Chapter I

Introduction

Cell death is critical for a variety of processes during development and maintenance of total cell number in metazoans, and in no system is it more crucial than the immune system. Specifically, programmed cell death, or apoptosis, is absolutely required for both the normal development and homeostasis of the immune system. Maintenance of an appropriate cell number by a delicate balance between proliferation and apoptosis prevents disorders as diverse as cancer and lymphopenia. Apoptosis is an orderly biologic process with classic characteristics such as membrane blebbing, DNA fragmentation and chromosome condensation (1). Unlike cellular necrosis, apoptosis is considered to be immunologically silent, meaning that this form of cell death can dampen immune responses, rather than causing inflammation (2-6).
**Apoptosis in the Immune System**

Apoptosis is critical during development of T lymphocytes in the thymus (7), for honing the repertoire of antigens to which T cells respond. Cells that respond either too strongly or not strongly enough are clonally deleted by apoptosis during two rounds of selection, resulting in T cells that will respond appropriately only to foreign antigens in the periphery. When T cell precursors arrive in the thymus from the bone marrow, they do not express a T cell receptor (TCR) or either of the cofactors CD4 or CD8, are termed double negative (DN) (8) and are in the process of rearranging their TCR loci (9, 10). Upon successful coexpression of a complete TCR with both CD4 and CD8 coreceptors, they become double positive (DP) thymocytes. DP cells encounter thymic stromal epithelial cells and circulating antigen presenting cells (APCs) expressing major histocompatibility (MHC):antigen complexes on their surfaces (11). Positive selection allows thymocytes to continue development as long as they express a TCR which interacts at a minimum avidity with MHC:antigen complexes (12, 13). Unselected cells in this round undergo apoptosis due to a lack of anti-apoptotic factors which are upregulated only in cells that interact with MHC (14, 15). Conversely, negative selection is the process by which cells that react too strongly with self antigens are deleted (16, 17). If the interaction of the TCR with the MHC:antigen is too strong, these cells could be dangerous in the periphery, as they would likely be activated by recognition of self antigens, causing autoimmune diseases (18), and thus, selected cells in this round are actively induced to undergo apoptosis (19).
Homeostasis of the adaptive immune system also requires apoptosis following development. When peripheral T cells encounter their cognate antigen along with a costimulatory signal, they are activated and begin to clonally expand in order to effectively eradicate the pathogen. Once the pathogen has been eliminated and the activated T cells are no longer required, most are deleted (20) by a process termed activation-induced cell death (AICD), employing apoptotic pathways (21-23). If these cells have a defect in the deletion mechanism, many more activated T cells remain than are needed, which can pose a threat itself. Conversely, if apoptosis is overly efficient and every cell is deleted, the memory required to efficiently respond to similar threats in the future is absent. Thus, by necessity, apoptosis is a very tightly regulated process, which will be discussed in greater detail below.

Extrinsic Apoptosis

There are two basic pathways by which apoptosis occurs, one initiated by receptor signaling, and the other mediated by mitochondrial permeabilization (shown in Figure 1.1). The key receptors and ligands involved in the receptor-initiated, or extrinsic, pathway of apoptosis are a subset of the tumor necrosis factor (TNF) ligand and TNF receptor (TNFR) superfamily, namely those receptors that contain death domains (DDs) (24, 25). The TNFR subset includes TNFR1 (24), Fas (26, 27), and the receptors for TNF-related apoptosis-inducing ligand (TRAIL) (28, 29), which can activate apoptotic pathways when bound by their cognate ligands. The ligands themselves may be able to transduce different signals based on whether they are found in the membrane-bound or soluble form (30-34).
An apoptotic signal in the extrinsic pathway originates with binding of a receptor such as Fas by its cognate ligand, FasL, stimulating the oligomerization of the death-inducing signaling complex (DISC) (35). The receptor’s DD, a conserved intracellular region of the receptor of approximately 80 amino acids (24) that binds to specific adaptor proteins which also contain DDs, catalyses the oligomerization of the DISC. The DD of Fas recruits Fas-associated DD (FADD) (36), which in turn recruits pro-Caspase-8 (37). The proximity of multiple pro-Caspase-8 molecules results in their autoproteolytic cleavage and activation to Caspase-8 (38, 39). Caspases are a family of aspartic acid-specific cysteine proteases (40) that form a cascade of proteolytic events resulting in cell death (41-45). In the extrinsic pathway, Caspase-8 is an initiator caspase, cleaving and activating the downstream effector Caspase-3 (46, 47), which ultimately leads to cleavage of cell-death substrates (48).

Other cell death receptors follow similar pathways with the same key elements, including death domains which recruit other molecules and a cascade of caspase activation. However, receptors such as TNFR1 are more complicated, and can initiate either pro- or anti-apoptotic signals, depending on the context of the signal. Ligation of TNFR1 initiates the formation of a complex (complex I) at the cell surface that includes TNFR-associated DD (TRADD) (49), TRAF2 (50), and receptor-interacting protein (RIP1) (51), but not Caspase-8. Upon TNF binding to its receptor, the TNFR transduces signals through its adaptors which result in cellular proliferation and differentiation. When such pro-survival signals are blocked by regulatory events which will be discussed later, TNFR1 can alternatively promote death signals through its adaptor proteins. The TRADD/RIP/TRAF2 complex dissociates from the intracellular domain of the receptor.
and then pro-Caspase-8 is recruited (to form complex II) and activated, followed by activation of Caspase-3 and subsequent apoptotic cell death (52).

**Figure 1.1: Diagram of apoptotic pathways.**
Intrinsic (lower) and extrinsic (upper) apoptotic signals converge at caspase-3 (green arrows). These signals are tightly regulated (red lines).

