and Caco2 binding proteins isolated on Coomassie-stained gels.

Listeria monocytogenes associated cytosolic proteins were isolated by an affinity-based assay. Cytosolic proteins were isolated from host cells that were uninfected or infected with *Lm* to enhance expression of any proteins upregulated upon infection. Lysates were incubated with *Lm* or protein A/G agarose beads. The bacteria were pelleted and washed to remove any proteins that were not associated with the bacterial pellet. Isolated proteins were run on a 10% acrylamide gel and stained with Coomassie blue. Agarose beads were used as a negative control, to enhance stringency of the assay since anything that bound to agarose beads would likely be inherently sticky and not likely to regulate the immune response to cytosolic *Lm*. Bands identified with arrows were isolated and identified by mass spectroscopy.

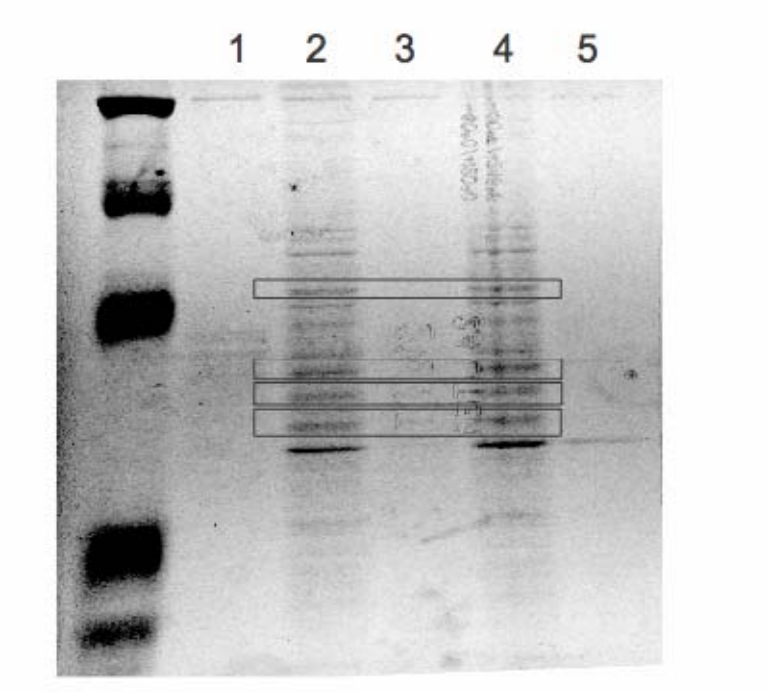


Figure 2.3. Bone marrow macrophage *Listeria monocytogenes* binding proteins.

Listeria monocytogenes associated cytosolic proteins were isolated by an affinity-based assay. Cytosolic proteins were isolated from host cells and incubated with *Lm*. The bacteria were pelleted and washed to remove any proteins that were not associated with the bacterial pellet. Isolated proteins were run on a 10% acrylamide gel and stained with Coomassie blue. (1) *Lm* proteins. (2) Cytosolic proteins from uninfected macrophages incubated with *Lm*. (3) Cytosolic proteins from uninfected macrophages incubated with agarose beads. (4) Cytosolic proteins from macrophages infected for 1h incubated with *Lm*. (5) Cytosolic proteins from macrophages infected for 1h incubated with agarose beads. Bands identified with boxes were isolated and identified by mass spectroscopy.

	Protein Name	Accession Number	Molecular Weight	% Coverage	% Identity
HeLa					
Band 1-55000	Suppressor of cytokine signaling 6	O14544	59525	16%	7/15=46%
	T-complex protein 1 beta subunit	P78371	57489	15%	6/15=40%
	Cytochrome P450 2C10	P11713	55626	10%	6/15=40%
Band 2-53000	Zinc finger protein HRX	Q03164	431895	1%	6/37=16%
	Ryanodine Receptor 3	Q15413	551937	2%	6/37=16%
Band 3-48000	Low density lipoprotein receptor related protein 2 precursor (megalin)	P98164	52193	0%	2/16=12%
	Hurpin	Q9UIV8	44276	16%	5/7=71%
	Arfaptin 1 ADP ribosylation factor interacting protein 1	P53367	42739	17%	4/7=57%
Band 4-45000	TRAF 4 associated factor 2 (sorting nexin 6)	Q9UNH7	46649	20%	7/18=38%
	ubiquitin carboxyl terminal hydrolase 12	O75317	41807	10%	6/18=33%

Figure 2.4 Candidate proteins identified from HeLa cytosolic proteins incubated with *Listeria monocytogenes*

Proteins identified by mass spectroscopy from the bands submitted from HeLa cell *Listeria* associated proteins. Proteins in red are of particular interest based upon their known identify and function. The approximate size of the band isolated is located in the first column next to the band name. The % coverage indicates the portion of the protein that matched the peptides identified by mass spectroscopy. The % identity indicates how similar the peptide sequences were to the protein sequence.

	Protein Name	Accession Number	Molecular Weight	% Coverage	% Identity
Caco2					
Band 1-46000	TRAF 4 associated factor 2 (sorting nexin 6)	Q9UNH7	46649	23%	7/17=41%
	Aspartyl-RNA synthetase	P14868	57137	24%	7/17=41%
	Polyadenylate-binding protein 2 (poly(a) binding protein 2) PABP2	Q15097	58519	22%	7/17=41%
	Zinc finger imprinted 3	Q96PE6	54498	18%	7/17=41%
Band 2-50000	EH domain containing 4	7212811	54565	45%	8/21=38%
	sphingosine-1 phosphate phosphatase	41700844	44741	32%	7/21=33%
	Bile Acid Receptor (Farnesoid X activated receptor) Retinoid x receptor i	Q96RI1	54862	27%	6/21=28%
	DNAJ homolog subfamily A member 3 (hTid-1)	Q96E1	52538	23%	6/21=28%
Band 3-53000	snRNA activation protein complex 43 kDA subunit (SNAPc)	Q16533	42995	22%	7/21-33%
	Telomeric repeat binding factor 2 interacting protein 1 (hRAP1)	Q9NYB0	44260	24%	7/21-33%
	SMAD 8	O15198	52494	19%	7/21-33%
	Heme oxygenase 2 (HO-2)	P30519	36033	15%	7/21-33%

Figure 2.5. Table of *Listeria* associated proteins from Caco2 cytosolic proteins.

Proteins identified by mass spectroscopy from the bands isolated from cytosolic Caco2 proteins by affinity association assays with *Listeria monocytogenes*. Proteins in red are of particular interest based upon their known identity and function. The % coverage indicates the portion of the protein that matched the peptides identified by mass spectroscopy. The % identity indicates how similar the peptide sequences were to the protein sequence.

	Protein Name	Accession Number	Molecular Weight	% Coverage	% Identity
BMMO					
Band 1-55000	XPG domain like KIAAI 838 human protein from brain	26341048	55822	35%	13/37=35%
	Nucleobindin 1 precursor (CALNUC) Calcium binding protein (golgi)	Q02819	53409	22%	11/37=29%
Band 2-70000	Nef-associated factor 1 (A20 binding inhibitor of NFKB) ABIN	Q9WUU8	73050	13%	8/16=50%
	TNFAIP3 interacting protein 1 (Abin1)	14198253	73107	13%	8/16=50%
Band 3-72000	Zinc finger protein ZFP109	5640009	73457	28%	11/33=33%
	T-BRAIN 1 protein (T-box brain protein 1) brachyury homologue	2501123	73941	23%	9/33=27%
	MAIP2 (c-IAP2)	Q62210	69677	21%	8/33=24%
Band 4-75000	MAIP1 (c-IAP1)	O08863	67199	21%	9/29=31%
	Ring finger protein 17	Q99MV7	69627	14%	9/29=31%
	G protein coupled receptor kinase 4	O70291	66912	11%	9/29=31%

Figure 2.6 Table of *Listeria* associated cytosolic proteins from bone marrow derived macrophages.

The proteins were identified by mass spectroscopy, after isolation by affinity association with the surface of *Listeria monocytogenes*. Proteins in red are candidates of interest based on their identity and function. The % coverage indicates the portion of the protein that matched the peptides identified by mass spectroscopy. The % identity indicates how similar the peptide sequences were to the protein sequence.

of the components of the TNF pathway in mammalian cells have been implicated in cytosolic immunity. Our results suggest that we did not simply isolate proteins that are highly abundant in the host cell cytosol, but identified proteins that could be specifically associated with inflammation and immunity.

FXR is not required for innate immunity to *Listeria monocytogenes*

We first examined FXR (farnesoid X-activated receptor) as it is a member of the nuclear bile acid receptors, and LXR, another member of that family, is rapidly upregulated upon cytosolic *Lm* infection[65]. Additionally, mice deficient in LXR are highly susceptible to *Lm* infection, due to increased apoptosis of macrophages. FXR regulates bile acid and cholesterol homeostasis, however its role during the innate immune response to *Lm* is unknown. Interestingly, both LXR and FXR regulate *apoE* gene expression; ApoE-deficient mice are highly susceptible to *Lm* infection, displaying a defect in the innate immune response[66]. We identified FXR from the Caco2 cytosolic proteins that associated with *Lm*; the peptides obtained from mass spectroscopy covered 27% of the protein (**Fig. 2.7**). To examine the ability of FXR to control *Lm* infection, we obtained FXR-deficient mice. FXR-deficient and wildtype mice were infected intraperitoneally (i.p.) with $5x10^5$ CFU of *Lm*. We examined the innate immune response to infection by harvesting at 48h, an early time point during *Lm* infection when the innate immune response plays a critical role in controlling the infection, but prior to the development of adaptive immunity. The spleens and livers were harvested from infected mice at 48hpi and enumerated the recovered CFU (**Fig 2.8**). The wildtype and FXR-deficient mice were able to control infection equally well. Since the LXR-deficient mice were more

Figure 2.7 FXR peptides identified by mass spectroscopy.

Alignment of the 6 peptides of FXR identified from mass spectroscopy of band 2 from Caco2 cells. Several of the identified peptides overlap covering greater portions of the protein.

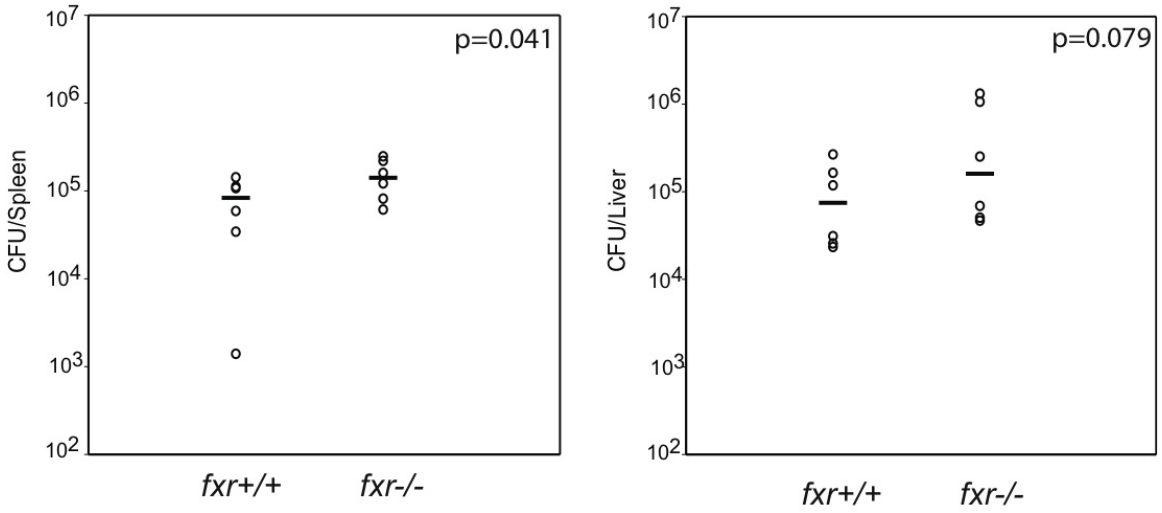


Figure 2.8 FXR is not required for the innate immune response to *Lm* infection

FXR-deficient mice were infected with $5x10^5$ *Lm* i.p., the liver and spleen harvested at 48hpi and CFU were enumerated. Each spot represents an individual animal. The bar indicates the geometric mean of each group. The results are pooled from two individual experiments, and are representative of 3 individual experiments.

susceptible to *Lm* infection, these results suggest that FXR and LXR do not function similarly during *Lm* infection. We concluded that FXR does not play a major role in the innate immune response to *Lm* infection.

c-IAP1 is not required to regulate innate immunity to *Listeria monocytogenes*

The IAP protein family, originally identified as inhibitors of apoptosis, has recently been implicated in a number of other signaling pathways. Further introduction to the IAP proteins can be found in Appendix 1. There is a wealth of literature that implicates c-IAP1, c-IAP2 and XIAP in regulating the NF-κB and MAP kinase signaling pathways, which among other functions are critical regulators of proinflammatory cytokine production by the innate immune system[67,68]. The c-IAP1 and c-IAP2 proteins are components of the TNF signaling pathway responsible for activating NF-κB. Their genes lie in tandem in the genome, and due to their high degree of similarity, likely originated from a gene duplication event[62]. As such, their functions may be partially redundant, although deletion of each gene in animals has uncovered unique roles for these proteins in defined experimental contexts. In mice, c-IAP2 regulates the endotoxic shock response, possibly by preventing apoptosis of macrophages[69]. Both of the c-IAP proteins were identified in our affinity-based assay in macrophages and found peptides that align in both proteins due to their extensive homology (**Fig. 2.9**). We decided to first examine the innate immune response in c-IAP1-deficient animals, as we did not have access to the c-IAP2-deficient animals at that time[70]. Wildtype and c-IAP1-deficient mice were infected i.p. with $5x10^5$ CFU of *Lm*. The liver and spleen were harvested from these animals at 48hpi, and enumerated the CFU recovered (**Fig. 2.10**). We observed

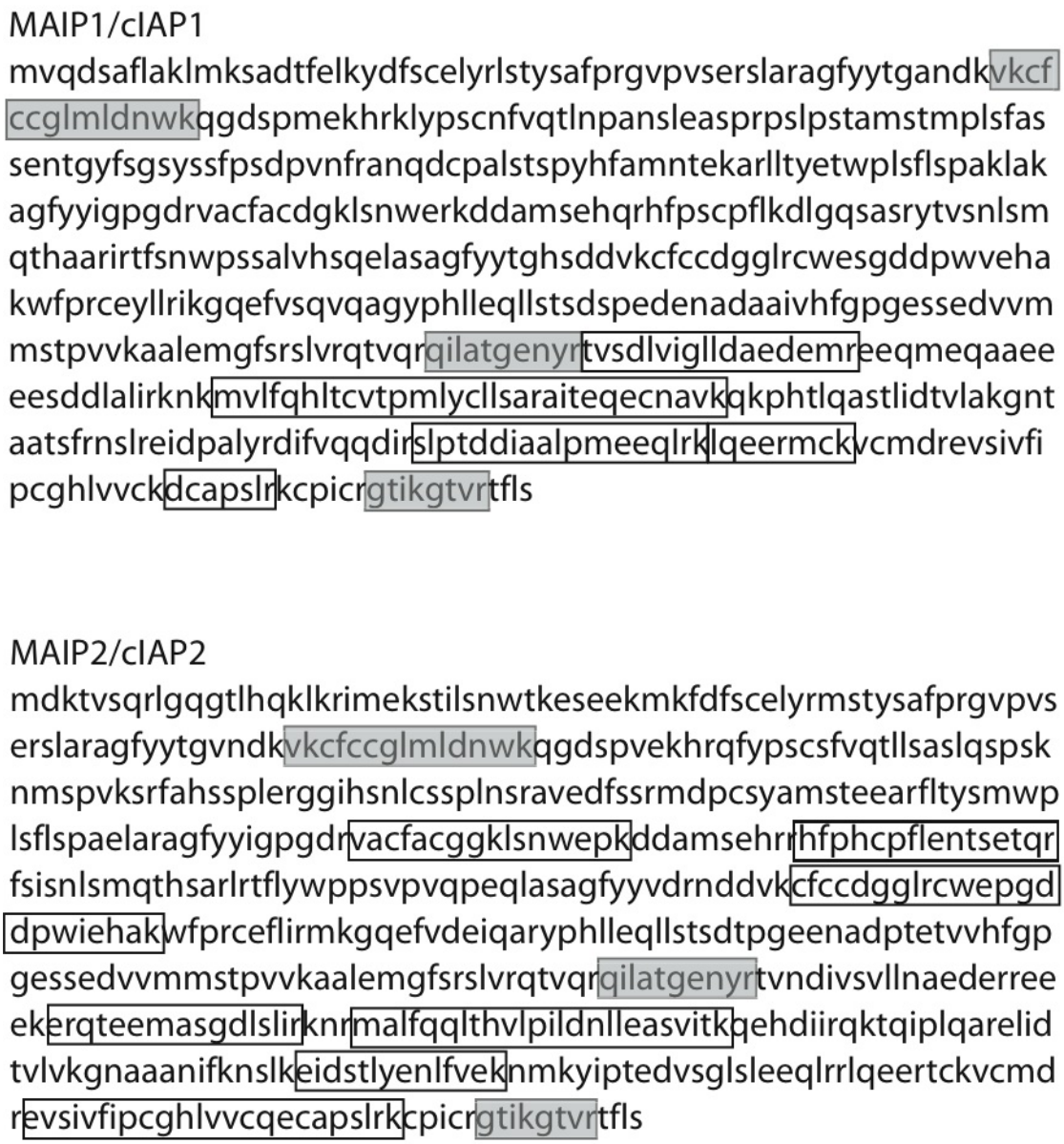


Figure 2.9 Alignment of the peptides identified in bone marrow derived macrophages that correspond to the IAP proteins.

There were 9 peptides identified that corresponded to c-IAP1, several of which overlap. There were 8 peptides that corresponded to c-IAP2. Due to substantial homology between the two proteins, 3 peptide sequences were found in both genes (they are shaded in gray).

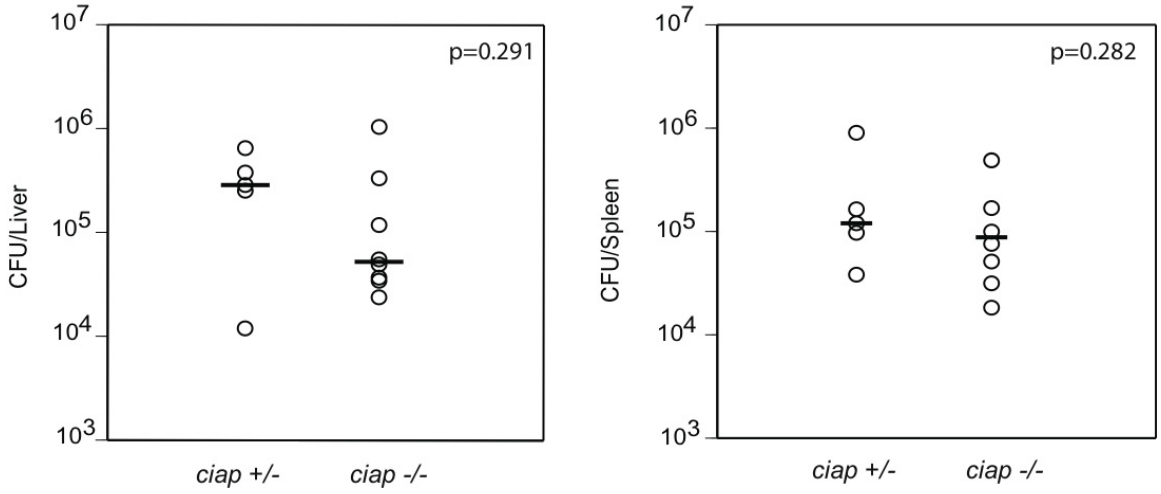


Figure 2.10 c-IAP1 is not required for the innate immune response to *Lm* infection

c-IAP1-deficient mice were infected with $5x10^5$ *Lm* i.p., the liver and spleen harvested at 48hpi and CFU were enumerated. Each spot represents an individual animal. The bar indicates the geometric mean of each group. The results are representative of 2 individual experiments with at least 5 pairs of animals.

similar numbers of *Lm* in the wildtype and c-IAP1-deficient mice, indicating that a deficiency in c-IAP1 does not impair the innate immune response to *Lm* infection. However, these mice still contain an intact c-IAP2 gene, which may compensate for loss of c-IAP1. The c-IAP1/c-IAP2 double deficient mice have not been generated, as it would be very difficult to disrupt both of these tightly linked genes. From this data, we can conclude that in the presence of c-IAP2, c-IAP1 is not required for the innate immune response to *Lm*.

The innate immune response to *Listeria monocytogenes* is regulated by XIAP

Another IAP family protein, XIAP, has a well established role in regulating the NF-κB and MAP kinase pathways and was recently implicated in X-linked lymphoproliferative disease (XLP) in humans, a primary immunodeficiency that is characterized by susceptibility to Epstein Barr virus. We wanted to determine if XIAP might regulate the cytosolic innate immune response to *Lm* infection[71]. XIAP-deficient animals were previously described, and did not display any striking phenotype, thus the role of XIAP was unclear[72]. We infected wildtype and XIAP-deficient animals with $5x10^5$ CFU *Lm* i.p. and harvested the spleens and livers at 2 and 4 days post infection (dpi) to enumerate CFU (**Fig 2.11**). We observed a 10-fold increase in bacterial CFU in the XIAP-deficient animals compared to the wildtype animals at 2dpi, indicating that XIAP regulates the innate immune response to *Lm* infection. At 4dpi, although the bacterial numbers were decreasing, there were still approximately 10 times more bacteria in the XIAP-deficient animals than the wildtype animals. We conclude that XIAP regulates the innate immune response to *Lm* infection.

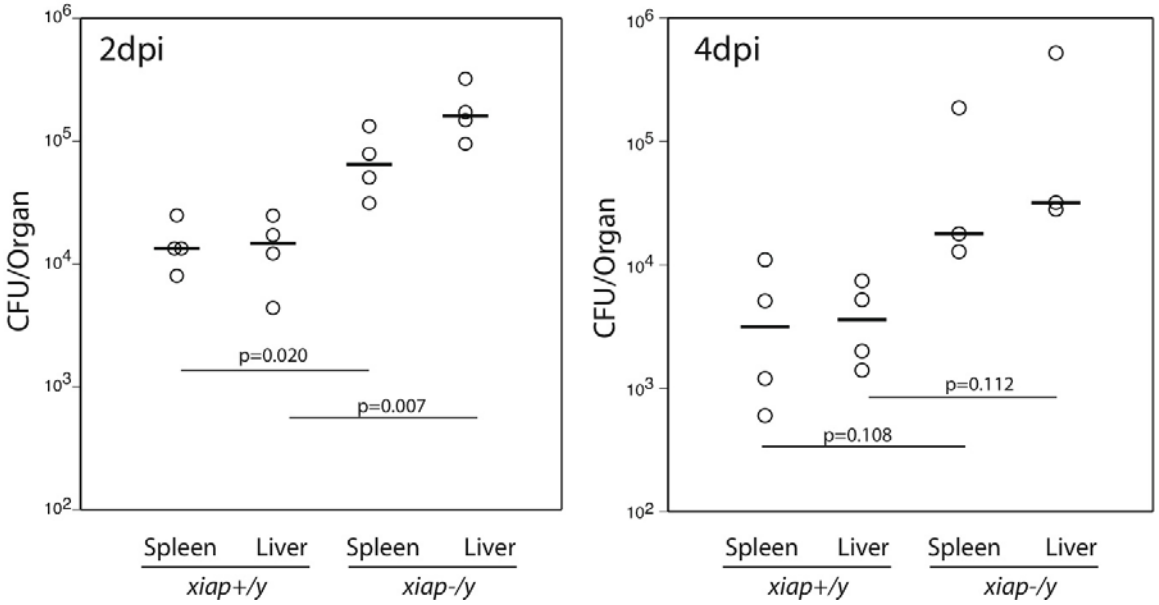


Figure 2.11 XIAP regulates the innate immune response to *Lm* infection

XIAP-deficient mice were infected with $5x10^5$ *Lm* i.p., the liver and spleen harvested at 2 and 4 dpi and CFU were enumerated. Each spot represents an individual animal. The bar indicates the geometric mean of each group.

SAP is not required for innate immune control of *Listeria monocytogenes*

A subset of human patients with XLP have mutations in either XIAP or in SLAM associated protein (SAP), a gene located very close to XIAP on the X chromosome[73]. SAP is an adaptor protein that recruits FYNT, a tyrosine kinase, to the SLAM receptors, resulting in activation of numerous immune signaling pathways in lymphocytes[74]. Since mutations in either SAP or XIAP can cause XLP, and XIAP is critical for regulating the innate immune response to *Lm* infection, we tested whether SAP was also important for the immune response to *Lm*. Wildtype and SAP-deficient mice were infected i.p. with $5x10^5$ CFU *Lm*; at 48hpi, spleens and livers were harvested from these animals to enumerate CFU (**Fig 2.12**). We observed no significant difference in the numbers of CFU recovered from wildtype and SAP-deficient animals. These data indicate that SAP is not a critical contributor to the innate immune response to *Lm* infection. Therefore, we infer that the mechanisms of innate immune regulation by SAP and XIAP are likely different in the mouse model.

XIAP is not required for immunity to *Salmonella enterica Typhimurium*

To determine if the XIAP-deficient mice displayed a general immunodeficiency, we examined the immune response to another intracellular bacterial pathogen, *Salmonella enterica* serovar *Typhimurium (St). St* is a Gram-negative organism that largely resides in the vacuole and causes typhoid disease in mice. We infected wildtype and XIAP-deficient mice with 250 CFU of *St* i.p. and harvested spleens and livers at 48hpi to enumerate the bacterial burden (**Fig 2.13**). We recovered similar numbers of

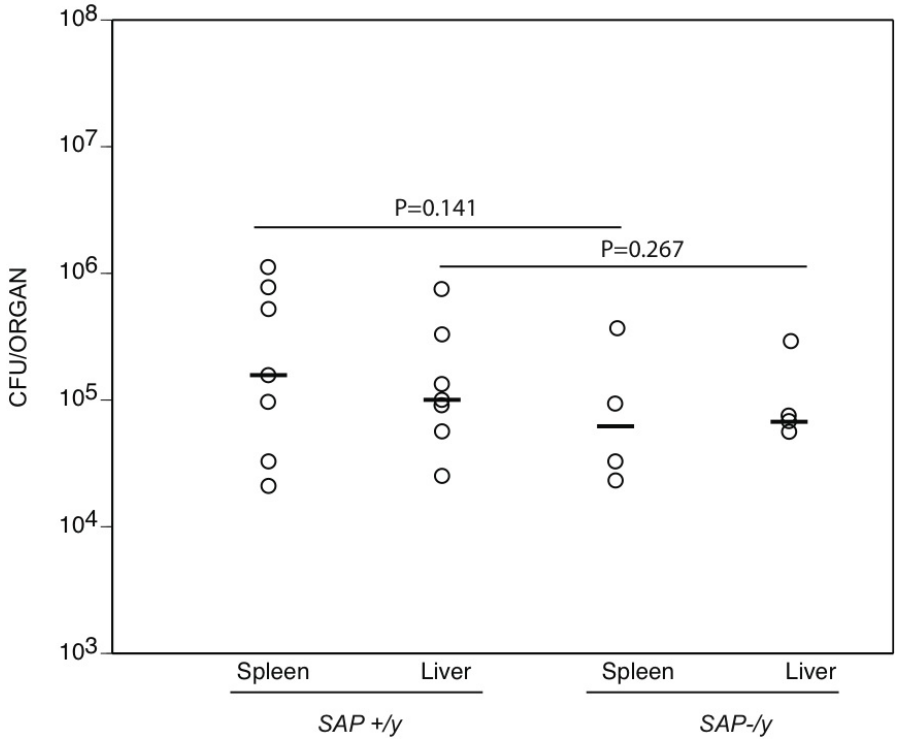


Figure 2.12 SAP is not required for innate immunity to *Lm* infection

SAP-deficient mice were infected with $5x10^5$ *Lm* i.p., the liver and spleen harvested at 48hpi and CFU were enumerated. Each spot represents an individual animal. The bar indicates the geometric mean of each group. The results are representative of 2 independent experiments with at least 5 pairs of animals.

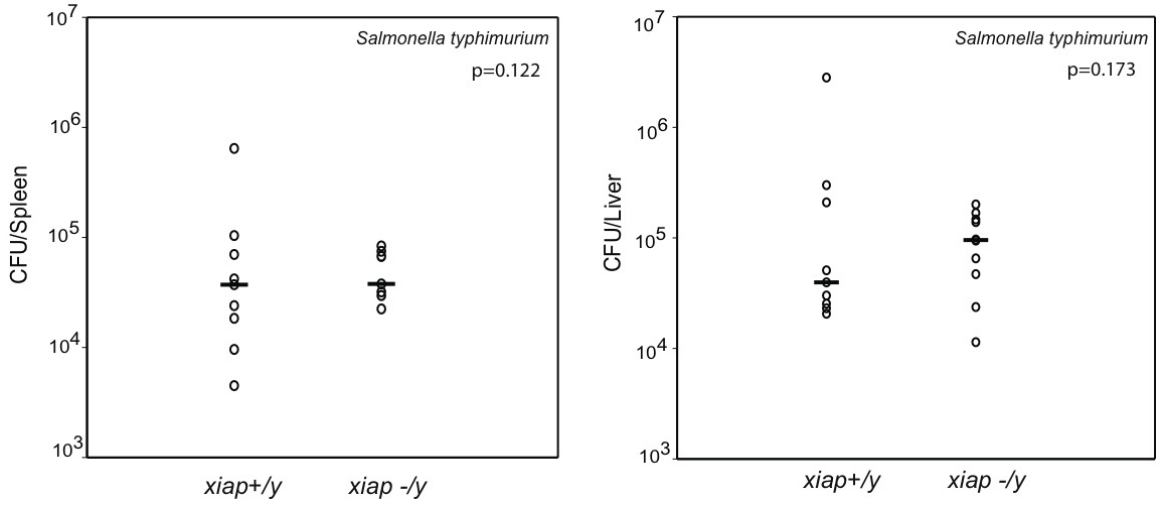


Figure 2.13 XIAP is not required for innate immunity to *St* infection

XIAP-deficient mice were infected with 250 *St* i.p., the liver and spleen harvested at 48hpi and CFU were enumerated. Each spot represents an individual animal. The bar indicates the geometric mean of each group.

CFU from both wildtype and XIAP-deficient mice at 48hpi. Thus, we conclude that XIAP is not required for the innate immune response to systemic *St* infection.

Characterization of the association between XIAP and *Listeria monocytogenes*

Since we originally identified the IAP family through our affinity-based assay for proteins that associate with the surface of *Lm*, we wanted to determine if XIAP associated with the surface of *Lm in vivo*. To do this we took several approaches: visualization of colocalization by immunofluorescence, *in vitro* association and intracellular association. We were unable to observe colocalization of XIAP and *Lm* by immunofluorescence or using *in vitro* association techniques, due to technical reasons. To examine if XIAP interacts with *Lm in vivo,* we infected primary bone marrow derived macrophages with *Lm* and harvested the bacteria at various time points after infection. We observed enhanced association of XIAP with the *Lm* pellet at 60mpi (**Fig 2.14**). While this experiment was not quantitative, the association of XIAP with *Lm* at 60mpi was intriguing because until approximately 30mpi *Lm* is trapped in the vacuole. The bacteria therefore would not be able to interact with cytosolic components. Thus, we would predict there would be very little association of *Lm* with XIAP at that time point. These results suggest that once *Lm* gains access to the cytosol, XIAP can associate with the bacteria.

DISCUSSION

We developed an affinity-based assay to identify host cytosolic proteins that can associate with the surface of *Lm*. Through the use of this method, we identified several

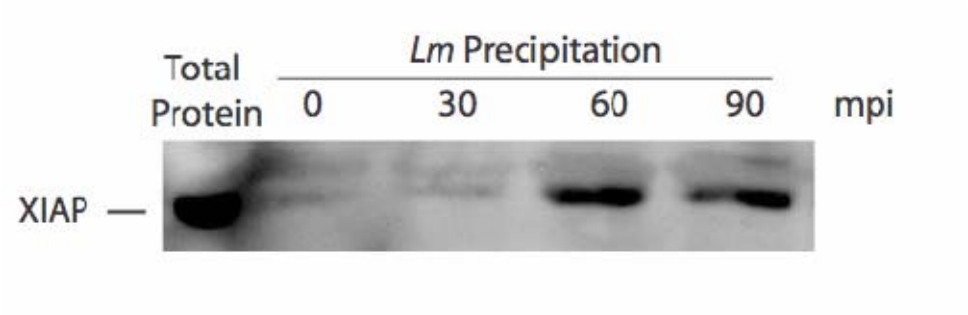


Figure 2.14 XIAP Associates with *Listeria monocytogenes* in cells.

Bone marrow derived macrophages were infected with *Lm*. At indicated time points, *Lm* was isolated from the host cells by differential centrifugation. The resulting pellet was analyzed by SDS-PAGE and immunoblotted with an anti-XIAP antibody.

candidate proteins that may regulate the innate immune response to cytosolic *Lm*. Many of the protein candidates identified modulate TGFβ or TNF signaling. We determined that neither FXR nor c-IAP1 are required for the innate immune response to *Lm* infection, as mice deficient in either of these proteins were as resistant as wildtype mice to *Lm* infection. Finally, we identified a new candidate through family association, XIAP, which regulates the innate immune response to *Lm* infection.

TGFβ signaling during *Lm* infection broadly suppresses many aspects of the immune response including decreasing leukocyte migration, reducing macrophage microbicidal activity and reactive oxygen species generation, limiting NK cell activation, antibody production, T cell proliferation and cytokine production[75]. We speculate that the identification that multiple protein components of this pathway associate with *Lm* may indicate a larger role for this pathway in innate immune signaling in response to cytosolic pathogens. It is intriguing to hypothesize that, for proper innate immune signaling to occur during cytosolic bacterial infection, the TGFβ pathway components may be used, thus limiting the amount of immune suppression that occurs early. Later during infection, when too much immune signaling becomes detrimental to the host, these proteins may no longer be needed for immune activation but can mediate suppression.

The mammalian TNF pathway is largely homologous to the Imd pathway in *Drosophila*. The Imd pathway is responsible for detecting Gram-negative pathogens and results in activation of NF-kB and MAP kinase homologs[4]. There is evidence in mammalian systems that several of the components of the TNF pathway are important for the innate

immune response to intracellular pathogens. For example, FADD, a death domain containing adaptor protein critical for the Imd pathway, regulates signaling in response to viral infection in mammalian cells[6]. Innate immune signaling pathways are very well conserved from *Drosophila* to mammals; thus, it was encouraging to identify several components of the TNF signaling pathway in our affinity-based assay for cytosolic innate immune signaling components.

Drosophila has two IAP proteins: dIAP1, which functions to inhibit apoptosis in flies and dIAP2, which was identified as a member of the Imd signaling pathway[76,77,78,79]. Genetic epistasis experiments place dIAP2 upstream of NF-κB and MAP kinase pathways but in parallel to the Imd protein, which is homologous to the mammalian RIP protein. c-IAP1 and c-IAP2 associate with and modify RIP1 during TNF signaling to regulate NF-κB activation[62]. Thus, it is likely that the IAP proteins have evolved to regulate signaling as well as apoptosis. In our studies, we determined that XIAP, but not c-IAP1, is required for the innate immune response to *Lm* infection. It is possible that c-IAP2 was able to compensate for loss of c-IAP1 during *Lm* infection and that the c-IAP proteins may also regulate the innate immune response to cytosolic bacterial infection.

It has become evident that ubiquitin is a very important protein modification that is critical for innate immune signaling[80,81,82]. One protein candidate identified, ABIN, is an inhibitor of the A20 protein, which functions as a deubiquitinating enzyme. A20 deubiquitinates TRAF6, among others, to inhibit both TNF and TLR signaling[83]. Thus, ABIN inhibition of A20 during *Lm* infection may allow these innate immune

signaling pathways to induce proinflammatory cytokines. Additionally, many IAP family members, including both c-IAPs and XIAP, have a RING finger domain, which functions as an E3 ubiquitin ligase. The addition of ubiquitin to proteins can serve as a signaling moiety or can target proteins for proteasomal degradation. The identification of several ubiquitin modifying proteins in our affinity-based method suggests this modification is important for the innate immune response to cytosolic *Lm* infection.

The result that XIAP-deficient mice are more susceptible to *Lm* infection than *St* infection, suggests two possible roles for XIAP: that XIAP regulates the immune response to Gram-positive bacteria and not Gram-negative bacteria, or that it regulates the immune response to cytosolic but not vacuolar pathogens. It is possible that XIAP may aid in detecting a component of *Lm* that is not exposed in *St*, due to the peptidoglycan being covered by an outer membrane in Gram-negative organisms. Additionally, it could be that XIAP aids in detecting a similar component on each bacteria, but the localization of the bacterial components is important for inducing the immune response.

Our affinity-based method to identify cytosolic host proteins that associated with the surface of *Lm* proved fruitful as we identified components of the TGFβ, TNF and ubiquitin modifying pathways that associate with the surface of *Lm*. At the time this thesis research was initiated, XIAP was not known to be a regulator of the innate immune response. While XIAP regulates both NF-κB and MAP kinase pathways, its ability to regulate these pathways during an immune response *in vivo* was undefined. Chapter 3

and 4 will focus on characterizing the role of XIAP during the innate immune response to cytosolic *Lm* infection.

MATERIALS AND METHODS

Antibodies and Reagents.

Anti -XIAP (BD Transduction 610716), anti-HA (Abcam Ab9110). SNX6-HA PEBB construct was obtained from Tony Parks at University of Washington[84]. XIAP-HA-PEBB, XIAP-GFP PEBB constructs were a gift from Dr. Colin Duckett. For *in vitro* protein expression, we used the TNT coupled wheat germ (L5030) and TNT Quick coupled transcription/translation system (L5020) from Promega, according to the manufacturers protocol. One tenth of the reaction was incubated with $1.2x10^8$ bacteria, in 500 ul Buffer A (50mM Tris pH 8.0, 5mM EDTA pH 8.0, 150mM NaCl, 0.05% NP-40, 1 EDTA-free protease tablet/10mls (Roche)) with 1x cytosolic salts(10x= 0.3M HEPES, 0.03M $MgCl_2$, 1.4M KCl, pH 7.9). Samples were incubated for 4h at 4C nutating, followed by 2 washes in bufferA+ 1x cytosolic salts. The bacterial pellet was then run on an SDS-PAGE gel to visualize any bound proteins. Protein A/G agarose beads were purchased from Santa Cruz Biotechnology (sc-2003).

Affinity Assay

Cytosolic protein was isolated from host cells by treatment with Lysis buffer (50mM Tris pH 8.0, 5mM EDTA pH 8.0, 150mM NaCl, 0.05% NP-40, 1 EDTA-free protease tablet/10mls (Roche)). Cells in lysis buffer were incubated on ice for 5 minutes, vortexed for 10 sec and incubated on ice again for 5 minutes. Lysed cells were centrifuged at

1500rpm for 2 min to remove nuclei, the supernatant was transferred to a new tube and centrifuged at 14k rpm for 10 minutes to pellet cellular debris. The supernatant was removed and incubated with 30ul protein A/G agarose beads for 1hr at 4C nutating. Beads were pelleted by centrifugation at 7500rpm for 2 min, supernatant was removed and incubated overnight at 4C nutating with *Lm* or agarose beads. Beads or *Lm* were pelleted by centrifugation at 7500 or 13000 rpm respectively for 2 min. Supernatant was removed and beads or *Lm* were washed 3x in 200mM NaCl+0.5% NP40. The beads or bacteria were resuspended in 2x SDS-PAGE buffer and boiled before running on an SDS-PAGE gel and immunoblotted. To remove *Lm* surface proteins, *Lm* was boiled in 2x SDS-PAGE sample buffer for 30 min. For mass spectrometry analysis, SDS-PAGE gels were stained in 0.25% Coomassie stain (Brilliant blue R-250) in destain solution (50% MeOH, 10% acetic acid) for 30 min shaking. Gels were then destained with several washes of diH_2O. Protein bands were identified by mass spectrometry (LC-MS/MS) at the University of Michigan Protein Structure Facility.

Animals, bacterial strains and infections. Mice deficient in XIAP (accession #U88990) were originally generated on a 129/Sv × 129/SvJ background as described [72]. The XIAP-deficient mice were backcrossed onto the C57Bl/6 background for more than 10 generations. Mice were genotyped using the following primers, for wildtype F1-ctcaagtggtttggtaatgtacgac and R1-acagctgagtctccatactgccat and for the knockout allele: F2-agtgtatgtggaacagaggctgct and R4-acatagcgttggctacccgtgata. Mice deficient in FXR were obtained from the Jackson laboratories (007214). c-IAP1 and SAP-deficient mice were obtained from Dr. Colin Duckett (University of Michigan) and Dr. Pamela

Schwartzberg (NIH) respectively[70,85]. Six to twelve week old male knockout mice or wildtype littermate controls were used for infection experiments. All animals received humane care as outlined by the Guide for the Care and Use of Laboratory Animals (University of Michigan Committee on Use and Care of Animals). Caco2 and HeLa cells were obtained from ATCC, # HTB-37 and CCL-2 respectively. Epithelial cells were cultured in MEMα with 10% FBS, 1mM L-Glutamine, 1mM Sodium Pyruvate, 1% Non-essential Amino Acids. Cells were passaged by removing media, rinsing in PBS without Ca++ or Mg++, finally cells were removed by adding Tryple Express. After 2-5 minutes of incubation 10mls of media was added to dilute out the trypsin. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturers protocol. For cell culture infections, *Listeria monocytogenes* strains 10403S (wildtype) was inoculated into liquid brain-heart infusion (BHI) broth and incubated at 30°C overnight without shaking[86]. Prior to infection, bacterial cultures were washed and resuspended in PBS. Epithelial cells were infected at an MOI of 100 for 1h, while macrophages were infected at an MOI of 1 for 30 minutes. For animal infections, *L. monocytogenes* and *S. enterica Typhimurium* (SL1344) were grown to log-phase in BHI and aliquots were stored at -70°C. For each experiment, a vial was back-diluted and allowed to grow to OD_{600} 0.5. The bacteria were washed in PBS and diluted before injection. Mice were injected i.p. with $5x10^5$ *L. monocytogenes* equivalent to 0.5 LD_{50} for infection by the i.p. route in C57Bl/6 mice [87]. Mice were injected i.p. with 250 *S. enterica Typhimurium*. The number of viable bacteria in the inoculum and organ homogenates was determined by plating 10-fold serial dilutions on Luria broth (LB) agar plates.

BMDM culture. Bone marrow macrophages were differentiated in DMEM supplemented with 20% heat inactivated FBS, 2mM L-glutamine, 1mM sodium pyruvate, 0.1% β-mercaptoethanol and 30% L929 conditioned medium. Bone marrow cells were cultured at an initial density of 10^7 cells per 150mm non-tissue culture treated dish for 6 days, with fresh medium added at day three. Cells were harvested with cold PBS without calcium and magnesium.

Immunoblot analysis. Whole cell lysates were generated by adding 2x SDS-PAGE sample buffer directly to cell monolayers. Protein samples were separated by SDS-PAGE and transferred to PVDF. Blots were blocked in 5% BSA, incubated with primary antibodies, followed by a horseradish peroxidase conjugated secondary antibody. The following antibodies were used: XIAP (BD Transduction Laboratories #610717), goat anti-mouse IgG-HRP (MP Biomedical #67429).

Chapter 3 : XIAP Regulates Cytosol-specific Innate Immunity to *Listeria* Infection

ABSTRACT

The Inhibitor of Apoptosis Protein (IAP) family has been implicated in immune regulation, but the mechanisms by which IAP proteins contribute to immunity are incompletely understood. We show here that X-linked IAP (XIAP) is required for innate immune control of *Listeria monocytogenes* infection. Mice deficient in XIAP had a higher bacterial burden 48 hrs after infection than wildtype littermates, and exhibited substantially decreased survival. XIAP enhanced NF-κB activation upon *L. monocytogenes* infection of activated macrophages, and prolonged phosphorylation of Jun N-terminal kinase (JNK) specifically in response to cytosolic bacteria. Additionally, XIAP promoted maximal production of pro-inflammatory cytokines upon bacterial infection *in vitro* or *in vivo*, or in response to combined treatment with Nod2 and TLR2 ligands. Together, our data suggest that XIAP regulates innate immune responses to *L. monocytogenes* infection by potentiating synergy between Toll-like receptors (TLR) and Nod-like receptors (NLR) through activation of JNK and NF-κB dependent signaling.

INTRODUCTION

The Inhibitor of Apoptosis (IAP) family of proteins plays a key role in cellular signaling, such as apoptosis, by binding to pro-apoptotic proteins, interrupting the intrinsic programmed cell death pathway and activating anti-apoptotic mechanisms [88,89,90]. In addition to modulating apoptosis, recent genetic studies have revealed that a *Drosophila* IAP protein, *diap2,* acts as a regulator of anti-microbial immunity [76,77,78,79]. Innate immune signaling pathways are well conserved from *Drosophila* to humans, suggesting that IAP proteins may also play a role in mammalian innate immunity [4]. This hypothesis is consistent with a study demonstrating that c-IAP2 exacerbates endotoxic shock in mice by controlling macrophage apoptosis [69]. Furthermore, a cohort of patients with X-linked lymphoproliferative syndrome (XLP) were found to have mutations in the gene encoding XIAP, resulting in a primary immunodeficiency [71]. XIAP, also known as BIRC4 and hILP, contains three baculoviral IAP repeat (BIR) domains, the characteristic protein-protein interaction domain of the IAP family[91]. XIAP also has a carboxy-terminal RING domain with E3 ubiquitin ligase activity that directs proteasomal degradation of target proteins [92]. Multiple signaling pathways can be modulated by XIAP, including NF-κB, MAP kinase and TGFβ signaling [93,94,95,96]. Moreover, XIAP can integrate cellular responses to diverse stimuli by interacting directly with ligands such as copper to regulate copper homeostasis [97]. XIAP has been predominantly characterized as an inhibitor of apoptosis, and interacts with many known mediators of programmed cell death, such as JNK, TAK1, TAB1, TRAF6, caspases-3, 7 and 9 [90,93,98,99]. However, XIAP-deficient mice do not appear

to have striking defects in apoptosis, thus the role of XIAP *in vivo* is not yet clearly understood [72].

The innate immune response protects host organisms against invading pathogens prior to the onset of adaptive immunity. Pathogens stimulate innate immune signaling through pattern recognition receptors (PRR), which recognize well-conserved pathogen-associated molecular patterns (PAMPs) [100]. PAMPs are detected at the host membrane by TLRs, and in the cytosol by the NLR and the RIG-I-like helicase (RLR) sensors [101,102]. Stimulation of either extracellular or intracellular PRR can result in activation of NF-κB and MAP kinase signaling pathways, leading to production of inflammatory mediators such as cytokines and costimulatory molecules [10]. Activation of TLRs and NLRs together can induce synergy between the signaling pathways, resulting in enhanced activation of innate and adaptive immunity [53,103]. *Listeria monocytogenes* is a cytosolic bacterial pathogen used extensively to probe aspects of innate and adaptive immunity [25]. *L. monocytogenes* is recognized by TLRs expressed on the surface of phagocytes [25]. After phagocytic uptake, *L. monocytogenes* escapes from host vacuoles by secreting a pore-forming toxin, listeriolysin O (LLO) [104]. Once in the cytosol, *L. monocytogenes* can trigger oligomerization and signaling by Nod1 and other NLRs [57]. Here we show that XIAP plays a protective role during infection by *L. monocytogenes*. We present evidence that amplifying JNK activation and subsequent pro-inflammatory cytokine production in response to cytosolic bacteria is one mechanism by which XIAP modulates innate immunity.

RESULTS

XIAP regulates innate immunity to *L. monocytogenes*

We first tested the hypothesis that XIAP contributed to anti-microbial immunity by infecting *xiap*$^{+/y}$ and *xiap*$^{-/y}$ mice with 1x10^5 *L. monocytogenes* and determining survival over time (**Fig. 3.1**). At 7d pi (days post infection), 60% of the XIAP-deficient mice had succumbed to infection, whereas all wildtype mice survived. Similarly, at higher doses of *L. monocytogenes* more *xiap*$^{-/y}$ than *xiap*$^{+/y}$ mice succumbed to infection, although some *xiap*$^{+/y}$ mice also became moribund (unpublished data). Depending upon the inoculum, morbidity and mortality of *xiap*$^{-/y}$ animals occurred between 2 and 5d pi, prior to peak development of adaptive immunity, suggesting that XIAP had a protective effect during the innate response to bacterial infection. To better define the role of XIAP during innate immunity to intracellular bacterial infection, we infected wildtype and XIAP-deficient mice intraperitoneally with 5x10^5 *L. monocytogenes,* and harvested spleen and liver to enumerate bacterial burden at 24, 28 and 72h pi (**Fig. 3.2**). By 48h, *xiap*$^{-/y}$ mice had approximately 10-fold more *L. monocytogenes* in liver and spleen at 48h pi compared to the *xiap*$^{+/y}$ mice, consistent with our observation of their decreased survival. At 72h pi, the difference between the *xiap*$^{+/y}$ mice and the *xiap*$^{-/y}$ was even more pronounced, with the *xiap*$^{-/y}$ mice supporting 100-fold greater bacterial numbers. These results indicate that XIAP mediates innate resistance to *L. monocytogenes* infection.

Mutations in XIAP have been associated with the human immunodeficiency syndrome, XLP [71]. One feature associated with this disease is an abnormally low number of natural killer T-cells (NKTC), although it is not yet clear how much this phenotype

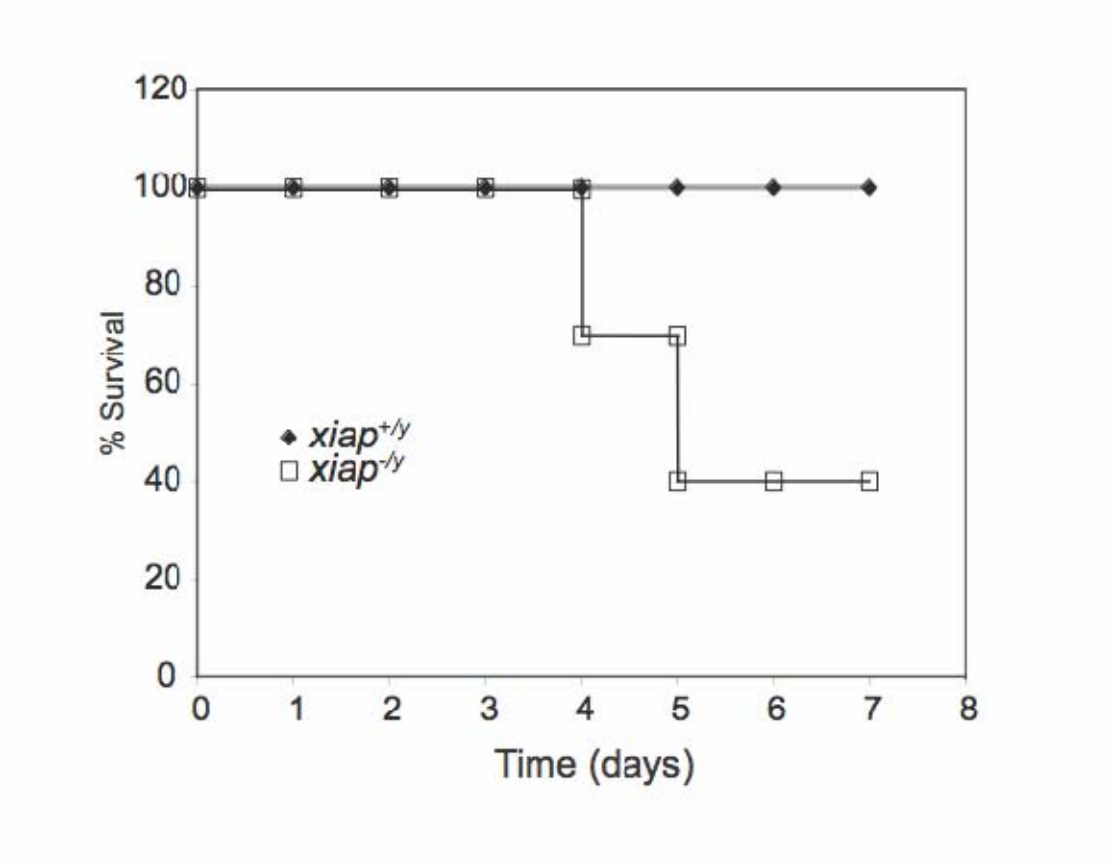


Figure 3.1 XIAP enhances the survival of mice during *L. monocytogenes* infection.

Survival curve of *L. monocytogenes* in *xiap*$^{+/y}$ and *xiap*$^{-/y}$ mice. Mice were injected with $1x10^5$ *L. monocytogenes* intraperitoneally, and survival was monitored daily (n=10 animals per group).

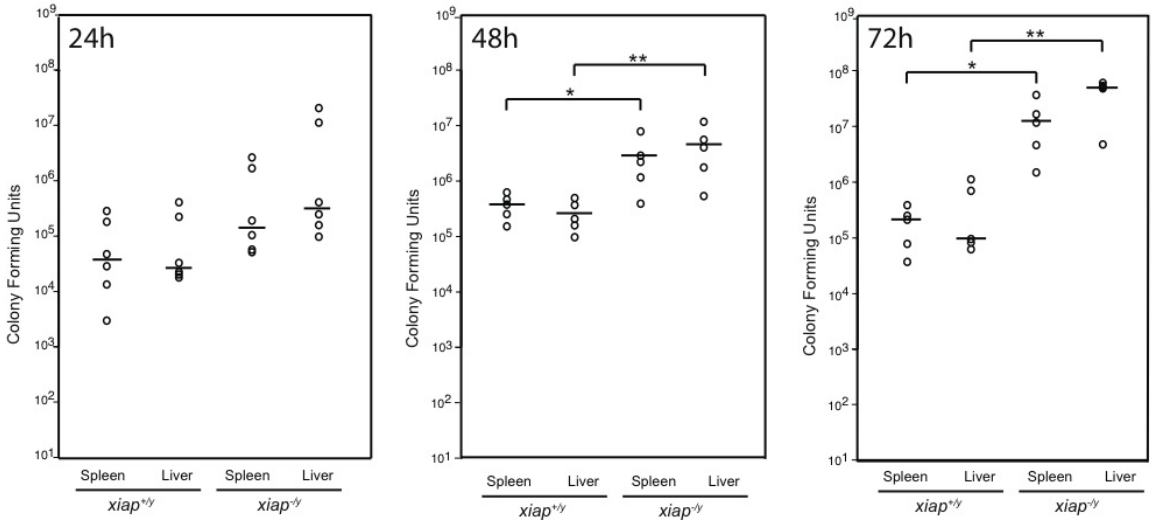


Figure 3.2 XIAP regulates the innate immune response *in vivo*

CFU isolated from the liver or spleen of mice infected with 5 x10^5 *L. monocytogenes* i.p. at 24h, 48h and 72h pi. Each point represents one animal. Mean CFU is indicated by a horizontal line. * indicates $p \leq 0.05$ and ** indicates $p \leq 0.005$.

contributes to immunodeficiency. To determine if mice lacking XIAP exhibit a similar phenotype to XLP patients, we quantitated the percentage of NKTC in the spleen of *xiap*$^{+/y}$ and *xiap*$^{-/y}$ mice (**Fig. 3.3A**). No significant difference in the number of splenic NKTC was observed between *xiap*$^{+/y}$ and *xiap*$^{-/y}$ mice, indicating that survival of NKTC in uninfected mice is not affected by a deficiency in XIAP, consistent with a previous report [71]. To determine if NKTC survival or activation was dependent on XIAP during *L. monocytogenes* infection, we infected animals and determined the number of splenic NK1.1^{+}CD3^{+} NKTC that expressed CD69, a marker of activation (**Fig. 3.3B**). We observed similar numbers of activated NKTC in *xiap*$^{+/y}$ and *xiap*$^{-/y}$ mice. These data suggest that XIAP does not play an important role in NKTC survival or activation in a murine model of listeriosis.