*Intrinsic Apoptosis*

The intrinsic apoptotic pathway is dependent on permeabilization of the outer membrane of the mitochondria, and is initiated by various insults to the cell, ranging from withdrawal of growth signals to DNA damage (53, 54, 54). These insults ultimately activate the pro-apoptotic subset of the Bcl-2 family, primarily Bax and Bak (Figure 1.1) (55-57), which permeabilize the mitochondrial outer membrane, leading to the release of...
a variety of pro-apoptotic molecules, including cytochrome c (58, 59). When cytochrome c is released, it binds to the cytoplasmic protein apoptotic protease activating factor 1 (Apaf1), inducing a conformational change in Apaf1. This causes oligomerization of multiple cytochrome c and Apaf1 molecules to form the apoptosome (60, 61), which recruits pro-Caspase-9 (62). In a similar manner to Caspase-8, the proximity of multiple molecules of pro-Caspase-9 initiates their autoactivation (63), resulting in the cleavage of the same downstream effector caspases as the extrinsic pathway and subsequent apoptosis (64).

In addition to the convergence of the extrinsic and intrinsic pathways at the level of the effector caspases, there is some built-in crosstalk between the two pathways. For instance, in some cell types (termed Type II) (65), Caspase-8 activated by the receptor pathway may not be strong enough to activate Caspase-3 on its own. In this case, Caspase-8 can cleave a member of the Bcl-2 family called Bid to truncated Bid (tBid) (66, 67), which exerts pro-apoptotic effects through activation of Bak or Bax (68). The intrinsic pathway then acts as an activation loop, as Caspase-9 may be able to cleave more Caspase-8 and further amplify the caspase activation cascade.

Regulation of the Apoptotic Pathways

Given that aberrant apoptosis can be detrimental for an organism, tight regulation of the process is necessary. One protein that only regulates the extrinsic pathway is cellular FLICE-like inhibitory protein (c-FLIP), which binds pro-Caspase-8 at the DISC to inhibit its processing and activation (69, 70). c-FLIP is controlled first at the transcriptional level, as it is upregulated when the TNF receptor signals through the
activation of NF-κB for survival (71). Second, when JNK signaling is activated, which itself can be either pro-survival or pro-apoptotic, the ubiquitin ligase Itch can be activated to degrade c-FLIP (72). This allows pro-Caspase-8 processing and the remainder of the caspase cascade to proceed.

The Bcl-2 family of proteins primarily controls the intrinsic pathway of apoptosis through a careful balance of pro- and anti-apoptotic factors. This family consists of three groups: 1) the pro-survival Bcl-2 homologs, 2) the “BH3 only” pro-apoptotic proteins, such as Bid, described above, and 3) the pro-apoptotic proteins including Bax and Bak. The third group is directly responsible for forming pores in the mitochondrial membrane (55-57), and are likely held in an inactive conformation by the pro-survival group (73). The BH3-only proteins can displace these pro-survival proteins from the pro-apoptotic proteins to allow pore formation (74, 75). These are complex interactions, reviewed well in (76), and illustrate the many levels of control over apoptosis.

The furthest downstream control of apoptosis is exerted by a family of proteins, called the inhibitors of apoptosis (IAPs) (77-79). IAPs are defined by the presence of at least one 65 amino acid domain originally identified in baculoviruses (80), and thus called a baculovirus IAP repeat (BIR) (81). The IAPs are a multifunctional family of proteins, originally described to inhibit apoptosis by directly binding and inhibiting the activation of caspases. Recent findings also suggest that members of the IAP family can participate in many other cellular processes.
Some IAPs are Inhibitors of Apoptosis

The human genome encodes eight members of the IAP family, pictured in Figure 1.2. The IAP BIR domains were originally described as selective caspase inhibitors (82, 83). Specifically, the intrinsic pathway initiator Caspase-9 and the downstream Caspase-3 and -7 have been shown to be inhibited by some members of the IAP family. X-linked IAP (XIAP) is the most potent human inhibitor of caspases, and most well-characterized IAP (84). XIAP contains three BIR domains, two of which are described to inhibit caspases: BIR3 binds Caspase-9 (85), while BIR2 and its N-terminal flanking sequence bind the effectors, Caspase-3 and -7 (86-89). Two closely related IAP family members, c-IAP1 and c-IAP2, have also been suggested to bind and inhibit caspases, but while they can bind, they appear to lack the residues required for inhibition (90). The rest of the IAP family members also lack various components needed for caspase binding and/or
inhibition, making it unlikely that they function to inhibit caspases (91-93). This has prompted a suggestion to rename the family BIR-containing proteins (BIRPs), though for the remainder of this dissertation, the family will continue to be referred to as IAPs.

The binding and inhibition of caspases by XIAP is accomplished by a complex set of residues in the XIAP molecule, made more intricate by the fact that each BIR domain accomplishes this in a different manner. The two caspase-inhibitory BIR domains contain grooves that act as anchors in which the IAP binding motif (IBM) of the caspase binds, though binding is not what inhibits the caspase (94). Caspase-3 binds within BIR2 of XIAP; however the residues important for inhibiting the caspase are found toward the amino-terminus from the BIR itself. Specifically, amino acids L141 and D148 in XIAP sterically block the catalytic region of bound Caspase-3 (95). Interestingly, Caspase-9 inhibition is performed by an alpha helical structure following BIR3 of XIAP, which forces Caspase-9 to acquire an inactive monomeric conformation (85, 96).

Caspases are not the only proteins that bind to the grooves within XIAP’s BIR domains. A potentially potent protein such as an apoptosis inhibitor must necessarily be tightly regulated itself. As an example of this, a nuclear-encoded, mitochondria-localized protein called second mitochondrial activator of caspases (Smac) can bind and inhibit XIAP (97, 98). Smac (also called DIABLO), which loses its localization signal during mitochondrial localization (99), contains an IBM at its amino terminus similar to those within the caspases (97, 100, 101). Upon mitochondrial permeabilization, Smac is released along with cytochrome c and binds to XIAP in the same groove as caspases, displacing the XIAP:caspase interaction and allowing apoptosis to proceed. It has been suggested that some of the other members of the IAP family, namely those that contain
an IBM-binding groove but no caspase-inhibition residues, may contribute to apoptotic inhibition by binding and sequestering Smac (102, 103). This binding would effectively leave XIAP free to directly bind caspases and inhibit apoptosis.

The overexpression of XIAP in some neoplasms has led to speculation that XIAP contributes to the transformation or maintenance of tumors (104-106). XIAP has therefore been an attractive target for chemotherapeutics (107-110). The inhibition of XIAP by Smac has been mimicked by small molecules aiming to potentiate apoptosis, perhaps by relieving the binding of XIAP to Smac (111-114). These Smac mimetics have demonstrated some success in vitro, killing some transformed cell lines directly, and others in combination with other chemotherapeutics such as TRAIL (115).

*Apoptotic Mediators in the Immune System*

While the overall mechanisms of cell death are very well understood in culture, it is as yet not clear how apoptosis is mediated in physiological systems, such as in the context of the adaptive immune system. In the periphery, the primary mode of deletion of activated cells is through the death receptor Fas. Upon activation, T cells upregulate FasL expression, which can then be engaged by Fas to signal death as described above (116-119). This results in the downregulation of the immune response coincident with the killing of the specific pathogen. However, this mechanism seems to differ significantly from apoptosis induced during negative and positive selection in the thymus.

It is apparent that Fas is not required for selection of thymocytes (120), as deletion of the receptor or its signaling intermediates has no effect on the number of T cells maturing (121, 122). Positive selection of thymocytes has been widely described to
upregulate the anti-apoptotic factor Bcl-xL, thus allowing survival of cells with an effective TCR (14, 15). Those that do not transduce a signal, then, do not upregulate anti-apoptotic factors and essentially undergo “death by neglect.” It has been suggested that negative selection may instead be dependent on TRAIL, as TRAIL-/- mice exhibit some defects in thymic deletion (123). The defects are not complete however, which led to the subsequent identification of another factor. Bim, a BH3-only member of the Bcl-2 family, is similarly required for normal deletion of autoreactive T cells (124). Interestingly, expression of Bcl-2, the inhibitor of mitochondrial death, does not reverse this phenotype in Bim-/- mice (125, 126). It is likely that a complex interplay between Bim and TRAIL mediates the finely tuned negative selection of thymocytes. Additionally, it is apparent that much work is still needed to fully understand the mechanisms of apoptosis in the immune system.

IAPs Have Functions Beyond Apoptosis Inhibition

It is becoming increasingly evident that though the IAP family-defining BIR domain is highly conserved, distinct BIRs likely have different functions in cell signaling. Other domains are also contained within the IAP family, which diversifies its functions. RING domains, which have been described in many proteins to have E3 ubiquitin ligase function (127), are present in several IAP family members (128). While few targets of the E3 ubiquitin ligases XIAP and c-IAP1/2 have been identified, it is clear that they are capable of autoubiquitination (129). Two IAPs, c-IAP1 and c-IAP2, each contain a caspase activation and recruitment domain (CARD), which has complex roles in caspase interactions and NF-κB regulation (130). Furthermore, some unique protein domains are
Figure 1.3: Non-apoptotic IAP signaling. The IAP family has recently been demonstrated to participate in diverse signaling functions beyond apoptosis.
interactions and NF-κB regulation (130). Furthermore, some unique protein domains are found within the IAP family, including a nucleotide-binding oligomerization domain (NOD) and a leucine rich repeat (LRR) in neuronal apoptosis inhibitory protein (NAIP) (131), and a ubiquitin-conjugating domain (UBC) in Apollon (132). Here, I will discuss some of the non-apoptotic functions of IAP family members, which are depicted in Figure 1.3.

**Survivin** The smallest member of the mammalian IAP family, Survivin, contains only one BIR and no other functional domains (133). Its simplicity belies its apparent importance for two separate processes critical for cell homeostasis: cell cycle regulation and apoptotic inhibition. Interestingly, Survivin has been reported to be upregulated in cancer cells (134), though it is possible that this upregulation is simply indicative of cancer cells cycling at a greater rate than the rest of the cell population, as Survivin is regulated in a cell cycle-dependent manner (135, 136). The role of Survivin in cell cycle regulation is evidenced by the cell cycle arrest or mitotic catastrophe that results from loss of Survivin in cycling cells (137, 138). Survivin binds to AuroraB and INCENP during cytokinesis, and may have other functions in the promotion of cell division (139, 140). Additionally, studies have suggested that Survivin can inhibit cell death, possibly by sequestering Smac, thus leaving XIAP free to inhibit caspases (141).

**NAIP and BRUCE/Apollon** Two members of the IAP family whose functions are not yet fully understood are NAIP and BRUCE/Apollon, that each contain domains unique to the IAP family. NAIP was the first mammalian member of the IAP family to be
identified, and it was found in connection with spinal muscular atrophy, since it is contained within the locus responsible for the disease (142). Mice deficient in NAIP5, one of the several paralogs of NAIP, are defective in the detection of bacterial flagellin and subsequent activation of Caspase-1 for pyroptotic cell death (143-145). This has been supported by work with the human protein (146), and work continues to clarify the role of NAIP in bacterial detection, while the anti-apoptotic function remains unclear. BRUCE/Apollon, the largest member of the mammalian IAP family (147), is less well understood. Interestingly, murine deficiency in BRUCE/Apollon leads to embryonic lethality due to excessive apoptosis of the placenta, which is a far more striking phenotype than that caused by loss of the better-characterized XIAP. It appears that the antiapoptotic function of BRUCE/Apollon resides in the C-terminal UBC domain, rather than the single BIR (148). BRUCE/Apollon has also recently been described to participate as a coordinator of multiple processes during cytokinesis (149), which again illustrates the diversity of functions within the IAP family.