We then tested the role of XIAP during infection of primary macrophages, an innate immune effector cell and a well-characterized host for *L. monocytogenes* replication. We infected unactivated bone marrow derived macrophages (BMDM), BMDM activated with LPS and IFNγ or peritoneal macrophages with *L. monocytogenes* and measured intracellular bacterial growth over time (**Fig. 3.4**). All types of *xiap*$^{+/y}$ and *xiap*$^{-/y}$ macrophages controlled *L. monocytogenes* infection equally well. We conclude from these data that XIAP does not contribute directly to restriction of *L. monocytogenes* growth in macrophages, even though XIAP–deficient mice exhibited an increased bacterial burden compared to wildtype mice. Taken together, our results demonstrate that XIAP is required for a protective immune response to *L. monocytogenes* infection *in vivo*.

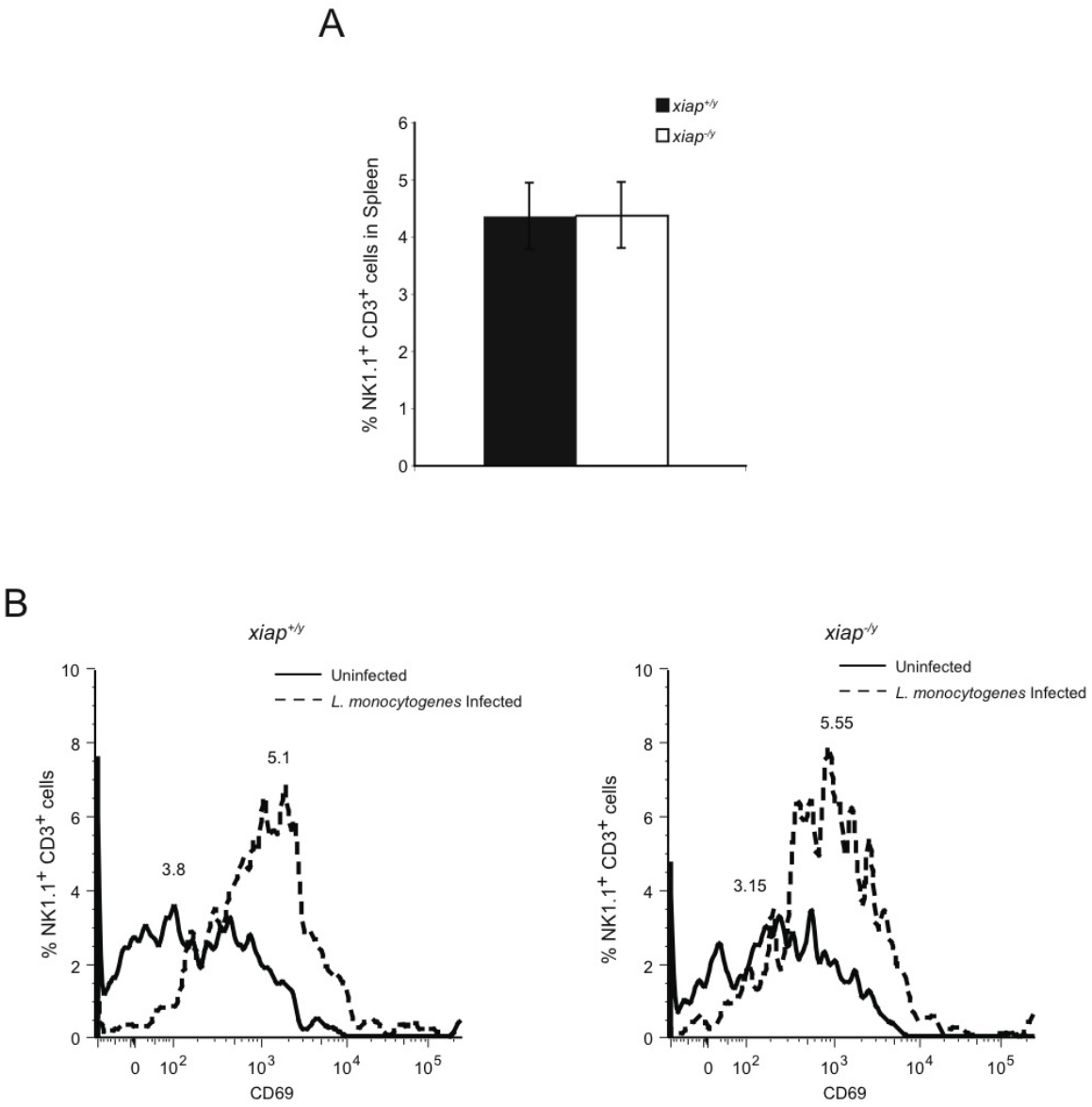


Figure 3.3 XIAP does not regulate the survival or activation of NKTCs *in vivo*

(A) Flow cytometry analysis of $NK1.1^{+}CD3^{+}$ NKTC in the spleens of uninfected $xiap^{+/y}$ and $xiap^{-/y}$ animals (error bars represent s.d.). (B) Splenocytes were harvested from infected animals at 48h pi, and stained with NK1.1-biotin, CD3-FITC and CD69-PE fluorescent-coupled antibodies for flow cytometry analysis. Results are representative of three independent experiments (n=9 animals).

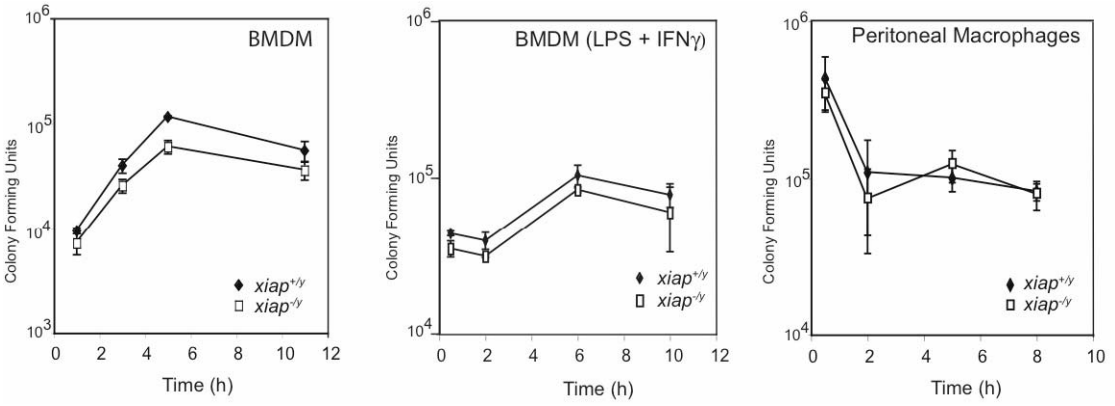


Figure 3.4Intracellular growth of *L. monocytogenes* in unactivated, activated and peritoneal macrophages.

Unactivated macrophages were infected at an MOI of 1. Activated macrophages were stimulated overnight with 10ng/ml LPS and 10ng/ml Interferon-γ. Activated and peritoneal macrophages were infected with an MOI of 10 (error bars represent s.d.).

Translocation of NF-κB in response to *L. monocytogenes* is enhanced by XIAP

XIAP can activate NF-κB-dependent transcription in response to apoptotic stimuli [94]. In addition to regulating apoptosis, the canonical NF-κB p50/p65 heterodimer has a well-established role in proinflammatory cytokine transcription stimulated by TLR and NLR signaling [100]. Expression profiling of unactivated macrophages infected with *L. monocytogenes* did not reveal reproducible differences between wildtype and XIAP-deficient macrophages (**Appendix 2**). We then reasoned that activated macrophages might be a more relevant environment for studying XIAP function. We therefore investigated whether XIAP regulated NFκB-dependent processes during *L. monocytogenes* infection in activated macrophages by measuring translocation of p50 to the nuclear compartment. Activated BMDM were infected with wildtype *L. monocytogenes,* and translocation of the p50 subunit of NF-κB was analyzed by immunoblot (**Fig. 3.5A**). As early as 0.5h pi, p50 was detected in the nuclear fraction of both *xiap*$^{+/y}$ and *xiap*$^{-/y}$ cells, however, in the presence of XIAP there was substantially more p50 in the nuclear fraction over time. We also measured DNA binding activity of the p65 subunit of the p50/p65 heterodimer in the nuclear fraction of uninfected and *L. monocytogenes* infected activated macrophages (**Fig. 3.5B**). At 1 and 2h pi, infected *xiap*$^{+/y}$ macrophage nuclear lysates contained significantly more NF-κB DNA binding activity than infected *xiap*$^{-/y}$ nuclear lysates, suggesting that XIAP might enhance signaling of NF-κB-dependent pathways stimulated by bacterial infection.

In some contexts, XIAP-dependent NF-κB activation can protect against apoptotic stimuli; therefore we tested if XIAP modulated apoptosis during *L. monocytogenes*

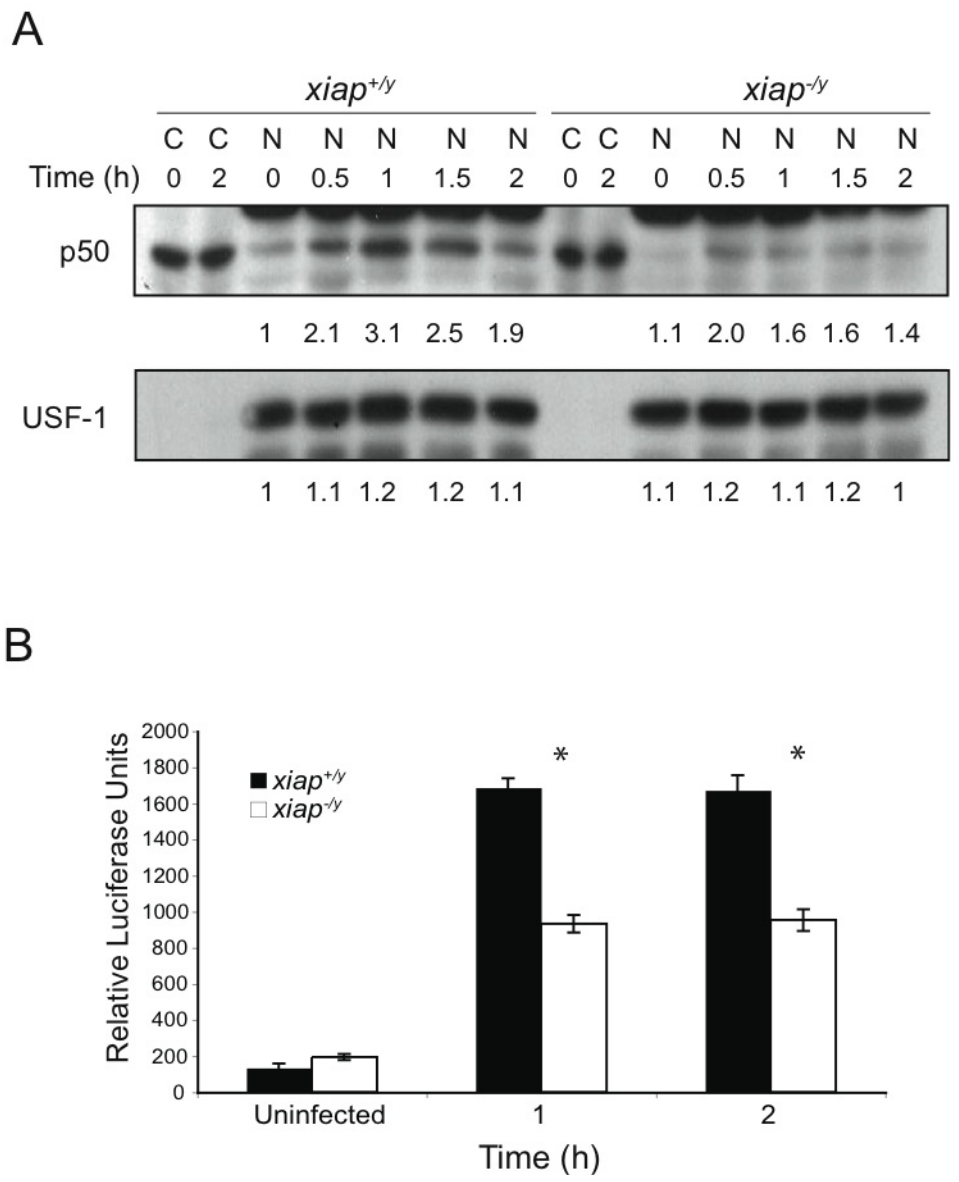


Figure 3.5 XIAP enhances NF-κB translocation during *L. monocytogenes* infection.

(A) Nuclear translocation of p50 in xiap+/y and xiap-/y activated BMDM in response to wildtype *L. monocytogenes* infection. Cells were activated with 10ng/ml LPS and 10ng/ml interferon-γ overnight, and infected at an MOI of 10 for 30min. Upon lysis, the nuclear fraction (N) was separated by centrifugation from the cytosolic fraction (C). Data are representative of at least 3 independent experiments. (B) DNA binding activity of p50/p65 as measured by ELISA. Nuclear extracts from xiap+/y and xiap-/y activated BMDM that were uninfected or infected with wildtype *L. monocytogenes* were added to 96 well dishes coated with a canonical NF-kB consensus DNA binding sequence, followed by detection with a p65-specific antibody. Results are representative of at least 3 independent experiments (error bars represent s.d.)

infection. We first examined apoptosis in activated macrophages during *L. monocytogenes* infection by flow cytometry of infected cells using Annexin V (AnnV), an indicator of apoptosis (**Fig. 3.6A**). A modest but reproducible increase in apoptosis was observed by 3h pi in XIAP-deficient macrophages compared to wildtype macrophages, which remained consistent throughout infection (**Fig. 3.6 B**). We also examined apoptosis in infected liver and spleen at sites of *L. monocytogenes* replication 48h pi by performing TUNEL staining (**Fig. 3.6C**). Although the extent of apoptosis at foci of infection were heterogeneous, there did not appear to be any notable difference in the number or distribution of apoptotic cells per focus in $xiap^{+/y}$ compared to $xiap^{-/y}$ livers or spleens. We did not observe any XIAP-dependent difference in the numbers of $AnnV^{+}$ T or B cells present in the spleens of mice at 48h pi (**Fig 3.6D**). In addition, caspase-3 cleavage in infected activated macrophages was not significantly altered (unpublished data). While the infected $xiap^{-/y}$ macrophages exhibited a modest increase in cell death, we found no striking evidence for regulation of apoptosis by XIAP in the context of *L. monocytogenes* infection *in vivo*. Thus, XIAP regulates NF-κB activation during *L. monocytogenes* infection, but may enhance innate immunity by modulating cellular responses other than apoptosis in infected macrophages.

XIAP modulates JNK activation in response to cytosolic *L. monocytogenes*

In addition to NF-κB activation, TLR and NLR sensing of microbial infection stimulate MAP kinase phosphorylation, leading to activation [81]. Previous reports suggested that XIAP could promote JNK phosphorylation via interaction with TAB1 and the MAP3K, TAK1[94,96,105]. To determine if XIAP affected JNK phosphorylation during *L.*

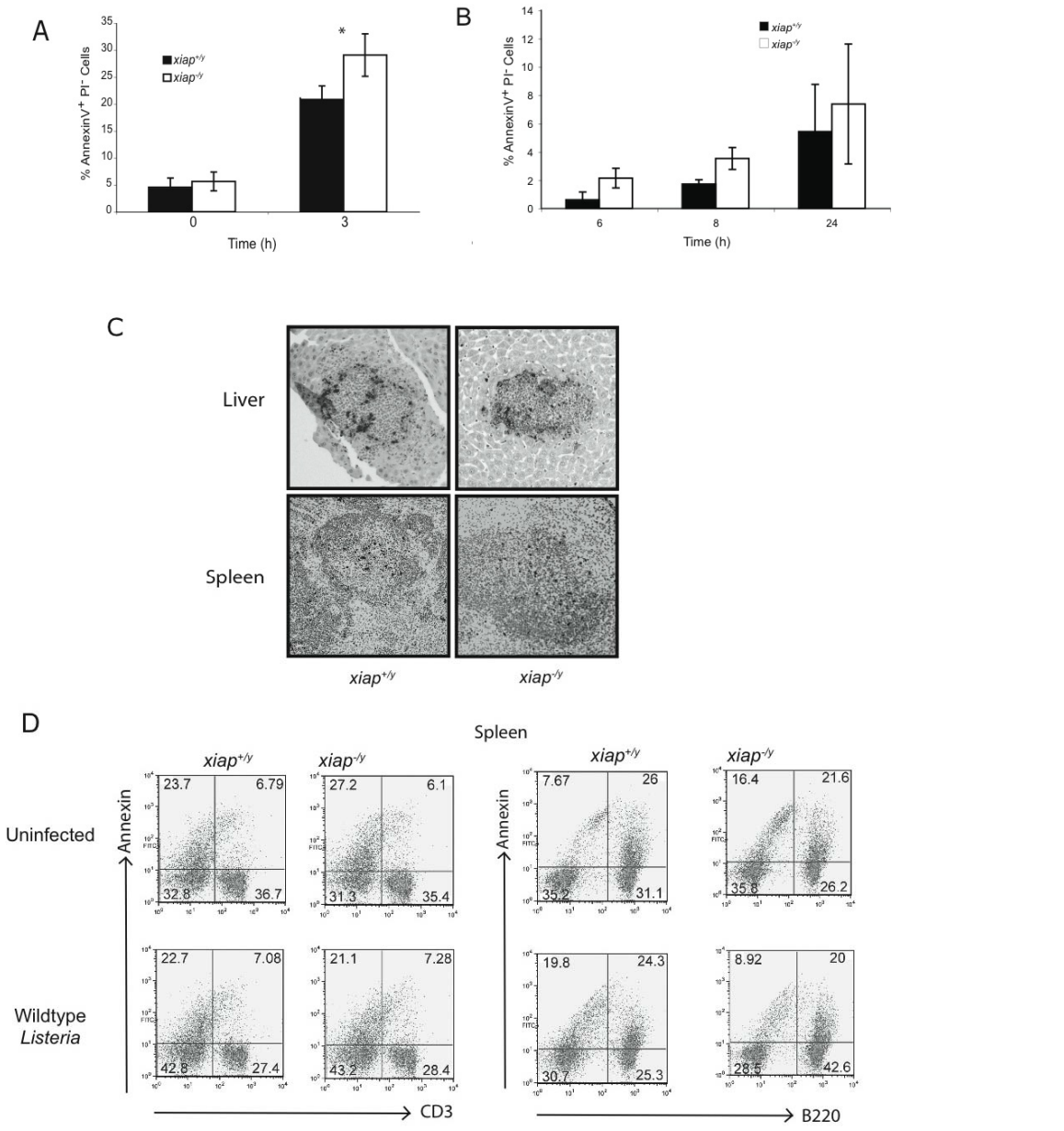
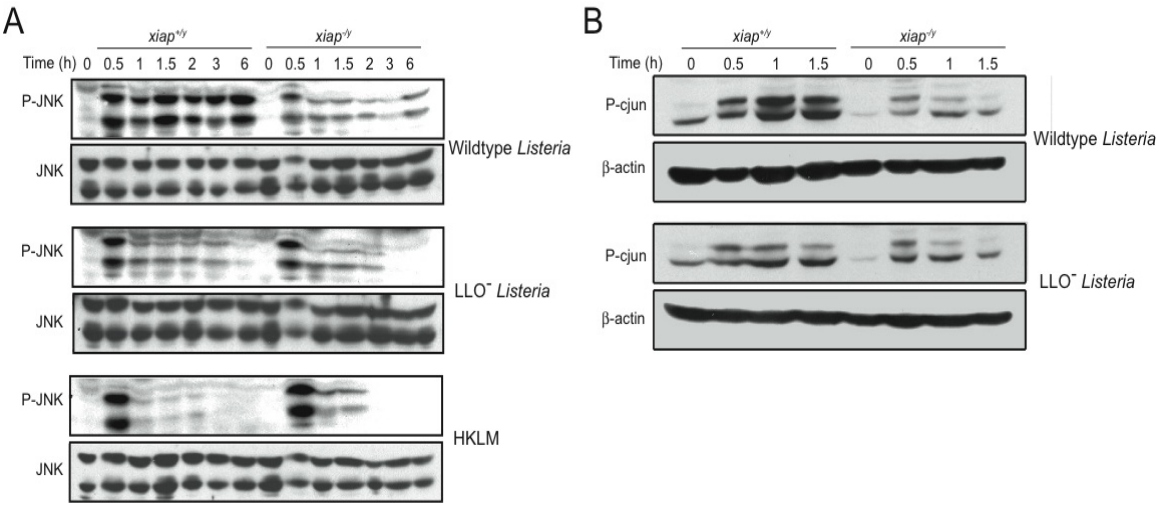


Figure 3.6 XIAP has a modest affect on apoptosis during *L. monocytogenes* infection

(A,B) Flow cytometry analysis of apoptosis in activated BMDM infected with *L. monocytogenes* at 3h pi (A), 6, 8 and 24hpi (B). Macrophages were stained at the indicated times post infection with Annexin V-FITC and propidium iodide. Results are representative of at least 3 independent experiments (error bars represent s.d. of macrophages from 3 mice). (C) TUNEL staining of histological sections of livers and spleens from *xiap*$^{+/y}$ and *xiap*$^{-/y}$ mice infected with *L. monocytogenes* for 48 h (n=3 animals/genotype). Ten sections per animal were examined. (D) Apoptosis of T cells (CD3^{+}) and B cells (B220^{+}) from uninfected and *L. monocytogenes* infected splenocytes, as determined by Annexin V and PI staining. Results are representative of at least 3 independent experiments (n=9 animals/ genotype).

monocytogenes infection, we performed immunoblot analysis of infected lysates from *xiap*$^{+/y}$ and *xiap*$^{-/y}$ activated macrophages using a phospho-JNK specific antibody (Fig. 3.7, 3.8). Upon infection with wildtype *L. monocytogenes,* JNK phosphorylation occurred as early as 0.5h pi in both *xiap*$^{+/y}$ and *xiap*$^{-/y}$ cells. In the *xiap*$^{-/y}$ macrophages, JNK phosphorylation peaked at 0.5h pi. However, in the presence of XIAP, enhanced JNK activation was prolonged up to 6 hours. This suggests that XIAP augments JNK signaling during wildtype *L. monocytogenes* infection. To determine the contribution of XIAP to cytosol-specific signaling, we compared wildtype *L. monocytogenes* infection with a strain deficient in LLO or heat killed *L. monocytogenes* (HKLM), which both remain trapped in the vacuole. The LLO^{-} bacteria and HKLM induced JNK phosphorylation at 0.5h pi similarly to infection by wildtype bacteria, suggesting that this early JNK phosphorylation was linked to signaling from the vacuole, most likely through TLRs. However, JNK phosphorylation in response to vacuolar bacteria quickly diminished after 30 min, in contrast to the extended XIAP-dependent JNK activation observed during wildtype bacterial infection. To confirm that enhanced JNK phosphorylation in *xiap*$^{+/y}$ activated macrophages resulted in downstream signaling, we examined phosphorylation of c-jun, a target of JNK, by immunoblot (**Fig. 3.7**) [106]. Upon infection by wildtype *L. monocytogenes*, c-jun phosphorylation was prolonged in *xiap*$^{+/y}$ but not *xiap*$^{-/y}$ cells, similarly to JNK phosphorylation. Moreover, activation of c-jun upon infection by LLO^{-} bacteria was considerably decreased compared to wildtype bacteria. To determine how XIAP promotes prolonged JNK phosphorylation we examined the protein levels of MKP1 and MKP5, two MAP kinase phosphatases (**Appendix 3**). To determine if XIAP also stimulated activation of other



C

	xiap+/y							xiap-/y						
	0	0.5	1	1.5	2	3	6	0	0.5	1	1.5	2	3	6
P-JNK Wildtype *Listeria*	1.0	5.5	3.6	5.8	3.4	3.0	5.0	0.4	3.0	2.0	1.6	1.1	0.5	1.5
JNK Wildtype *Listeria*	1.0	1.0	0.8	1.0	0.8	0.7	0.9	1.1	0.9	1.0	1.2	1.0	1.1	1.2
P-JNK *LLO- Listeria*	1.0	8.8	5.2	4.7	3.4	3.5	0.9	0.2	7.8	5.0	3.9	2.1	0.0	0.0
JNK *LLO- Listeria*	1.0	0.9	0.9	1.0	1.0	0.8	0.9	1.0	0.6	0.9	1.1	1.1	0.9	0.8
P-JNK HKLM	1.0	17.9	5.4	3.1	3.9	2.1	1.9	0.3	20.7	6.9	4.6	0.1	0.0	0.0
JNK HKLM	1.0	1.5	1.4	1.9	1.4	1.1	1.4	1.9	1.1	1.3	1.3	1.2	1.2	1.0

D

	xiap+/y				xiap-/y			
	0	0.5	1	1.5	0	0.5	1	1.5
P-cjun Wildtype *Listeria*	1.0	3.0	5.3	4.6	1.0	2.1	1.6	1.0
B-actin Wildtype *Listeria*	1.0	0.9	1.0	1.0	1.0	0.9	0.9	0.8
P-cjun *LLO- Listeria*	1.0	2.6	2.6	1.9	1.0	2.2	1.5	1.1
B-actin *LLO- Listeria*	1.0	1.0	0.9	1.0	0.9	1.0	1.0	0.9

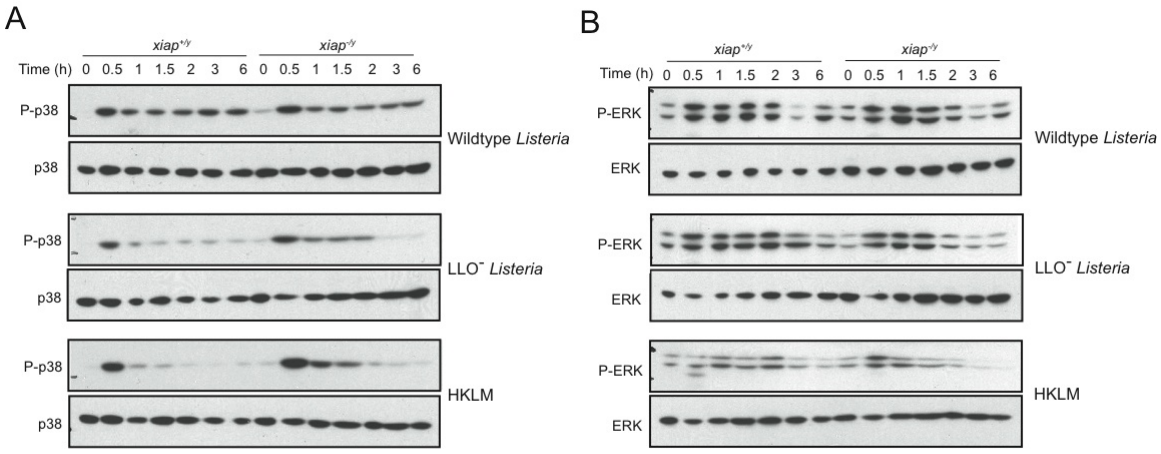
Figure 3.7 XIAP prolongs JNK signaling in response to cytosolic *L. monocytogenes*.

Immunoblot of lysates from *xiap*$^{+/y}$ and *xiap*$^{-/y}$ activated BMDM that were uninfected or infected with wildtype, or LLO$^-$ *L. monocytogenes* or HKLM. Cells were activated overnight with 10ng/ml LPS and 10ng/ml interferon-γ, followed by infection at an MOI of 10 for 30 min. Cells were lysed and subjected to immunoblot analysis using anti-JNK, anti-phospho-JNK, anti-phospho-c-jun, and anti-c-jun. Data are representative of at least 3 independent experiments. (A) JNK phosphorylation, (B) c-jun phosphorylation (C,D) Blots were quantitated based upon band density, as determined by ImageJ software. Band intensities were compared to the first sample of the blot, whose value was arbitrarily set to 1. (C) P-JNK blot quantitation. (D) P-cjun quantitation.