*c-IAP1* Interestingly, when Smac binds to some IAP family members, it can trigger degradation of the IAP, which is also accomplished by Smac mimetics (150-152). These Smac mimetics have elucidated functions of c-IAP1, showing that under normal conditions, c-IAP1 function as an E3 ubiquitin ligase (likely through its RING domain) for NF-κB inducing kinase (NIK), thereby maintaining low basal levels of NIK and preventing NF-κB signaling. The loss of c-IAP1 (through degradation by Smac or Smac mimetics, for example) results in phosphorylation and activation of downstream signaling molecules including NIK, leading to the translocation of NF-κB dimers into the nucleus.
and subsequent activation of κB-dependent transcription. NF-κB activation induces the expression of TNF, which can exert an autocrine effect on the cell. In the absence of c-IAP1, TNF receptor ligation results in the deubiquitination of RIP1 by the tumor suppressor CYLD and induction of a death-inducing complex, which includes RIP, FADD and activated Caspase-8 and culminates in cell death through Caspase-3 activation (153). These recent studies have elucidated signaling functions for c-IAP1 that may be related to the function of the RING domain and suggest that BIR domains may not always be the primary functional domains for IAP proteins.

**XIAP**

XIAP has also been implicated in other cellular processes besides the suppression of apoptosis, including the control of JNK- and NF-κB-dependent transcription. A recent report connects XIAP with innate immunity (154), albeit through a different mechanism from NAIP. In response to *Listeria monocytogenes* infection, XIAP aids in the transduction of signals from Toll-like receptors (TLRs) and NOD-like receptors (NLRs), pattern-recognition receptors that are generally considered the first line of defense. Specifically, XIAP activates JNK and NF-κB-dependent transcription that subsequently upregulates cytokine production and enhances the immune response. Notably, *Xiap*-null mice are deficient in the ability to respond to *L. monocytogenes* infection, which results in a higher frequency of death than in wildtypes.

Previous studies have also implicated XIAP in NF-κB activation. TNF receptor ligation activates the canonical pathway of NF-κB by phosphorylation of the IKK complex, which results in phosphorylation and degradation of the inhibitor of κB (IκB) and release of NF-κB dimers into the nucleus (reviewed in (155)). The phosphorylation
of the IKKs is often performed by TAK1, a MAP3K also involved in JNK signaling (156, 157). XIAP has been shown to activate TAK1 binding protein 1 (TAB1) through BIR1, which in turn activates TAK1 (158-160). Activation of TAK1 results in both NF-κB and JNK activation and is thought to enhance the anti-apoptotic function of XIAP (158). The BIR1 domain of XIAP, though homologous to BIR2 and 3, does not have caspase-inhibitory activity. Other studies using ectopic expression of XIAP demonstrate that the RING domain is required for activation of a κB reporter construct (161), indicating that there may be more than one way that XIAP can promote NF-κB activation.

XIAP has also been shown to interact with Copper Metabolism (Murr1) Domain Containing 1 (COMMD1), a copper metabolism protein, and through this interaction has effects on two distinct signaling pathways. COMMD1 is an inhibitor of NF-κB-dependent transcription, and the interaction of XIAP with COMMD1 may point to its second effect on the NF-κB pathway. COMMD1 has been shown to bind and catalyze the ubiquitination of DNA-bound RelA, a key NF-κB subunit, thus suppressing NF-κB-dependent transcription (162). XIAP binds COMMD1 through its BIR3 domain and catalyzes its ubiquitination through the RING (163). Thus XIAP can prevent the inhibition of RelA by COMMD1 and allow continuation of NF-κB-dependent transcription. The physiological consequences of the XIAP-COMMD1 interaction remain to be elucidated, but may provide a mechanism by which the RING domain can affect NF-κB signaling.

A completely different cellular process in which the interaction of XIAP with COMMD1 appears to play an important role is copper homeostasis. The importance of COMMD1 in copper export from the cell is evidenced by genetic loss of COMMD1
resulting in a copper toxicosis syndrome common in Bedlington terriers (164). COMMD1 likely promotes copper export from the cell through its interaction with the copper export protein ATP7B (165). XIAP can catalyze the ubiquitination of COMMD1, as described above, increasing intracellular copper levels (163). Homeostasis of copper metabolism is achieved through a negative feedback loop, in which higher copper levels induce XIAP degradation (166).

Additional studies support a role for XIAP in TGF-β/BMP signaling, though the mechanism has not been established and the domains of XIAP involved are unclear. It has been suggested that the role for XIAP in the TGF-β pathway may be related to its role in NF-κB induction, through TAK1 activation upon TGF-β receptor ligation (158, 160). This activation would require interaction of XIAP’s BIR1 with TAB1. However, other studies indicate that XIAP interacts directly with the type 1 TGF-β receptor and signals through Smad4, which requires BIR3 and the downstream loop region of XIAP (161, 167). TGF-β is a pleiotropic cytokine, which elicits diverse effects in the different cell types in which it signals. It is possible that this can account for the discrepancies between the different studies, though most of this work is performed using overexpression systems, and the physiological role for XIAP in TGF-β signaling remains unclear.

**X-linked Lymphoproliferative Syndrome**

While the physiologic relevance of the attributed functions of XIAP remains unclear, the recent description of causative mutations in XIAP in patients with a rare primary immunodeficiency, X-linked lymphoproliferative syndrome (XLP) provides
insight into the importance of a role for XIAP in humans (168). XLP is a rare immunodeficiency, which is often initiated upon or exacerbated by Epstein-Barr virus (EBV) infection. The viral infection is uncontrolled due to defects in immune responses, and frequently (50% of patients) results in fatal infectious mononucleosis. Other common manifestations of the disorder include B cell lymphomas and hypogammaglobulinemia (169) [reviewed in (170)]. XLP affects approximately 1-3 in 1,000,000 males, though it may be under-diagnosed due to the heterogenous nature of the disorder and similarity to other disorders. Age of onset varies by primary feature of the disease, but 70% of patients die by the age of ten. Bone marrow transplant before fatal infectious mononucleosis onset has emerged as a promising treatment (171).

XLP was first described as a clinical entity (initially as Duncan’s Syndrome) in the early 1970s (172), but it was not until 1998 that the gene responsible for the majority of cases was identified by three independent groups (173-175). The defect was mapped to a single locus within the Xq25 band of the X chromosome, which encodes signaling lymphocytic activation molecule (SLAM)-associated protein (SAP), an adaptor molecule for the SLAM family of receptors, a subset of the CD2 subfamily of immunoglobulin receptors (176-179). The majority of patients have a variety of described mutations in the SAP coding region leading to loss of protein expression (173-175). This correlates closely with patients having a family history of XLP. A minority of patients do not exhibit loss-of-protein mutations within SAP, and these cases of XLP have primarily been explained by misdiagnosis of XLP or the existence of mutations outside the coding sequence which affect SAP expression levels (180, 181).
The generation of mice lacking *Sap/Sh2d1a*, the murine *SAP* homolog, has provided a valuable tool to study the roles of SAP in XLP (182, 183). The most striking defect in *Sap*-null mice is a defect in the development of natural killer T (NKT) cells, which is consistent with the phenotype of XLP patients (178, 184, 185). It is unclear which receptor is required for this function, though the Src family tyrosine kinase Fyn, which SAP recruits to SLAM-family receptors is also required for the signal that is transduced through SAP (186). In addition, some T cell functions are severely impaired in *Sap*-null mice, including T cell help for humoral immunity (187, 188) and Th2 cytokine expression (189). The mechanism by which SAP is involved in these responses is also unclear, though Fyn appears to be involved in cytokine expression but not germinal center formation in response to T cell-dependent antigens. Natural killer (NK) cell cytotoxicity is also reduced in *Sap*-deficient mice, as SAP is an important adaptor for the SLAM family receptor 2B4 (190, 191). For reasons that are as yet unclear, *Sap*-null mice are also unable to respond appropriately to an infection with MHV-68, a murine γ-herpesvirus closely related to EBV (192). An uncontrolled proliferation of nonspecific CD8 T cells is observed which are unable to clear the virus, which phenotype may mimic the response to EBV infection in human XLP patients. While these defects described in *Sap*-null mice have greatly improved the understanding of the role of SAP and its possible contribution to XLP, an effective treatment has yet to emerge from these studies.

**XIAP in XLP**

Rigaud *et al.* recently published a report describing three XLP families that express normal SAP and share the majority of their symptoms with patients having
mutations in $SAP$ (168). Interestingly, these patients have different mutations mapping to the same locus, but the mutated gene was found to be $XIAP$ and the described mutations result in loss of functional XIAP protein. At the immunologic level, this cohort of XLP patients exhibits a loss of NKT cells, similar to patients lacking SAP. Additionally, lymphocytes from these patients demonstrate enhanced apoptosis in response to extrinsic apoptotic stimuli (168). This study was the first to identify XLP patients with mutations in a gene other than $SAP$. Remarkably, though both demonstrate a wide array of functions, SAP and XIAP show no obvious structural or functional similarity beyond a common chromosomal locus and their involvement in XLP.

**Goals of This Dissertation:**

XIAP has been proposed to be a multifunctional protein. It can inhibit apoptosis initiated by a variety of stimuli through direct interaction with caspases and prevention of their activation. In addition, studies have connected XIAP to NF-κB-dependent transcription, suggesting that XIAP is a cofactor for the activation of the signaling mediator TAK1. TGF-β-mediated signaling is yet another pathway in which XIAP is thought to participate, possibly through direct interactions with the type 1 receptor. However, little data currently exists showing non-apoptotic roles for XIAP in physiological systems. The apoptotic role for XIAP has also made it an attractive target for anti-cancer therapy, with the goal of ablating its expression to increase tumor cell sensitivity to apoptosis. However, if all of the roles XIAP plays in a cell are not fully understood, some serious side effects could potentially result from drugs designed to target this protein. This was highlighted with Smac mimetics, which not only targeted
XIAP, but also its closely related family members c-IAP1 and c-IAP2 with dramatic effects. Therefore, understanding XIAP (and other IAPs) is paramount for the development of future therapies targeting this protein. This becomes even more important with the recent identification of XLP patients with mutations in XIAP. It is unclear how XIAP is responsible for this phenotype, and clarifying this could help to understand the pathogenesis of the disease and lead to treatments.

One confounding aspect of previous XIAP studies is that despite its apparent importance, at least for apoptotic inhibition, mice with genetically targeted disruption of XIAP have exhibited minimal defects in apoptosis (193). Both receptor-mediated and mitochondrial apoptosis appear to proceed essentially normally in these mice, and it was suggested that the c-IAPs may be compensating, even though they are not thought to be able to directly inhibit caspases. One study showed that cytochrome c injected into neurons induced less death in wildtype cells than Xiap-null cells (194). Additionally, mammary gland development was altered in Xiap-null mice, which may be related to NF-κB signaling (195). These findings lead to some questions: Does XIAP have different functions in a mouse than a human? Is XIAP’s role to modulate apoptotic responses, rather than outright inhibition? And, how does XIAP participate in XLP pathogenesis? These questions will be addressed in this dissertation in the form of the following Aims:

**Aim 1 (Chapter 2):** Examination of the role of XIAP in signal transduction pathways

**Aim 2 (Chapter 3):** Apoptotic sensitivity of murine IAP-deficient cells

**Aim 3 (Chapter 4):** Distinct functional and biochemical properties of XIAP and SAP, two factors targeted in X-linked Lymphoproliferative Syndrome (XLP)
Chapter II

Examination of the Role of XIAP in Signal Transduction Pathways

Summary

The IAP family is made up of many multifunctional proteins, the roles of which are only beginning to be elucidated. XIAP has been suggested to be a cofactor in several signaling pathways, including NF-κB-dependent transcription and TGF-β signaling. The exact roles XIAP plays in these signaling events is unclear, however. We took advantage of recent advancements in real-time semiquantitative RT-PCR as well as Xiap-null mice to investigate the necessity of XIAP for these signaling pathways. Interestingly, we found that, under the conditions tested, neither overexpression nor loss of XIAP affected gene expression or overall outcome upon treatment with stimuli activating NF-κB or TGF-β, suggesting there is not an absolute requirement for XIAP in signaling.
Introduction

X-linked inhibitor of apoptosis (XIAP) is interestingly the only member of the inhibitor of apoptosis (IAP) family of proteins actually able to inhibit apoptosis by directly binding caspases (196). Through its family-defining baculoviral IAP repeat (BIR) domains, it can bind to the intrinsically-induced Caspase-9 (BIR3) as well as the effector caspases-3 and -7 (BIR2). Thus, XIAP is capable of inhibiting apoptosis induced through either the mitochondrial or receptor-mediated pathway, making it a powerful apoptotic inhibitor. This also distinguishes it from other inhibitors that work upstream of the mitochondria only.

In addition to its importance as an inhibitor of apoptosis, XIAP is a multifunctional protein. XIAP has been suggested to be involved in various signaling pathways, such as TNF receptor-mediated activation of the canonical pathway of NF-κB activation. Upon receptor ligation, the pathway proceeds through phosphorylation of the IKK complex, which results in phosphorylation and degradation of the inhibitor of κB (IκB) and release of NF-κB dimers into the nucleus (155). The phosphorylation of the IKKs is often performed by TAK1, a MAP3K also involved in JNK signaling (156, 157). XIAP has been shown to interact with TAK1 binding protein 1 (TAB1) through the most amino-terminal BIR (BIR1), which in turn activates TAK1. Activation of TAK1 results in both NF-κB and JNK activation and is thought to enhance the anti-apoptotic function of XIAP (158).

Another interaction partner through which XIAP may be able to affect function of NF-κB is COMMD1, a protein involved in copper homeostasis. COMMD1 has been shown to bind and catalyze the ubiquitination of DNA-bound RelA, a key NF-κB
subunit, thus suppressing NF-κB-dependent transcription. COMMD1 is bound by BIR3 of XIAP, which catalyzes ubiquitination of COMMD1 through the RING domain at XIAP’s carboxyl terminus (128), preventing this inhibition and allowing continuation of NF-κB-dependent transcription (197). These studies, together with those performed on the interaction of XIAP with TAK1, suggest that there may even be multiple pathways through which XIAP can affect NF-κB-dependent transcription.

In addition to NF-κB-dependent transcription, XIAP has been suggested to be a cofactor for TGF-β signaling (161, 167). Ectopically expressed XIAP can bind to TGF-βR1 and participate in downstream signaling events through Smad4 to activate transcription from a TGF-β-responsive reporter. The domain responsible for this function of XIAP has yet to be elucidated, though it is evident that it is unrelated to the apoptosis-inhibitory function. BIR3 and the carboxy-terminal loop region have been implicated through reporter assays, while crystallographic studies have suggested that BIR1 is responsible (198).

Surprisingly, given the apparent importance of XIAP to this variety of different signaling pathways, no gross defect has been reported upon loss of the protein, namely in Xiap-null mice (193). One study described a slight delay in mammary gland development, possibly related to NF-κB activation (195). However, most of the previous studies have focused on whether overexpression of XIAP is capable of transducing signals rather than whether it is necessary for such. We have exploited the recent advancements in semiquantitative realtime RT-PCR to investigate whether expression or loss of XIAP in human and murine cells can affect various signaling pathways.
Materials and methods

Reagents:
Recombinant human and murine TNF and human TGF-β1 were from Roche, PMA and ionomycin were from Sigma-Aldrich. Antibody to mouse CD3 was obtained from BD Pharmingen.

Cells:
All cells were cultured in RPMI or DMEM (Mediatech) supplemented with 10% fetal bovine serum (FBS) (Mediatech), 2mM glutamine (Gibco) with or without 1% penicillin/streptomycin (Gibco) at 37°C, 5% CO₂. XIAP (day 12.5) MEFs were isolated from individual embryos from timed matings of XIAP WT male with XIAP heterozygous female according to standard procedures. After two passages, WT and KO embryos were transformed by serial infection with lentiviruses expressing Ras and E1A (199). Experiments were performed with male XIAP WT and KO. Stable reconstitution of Xiap-null MEFs was accomplished by infection with a lentivirus expression system (manuscript in preparation) encoding full length XIAP.

Mice:
XIAP KO mice (193) were backcrossed in the C57BL/6 strain for at least 12 generations. All mice were housed under specific pathogen-free conditions within the animal care facility at the University of Michigan. The University of Michigan Committee on the Use and Care of Animals approved all experiments.
Real-time RT-PCR

RNA was isolated using the RNeasy Kit (Qiagen). Each 25µl reverse transcription reaction was performed with 100 ng RNA and reagents from Applied Biosystems, Inc according to standard procedures. Semiquantitative PCR was performed on a Taqman7500 machine using Taqman Gene Expression Assays to IkBα, TNF and PAI-1 from Applied Biosystems, Inc.