MAP kinase family members, we analyzed phosphorylation of p38 and ERK by immunoblot of infected macrophage lysates (**Fig. 3.8**). ERK1 and ERK2 were phosphorylated equivalently in *xiap*$^{+/y}$ and *xiap*$^{-/y}$ macrophages in response to infection by all *L. monocytogenes* strains. As previously shown, p38 phosphorylation was decreased during infection by vacuole restricted bacteria compared to wildtype bacteria [34]. Phosphorylation of p38 upon infection with wildtype *L. monocytogenes* was not significantly affected by XIAP. These data demonstrate that XIAP prolongs JNK activation specifically in response to cytosolic *L. monocytogenes*.

L. monocytogenes induced proinflammatory cytokine expression is enhanced by XIAP

Since XIAP modulated JNK and NF-κB signaling in the context of infection, we hypothesized that induction of proinflammatory cytokines through these pathways would also depend on XIAP. Activated macrophages were infected with *L. monocytogenes* for 3h, and RNA was analyzed by qRT-PCR to determine the expression of a subset of genes involved in innate immunity (**Fig. 3.9**). Transcription of *il6, tnf*, *il10*, *mip2* and *kc* was strongly upregulated upon infection in the presence of XIAP, while induction of *ifnb, il1b*, *ido* and *inos* was not significantly altered. To assess if XIAP-dependent gene expression correlated to increased protein production, we compared the secretion of IL-6 and TNF from uninfected and infected activated macrophages (**Fig. 3.10**). Upon infection by wildtype *L. monocytogenes*, IL-6 and TNF secretion was induced to a greater extent in *xiap*$^{+/y}$ macrophages than in *xiap*$^{-/y}$ macrophages, while infection with the LLO^{-} mutant induced little IL-6 and TNF secretion by either genotype. To determine if JNK



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	$xiap^{+/y}$							$xiap^{-/y}$						
	0	0.5	1	1.5	2	3	6	0	0.5	1	1.5	2	3	6
P-p38 Wildtype *Listeria*	1.0	4.1	2.7	2.7	2.9	3.4	3.2	1.4	4.3	2.5	2.7	2.4	2.5	2.7
p38 Wildtype *Listeria*	1.0	1.4	1.1	1.1	1.3	1.2	1.2	1.4	1.3	1.3	1.4	1.5	1.3	1.5
P-p38 *LLO- Listeria*	1.0	3.5	1.6	1.3	1.3	1.4	1.2	1.2	4.3	2.5	2.5	2.2	0.9	0.9
p38 *LLO- Listeria*	1.0	1.0	0.7	1.0	0.7	0.6	0.8	1.0	0.7	0.8	1.0	1.0	1.1	1.1
P-p38 HKLM	1.0	4.1	1.4	1.2	1.0	0.9	1.0	1.1	4.9	3.2	2.4	1.2	1.0	0.9
p38 HKLM	1.0	1.1	0.8	1.1	1.0	0.8	0.7	1.0	0.9	1.0	1.0	0.9	1.0	0.9

D

	$xiap^{+/y}$							$xiap^{-/y}$						
	0	0.5	1	1.5	2	3	6	0	0.5	1	1.5	2	3	6
P-ERK Wildtype *Listeria*	1.0	2.1	2.1	2.1	1.6	0.6	1.7	1.1	1.8	2.5	2.1	1.3	0.7	1.3
ERK Wildtype *Listeria*	1.0	1.0	1.0	1.1	0.9	0.9	1.1	1.4	1.3	1.5	1.6	1.5	1.4	1.5
P-ERK *LLO- Listeria*	1.0	1.7	1.8	1.8	2.0	1.7	1.1	0.7	1.6	1.7	1.7	0.8	0.6	0.5
ERK *LLO- Listeria*	1.0	0.9	0.9	1.3	1.5	1.6	1.6	1.8	1.2	1.6	2.1	2.0	1.8	2.0
P-ERK HKLM	1.0	1.3	1.9	1.4	1.8	0.9	0.6	1.0	1.8	1.7	1.0	0.6	0.4	0.3
ERK HKLM	1.0	0.7	0.9	1.1	1.1	1.0	0.9	1.0	0.8	0.9	1.0	1.0	1.0	0.8

Figure 3.8 XIAP does not regulate ERK or p38 MAPK signaling in response to cytosolic *L. monocytogenes*

Immunoblot of lysates from *xiap*$^{+/y}$ and *xiap*$^{-/y}$ activated BMDM that were uninfected or infected with wildtype, or LLO^{-} *L. monocytogenes* or HKLM. Cells were activated overnight with 10ng/ml LPS and 10ng/ml interferon-γ, followed by infection at an MOI of 10 for 30 min. Cells were lysed and subjected to immunoblot analysis using anti-phospho-ERK, anti-ERK-1, anti-phospho-p38, and anti p38 antibodies. Data are representative of at least 3 independent experiments. (A) p38 phosphorylation, (B) ERK phosphorylation (C,D) Blots were quantitated based upon band density, as determined by ImageJ software. Band intensities were compared to the first sample of the blot, whose value was arbitrarily set to 1. (C) P-38 blot quantitation (D) P-ERK blot quantitation.

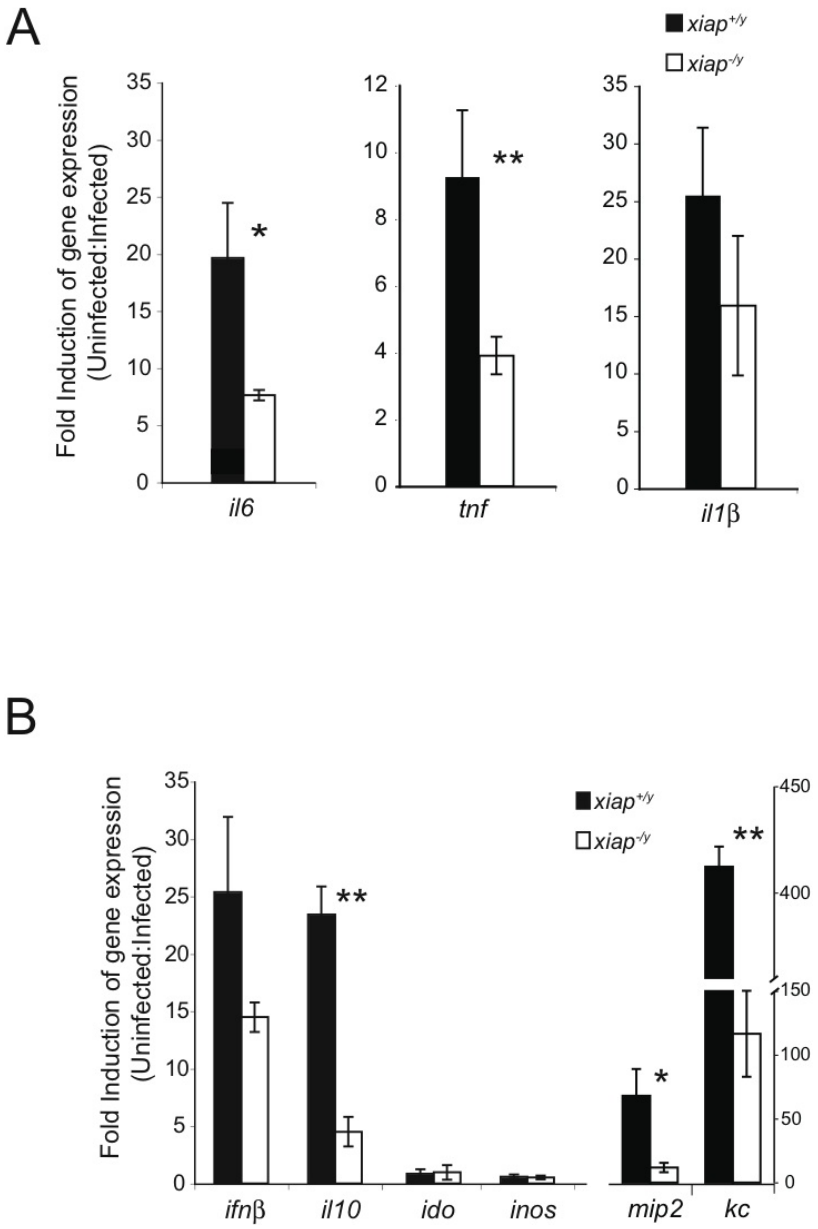


Figure 3.9 XIAP enhances proinflammatory cytokine gene expression

(A,B) qRT-PCR of genes associated with innate immune activation. BMDM were activated overnight with 10 ng/ml LPS and 10 ng/ml interferon-γ, infected with *L. monocytogenes* for 30 min, and harvested at 3h pi for RNA isolation and production of cDNA. Fold induction was calculated using the $\Delta\Delta C_t$ method where uninfected samples were compared to infected samples, relative to β-actin levels. (A) *il6, tnf and il1b*, (B) *ifnb, il10, ido, inos, mip2* and *kc.*

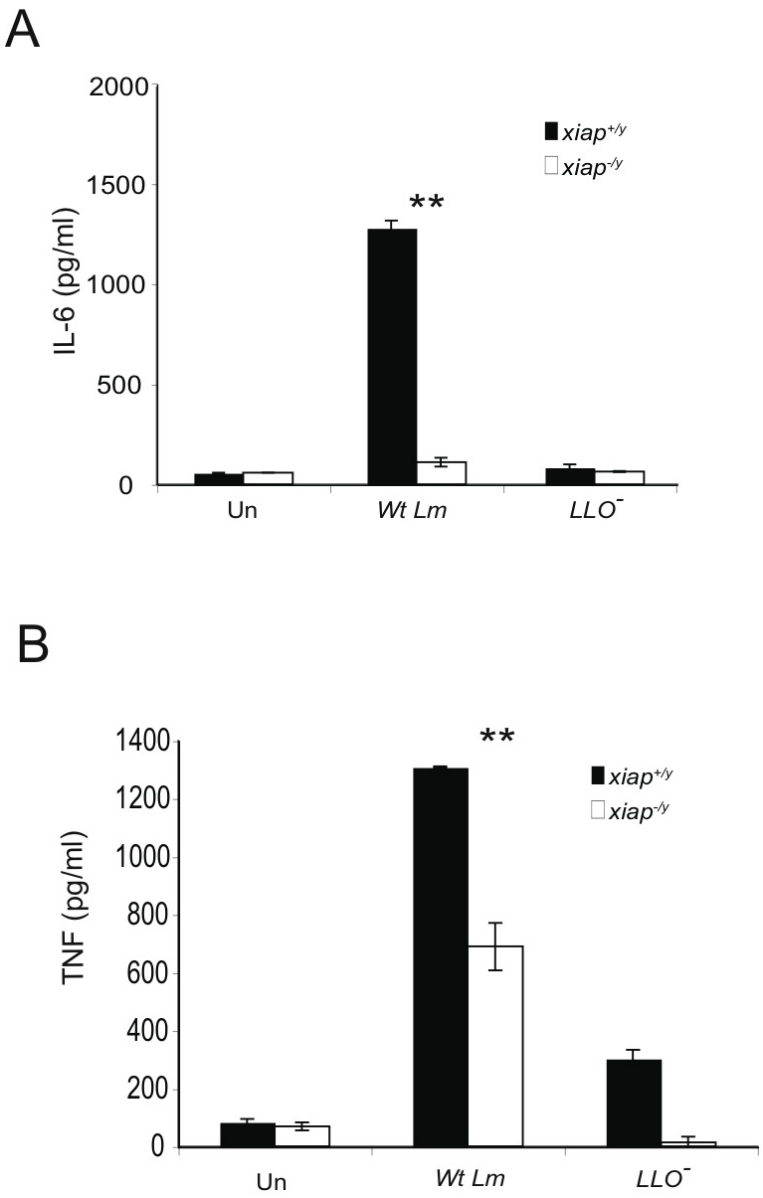


Figure 3.10 XIAP promotes secretion of proinflammatory cytokines

(A,B) ELISA of IL-6 (A) and TNF (B) secretion from activated BMDM infected with wildtype or LLO$^-$ *L. monocytogenes.* Cells were infected with *L. monocytogenes* at an MOI of 10 for 30 min. Supernatants were collected at 8h pi. Error bars represent the s.d. of macrophages from 3 animals. Results are representative of at least 3 independent experiments. * indicates $p \leq 0.05$ and ** indicates $p \leq 0.005$.

activation was required for induction of IL-6 gene expression and secretion in response to wildtype *L. monocytogenes* infection, we treated activated macrophages with the JNK inhibitor, SP600125 (**Fig. 3.11**). IL-6 secretion by infected macrophages was markedly diminished by JNK inhibition, indicating that JNK activation is required for IL-6 induction by *L. monocytogenes.* Moreover, since LLO- mutant bacteria stimulated robust but temporally limited JNK phosphorylation and little IL-6 secretion, we infer that prolonged JNK activation is necessary for maximal IL-6 production during intracellular infection by *L. monocytogenes.* When *L. monocytogenes* infected cells were treated with an ERK-specific inhibitor, IL-6 secretion was similar to the untreated infected control cells. These results collectively suggest that the presence of XIAP enhances JNK activation in response to cytosolic bacteria, resulting in increased production of proinflammatory cytokines.

XIAP enables synergy between TLR and NLR signaling

Innate immune signaling mediated by pattern recognition receptors, located on cellular membranes or in the host cytosol, stimulates transcription and secretion of proinflammatory cytokines. We used purified TLR and NLR ligands to better define a role for XIAP in innate immune signaling. Wildtype and XIAP-deficient activated macrophages were treated with TLR ligands, and secretion of IL-6 and TNF was measured after 24h (**Fig. 3.12**). While some PAMPS, such as the lipoprotein Pam_3CSK_4 could induce high levels of IL-6 and TNF, we found no XIAP-dependent differences in proinflammatory cytokine induction. These results suggest that XIAP does not contribute to cytokine output in response to TLR stimulation alone.

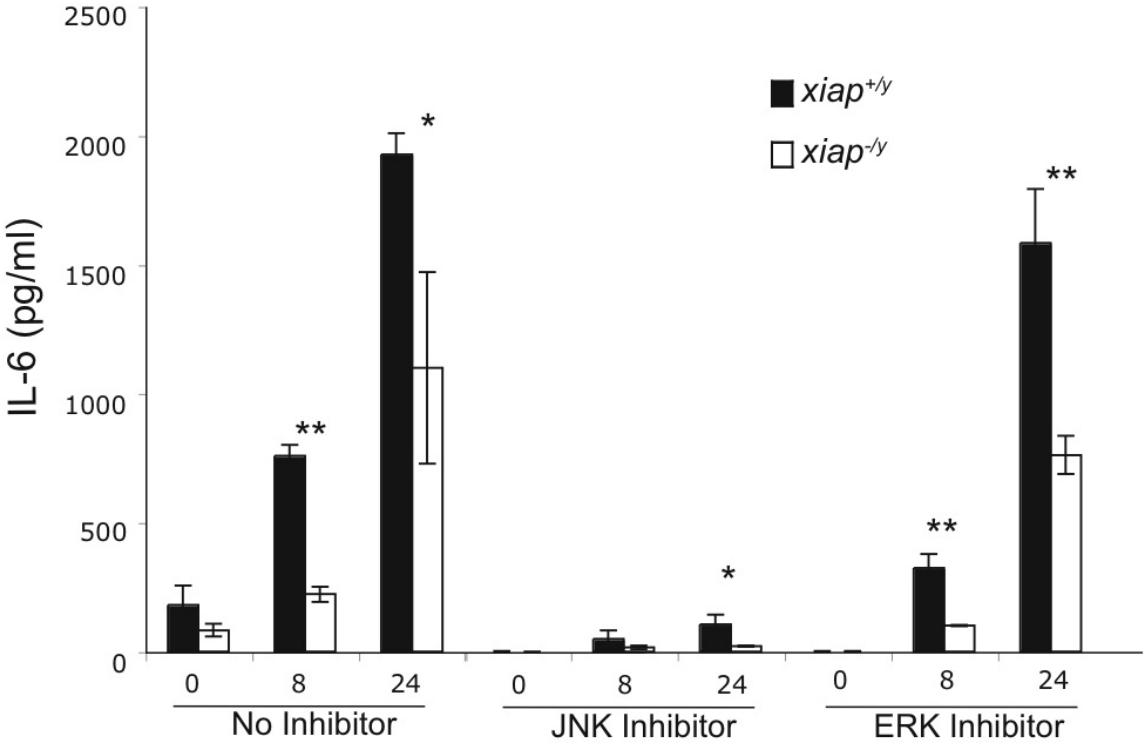


Figure 3.11 JNK activation is required for maximal production of IL6 by activated macrophages infected with *L. monocytogenes*

ELISA of IL-6 secretion from activated BMDM infected with *L. monocytogenes* and treated with the indicated inhibitors. JNK inhibitor (SP600125) was used at 20 μM, and the ERK inhibitor (U0126) was used at 10 μM. Cells were treated with inhibitors for 1h, infected at an MOI of 10 for 30 min, washed with PBS and fresh medium with 50mg/ml gentamicin and the indicated inhibitor was added. Supernatants were collected at 8 and 24h pi. Error bars represent the s.d. of macrophages from 3 animals. Results are representative of at least 3 independent experiments. * indicates $p \leq 0.05$ and ** indicates $p \leq 0.005$.

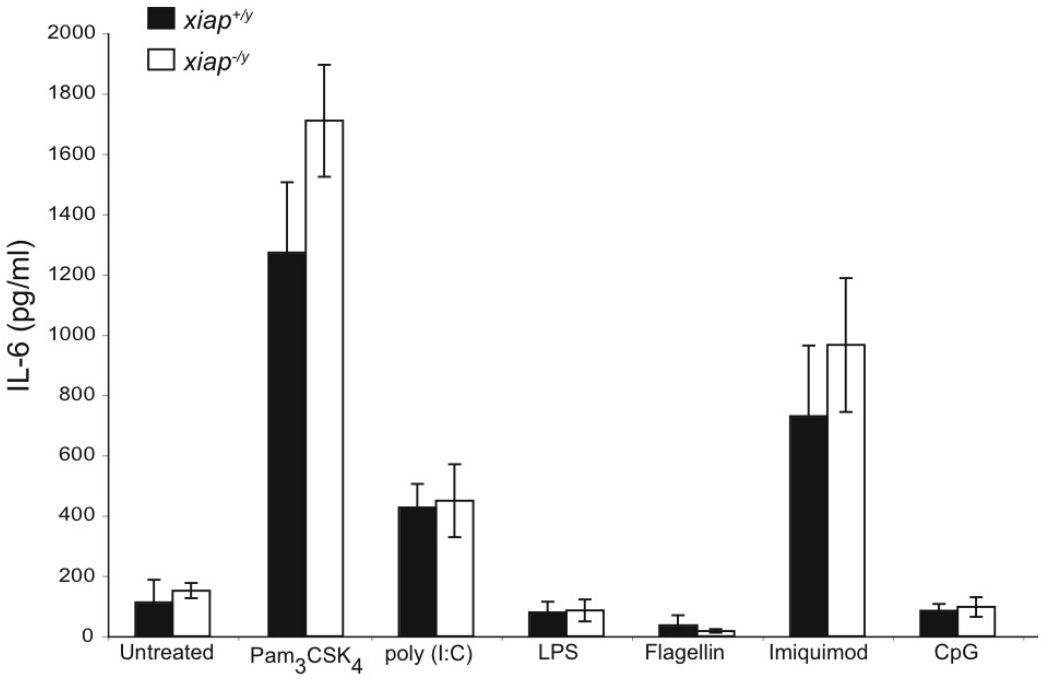


Figure 3.12 XIAP does not appear to enhance TLR signaling

IL-6 secretion from *xiap*$^{+/y}$ and *xiap*$^{-/y}$ activated BMDM treated with the indicated TLR ligands as measured by ELISA. Macrophages were activated overnight with 10 ng/ml LPS and 10 ng/ml interferon-γ. Cells were left untreated or were treated for 24h with Pam_3CSK_4 (2 μg/ml), poly (I:C) (10 μg/ml), LPS (10 ng/ml), Flagellin (10 ng/ml), Imiquimod (5 μg/ml) or CpG DNA (1μg/ml). Results are representative of at least 3 independent experiments (error bars represent s.d.).

During a physiological infection, intracellular pathogens activate both extracellular and cytosolic innate immune pathways resulting in a coordinated immune response [25]. One well-characterized consequence of microbial sensing by cytosolic NLR proteins is activation of caspase-1, which cleaves pro-IL-1β into its mature form [19]. Since XIAP can regulate the activity of some caspases, we tested whether XIAP contributed to IL-1β production, measured by ELISA, as an indicator of caspase-1 activation (**Fig. 3.13**). Consistent with previous reports, IL-1β production was induced by cytosolic *L. monocytogenes*, but was not dependent upon XIAP [107]. We next examined the activation of NLR signaling using MDP, a ligand for Nod2 (**Fig. 3.14**). No differences in cytokine secretion were observed by treatment with MDP alone, however, during a physiological infection bacteria likely present both TLR and NLR ligands to an infected host cell. PAMPs contained by *L. monocytogenes* include lipoprotein, muramyldipeptide, bacterial DNA and flagellin[25]. To better understand the role of XIAP in Nod2 signaling we examined the ability of XIAP to affect the stability of RICK, a Nod adaptor protein (**Appendix 4**). To determine if XIAP enhanced synergy between TLRs and NLRs, we examined IL-6, TNF and IL-1β secretion from xiap+/y and xiap-/y activated macrophages in response to the lipopeptide, Pam_3CSK_4, the Nod2 ligand, MDP, or both (**Fig. 3.14**). When Pam_3CSK_4 and MDP were used in combination, we saw a substantial increase in IL-6 and TNF secretion by xiap+/y but not xiap-/y activated macrophages. We did not see any XIAP-dependent enhancement of IL-1β secretion in response to Pam_3CSK_4 and MDP in combination. To better deconstruct how XIAP might participate in integrating TLR and NLR signaling, we analyzed transcription of the

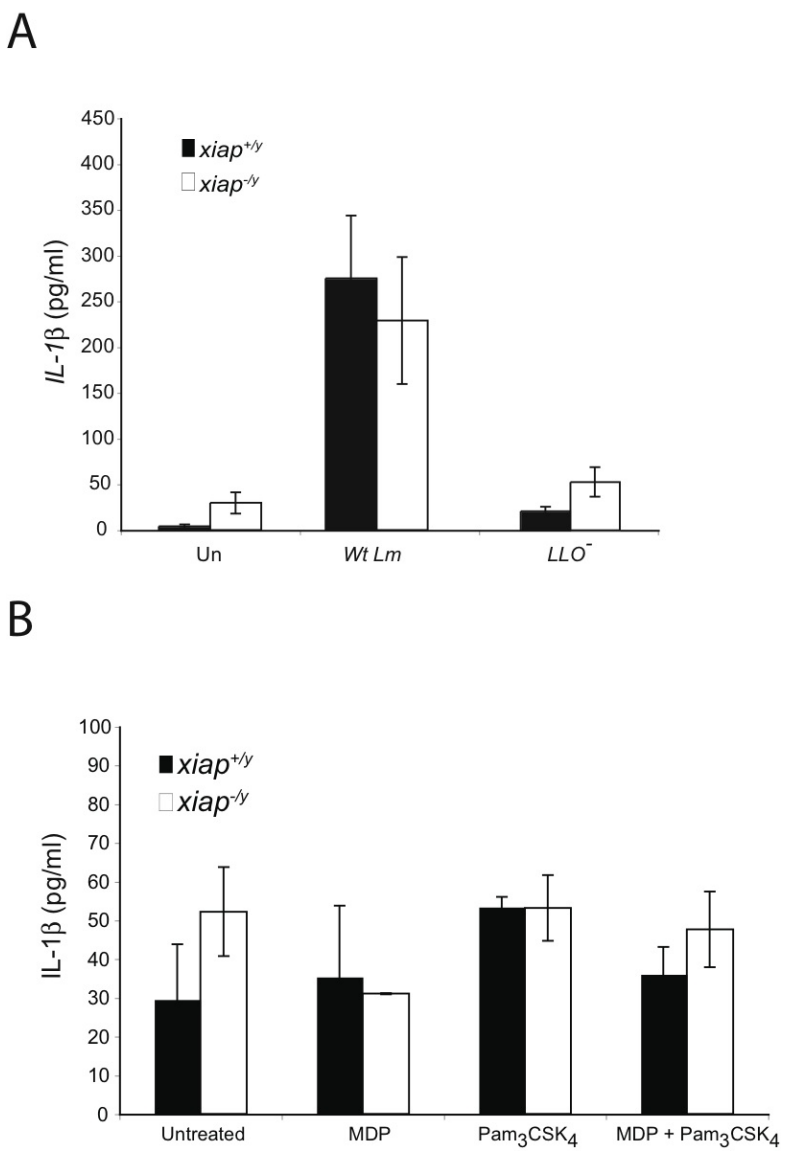
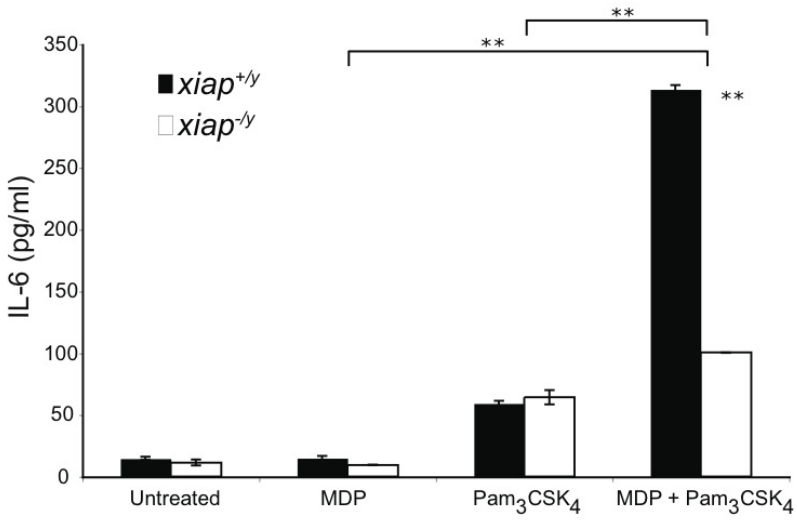


Figure 3.13 XIAP does not regulate Inflammasome signaling

(A) IL-1β from the supernatants of *xiap*$^{+/y}$ and *xiap*$^{-/y}$ activated BMDM left uninfected or infected with wildtype or LLO^{-} *L. monocytogenes* as measured by ELISA. Supernatants were collected at 8h pi. Results are representative of 3 independent experiments (error bars represent s.e.m. of cells from 6 animals). (B) ELISA of IL-1β secretion from *xiap*$^{+/y}$ and *xiap*$^{-/y}$ activated BMDM left untreated or treated for 8h with MDP (10 μg/ml) and/or Pam_3CSK_4 (0.5 μg/ml). Data are representative of 3 independent experiments with 3 mice each (error bars represent s.d.). * indicates $p \leq 0.05$ and ** indicates $p \leq 0.005$.

A



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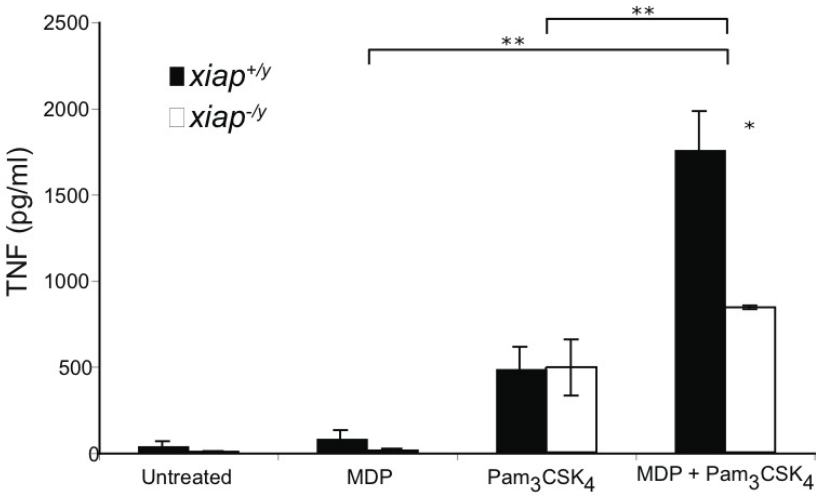

Figure 3.14 XIAP promotes synergistic proinflammatory cytokine production in response to TLR and NOD ligands

ELISA of IL-6 (A) or TNF (B) secretion from *xiap*$^{+/y}$ and *xiap*$^{-/y}$ activated BMDM left untreated or treated for 8h with MDP (10 μg/ml) and/or Pam_3CSK_4 (0.5 μg/ml). Data are representative of 3 independent experiments with 3 mice each (error bars represent s.d). * indicates $p \leq 0.05$ and ** indicates $p \leq 0.005$.

il6 gene from xiap+/y and xiap-/y activated macrophages treated with MDP, Pam_3CSK_4, or both ligands (**Fig. 3.15**). Pam_3CSK_4 induced expression of the *il6* gene in an XIAP-independent manner. Upon treatment with MDP, $xiap^{+/y}$ but not $xiap^{-/y}$ macrophages, responded by upregulating *il6* transcript levels approximately 5-fold. When macrophages were treated with both ligands, $xiap^{+/y}$ macrophages exhibited enhanced expression of il6 compared to treatment of Pam_3CSK_4 alone, but xiap-/y macrophages did not. These results demonstrate that XIAP promotes synergy between the TLR and NLR pathways, resulting in increased production of pro-inflammatory cytokines.

DISCUSSION

Here we show that XIAP can regulate innate immunity to the bacterial pathogen, *L. monocytogenes* by modulating JNK and NF-κB signaling, resulting in enhanced cytokine production. We found little evidence to suggest that XIAP regulated apoptosis of bacterially infected cells *in vitro* or *in vivo*, but instead found that XIAP promoted synergistic inflammatory cytokine expression induced by extracellular and cytosolic innate immune signaling upon bacterial infection of activated macrophages. Specifically, XIAP amplified the cytosolic response to MDP or wildtype *L. monocytogenes*. These data identify XIAP as a regulator of cytosolic innate immune signaling. Notably, another IAP family member NAIP5 was found to mediate caspase-1 activation in response to cytosolic bacterial flagellin [108,109,110]. NAIP5 function in innate immunity could be attributed to the atypical domain structure of this IAP protein that exhibits similarities to the NLR family of cytosolic sensors [111]. However, these data taken together with our

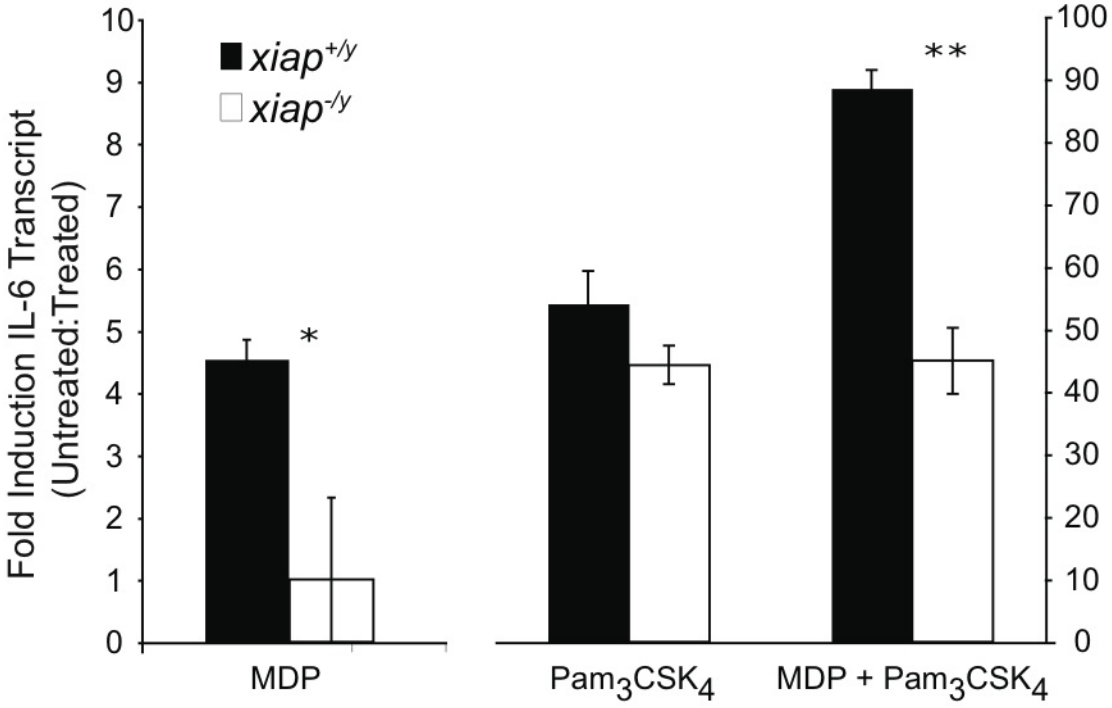

Figure 3.15 Transcription of proinflammatory cytokine genes is synergistically enhanced by XIAP in response to TLR and NOD ligands

qRTPCR analysis of IL-6 gene expression at 3h in *xiap*$^{+/y}$ and *xiap*$^{-/y}$ activated BMDM treated with MDP (10 μg/ml) and/or Pam_3CSK_4 (0.5 μg/ml). The data shown are form the same experiment, but are represented on different graphs to show y values more accurately. Data are representative of 3 independent experiments with 3 mice each (error bars represent s.d). * indicates $p \leq 0.05$ and ** indicates $p \leq 0.005$.

results lead us to speculate that regulation of innate immune signaling is an important role of mammalian IAPs.

The IAP family appears to play multiple roles in mammalian biology, including protecting cells from apoptotic stimuli, regulating the cell cycle and modulating innate immune signaling. As a whole, these studies are consistent with genetic evidence in *Drosophila* demonstrating that dIAP1 primarily protects insect cells from programmed cell death, while dIAP2 is required for anti-microbial function of the Imd pathway [76,77,78,79]. The Imd pathway in *Drosophila* is activated by peptidoglycan recognition proteins (PGRPs), while functionally analogous innate immune sensing of peptidoglycan in mammalian cells occurs in the cytosol by Nod1, Nod2 and Nalp3 [112]. The Imd protein in *Drosophila* shares sequence homology with the mammalian RIP proteins, and a mammalian paralog, RIP2, is an essential signaling adaptor for the cytosolic peptidoglycan sensors, Nod1 and Nod2 [4,113,114,115]. Thus, the Imd/RIP innate immune signaling module appears to have been co-opted for mammalian cytosolic surveillance for peptidoglycan. Genetic epistasis experiments in *Drosophila* place dIAP2 in parallel to TAK1 upstream of JNK and NF-kB signaling pathways [77]. Similarly, in mammalian cells, XIAP can modulate JNK and NF-kB signaling through TAK1 in endothelial cells and fibroblasts[93,116]. Activation of either Nod1 or Nod2 activates TAK1, leading us to hypothesize that during bacterial infection, XIAP may facilitate this key association, linking cytosolic sensors to downstream signaling mediators [117,118].

During infection, microbial pathogens present multiple PAMPs recognized by the innate immune system, eliciting a coordinated protective response. This concept is illustrated by the paradigm of IL-1β processing, where TLRs mediate transcription of pro-IL-1β however, cleavage and secretion are dependent upon activation of the caspase-1 inflammasome by cytosolic PAMPs [119]. However, IL-1β deficient mice are as resistant to *L. monocytogenes* infection as wildtype mice, suggesting that other inflammatory cytokines mediate innate immune control of this infection [120]. In contrast, IL-6-, TNF- and IFNγ-deficient mice are more susceptible to *L. monocytogenes* infection at 48h pi than wildtype mice, demonstrating a requirement for IL-6, TNF and IFNγ in protection from this particular pathogen [47,48,121,122,123]. IFNγ is largely produced by innate immune effector cells other than macrophages, thus our observation that *ifng* transcription is decreased in the spleens of *L. monocytogenes*-infected XIAP mutant mice must be due to either a XIAP-dependent cell autonomous defect in a different cell type or a non-autonomous defect in an IFNγ producing cell resulting from a defect in macrophages [124]. Since XIAP is expressed in many different tissues, it is reasonable to suppose that XIAP may have pleiotropic effects in the innate immune response to *L. monocytogenes* [125]. However, macrophages are primary producers of IL-6 and TNF, and notably, Nod2 signaling is known to stimulate production of IL-6 and TNF [16,113]. The deficit in IL-6 and TNF production we observed in infected *xiap*$^{-/y}$ activated macrophages, and the defect in gene expression *in vivo* likely contributes to the enhanced susceptibility of XIAP-deficient animals to *L. monocytogenes* infection. Recent reports indicate that macrophages treated with LPS become tolerized to re-stimulation with TLR ligands [126,127]. Additionally, when macrophages are tolerized by LPS, the role of

Nod1 and Nod2 in cytosolic surveillance becomes more critical during infection [59]. In our model, macrophages are activated with LPS and IFNγ prior to infection. We examined the role of LPS and IFNγ in XIAP-dependent signaling in activated macrophages in **Appendix 5**. When activated macrophages are infected with *L. monocytogenes,* the induction of proinflammatory cytokines is XIAP-dependent, indicating that XIAP plays a more critical role in regulating the innate immune response to cytosolic pathogens in macrophages where the TLR pathway may be tolerized and an inflammatory gene expression program initiated. We use these data to integrate XIAP into a cytosolic surveillance model whereby upon recognition of microbial ligands in the cytosol by innate immune sensors such as Nod2, XIAP enhances association and function of signal transducers such as TAK1 and JNK [93,98]. Recruitment of signaling molecules by XIAP upon NLR stimulation would potentiate signaling pathways activated by TLRs, leading to maximal proinflammatory cytokine production.

Apoptotic and microbial stimuli activate similar signaling pathways, but may lead to different outcomes. Macrophages as innate immune effector cells can control microbial infection by secreting cytokines and other pro-inflammatory molecules or by carrying out programmed cell death[128]. It has been hypothesized that when macrophages receive a strong inflammatory stimulus, they undergo apoptosis rather than secreting cytokines as a means of protecting the host [108,129,130]. Although previous data implicated XIAP in modulating apoptosis, our data demonstrate that XIAP also has an important role in proinflammatory cytokine production. However, we suggest that these two functions for XIAP may not be completely distinct, as the outcome of XIAP-dependent modulation of

JNK and NF- κB pathways may depend on the quality and intensity of the stimulus [105]. Additionally, the ability of XIAP to regulate innate immunity is likely cell type and context dependent, as we did not see reproducible XIAP-dependent transcriptional regulation in unactivated macrophages. Future studies in Chapter 4 will help to elucidate the complex role of XIAP in the mammalian immune response.

MATERIALS AND METHODS

Animals, bacterial strains and infections. For description of the XIAP-deficient mice see Chapter 2 methods. For cell culture infections, *Listeria monocytogenes* strains 10403S (wildtype) and hly- (LLO-) were inoculated into liquid brain-heart infusion (BHI) broth and incubated at 30°C overnight without shaking[86]. Prior to infection, *L. monocytogenes* cultures were washed and resuspended in PBS. HKLM was prepared by incubating bacteria at 70°C for 1h. For animal infection protocols, see Chapter 2 methods. For evaluation of survival, animals were infected with $1x10^5$ or $5x10^5$ *L. monocytogenes*, and observed every 24h post-infection. For histology, the spleen and liver from infected mice were harvested at 48h pi and fixed in 10% neutral buffered formalin. Paraffin sections were prepared and stained with ApopTag by the Cancer Center Research Histology and Immunoperoxidase Lab at the University of Michigan.

BMDM culture. See Chapter 2 methods for BMDM culture. BMDM were activated overnight in 10 ng/ml LPS (Sigma #L6143) and 10 ng/ml (100units/ml) interferon-γ (Peprotech #315-05). Activated macrophages were infected with *L. monocytogenes* at an MOI of 10, such that bacteria were observed in the cytosol in approximately 99% of the

macrophages. Peritoneal macrophages were harvested by peritoneal lavage. Cells were pooled from two mice prior to plating. For *L. monocytogenes* growth curves, cells were plated on coverslips at a density of 1.7 x10^5 cells/ ml in 24 well plates. Macrophages were infected with *L. monocytogenes* for 0.5h, washed 3 times with PBS, followed by addition of fresh medium with 50 μg/ml gentamicin. At each time point, 3 coverslips were lysed in water and plated on LB agar plates for to determine CFU. IL-6 (R&D systems), IL-1β (R&D systems) and TNF (University of Michigan Cellular Immunology Core) in the culture medium were measured by ELISA. Where indicated, cells were treated for 30 min with TLR ligands as follows: MDP 10 μg/ml (Bachem #4009623), Pam_3CSK_4 2 μg/ml (Invivogen #tlrl-pms), poly (I:C) 10 μg/ml, LPS 10 ng/ml (Sigma #L6143), Flagellin 10 ng/ml (Invivogen #tlrl-flic), Imiquimod 5 μg/ml (Invivogen #tlrl-imq), CpG DNA 1 μg/ml (IDT CpG F (5'-TCCATGACGTTCCTGACGTT), CpG R (5'-AACGTCAGGAACGTCATGGA)). At 8 and 24h post treatment, supernatants were harvested for measurement of cytokines by ELISA. Inhibition experiments were conducted as described above, except cells were treated with 20 μM JNK inhibitor, SP600125 (Sigma #S5567), or 10 μM ERK inhibitor U0126 (Cell Signaling #9903) for 1h prior to infection. For nuclear and cytoplasmic fractionation, cells were lysed in NP-40 lysis buffer (50mM Tris pH 8, 5mM EDTA pH 8, 150mM NaCl, 0.05% NP-40 (Igepal), EDTA-free protease inhibitor cocktail (Roche)). Nuclei were pelleted by centrifugation at 1000 rpm for 5 min; the cytosolic fraction was further clarified by centrifugation at 14000 rpm for 10 min. Nuclei were washed and either resuspended in 2x SDS-PAGE lysis buffer for immunoblot or lysed for NF-kB ELISA by resuspension in nuclear lysis buffer (20mM HEPES pH 7.9, 400mM NaCl, 1mM EDTA, 10% glycerol,

0.1mM DTT, EDTA-free protease inhibitor cocktail (Roche)) and incubated at 4 ºC for 30min. Nuclei were flash frozen and used for NF-κB p65 ELISA analysis (Stressgen EKS-446).

Apoptosis assays. BMDM were plated and activated overnight in 10ng/ml LPS and 10ng/ml interferon-γ. Cells were infected for 30 min at an MOI of 10, bacteria were removed by 3 washes with PBS, and fresh medium containing 50 μg/ml gentamicin added. At 3h pi, the medium was removed and spun to collect any non-adherent cells; the remaining cells were removed from the dish by incubating with ice cold PBS without calcium and magnesium for 20 min at 4°C. Cells were stained with Annexin V and propidium iodide according to the manufacturer's protocol (BD Biosciences #556420). Flow Cytometry. Splenocytes were harvested from uninfected or L. monocytogenes infected mice. BMDM were harvested from plates with ice cold PBS without Ca+ or Mg+. Cells were blocked with F_c block (BD Pharmingen 553142) for 15 min on ice. Cells were incubated in staining buffer (PBS, 10%FBS) with the indicated antibodies for 20 min on ice, followed by 3 washes in staining buffer. When necessary cells were incubated with secondary antibodies in staining buffer on ice for 20 min, and washed 3 times in staining buffer. Flow cytometric acquisition was performed on a FACSCanto. The data was analyzed using FlowJo software. The following antibodies were used: from BD Pharmingen; B220-PE (553089), NK1.1-biotin (553163), CD69-PE (553237); from Southern Biotech CD3 (1530-02), Streptavidin-APC (7100-11L).

Immunoblot analysis. See Chapter 2 methods for detailed protocol. The following antibodies were used: β-actin (Sigma #A5441), NF-κB p50 (Santa Cruz Biotechnology #8414), USF-1 (Santa Cruz Biotechnology #8983), Phospho-JNK (Cell Signaling 9251), JNK1 (Santa Cruz Biotechnology #571), Phospho-p38 kit (Cell Signaling 9210), Phospho-c-jun (Santa Cruz Biotechnology #822), Phospho-ERK (Cell Signaling 4377), ERK-1 (Santa Cruz Biotechnology #94), goat anti Rabbit IgG-HRP (MP Biomedical #67438), goat anti-mouse IgG-HRP (MP Biomedical #67429).

RNA isolation and quantitative RT-PCR analysis. For RT-PCR, total RNA was harvested from infected or treated cells at 3h pi with the RNeasy Mini Kit (Qiagen). The RNA was used in a reverse transcriptase (RT) reaction with Moloney murine leukemia virus (MMLV) RT (Invitrogen). cDNA obtained from the RT reaction was used for qRT-PCR amplification and quantitation by SYBR Green (Stratagene MX3000p). Data was analyzed using the ΔΔCt method (ΔΔCt = 2(ΔCt sample-ΔCt normalizer)) with b-actin used as a normalizer for in vitro experiments and *gapdh* used as a normalizer for *in vivo* experiments.

Statistical Analysis. A two-tailed t-test was used for statistical analysis; p values of ≤0.05 were considered significant, while p values ≤0.001 were considered highly significant.

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Chapter 4 : Regulation of innate immunity *in vivo* by XIAP

ABSTRACT

The success of the innate immune response relies on a series of complex interactions between the cells of the innate immune system to coordinate both control of the pathogen and effective instruction of the adaptive immune response. Detection of cytosolic bacteria is required during a *Listeria monocytogenes (Lm)* infection to promote protective immunity. Immunization with a strain of *Lm* deficient in listeriolysin O (LLO), which is trapped in the vacuole, does not protect against a secondary infection. Here we show that XIAP regulates the expression of proinflammatory cytokines *in vivo*. Additionally, XIAP is required for trafficking of *Lm* infected phagocytes to the white pulp of the spleen, where approximately 42% of the follicles exhibit disrupted T cell zones. Thus, we propose that XIAP promotes the proinflammatory cytokine environment necessary to effectively traffic *Lm* to the white pulp of the spleen.

INTRODUCTION

The ability of a vertebrate organism to clear infection relies upon both the innate and the adaptive immune response. During a *Lm* infection the innate immune system is critical for preventing rampant bacterial replication, while providing signals to the adaptive immune response to enable differentiation of CD8+ cytotoxic T cells[30]. Upon *Lm*

infection, phagocytes are recruited to engulf the bacteria; once taken up into a vacuole, *Lm* can be killed or it can mediate its own escape into the cytosol[131]. Exposure of host cells to *Lm* rapidly stimulates the immune response by activating immune cells, which is essential for host survival[25]. Activation of phagocytes induces expression of cytokines and cytokine receptors. Animals that are deficient in inflammatory cytokines or their receptors including, IFNγ, TNF or the TNFRp55 receptor, are very susceptible to *Lm* infection[122,132,133]. Another main function of phagocytes is to present antigen to the adaptive immune system. To do this, the phagocyte must traffic to the spleen or lymph nodes and display pathogen specific epitopes to T cells. Phagocytes respond to cytokine and chemokines gradients that direct localization in the spleen. In animals where the recognition of these chemical gradients are disrupted, such as in CCR2-deficient mice, phagocytes do not properly traffic and the animals succumb to *Lm* infection[134]. During a wildtype *Lm* infection, cells infected with cytosolic bacteria migrate to the T cell zone of the splenic white pulp, and then substantial apoptosis of the T cells occurs[41,135,136]. The specific role innate immune signaling may play in phagocyte trafficking and development of the adaptive immune response is unclear.

The location of bacteria in a phagocyte and the organization of innate immune cells in the spleen are both critical for the development of a productive immune response. When mice are infected with heat-killed *Lm*, or the LLO-deficient strain of *Lm,* neither of which can escape into the cytosol, the infected phagocytes do not localize to the white pulp of the spleen but instead concentrate in the marginal zone and red pulp. This results in an nonproductive adaptive immune response, which prevents long-term protective

immunity[50,137]. Additionally, the induction of early IFNγ *in vivo* during an *Lm* infection requires LLO expression[138]. LLO is a pore forming protein secreted by *Lm* that enables vacuolar escape, but in addition it is known to activate innate immune pathways, such as the inflammasome[139]. These results suggest that escape into the cytosol by the bacteria or expression of the LLO antigen, or both, are critical for splenic localization and development of a protective adaptive immune response[137].

Our work has implicated XIAP, an IAP family member, in regulating the innate immune response to cytosolic *Lm* infection. Specifically, we have shown that XIAP plays a critical role in promoting proinflammatory cytokine production in macrophages during cytosolic *Lm* infection. Additionally, our data suggest that XIAP regulates synergy between the TLR and NLR signaling pathways, resulting in enhanced proinflammatory cytokine production. However these results are from studies using primary macrophages in culture and may not reflect the complex interactions that occur between innate immune cells *in vivo*. To understand the role of XIAP *in vivo* during *Lm* infection, we have examined several aspects of the immune response including *in vivo* cytokine expression and production, and tissue morphology and composition in the infected organs. We have determined that XIAP regulates the localization of the bacteria during infection, which is required for control of bacterial replication.

RESULTS

XIAP promotes proinflammatory cytokine expression *in vivo* during *L. monocytogenes* infection

Our previous work has shown that when macrophages are infected with cytosolic *Lm*, XIAP promotes enhanced cytokine expression. We wanted to determine if immune cells obtained from infected animals would also display enhanced expression of proinflammatory cytokines. We first examined the expression of several proinflammatory cytokines by quantitative RT-PCR analysis of splenic RNA (**Fig. 4.1**). Mice were infected with $5x10^5$ CFU of *Lm* intraperitoneally (i.p.), and the spleens and livers were isolated at 48 hours post infection (hpi). Livers were homogenized and the bacterial CFU were enumerated to assess infection. Spleens were also homogenized and the resulting lysates were used to extract RNA for qRT-PCR analysis of proinflammatory cytokine gene expression. We examined the expression of several proinflammatory cytokines including, IL-6, TNF and IFN-γ produced during the innate immune response that are critical for clearing *L. monocytogenes* infection[47,48,122]. The expression of *il6* and *ifng* were significantly enhanced in the presence of XIAP during infection, while expression of *tnf* and *ifnb* were not altered. We also measured the expression of *il17*, which encodes a cytokine known to enhance expression of *il6*, but observed no reproducible differences in *il17* expression between wildtype and XIAP-deficient splenocytes[140]. These data support the results from our *in vitro* macrophage model and demonstrate that XIAP promotes the expression of proinflammatory cytokine genes in response to *L. monocytogenes* infection *in vivo.*

XIAP-deficient animals produce IL-6 at a level disproportionate to bacterial load

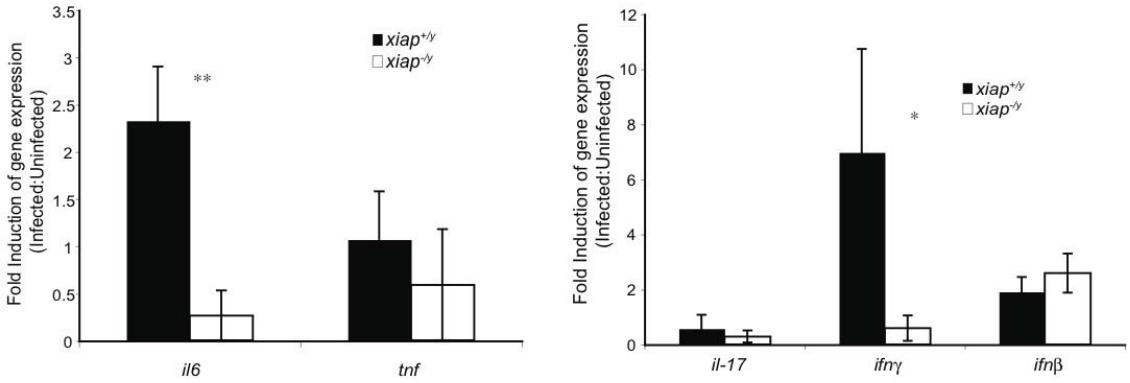

Figure 4.1 *In vivo L. monocytogenes* infection induces XIAP-dependent proinflammatory cytokine expression.

qRT-PCR of genes associated with innate immune activation. Mice were infected with *Lm*, splenocytes were harvested at 48h pi for RNA isolation and production of cDNA. Fold induction was calculated using the $\Delta\Delta C_t$ method where uninfected samples were compared to infected samples, relative to β2M levels. * indicates $p \leq 0.05$ and ** indicates $p \leq 0.005$. (error bars represent s.d.).

The XIAP-deficient animals have 10-fold more bacteria at 48hpi. To determine if the bacterial load affected the amount of IL-6 produced, we examined the production of IL-6 by macrophages in response to varied amounts of *Lm*. Primary bone marrow derived macrophages from wildtype and XIAP-deficient animals were activated overnight with LPS (10ng/ml) and IFNγ (10ng/ml) and then infected with *Lm* at an MOI of 1, 10 or 100 for 30 min (**Fig 4.2**). At 24hpi, the amount of IL-6 in the culture supernatant was quantified by ELISA. The XIAP-deficient macrophages produce less IL-6 than wildtype macrophages when stimulated with the same bacterial load. However, we observed similar levels of IL-6 in wildtype cells infected at an MOI of 10 and in XIAP-deficient cells infected at an MOI of 100. This suggests that per bacteria, the XIAP-deficient macrophages produce less IL-6. However, *in vivo*, the XIAP-deficient animals have 10-times more bacteria, therefore, we would predict that the amount of IL-6 produced would be similar in wildtype and XIAP-deficient animals. To determine the proinflammatory cytokine production *in vivo* during *Lm* infection, we quantitated the number of splenocytes producing IL-6 after a 48h *Lm* infection. Splenocytes were harvested from uninfected mice or animals infected with *Lm* for 24 or 48hpi. IL-6 secreting cells were quantitated by ELISPOT analysis. The number of IL-6 secreting cells was similar in the wildtype and XIAP-deficient animals at 24 and 48hpi (**Fig 4.3**). Taken together these data suggest that since the number of bacteria in the XIAP-deficient mice is 10-fold higher than the wildtype animals, the increase in bacterial burden likely accounts for the similar number of IL-6 secreting cells seen in wildtype and XIAP-deficient splenocytes.