Transfection:

Jurkat cells (10x10^6 cells/transfection) were electroporated with 200 volts, at infinite resistance and with siRNA oligos (Qiagen) to XIAP (AAGTGGTAGTCTGTGTTCACGC) or control (AAGACCGCGCCGAGGTGAAG) and placed into RPMI as above, but with 20% FBS. After 24 hours, live and dead cells were separated by Ficoll-Paque PLUS (GE Life Sciences), and live cells were resuspended in RPMI with 10% FBS and treated as described 48 hours after transfection. 293HEK cells were transfected by standard calcium phosphate procedure with pEBB empty or containing full-length human XIAP and treated as described 24 hours later.

In vivo injections of lipopolysaccharide (LPS):

Mice were anesthetized with ketamine (Hospira) and xylazine (Lloyd Laboraties), the neck was opened and 12.5 µg LPS (Sigma) or saline was instilled intratracheally. Six hours after surgery, mice were euthanized, and lungs were removed after washing with 1 ml saline to collect BAL fluid. Lung tissue was snap frozen, followed by homogenization using a Tissue Tearor (BioSpec Products, Inc.) in lysis buffer (1% Triton X-100 in PBS
with complete protease inhibitor tablets [Roche]). Lung lysates were diluted and BAL fluid was used undiluted in a standard sandwich ELISA procedure (200).
Results and Discussion

XIAP is dispensable for NF-κB activation

Since XIAP has been shown in reporter assays to activate NF-κB-dependent transcription when overexpressed (161), we examined the result of changes in XIAP expression levels upon the transcription of classical NF-κB target genes. XIAP was overexpressed in HEK293 cells, and the cells were stimulated with TNF to initiate NF-κB signaling. RNA was examined by semiquantitative realtime RT-PCR, using target assays for the NF-κB-dependent gene IκBα. Expression of IκBα (Figure 2.1A) and other genes (data not shown) was not found to be affected by the presence of elevated levels of XIAP protein, indicating that overexpression of XIAP may not affect NF-κB dependent gene expression. To address the possibility that the involvement of XIAP may be stimulus- or cell type-dependent, Jurkat cells were electroporated with siRNA to knock down the expression of XIAP. These cells were then stimulated with either PMA and ionomycin (Figure 2.1B) or antibody to CD3 (Figure 2.1C). Again, the expression of classical NF-κB-dependent genes IκBα and TNF was unchanged between the control and siXIAP samples, suggesting that XIAP may be dispensable for NF-κB activation by the T cell receptor.

However, overexpression and knockdown may not be the best method for determining the necessity of a factor for a certain signaling pathway, as knockdown does not completely ablate the expression and may have off-target effects. This leaves open the possibility that the remaining protein was simply above the threshold required for this signaling mechanism. A better tool would be cells that genetically lack XIAP, such as cell derived from targeted Xiap-null mice. Thus, Xiap-deficient and control embryonic
Figure 2.1: XIAP is dispensable for NF-κB activation by a variety of stimuli. A) HEK293 cells were transfected with either empty vector or XIAP. After 24 hours, cells were treated with media (control) or media with 200 U/ml hTNF. RNA was harvested 3 hours later and semiquantitative real-time PCR was performed for IκBα expression, normalized to GAPDH. B) Jurkat cells were electroporated with siRNA oligos targeting either GFP (control) or XIAP (siXIAP), and treated for 3 hours with media or PMA (20ng/ml) and ionomycin (1.4ug/ml) 48 hours later. Real-time PCR was performed as above. C) Jurkat cells were electroporated as above, and treated with plate-bound IgG control or anti-CD3 as indicated for 3 hours. Real-time PCR was performed as above, with TNF replacing IκBα. D) Xiap-null and littermate embryonic fibroblasts were treated for 3 hours with 500 U/ml mTNF. RNA was harvested and real-time PCR was performed as above. E) Thymocytes were harvested from Xiap-null and littermate mice and treated with 200 U/ml mTNF for 4 hours. RNA was harvested and real-time PCR was performed as above.
fibroblasts from were treated with TNF, and NF-κB-dependent transcription was again measured. Supporting the previous results, no difference in response to TNF was observed in transcription of IκBα (Figure 2.1D) or other genes (data not shown).

Primary thymocytes were also isolated from Xiap-null mice and controls and examined in the same way. TNF treatment somewhat increased NF-κB-dependent transcription, as observed by IκBα expression, but no difference was recorded between cells expressing normal levels of XIAP and those expressing none. Therefore, in the systems examined here, XIAP appears to be unimportant for the activation of NF-κB-dependent transcription.

**Figure 2.2: Loss of XIAP does not affect TGF-β-dependent transcription.** Xiap-null and littermate embryonic fibroblasts were treated for 3 hours with either low serum media (control) or 5 ng/ml human TGF-β1. RNA was harvested and real-time PCR was performed as above for PAI-1, normalized to GAPDH.

**XIAP is not required for TGF-β signaling**

In addition to NF-κB-dependent transcription, XIAP has been suggested to be involved in TGF-β signaling (167). Therefore, transcription of classically TGF-β responsive genes was investigated in the presence and absence of XIAP. Upon stimulation with recombinant TGF-β, control MEFs (XIAP WT) upregulated genes such as plasminogen activator inhibitor (PAI-1) as expected (Figure 2.2). However, MEFs
lacking XIAP (XIAP KO) upregulated PAI-1 to the same extent, and reconstitution of XIAP expression (KO+XIAP) did not affect gene expression. These results suggest that in mouse fibroblasts, XIAP is not universally required for TGF-β signaling. It remains possible that XIAP participates in signaling to a subset of TGF-β-responsive genes or that it is more crucial to other cell types.