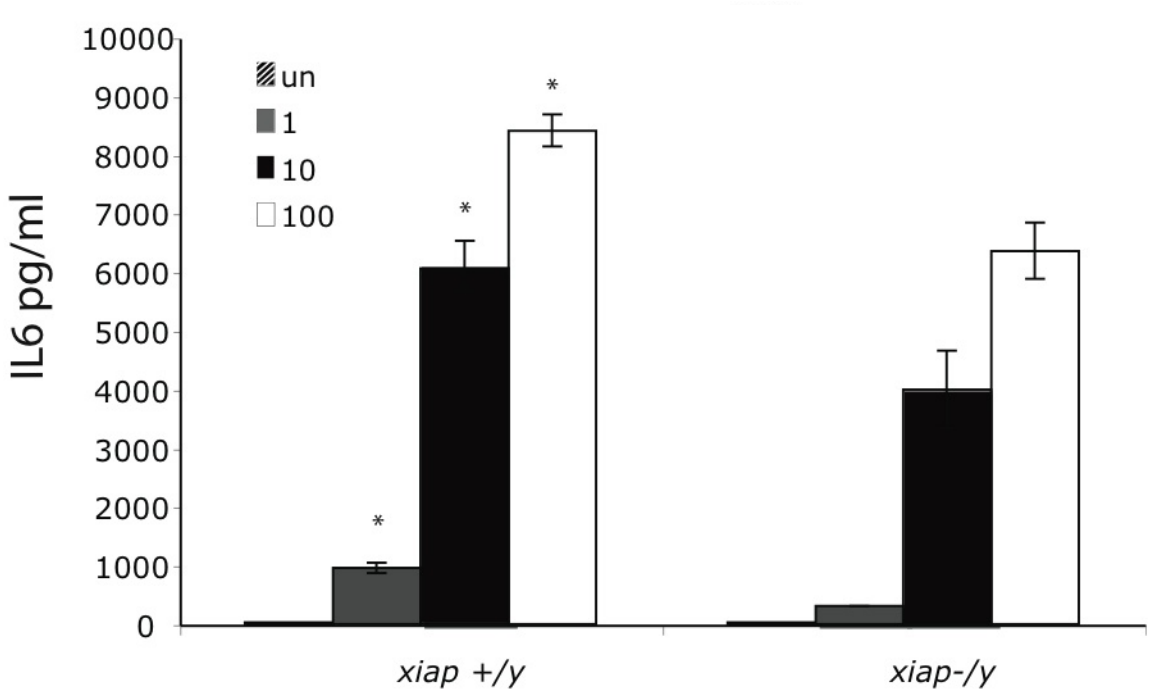

Figure 4.2 XIAP-deficient macrophages produce less IL-6 than wildtype cells

ELISA of IL-6 secretion from activated BMDM infected with wildtype *L. monocytogenes.* Wildtype or XIAP-deficient macrophages were infected with *Lm* at a MOI of 1, 10 or 100 bacteria per cell for 30 min. Supernatants were collected at 8h pi. * indicates $p \leq 0.05$, for comparison between wildtype and XIAP-deficient samples at the same MOI. The amount of IL-6 produced by the wildtype sample infected with an MOI of 10 and the XIAP-deficient sample infected at an MOI of 100 is not statistically different. (error bars represent s.d.).

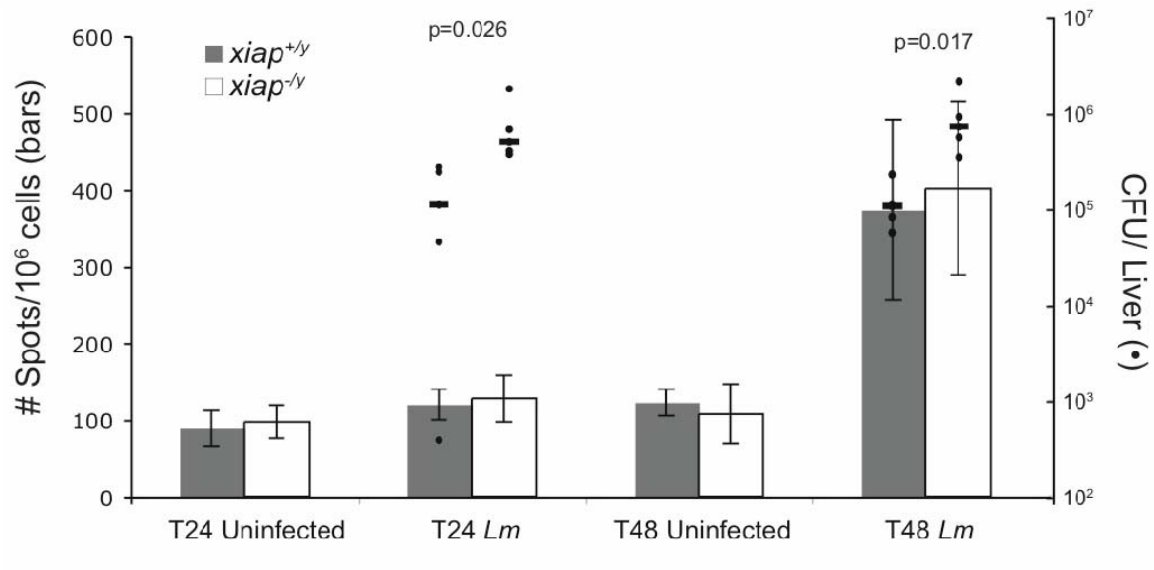

Figure 4.3 IL-6 production in XIAP-deficient mice is equal to wildtype due to a 10-fold increase in bacterial burden

ELISPOT analysis of IL-6 producing splenocytes harvested from uninfected or *Lm* infected animals. Mice were infected with $5x10^5$ Lm i.p., spleens were harvested at 48h, dissociated and plated in 96 well plates for ELISPOT analysis. IL-6 secretion was analyzed after a 24h incubation. Livers were also harvested and bacterial burden was enumerated. Left y-axis indicates ELISPOT analysis. Right y-axis indicates bacterial CFU. The median CFU of each group is indicated by a horizontal line. Each group contained 5 mice. This data is representative of 3 independent experiments. (error bars represent s.d.). P values indicated are for CFU data.

Decreased IL-12 expression in XIAP-deficient macrophages correlates with decreased IFNγ in XIAP-deficient mice

IFNγ activation of innate immune cells is critical for controlling *Lm* replication during infection[47]. IFNγ production is stimulated by IL-12 and IL-18 and is produced by natural killer dendritic cells, natural killer cells, dendritic cells and T cells[124,141]. We observed decreased *ifng* expression in splenocytes from XIAP-deficient infected animals compared to wildtype. Furthermore, activated macrophages infected with *Lm* depend upon XIAP to promote proinflammatory cytokine production. To determine if the reason for decreased *ifng* expression *in vivo* was due to decreased *il12* or *il18* expression by phagocytes, we examined the expression of *il12* and *il18* by qRT-PCR analysis of RNA isolated from uninfected or infected activated wildtype or XIAP-deficient macrophages (**Fig4.4**). We observed similar levels of *il18* gene expression but decreased levels of *il12* gene expression in the XIAP-deficient macrophages compared to wildtype cells. These results suggest that the decreased expression of *il12* in macrophages may contribute to the decreased expression of *ifng* observed *in vivo* and the increased susceptibility of the XIAP-deficient animals during *Lm* infection.

XIAP-deficient animals display altered splenic morphology after *Lm* infection

To better understand the role of XIAP *in vivo* during a *Lm* infection, we harvested spleens and performed histological analysis. Wildtype and XIAP-deficient animals were infected i.p. for 48h, at which time the spleens were isolated and prepared for cryosectioning. Spleens were also obtained from uninfected animals as a control. CFU were enumerated from the livers of infected animals to monitor the bacterial burden. Spleen sections were

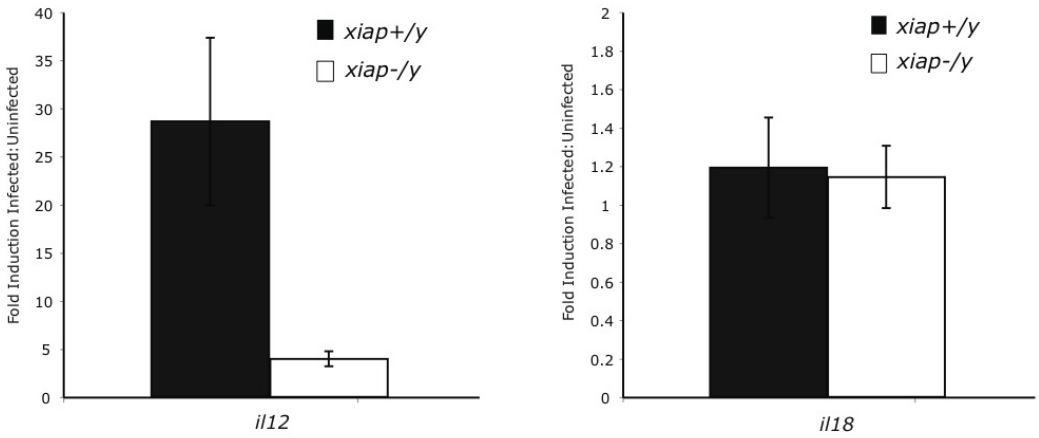

Figure 4.4 XIAP-deficient macrophages display decreased *il12* gene expression

qRT-PCR of genes associated with IFNγ expression. Wildtype or XIAP-deficient macrophages were infected with *Lm* at a MOI 10 bacteria per cell for 30 min. RNA was isolated 3hpi, and cDNA was synthesized to perform qRT-PCR. Fold induction was calculated using the $\Delta\Delta C_t$ method where uninfected samples were compared to infected samples, relative to β-actin levels. * indicates $p \leq 0.05$ (error bars represent s.d.).

stained with hematoxylin and eosin to visualize the splenic architecture (**Fig 4.5**). In the wildtype animals at 48hpi, the follicles of the spleen were enlarged due to lymphocyte recruitment. The follicles also contained a lesion, where cells were depleted. Previous work has shown an increase in the number of apoptotic cells in the area of clearing in the follicles, suggesting these cell are undergoing programmed cell death[135]. In the XIAP-deficient animals the follicles are smaller and there are fewer lesions compared to those observed in the wildtype animals.

XIAP-deficient spleens contain similar cell populations to wildtype animals

We reasoned that the lesions observed in the wildtype animals would likely result in an alteration of immune cell populations when compared to the XIAP-deficient animals. Therefore, we quantitated the splenocyte population in the wildtype and XIAP-deficient animals by flow cytometry. Splenocytes were isolated from uninfected mice and mice infected with *Lm* for 48h. Cells were with stained with various fluorescent antibodies to label different cell populations. We specifically examined T and B cells, phagocyte populations and activation of immune cells (**Fig 4.6, 4.7,** data not shown). We observed no significant differences in the cell populations of the wildtype or XIAP-deficient animals after *Lm* infection. This data indicates that the histological differences that were observed in H&E stained sections are likely not attributable to differences in the size of the cell populations between the wildtype and XIAP-deficient splenocytes.

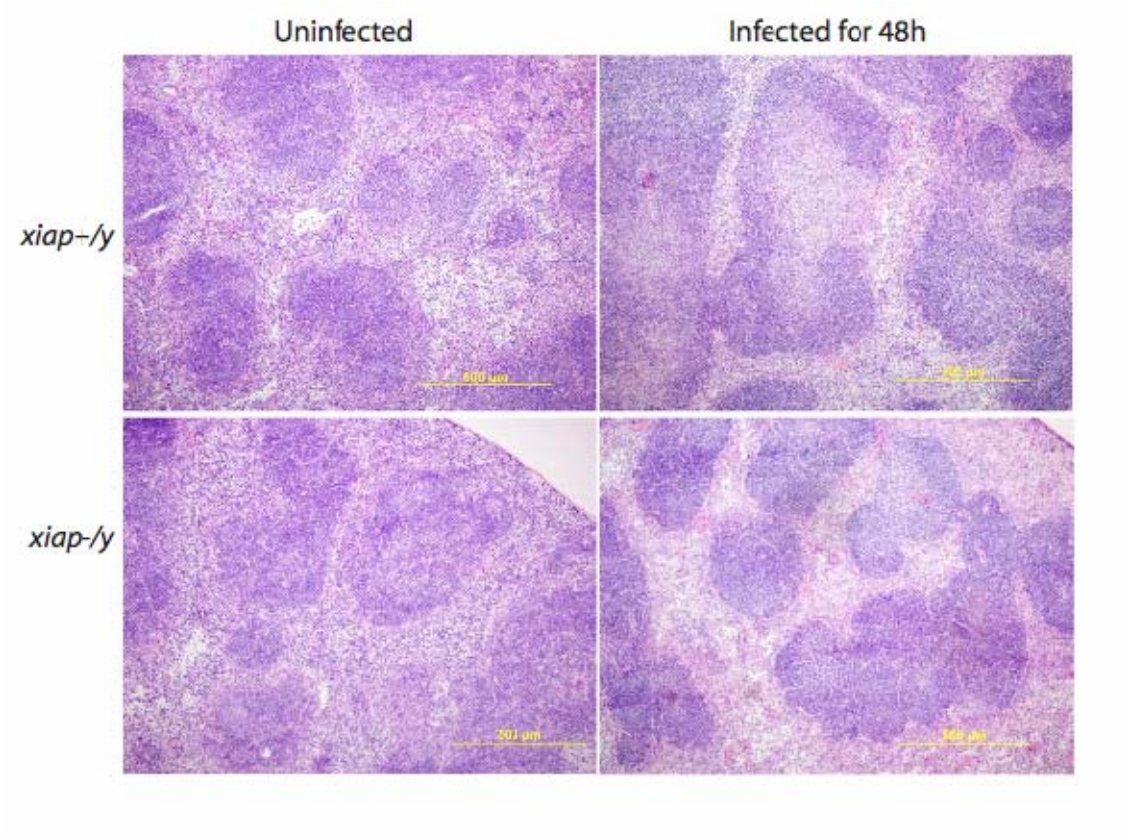

Figure 4.5 XIAP-deficient animals display altered splenic morphology after *Lm* infection

Hematoxylin and Eosin staining of histological sections of spleens from wildtype and XIAP-deficient mice infected with *Lm* i.p. at $5x10^5$ for 48 h (n=3 animals/genotype). Ten sections per animal were examined and representative sections are shown..

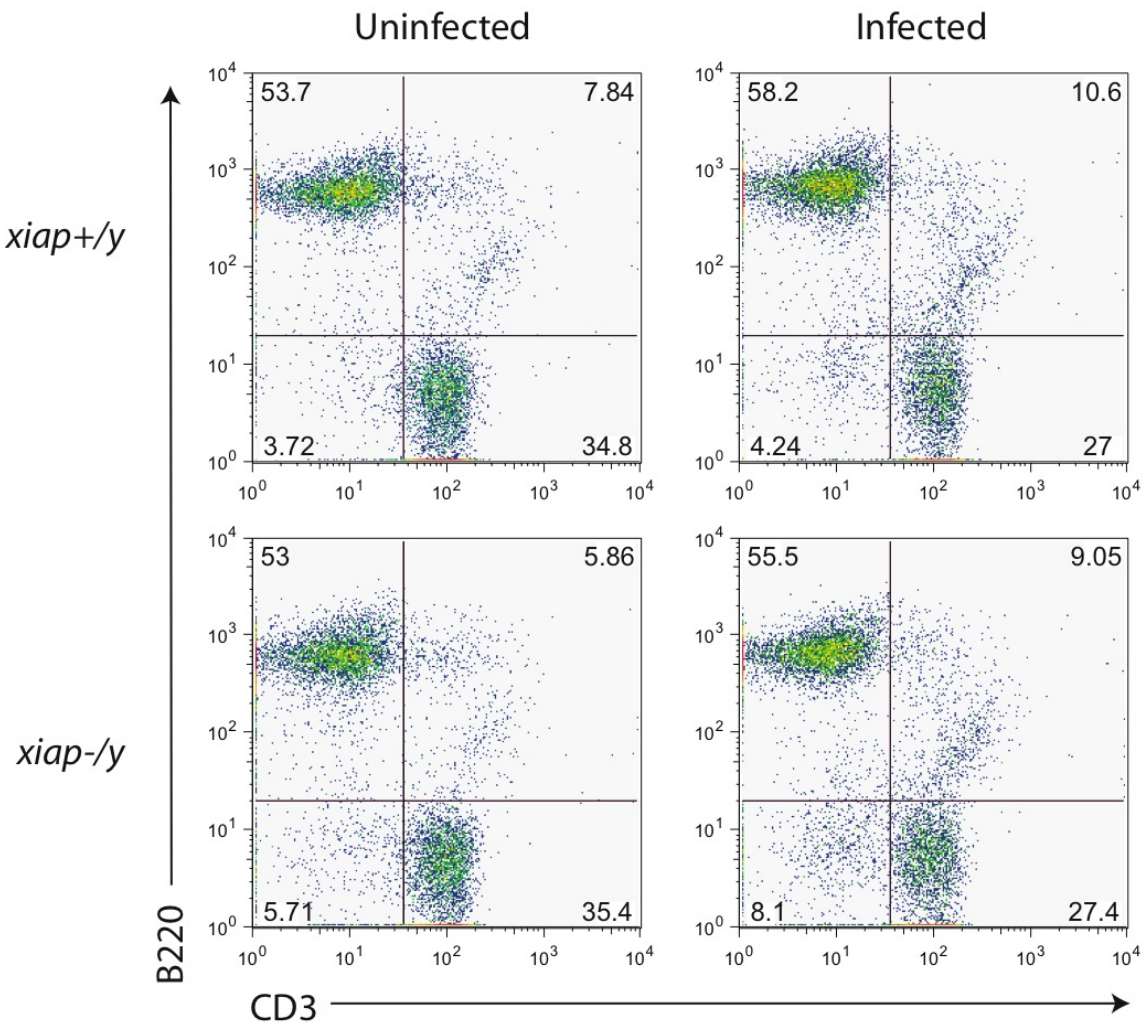

Figure 4.6 B and T lymphocyte populations are not altered in the XIAP-deficient animals

Flow cytometric analysis of B220+CD3+ splenocytes from uninfected and *Lm* infected animals. Splenocytes were harvested from infected animals at 48h pi, and stained with B220-PE and CD3-FITC antibodies for flow cytometry analysis. Results are representative of at least three independent experiments (n≥9 animals).

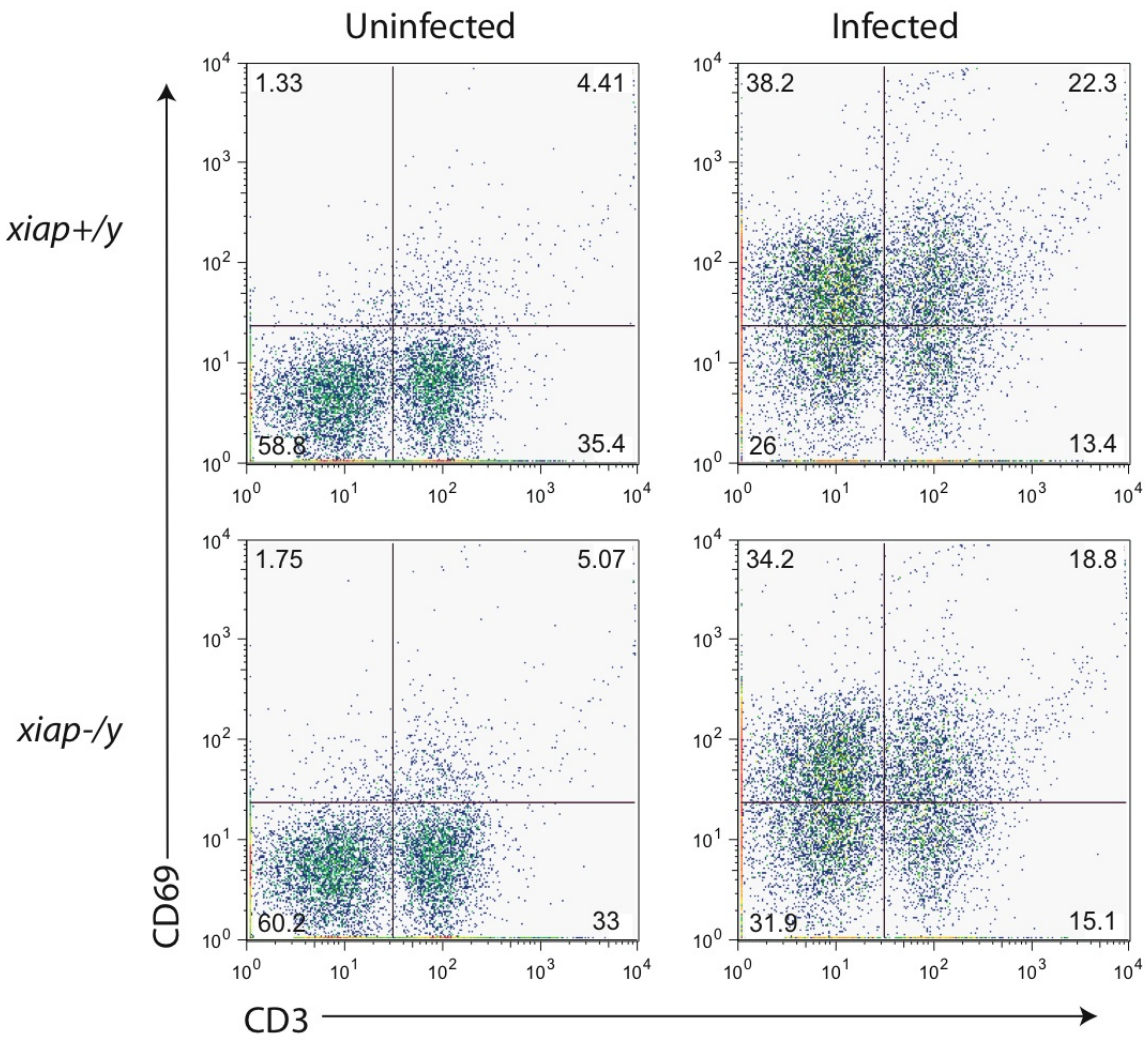

Figure 4.7 XIAP does not regulate expression of the CD69 activation marker by splenocytes during *Lm* infection

Flow cytometric analysis of CD69+ and CD3+ splenocytes from uninfected and *Lm* infected animals. Splenocytes were harvested from infected animals at 48h pi, and stained with CD69-APC and CD3-APC-Cy7 fluorescent-coupled antibodies for flow cytometry analysis. Results are representative of at least three independent experiments (n≥9 animals).

XIAP-deficient animals exhibit altered localization of intracellular *Lm* in the spleen

To determine which cell populations were involved in the clearance observed in the splenic follicles, we characterized the tissue morphology of the spleen sections by immunofluorescence histology. By analyzing the B and T cell populations of wildtype mice infected with *Lm* for 48h, we found a clearing of T cells at sites of bacterial replication (**Fig 4.8**). This alteration of the T cell zone was rarely observed in the XIAP-deficient animals. To further characterize the cell populations at the site of infection, we stained with antibodies to cell surface proteins characteristic of macrophages, dendritic cells and neutrophils. At sites of *Lm* replication, we observed recruitment of neutrophils, such that the area of T cell clearance was filled with neutrophils (**Fig 4.9**). Recruitment of neutrophils to sites of *Lm* replication was also observed in the XIAP-deficient animals, however the *Lm* were often not found in the follicles but instead located in the marginal zone around the follicles (**Fig 4.10**). We conclude that XIAP signaling promotes the transport of bacteria to the white pulp of the spleen. Additionally, T cell clearance was correlated with the localization of bacteria in the T cell zone of the follicle.

DISCUSSION

We show that XIAP promotes expression of proinflammatory cytokines in the spleen during *in vivo* infection with *Lm,* enabling a productive immune response. The XIAP-deficient phagocytes display decreased IL-6 production, however *in vivo*, perhaps due to enhanced bacterial loads, the secretion of IL-6 is similar to that of wildtype animals. XIAP-deficient animals also display altered splenic morphology. In wildtype animals, the bacteria were trafficked to the T cell zone and localized with neutrophils; a pattern

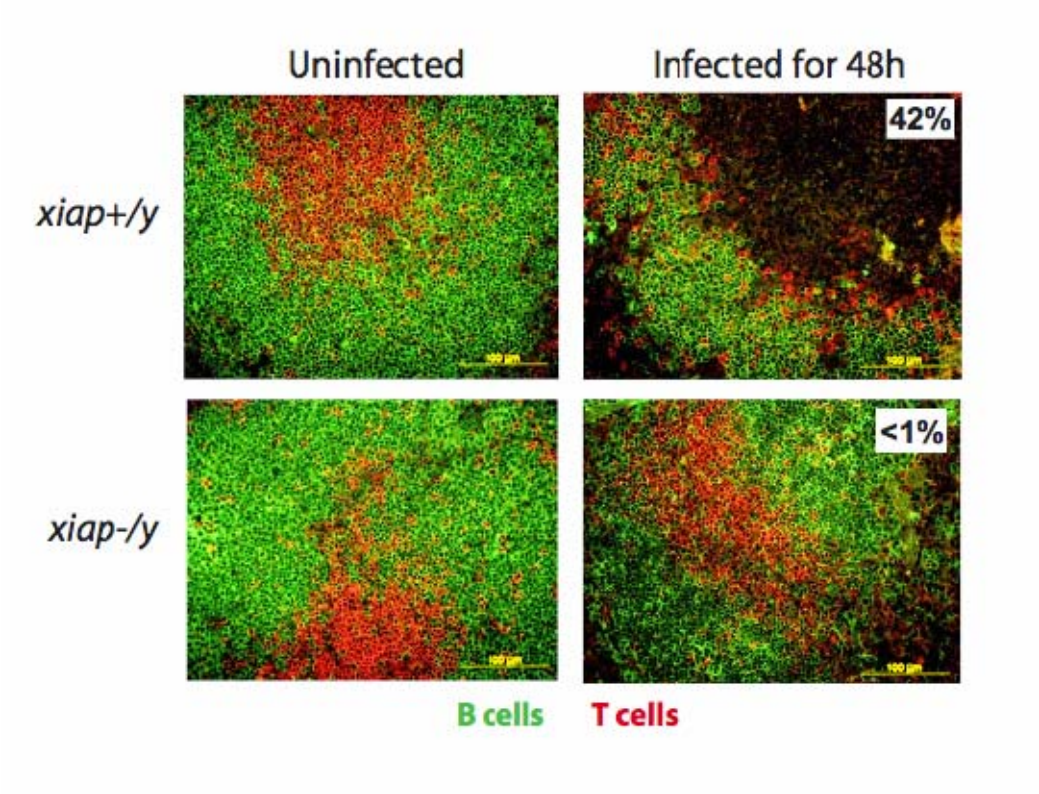

Figure 4.8 XIAP-deficient mice exhibit disrupted splenic T cell zones during *Lm* infection

Immunofluorescence microscopy of spleen samples from uninfected and *Lm* infected animals. Mice were infected with $5x10^5$ *Lm* i.p., spleens were harvested at 48hpi for immunofluorescence visualization and livers were harvested to enumerate the bacterial burden in the animals. Spleens were frozen in OCT and sectioned into 5uM sections for immunostaining. Sections were stained with anti-B220-FITC and anti-CD3-PE antibodies, followed by secondary antibodies to amplify the visible fluorescence. Number in top right indicates the percent of follicles that display disrupted structure.

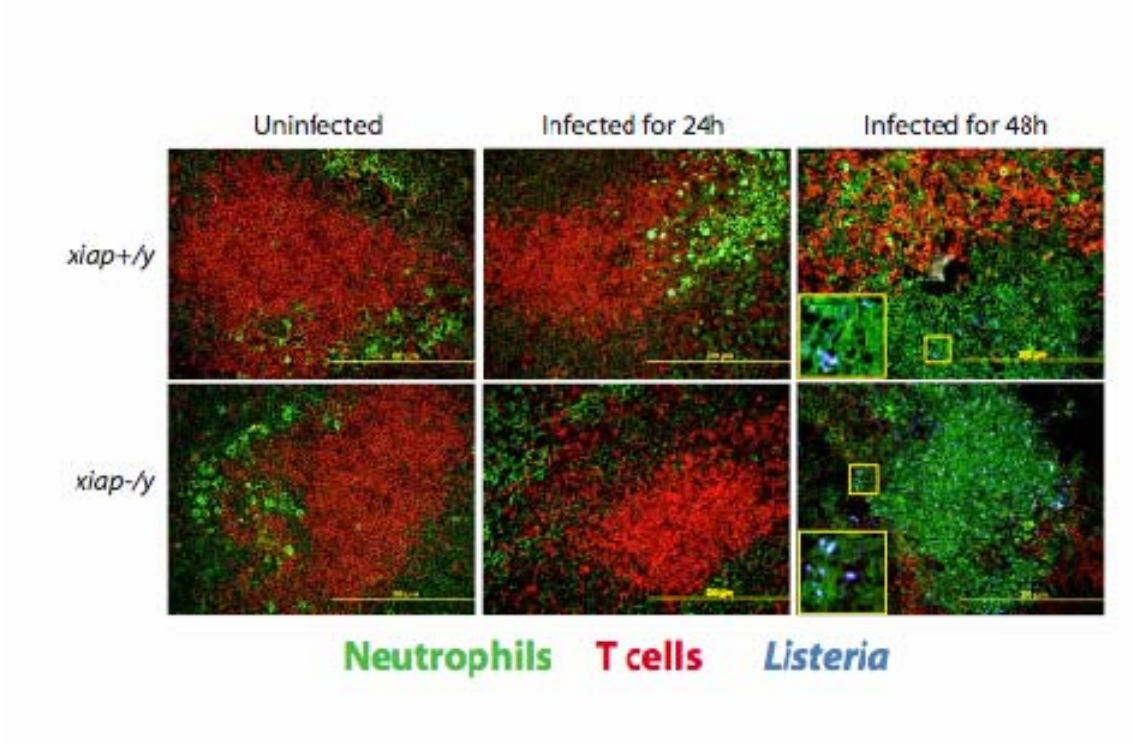

Figure 4.9 *L. monocytogenes* and neutrophils localize to the T cell clearing in the wildtype animals

Immunofluorescence microscopy of spleen samples from uninfected and *Lm* infected animals. Mice were infected with $5x10^5$ *Lm* i.p., spleens were harvested at 48hpi for immunofluorescence visualization and livers were harvested to enumerate the bacterial burden in the animals. Spleens were frozen in OCT and sectioned into 5uM sections for staining. Sections were stained with anti-GR-1-FITC, anti-Listeria-AMCA and anti-CD3-PE antibodies, followed by secondary antibodies to amplify the visible fluorescence. Yellow insets indicate areas that have been enlarged.

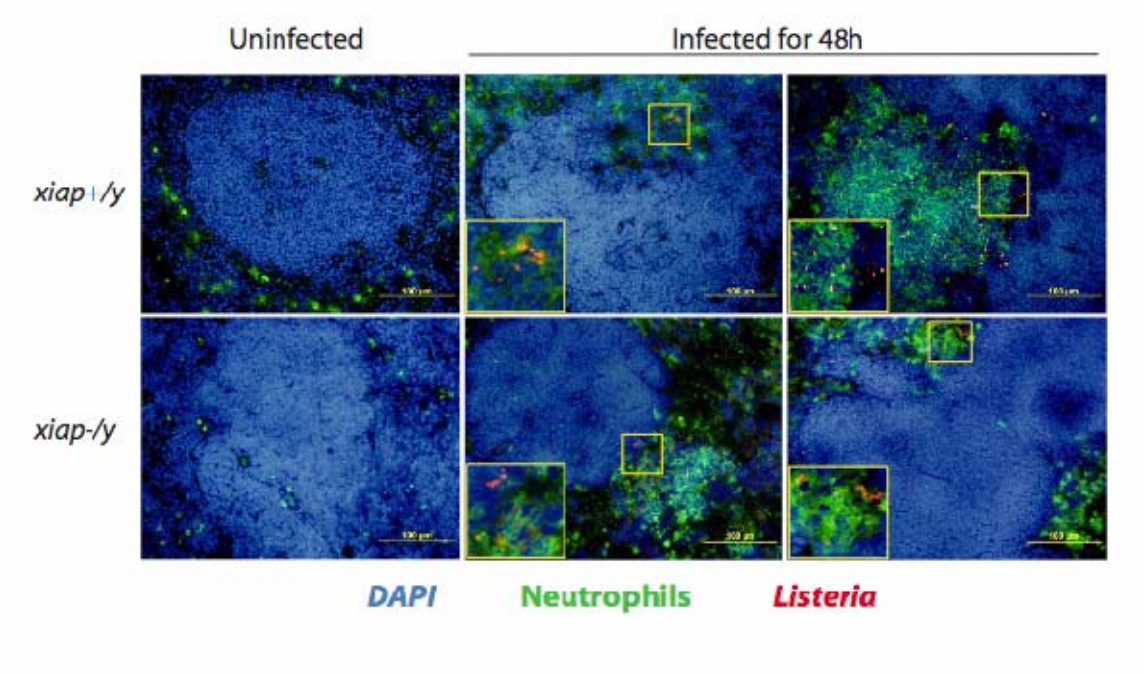

Figure 4.10 Altered localization of *L. monocytogenes* and neutrophils in the XIAP-deficient animals compared to wildtype animals.

Immunofluorescence microscopy of spleen samples from uninfected and *Lm* infected animals. Mice were infected with $5x10^5$ *Lm* i.p., spleens were harvested at 48hpi for immunofluorescence visualization and livers were harvested to enumerate the bacterial burden in the animals. Spleens were frozen in OCT and sectioned into 5uM sections for staining. Sections were stained with anti-GR-1-FITC and anti-Listeria-PE antibodies, followed by secondary antibodies to amplify the visible fluorescence. White pulp was determined by dense DAPI staining. Yellow insets indicate areas that have been enlarged.

that correlated with clearance of the T cells. In XIAP-deficient animals, the bacteria were not trafficked to the spleen, which may impact productive DC: T cell interactions that occur in the T cell zone[136].

XIAP regulates the production of proinflammatory cytokines by activated macrophages in response to cytosolic bacteria. In addition, it also regulates the expression of proinflammatory cytokines in the spleen during *in vivo* infection with *Lm*. When mice are infected with the LLO-deficient strain of *Lm,* which is unable to gain access to the cytosol, the bacteria are trafficked to the marginal zone of the lymphoid follicles in the spleen[142]. Since we observed altered localization of the bacteria in the XIAP-deficient mice, and our data suggest that XIAP regulates signaling in response to cytosolic *Lm*, we propose that XIAP-dependent cytosolic signaling is required for bacterial localization to the T cell zone in the spleen. Based on published studies with the LLO-deficient strain of *Lm*, development of a productive adaptive immune response to *Lm* infection requires cytosolic innate immune signaling. This suggests that the XIAP-deficient animals may not develop a fully functional adaptive immune response to *Lm* infection.

During a *Lm* infection in the wildtype animals, there is heterogeneity in the morphology of the spleen follicles. We observed three different situations: undisturbed follicles, follicles containing *Lm* and T cells, and follicles containing *Lm*, neutrophils but not T cells (**Fig 4.11**). Our model to explain this heterogeneity is that phagocytes that traffic *Lm* to the spleen from the site of infection can undergo three different immune

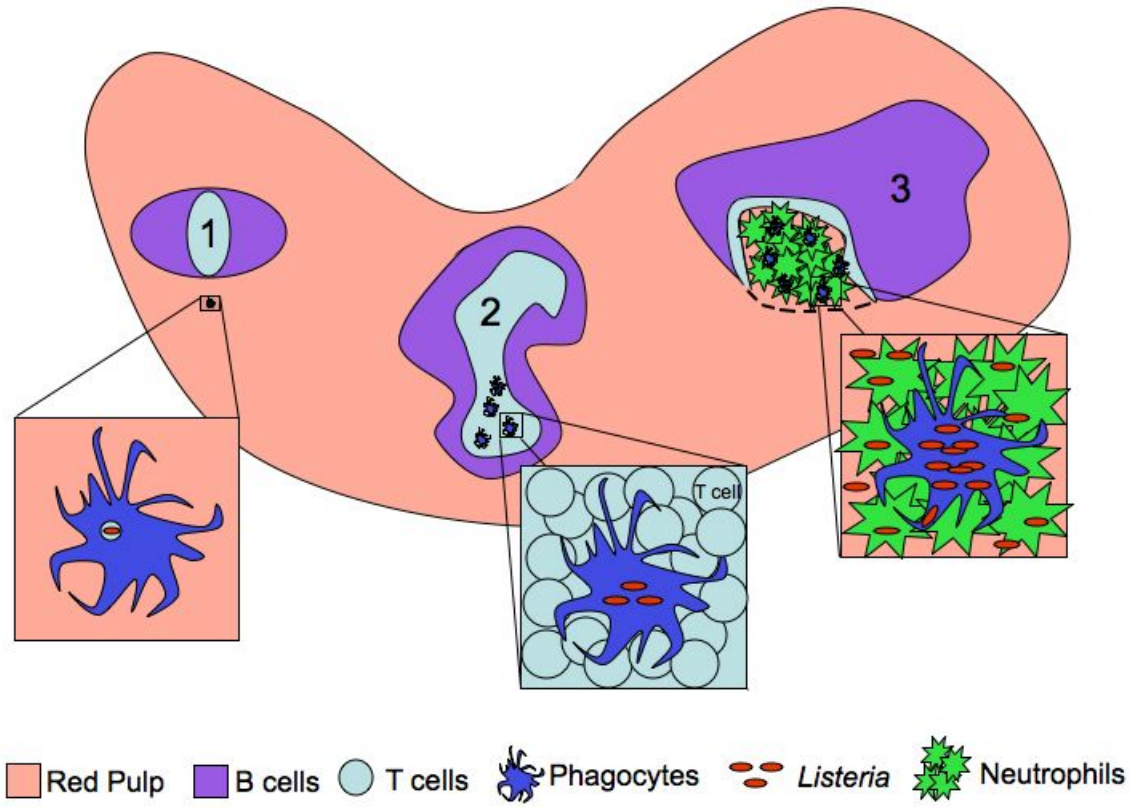

Figure 4.11 Heterogeneity of follicles observed in *Lm* infected wildtype animals

Depiction of the three different types of follicles observed in the spleens of wildtype animals infected with Lm for 48h. 1) Uninfected, unaltered follicle. 2) Follicle containing *Lm* and T cells without recruitment of neutrophils. 3) Follicle containing *Lm* and neutrophils in the absence of T cells.

responses: **1)** A non-productive infection where the bacteria are unable to escape from the vacuole, thus they do not get trafficked to the white pulp, **2)** A semi-productive infection where *Lm* is able to escape and replicate in the cytosol, however the phagocyte controls replication and presents antigen to T cells, **3)** A productive infection, where *Lm* gets into the cytosol, replicates and then spreads to surrounding cells, however the phagocyte can still present antigen to T cells. In the case of a semi-productive infection, it is likely that while the bacteria are properly localized to the white pulp, the bacteria are not extracellular, therefore, neutrophils are not recruited. During a productive infection, not only do the bacteria get to the proper location, but in addition, neutrophils are recruited to control bacterial spread and replication and in doing so, likely damage the T cells causing death and clearance.

Proinflammatory cytokines and chemokines regulate cell recruitment and localization during an infection. An explanation for the altered trafficking of *Lm* in the XIAP-deficient animals could be decreased chemokine production. Once such chemokine, CCR2, is required for recruitment of inflammatory monocytes to sites of bacterial infection; CCR2 and both of its ligands, MCP-1 and MCP-3, all contribute to optimal defense against *Lm* infection. Cytosolic infection of macrophages with *Lm* induces MCP-3 expression, allowing infected cells to recruit other immune cells, such as TipDCs, to help fight off infection[143]. Since these chemokines are regulated during a cytosolic bacterial infection it is possible that XIAP may function to modulate the expression of these chemokines as well.

The ability of the host to develop protective immunity depends upon the location of the innate immune cells during the immune response. Cells infected with *Lm* recruit innate immune cells to the site of infection to control bacterial replication as well as to collect antigens for presentation to the cells of the adaptive immune system. A recent study by Kang et al, shows that cytosolic signaling is critical for innate immune cell recruitment and activation at sites of *Lm* replication[144]. XIAP promotes production of proinflammatory cytokines in response to cytosolic bacteria, potentially regulating the recruitment of innate immune cells to the site of infection.

Our results suggest that XIAP-mediated signaling in response to cytosolic *Lm* infection is critical not only for the innate immune response to *Lm* infection, but also for the localization of *Lm* infected phagocytes to the splenic white pulp. Future studies will determine the precise role of XIAP in innate immune control of bacterial replication, as well as how XIAP specifically regulates cytosol-specific immunity. Additionally, we will determine if the adaptive immune response develops properly in the XIAP-deficient mice following primary *Lm* infection, providing protective immunity against *Lm* infection.

MATERIALS AND METHODS

Animals, bacterial strains and infections. Description of the animals used and methods used for infection can be found in Chapter 2. Methods for cell culture infections can be found in Chapter 3. For adaptive immune response experiments mice were infected with a primary dose of $5x10^4$ i.p. followed by a secondary infection of 5x106 i.p., mice were

harvested 48h after the second infection. The number of viable bacteria in the inoculum and organ homogenates was determined by plating 10-fold serial dilutions on Luria broth (LB) agar plates. For histology, the spleen and liver from infected mice were harvested at 48h pi and frozen in OCT media on dry ice. Blocks and sections were stored at -80C. 5uM sections were cut by the ULAM histology core, and stained with H&E. For immunofluorescence sections were fixed in acetone for 5 min at RT followed by 3 washes in PBS for 2 min each. Sections were blocked in sterile 1% BSA with 0.1% NaN3 in PBS for 45 min at RT. Slides were stained in PBS with 0.05% Tween-20 for 45 min at RT, antibodies were diluted 1:100. The slides were washed 3 times with PBS before secondary antibody staining (same as primary antibody staining). Slides were then washed 3 times with PBS and coverslips were mounted with Prolong Gold Antifade (Invitrogen). Slides were dried overnight at RT in the dark. Slides were visualized using a Olympus BX60 upright immunofluorescence microscope, photographs were taken using an Olympus DP70 color digital video camera and Olympus DP Controller/Manager software. The following antibodies were used: Anti-CD3-biotin (BDBiosciences 553059), Anti-CD19-FITC (Southern Biotech 1575-02), Anti-GR-1 (Southern Biotech Ly6G 1900-08), Anti-*Listeria* (Fisher DF2302-50-0), Streptavidin-PE (Jackson Immunoresearch 016-070-084), Anti-Rat FITC (Jackson ImmunoResearch 112-096-003), Anti-Rabbit AMCA (Jackson ImmunoResearch 111-156-003). DAPI (4',6-Diamidino-2-phenylindole, hydrochloride) was diluted 1:10,000 and was obtained from Fisher (46190).

BMDM culture. Methods can be found in Chapter 2 and 3.

Flow Cytometry. Splenocytes were harvested from uninfected or L. monocytogenes infected mice. BMDM were harvested from plates with ice cold PBS without Ca+ or Mg+. Cells were blocked with F_c block (BD Pharmingen 553142) for 15 min on ice. Cells were incubated in staining buffer (PBS, 10%FBS) with the indicated antibodies for 20 min on ice, followed by 3 washes in staining buffer. When necessary cells were incubated with secondary antibodies in staining buffer on ice for 20 min, and washed 3 times in staining buffer. Flow cytometric acquisition was performed on a FACSCanto. The data was analyzed using FlowJo software. The following antibodies were used: from BD Pharmingen; B220-PE (553089), NK1.1-biotin (553163), CD69-PE (553237); from Southern Biotech CD3 (1530-02), Streptavidin-APC (7100-11L).

RNA isolation and quantitative RT-PCR analysis. See Chapter 3 methods.

ELISPOT assay for cytokine-producing cells. An ELISPOT kit specific for IL-6 was purchased from eBioscience (88-7864-88). Polyvinylidene difluoride-backed microtiter plates (Fisher MAIPS4510) were coated with unlabeled capture antibody overnight. Plates were washed with ELISPOT coating buffer and blocked with complete RPMI-1640 (10% FBS, 1mM L-Glutamine) for 1h at RT. Splenocytes were serially diluted in complete RPMI and added in triplicate to the plate, plates were incubated at 37°C, in 5% CO2 for 24h. After washing, the detection antibody conjugated to biotin was added to the plates and incubated for 2h at RT. After washing, a Streptavidin-horseradish peroxidase reagent was added and incubated at RT for 45 min. Plates were washed and

spots were visualized by adding TMB solution (Fisher NC9779701) for 10-30 minutes until color develops, development was stopped by adding H2O. Plates were dried and spots were quantified with an Immunospot Series 1 ELISPOT analyzer (Cellular Technology Ltd.).

Statistical Analysis. As stated in Chapter 3.

Chapter 5 : Perspectives and Future Directions

XIAP regulates cytosol specific innate immune signaling

My studies have identified XIAP as a regulator of cytosolic innate immunity to *Lm* infection. Specifically, I have shown that XIAP-deficient animals are more susceptible than wildtype mice to *Lm* during the innate immune response. XIAP promotes the induction of proinflammatory cytokines by enhancing and prolonging JNK phosphorylation. Additionally, XIAP coordinates synergistic proinflammatory cytokine production resulting from simultaneous TLR and NLR stimulation. *In vivo* I have shown that XIAP regulates cytokine production and directs the localization of *Lm* to the white pulp of the spleen. Taken together, these data indicate that XIAP regulates innate immunity by regulating proinflammatory cytokine production, which directs the cells of the immune response to become activated and to traffic to the lymphoid follicles, which is required to clear *Lm* infection.

IAPs and Immunity

In addition to our identification of XIAP as a regulator of innate immunity, it has become clear that other IAP proteins also regulate immunity. In *Drosophila* there are two IAP proteins; dIAP1 is involved in protection against apoptosis, while the dIAP2 protein is part of the Imd innate immune signaling cascade[76,77,79]. Innate immune signaling

pathways are well conserved from *Drosophila* to humans, suggesting that IAP proteins may also play a role in mammalian innate immunity [4]. Several other mammalian IAPs have also been shown to be involved in immunity including NAIP5 and c-IAP2 in mice and XIAP in humans. Starting in the murine model, NAIP5 is involved in detecting cytosolic flagellin, resulting in proinflammatory cytokine production and restriction of *Legionella pneumophila* intracellular growth[108,109,110,145,146,147]. Studies in c-IAP2-deficient mice illustrate the role of c-IAP2 in promoting proinflammatory cytokines, exacerbating LPS induced endotoxic shock[69]. Recently XIAP has been implicated in the human disease, X-linked lymphoproliferative syndrome (XLP), resulting in primary immunodeficiency[71]. These studies indicate that IAP proteins regulate immunity as well as apoptosis.

What governs the requirement for XIAP?

Interestingly, when unactivated XIAP-deficient macrophages are infected, they are able to respond to *Lm* infection as well as unactivated wildtype macrophages. However, this response is decreased when compared to wildtype activated macrophages. Microarrays performed in unactivated wildtype or XIAP-deficient macrophages did not show any significant differential induction of genes upon infection. This suggests that for XIAP to enhance the innate immune response, macrophages must be activated to induce a state of readiness in the macrophage. The bactericidal environment in activated macrophages is critical for control of *Lm* replication[148]. We activated macrophages with both LPS and IFNγ, but found that it was the LPS stimulation that was critical to allow XIAP to enhance proinflammatory cytokine production. We and others have shown that treatment

of macrophages with LPS induces Nod2 gene expression[149]. The cytosolic sensors Nod1 and Nod2 are known to be critical for control of *Lm* infection in mice previously stimulated with LPS, indicating that the NLR receptors may be activated in response to TLR stimulation[59]. There are two hypothesis that can explain the role of XIAP in these TLR stimulated macrophages: 1) that in response to TLR stimulation, XIAP promotes the transcription or translation of proteins important for immune defense against cytosolic pathogens, or 2) that XIAP requires certain proteins induced by TLR stimulation to promote proinflammatory cytokine production. Since we have shown that XIAP promotes Nod2 transcription, as well as requiring the Nod2 protein for synergistic production of proinflammatory cytokines in response to TLR and NLR stimulation, it is likely that XIAP functions in both roles.

Are phagocytes *in vivo* less bactericidal in the absence of XIAP?

During the innate immune response to infection, the production of proinflammatory cytokines is critical for pathogen control and development of adaptive immunity. We observed decreased production of several proinflammatory cytokines, including IL-6 and TNF in the absence of XIAP, which would likely affect many aspects of the immune response *in vivo*. Proinflammatory cytokines serve to recruit innate immune cells as well as activate them to be more bactericidal[47,48]. Mice that are deficient in either IL-6 or the TNF receptor display enhanced susceptibility to *Lm* infection[48,122]. Among other roles, IL-6 is required for the production of IFNγ. In the XIAP-deficient animals, both *il6* and *ifng* expression is decreased *in vivo* in the spleen[49]. IFNγ is important for its role in activating phagocytes to become more bactericidal; a decrease in IFNγ could allow

rampant proliferation by the bacteria, overwhelming the innate immune response, as seen in the XIAP-deficient animals. While XIAP-deficient macrophages in culture were able to control *Lm* infection when stimulated with exogenous IFNγ and LPS, *in vivo* they may not receive the proper activation signals and thus may not be as bactericidal. This hypothesis could be tested by isolating phagocytes from infected animals and performing a growth curve *in vitro*, without additional *ex vivo* proinflammatory cytokine stimulation. If the cells from the XIAP-deficient mice are less bactericidal, we can conclude that the decreased proinflammatory cytokine environment of the XIAP-deficient animals affects the ability of the phagocytes to control *Lm* replication.

Does XIAP function as an E3 ubiquitin ligase during innate immune signaling?

XIAP, c-IAP1 and c-IAP2 all have a RING domain, which enables these proteins to function as E3 ubiquitin ligases. Additionally, they contain a ubiquitin-associated domain (UBA), which enables proteins to bind to ubiquitin[150,151]. Ubiquitylation is a mechanism used by cells to target proteins to participate in signaling networks, such as the TLR and NLR signaling pathways[152]. The role of ubiquitin modification during TLR and NLR signaling is very complex, and involves a number of E3 ubiquitin ligases, as well as deubiquitylating enzymes to properly control signaling[118,153,154,155,156,157,158]. However, there are still ubiquitin ligases involved in innate immune signaling that have not been identified. It is intriguing to hypothesize that XIAP may function as a ubiquitin ligase during innate immune signaling. In support of this hypothesis, the E3 ligase domain and the UBA domain of XIAP are both required for NF-κB activation[105,151]. Additionally, our preliminary

data suggests that XIAP stabilizes RIP2 during *Lm* signaling. Taken together, I propose that XIAP regulates the induction of proinflammatory cytokines by promoting ubiquitylation of the components of the TLR and NLR signaling pathways, possibly ubiquitylating MEKK2, NEMO or RIP2. Since many of these proteins feed into the same pathways, XIAP may be the node that coordinates activation of critical signaling molecules from both TLR and NLR stimulation.

Future Directions

After discussing the implications of my work and the role of XIAP in innate immunity, a number of hypothesis can be developed to further define how XIAP functions to regulate immunity. As discussed above, the role of the E3 ubiquitin ligase domain of XIAP in promoting ubiquitylation of proteins during the innate immune response is currently unknown. In addition, while the BIR domains of XIAP have been well characterized for their role in inhibiting apoptosis, little is known about their function during the immune response. The BIR1 domain of XIAP is known to associate with TAB1, allowing XIAP to interact with the TAK1 complex and induce NF-κB activation, however the role of the other BIR domains is unclear[94]. The role of XIAP in regulating Nod2 signaling is also currently unknown. Our preliminary data suggests that XIAP promotes stability of the RIP2 protein; it is possible that this stabilization enables signaling through Nod2. Finally, there is a great deal that is unknown about how XIAP functions during an *in vivo* response to *Lm* infection. We would like to determine if XIAP functions to regulate signaling primarily in phagocytes, or also in other immune cells during cytosolic bacterial infection, by performing adoptive transfer experiments. Additionally, we would like to

examine the trafficking patterns of wildtype and XIAP-deficient phagocytes during a *Lm* infection, to determine how a deficiency in XIAP affects localization. Finally, we would like to determine if the defect in the innate immune response in XIAP-deficient animals affects the development of a protective immune response to *Lm* infection. Overall my thesis work has implicated XIAP in cytosolic innate immune signaling, and suggests that the IAP proteins are multifunctional modulators of signaling.

Appendices

Appendix 1: Introduction to IAP proteins

Inhibitor of Apoptosis Proteins (IAPs)

The IAP (Inhibitor of Apoptosis) family of genes is well known for their role in regulating programmed cell death in organisms from insects to humans[90]. IAP proteins function to suppress apoptosis, specifically by binding to and inhibiting caspases, along with activating other pathways to promote cell survival, such as NF-κB and JNK1. The IAP protein family is characterized by BIR domains (baculoviral IAP repeats), which are protein-protein interaction domains. IAP proteins contain between one and three BIR repeats, many also contain a carboxyl-terminal RING finger domain that possess E3 ubiquitin ligase activity[105]. There are eight mammalian IAP proteins: XIAP (Birc4, MIHA, hILP), c-IAP1 (Birc2, MIHB, Hiap2), c-IAP2 (Birc3, MIHC, Hiap1), NAIP (Birc1), Livin (melanoma IAP (ML-IAP), Birc7), Survivin (Birc5, TIAP), Testis specific IAP (Ts-IAP, Birc8, hILP2), and Bruce (Birc6, Apollon, Bir containing ubiquitin conjugating enzyme) (**Fig. A.1.1**). XIAP is the most well characterized IAP protein, it has three BIR domains and a RING finger domain. XIAP is expressed ubiquitously in all normal tissues. It is able to directly bind to and inhibit caspases 3, 7 and 9[72]. XIAP is also involved in a number of other signaling pathways including JNK1, NF-κB and Smad signaling[159]. XIAP-deficient mice have been generated and characterized, however no altered phenotype was identified when compared to wildtype mice[72]. The c-IAP proteins are the closest homologs of XIAP in the IAP family. The c-IAP proteins contain

three BIR domains, a CARD domain and a RING finger domain. They bind to TRAF1 and TRAF2 proteins, regulating downstream NF-κB activity in response to TNF signaling[160]. When ectopically expressed, the c-IAP proteins can inhibit apoptosis, however their caspase binding activity does not result in inhibition[161]. Additionally, c-IAP1 can regulate XIAP and c-IAP2 protein levels by ubiquitylating and targeting these proteins for degradation[162].

XIAP

XIAP is the most well characterized IAP protein, possibly due to its ability to directly bind to and inhibit caspases[163,164,165]. XIAP is frequently overexpressed in cancers, allowing the cells to prevent apoptosis and continue to proliferate[166]. XIAP is involved in a number of signaling pathways including TGFβ and BMP receptor signaling, NF-κB and JNK activation. Additionally XIAP has been shown to regulate copper homeostasis by promoting the ubiquitylation and degradation of COMM1, a protein that promotes efflux of copper form the cell. XIAP can bind copper directly, which causes destabilization and degradation, leading to lower levels of XIAP. When XIAP is bound to copper it is unable to inhibit caspases[97]. Despite all of the functions of XIAP that have been described, there were no observable defects in the XIAP-deficient mice, however both c-IAP1 and c-IAP2 were shown to be upregulated, possibly compensating for the XIAP-deficiency[72]. During TGFβ and BMP signaling, XIAP bridges the BMP receptor to TAB1, a downstream signaling molecule. The BIR1 domain of XIAP is required for this interaction[94,95]. TAB1 recruits TAK1, which is responsible for activating the JNK and NF-κB signaling pathways. For JNK induction by

XIAP during TGFβ signaling, Smad4 is also required[159]. XIAP activation of the JNK pathway requires the ILPIP protein, which promotes the association of XIAP with TAK1 and TRAF6. XIAP does not activate JNK using MEKK1, MKK4, MKK7 or ASK1[93,96,98]. Ectopic expression of XIAP has also been shown to activate JNK and NF-κB signaling pathways independent of TGFβ signaling[93,96,116]. Additionally, point mutations preventing XIAP from inhibiting caspase activity do not affect XIAPs ability to activate JNK or NF-κB. XIAP requires the E3 ligase activity of its RING domain in order to activate NF-κB[105]. Possibly the mechanism used by XIAP to regulate NF-κB signaling is through interaction and ubiquitylation of MEKK2, this induces a second wave of NF-κB activation[167]. It is well established that NF-κB activation occurs in waves that are regulated by the IKB proteins. IKBα controls early/immediate NF-κB activation, while IKBβ mediates delayed activation. IKBα associates with MEKK3 and IKBβ with MEKK2. While the IAP proteins were initially characterized to primarily regulate apoptosis, the literature suggests that they play a much larger role in cells by regulating a variety of signaling pathways.

The c-IAP1 and c-IAP2 proteins

The c-IAP proteins are components of the TNF signaling pathway, where they promote activation of NF-κB while inhibiting induction of apoptosis [168]. Signaling through the TNFR1 receptor mainly results in induction of apoptosis, as the TNFR1 receptor contains a death domain in its cytoplasmic tail. TNFR2 however does not contain a death domain, and recruits TRAF1, TRAF2, c-IAP1 and c-IAP2[168]. Binding of TNF to the TNFR1 receptor recruits TRADD and FADD, two death domain containing proteins. Through

FADD, pro-caspase 8 is recruited. Subsequent oligomerization of pro-caspase 8 leads to activation by autoproteolytic cleavage and results in induction of apoptosis[169]. TNF signaling can also lead to NF-κB activation via TRADD, RIP, TRAF2 and MEKK3 recruitment. Both the c-IAP1 and c-IAP2 proteins have been shown to ubiquitylate RIP. When RIP is poly-ubiquitylated it functions to activate NF-κB, however ubiquitin also serves as a tag for degradation regulating RIP levels by targeting to the proteasome[62,170,171]. Ubiquitylated RIP associates with the TAB1 and TAB2 proteins, recruiting TAK1, which upon oligomerization autophosphorylates. The IKK complex is also recruited to ubiquitylated RIP, where activated TAK phosphorylates IKK. Active IKK phosphorylates IKBα releasing NF-κB allowing it to translocate to the nucleus where it can initiate target gene transcription[172]. It has been suggested that the c-IAP proteins function to promote cell survival during TNF signaling by limiting signaling through the TNFR1 receptor, possible through RIP degradation or by suppressing caspase 8 activity[172,173]. Work with Smac mimetics has found that non-ubiquitylated RIP associates with caspase 8 and FADD leading to caspase 8 activation suggesting that one mechanism the c-IAPs prevent apoptosis is by ubiquitylating RIP to prevent caspase 8 activation[174]. The physiological roles of c-IAP1 and c-IAP2 have been investigated by gene depletion in mice. While the c-IAP1-deficient mice have no obvious defects in their ability to respond to proapoptotic stimuli, they have marked increases in c-IAP2 protein levels, suggesting c-IAP2 can compensate for c-IAP1 function *in vivo*. c-IAP2 protein levels are regulated by c-IAP1 ubiquitylation[70]. The c-IAP2-deficient mice are resistant to LPS induced sepsis, due to an attenuated inflammatory response. LPS sepsis is characterized by a robust proinflammatory

cytokine burst that overwhelms the immune response. In the c-IAP2-deficient mice the levels of proinflammatory cytokines are reduced because the macrophages responsible undergo rapid cell death. The importance of the c-IAP proteins in promoting NF-κB activity and preventing apoptosis is clearly indicated by the ability of the c-IAP2-deficient mice to survive LPS induced sepsis[69].

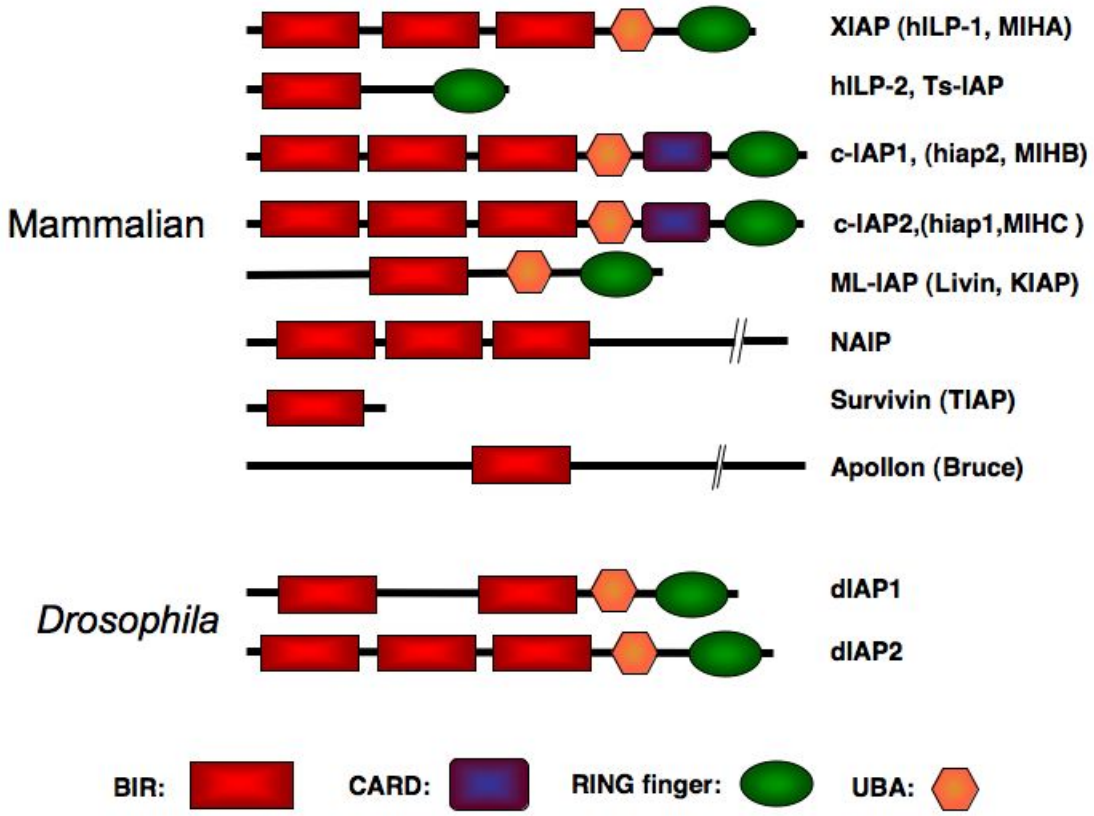

Figure A1.1 Domain structure of the IAP protein family.
The characteristic BIR domains are indicated by red rectangles, CARD domains by purple rectangles, RING domains by green ovals, and Ubiquitin binding domains in orange hexagons. Abbreviations: IAP, inhibitor of apoptosis; XIAP, X-linked IAP; hILP, human IAP-like protein; Ts-IAP, testis-specific IAP; c-IAP, cellular IAP; ML-IAP, melanoma-IAP; NAIP, neuronal apoptosis inhibitory protein; dIAP, *Drosophila* IAP; BIR, baculoviral IAP repeat; CARD, caspase recruitment domain; UBA, ubiquitin binding domain.

Appendix 2: Microarray Results of the gene expression of Wildtype and XIAP-deficient unactivated bone marrow derived macrophages induced by cytosolic bacteria

To determine if XIAP specifically regulated any genes during a cytosolic bacterial infection that would enable us to predict the role of XIAP during *Lm* infection, we performed a microarray. Unactivated bone marrow derived macrophages were infected with wildtype *Lm* or the LLO-deficient strain of *Lm* for 30 minutes. RNA was collected at 3hpi to determine gene expression by microarray. Using a two-fold cut off for differential gene expression we determined that while a number of genes were induced upon cytosolic *Lm* infection, there were very few genes that were differentially regulated between the wildtype and XIAP-deficient macrophages (**Fig. A2.1**). We also examined the ability of unactivated macrophages to produce IL-6 in response to *Lm* infection. Macrophages were infected with *Lm* for 30 min and supernatants were collected at 8hpi to quantitate IL-6 production. When the macrophages are not activated prior to infection, both the wildtype and XIAP-deficient cells produce equal levels of IL-6, however after activation, XIAP enhances production of IL-6 in response to *Lm* infection (**Fig. A2.2**). In order to determine if the reason for XIAP-dependent responses in the activated macrophages was due to an increased production of certain innate immune genes, we

examined the expression of the several key mediators of cytosolic immunity (**Fig. A2.3**). We did not observe a significant increase in c-IAP1 or c-IAP2 gene expression during activation of cells or due to *Lm* infection. Both Nod1 and Tak1 gene expression was induced by activation, but not infection in an XIAP-dependent manner. Interestingly Nod2 and RIP2 were induced by activation and infection and this was enhanced by XIAP. These data suggest that XIAP does not differentially regulate signaling in unactivated macrophages, but is necessary for enhancing expression of specific innate immune genes in response to cytosolic bacteria and macrophage activation.

Adhesion molecules/ Receptors		Cytokines & chemokines		Regulation of innate immunity			
Alcam	X+ 1.97	Ccl4	X- 1.80	Cd69	X- 1.06	Ifit1	X-1.81
Cnr2	X-3.14	Ccl5	X- 2.11	Dll1	X- 1.63	Klf10	X- 1.79
Ifnar1	X+ 2.85	Ccl8	X+ 2.5	E2f2	X-1.65	Map4k3	X+2.16
Il10ra	X- 2.90	Ccrl2	X- 2.07	Egr1	X-2.53	Nfatc1	X-3.78
Il10rb	X+ 2.28	Csf1	X- 1.77	Egr2	X+2.32	Pde7b	X+3.14
Itga5	X+ 3.05	Cxcl2	X-3.14	Exo1	X-3.05	Ptgs2	X- 2.08
Itgax	X- 3.68	Il1a	X-1.61	Gab1	X+ 2.99	Rgs1	X+ 1.86
Malt1	X+ 1.94	Il1b	X- 2.55	Gas5	X- 4.69	Socs3	X-1.53
Marco	X+ 1.17	Il6	X-3.31	Gbp2	X- 1.84	Tank	X+2.89
Plau	X+ 4.01	Il12b	X-1.40	Gdf15	X+3.04	Tgfb1i4	X-2.08
Sdc1	X- 2.83	Lgals3	X+2.16	Id2	X+ 3.03	Zfhx1b	X+ 3.01

Figure A.2.1 XIAP regulated genes involved in immunity
Macrophages were infected with *Lm* for 30min and RNA was harvested at 3hpi for microarray analysis. Genes that were differentially regulated by cytosolic bacteria compared to a vacuole-trapped strain were selected based upon a two-fold cut off. The table represents genes that are involved in the immune response. Bold indicates the genes were upregulated, non-bold text are genes that were downregulated by cytosolic bacteria. Genes that were more greatly altered in the wildtype cells are indicated by X+, while X- indicates the difference was greater in the XIAP-deficient macrophages. The number indicates the fold difference between wildtype and XIAP-deficient macrophages.

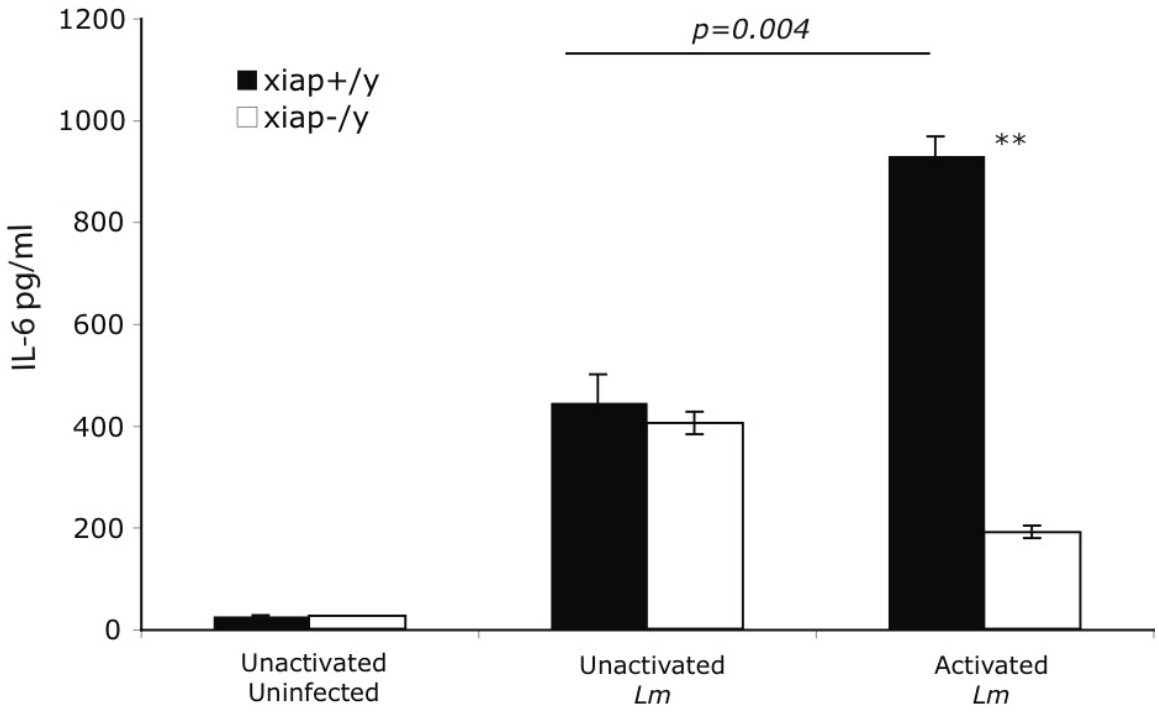

Figure A2.2 Activation of macrophages allows XIAP to promote proinflammatory cytokine production

ELISA of IL-6 production from unactivated and activated macrophages infected with *Lm* for 30 min. Supernatants were harvested at 8hpi. Data is representative of 3 independent experiments. ** indicates $p < 0.001$. P value indicated compares wildtype unactivated and activated macrophages.

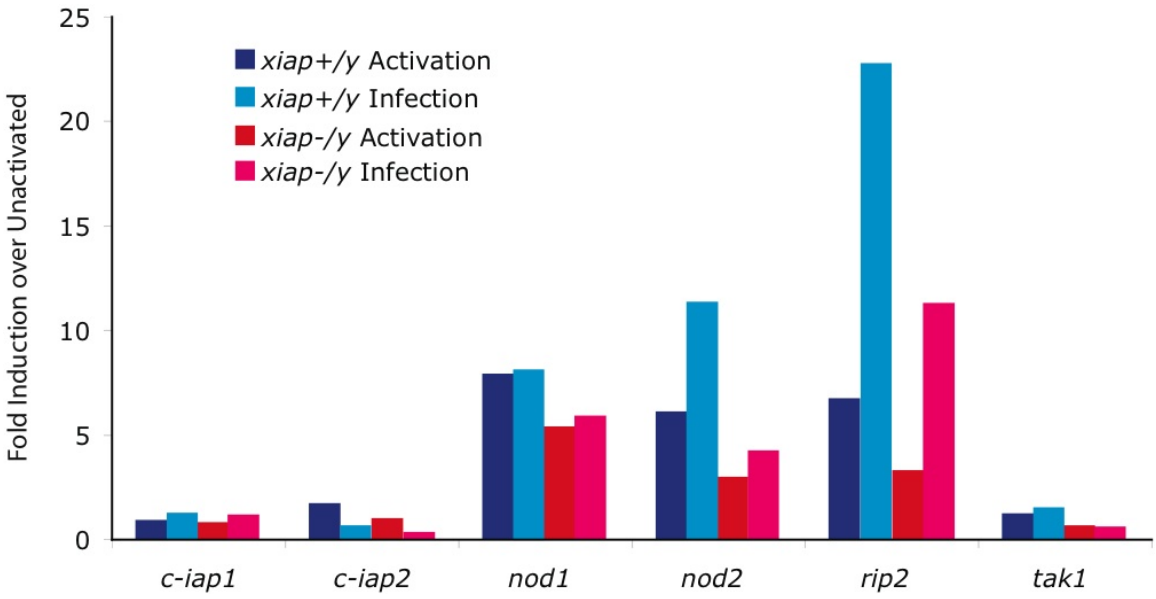

Figure A2.3 Gene expression of innate immune genes important for cytosolic immunity

qRT-PCR analysis on RNA from three sets of macrophages: unactivated, activated or activated and infected. Macrophages were activated overnight with LPS (10ng/ml) and IFNg (10ng/ml). Cells were infected with *Lm* for 30 min and RNA was collected at 3 hpi. Data represents the fold induction of each gene over the expression levels in the unactivated macrophages. Fold induction was calculated using the $\Delta\Delta C_t$ method where uninfected samples were compared to infected samples, relative to β-actin levels. Data is representative of 3 independent experiments.

Appendix 3 : The MAP kinase phosphatases 1 and 5 are not responsible for prolonging JNK activation during XIAP innate immune signaling.

The MAP kinase phosphatase family is responsible for dephosphorylating proteins to downregulate signaling. MKP1 and MKP5 have both been shown to dephosphorylate JNK; therefore, we investigated if XIAP affected the stability of these proteins during *Lm* infection. We hypothesized that XIAP would prolong JNK phosphorylation by targeting MKP1 or MKP5 for degradation by ubiquitylation, thus preventing JNK dephosphorylation. Macrophages were treated with cyclohexamide to prevent protein translation, or treated with LLNL to inhibit protein degradation during *Lm* infection (**Fig. A3.1**). We observed no decrease in protein levels of either MKP1 or MKP5 during *Lm* infection, in the wildtype or XIAP-deficient cells. Additionally, we did not observe any effect on the protein levels after addition of cyclohexamide, suggesting that infection with *Lm* does not induce translation. We were able to observe protein degradation due to the increase in protein after addition of LLNL, however, this was not altered in the absence of XIAP. These data suggest that XIAP does not promote JNK activity by targeting the MKP proteins.

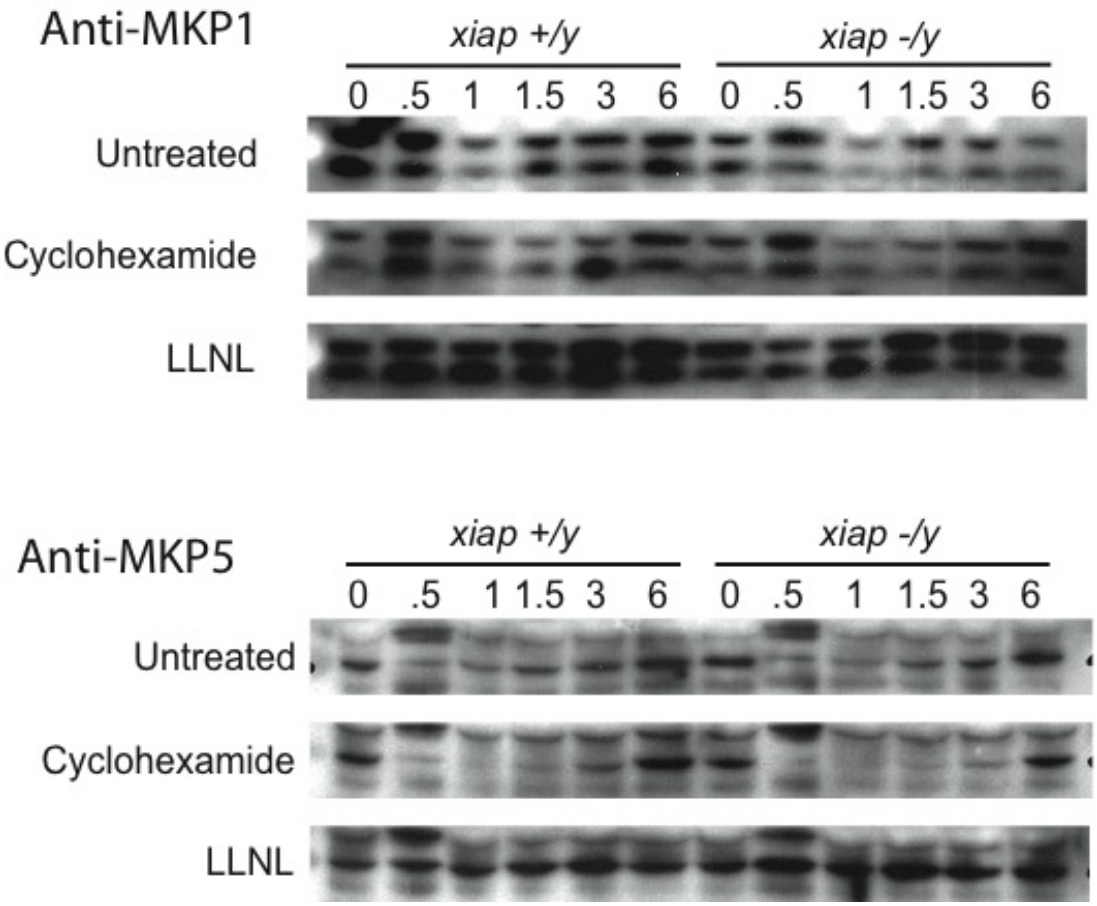

Figure A3.1 XIAP does not regulate MKP1 or MKP5 levels during *Lm* infection
Immunoblot of lysates from *xiap*$^{+/y}$ and *xiap*$^{-/y}$ activated BMDM that were uninfected or infected with *Lm*. Cells were activated overnight with 10ng/ml LPS and 10ng/ml interferon-γ. Cells were incubated with cyclohexamide or LLNL for 1h prior to infection to inhibit protein translation and degradation respectively. After 1 h pretreatment, cells were infection at an MOI of 10 for 30 min in the presence of inhibitors. Cells were lysed and subjected to immunoblot analysis using anti-MKP1 and anti-MKP5. Data are representative of at least 3 independent experiments.

Appendix 4 : XIAP stabilizes RIP2 protein levels

We observed an XIAP-dependent enhancement of proinflammatory cytokine production in response to MDP, the Nod2 ligand, and synergistically to MDP and Pam_3CSK_4, a TLR2 ligand. Therefore, we wanted to determine how XIAP affected Nod2 signaling. Nod2 uses the adaptor protein, RIP2 to induce NF-κB and MAP kinase activation. XIAP may modulate RIP2 to promote signaling in response to Nod2 ligands. We examined RIP2 protein levels during *Lm* infection in wildtype and XIAP-deficient cells to determine if XIAP affected RIP2 stability (**Fig. A4.1**). We observed enhanced protein stability in the wildtype cells compared to the XIAP-deficient cells. This stabilization was independent of *Lm* infection, as uninfected cells lacking XIAP also had less RIP2 protein.

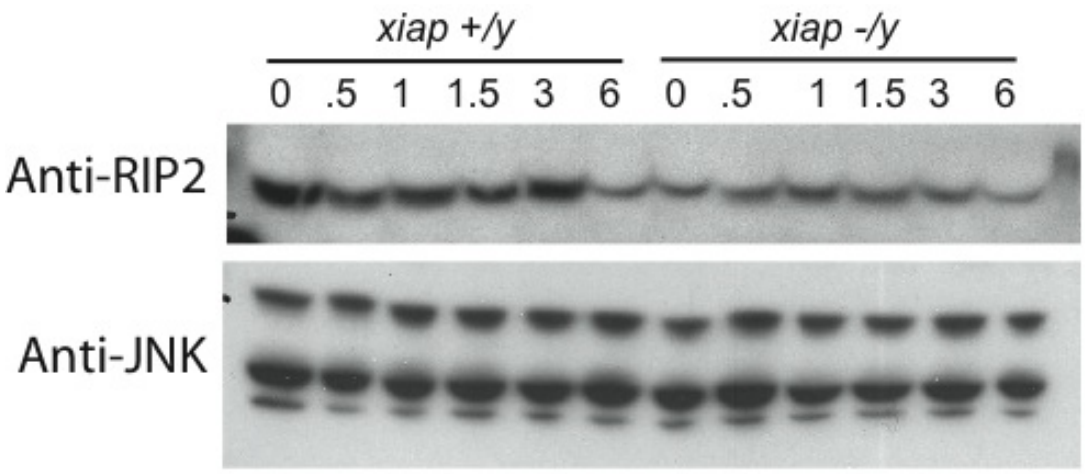

Figure A4.1 XIAP stabilizes RIP2
Immunoblot of lysates from *xiap*$^{+/y}$ and *xiap*$^{-/y}$ activated BMDM that were uninfected or infected with *Lm*. Cells were activated overnight with 10ng/ml LPS and 10ng/ml interferon-γ, prior to infection with *Lm* at an MOI of 10 for 30 min. Cells were lysed and subjected to immunoblot analysis using anti-RIP2 and anti-JNK. JNK is used as a loading control. Data are representative of at least 3 independent experiments.

Appendix 5 : XIAP-dependent proinflammatory cytokine production requires LPS activation of macrophages

Recent literature indicates that LPS stimulation causes tolerization of macrophages to restimulation by other TLR ligands[126,127]. Additionally, when macrophages are stimulated with LPS, the role of Nod1 and Nod2 in cytosolic immunity becomes more critical during infection with *Lm*[59]. Stimulation of macrophages with IFNγ activates them enhancing their bactericidal ability[148]. Due to these observations we wanted to determine if either TLR stimulation by LPS or IFNγ signaling was more critical to promoting a state of activation in the macrophages, allowing XIAP to promote cytosolic signaling. Therefore, we examined IL-6 production by macrophages stimulated with LPS, IFNγ, or both (**Fig. A5.1**). We determined that LPS stimulation of macrophages sets up a state of readiness allowing XIAP to promote cytosolic signaling. This is possibly due to enhanced expression of Nod2 and RIP2 in the wildtype cells compared to the XIAP-deficient macrophages. These data suggest that prior TLR stimulation of cells sets up a state of readiness that enables the cytosolic innate immune response to enhance production of proinflammatory cytokines.

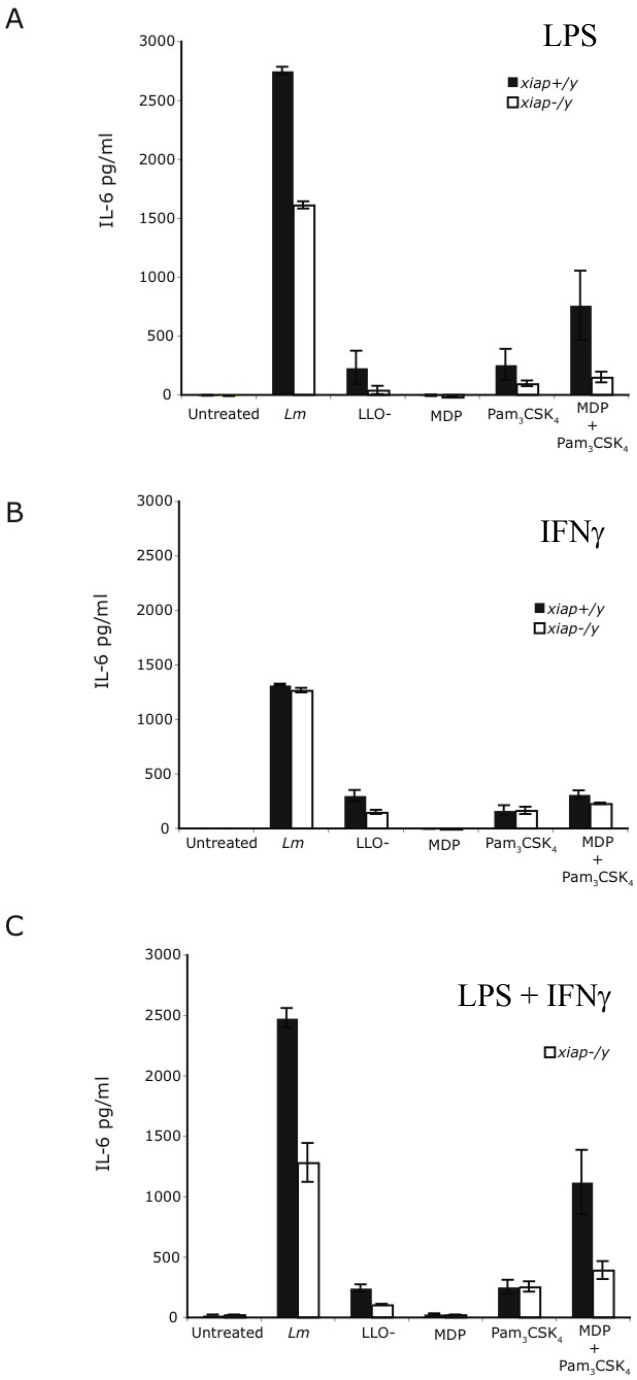

Figure A5.1 LPS stimulation enables XIAP to promote the cytosolic immune response to *Lm* infection.
ELISA of IL-6 secretion from *xiap*$^{+/y}$ and *xiap*$^{-/y}$ activated BMDM infected with wildtype Lm, LLO- Lm or treated with MDP (10 μg/ml) and/or Pam_3CSK_4 (0.5 μg/ml). (A) 10ng/ml LPS stimulation (B) 10ng/ml IFNg stimulation (C) 10ng/ml LPS and 10ng/ml IFNg. left untreated or treated for 8h Data are representative of 3 independent experiments with 3 mice each (error bars represent s.d). * indicates $p \leq 0.05$ and ** indicates $p \leq 0.005$.

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