**XIAP is dispensable for inflammatory response to LPS**

Since XIAP has been suggested to promote NF-κB transcription though activation of TAK1 (158), a signaling pathway in which TAK1 is necessary was examined for evidence of XIAP requirement. Bacterial lipopolysaccharide (LPS) is detected by TLR4, a member of the Toll-like family of pattern recognition receptors (201). TLR4 activates the inflammatory response to bacteria through NF-κB transcription of inflammatory factors, for which TAK1 is necessary, as demonstrated in TAK1-/- mice (157). Therefore, *Xiap*-null mice and littermate controls were injected intratracheally with LPS, and lung inflammatory responses were measured. Lung sections were cut and stained with hematoxylin and eosin to examine the total inflammation (Figure 2.3A). As shown in representative sections, six hour treatment with LPS increased the amount of inflammatory infiltrates visible in the lung, but no significant difference was observed between *Xiap*-null mice and littermate controls.

Inflammation is usually manifested by a significant increase in a subset of cytokines considered to be proinflammatory. Therefore, inflammatory factors were measured in bronchoalveolar lavage (BAL) fluid as well as whole lung lysates. Levels of CCL5 (RANTES), a chemokine involved in recruitment of leukocytes into inflamed
tissue (202), were measured by ELISA, as well as numerous other chemokines and cytokines. CCL5 (Figure 2.3B) and other inflammatory mediators (not shown) were elevated in both BAL fluid and the inflamed lung tissue. However, no difference was observed between Xiap-null mice and littermate controls. This indicates that XIAP is unimportant for the short-term response to LPS activation of TLR4 signaling. Taken with previous results, it further shows that XIAP is not absolutely necessary for NF-κB activation under a variety of signals.

Figure 2.3: XIAP does not affect response to LPS in vivo. Xiap-null and littermate mice were injected intratracheally with saline or 50ul of 0.25 µg/µl LPS. After 6 hours, mice were euthanized and lung and BAL fluid were collected for protein and histology. A) Representative lung sections, formalin fixed and stained with hematoxylin and eosin. B) Protein was extracted from lung tissue with Triton and entered into an ELISA for CCL5, along with BAL fluid.
Discussion

Previous studies have indicated a role for XIAP in multiple signaling pathways, including NF-κB-dependent transcription and TGF-β-initiated pathways (158, 161, 167). While it remains possible that XIAP may be capable of participating in a subset of these pathways, our results indicate that XIAP is not a universally required cofactor for these pathways. We examined NF-κB-dependent transcription by classical gene expression upon stimulation with a variety of signals that activate the NF-κB pathway. Stimulation of various cell types with TNF, anti-CD3 antibody, or PMA plus ionomycin in the presence or absence of XIAP showed no difference in the level of target gene expression. Additionally, XIAP was not necessary for the response to LPS in vivo, a response the involves NF-κB activation and requires TAK1, the factor through which XIAP was thought to affect NF-κB signaling. Interestingly, TAB1, the probable binding partner for XIAP, was also not required for NF-κB and JNK signaling stimulated by several pathways (157). This suggests that the TAB1-XIAP binding might serve as a redundancy, which could be tested by inhibiting other ways of activating TAK1. The previous work that suggested that XIAP may be involved in NF-κB activation was performed using overexpression and reporter systems, which is very different from knockdown or knockout systems we used, looking at real gene expression. It is possible that when overexpressed, XIAP can adopt functions that XIAP expressed at endogenous levels does not. It may be important to note that this seems to hold true for both mouse and human, as loss of XIAP in either species does not seem to effect signal transduction.
Similar results were obtained with TGF-β signaling, for which XIAP has been previously been shown to be a cofactor: in fibroblasts, XIAP absence had no effect on target gene expression upon stimulation with TGF-β. This suggests that XIAP is not necessary for signal transduction from the TGF-β receptor complex, even if it is capable of binding there and initiating signals when it is overexpressed (161, 167). TGF-β is a pleiotropic cytokine, however, and it is possible that XIAP is necessary for signaling in a small subset of cell types to influence some outcomes. It is apparent, however, that XIAP is not universally required for either NF-κB or TGF-β signaling.
Chapter III

Apoptotic Sensitivity of Murine IAP-Deficient Cells

Summary

Although numerous studies have implicated the Inhibitor of Apoptosis (IAP) proteins in the control of apoptotic cell death, analyses of murine Iap-targeted cells have not revealed significant differences in susceptibility to apoptosis. Here we show that under defined conditions, murine cells lacking XIAP and c-IAP2, but not c-IAP1, exhibit heightened apoptotic sensitivity to both intrinsic and extrinsic apoptotic stimuli.
Introduction

The inhibitor of apoptosis (IAP) family of proteins are thought to play a variety of physiological roles in addition to their initially described function as suppressors of programmed cell death. IAPs were first discovered in the genomes of baculoviruses, and cellular orthologs were subsequently identified in an evolutionarily diverse range of organisms (196). IAPs are thought to exert their pro-survival effects primarily through direct binding and inhibition of caspases, a family of aspartate-specific cysteine proteases that are the executioners of cell death. While several members of the IAP family have been shown to perturb caspase activity, X-linked Inhibitor of Apoptosis (XIAP) is a more potent inhibitor of caspases than any other family member. Nevertheless, two related IAP proteins, c-IAP1 and c-IAP2, have both been described to exhibit antiapoptotic activity (203, 204).

XIAP contains three BIR (baculoviral IAP repeat) domains, which are the defining elements of the IAPs, as well as a carboxy-terminal RING domain, which has been shown to catalyze ubiquitination of target proteins through its role as an E3 ubiquitin ligase. Two domains of XIAP are responsible for direct, high affinity binding to caspases. A region amino-terminal to the second, central BIR binds to effector caspases -3 and -7, while the most carboxy terminal BIR (BIR3) is specific for binding and inhibition of Caspase-9. Ectopic expression of XIAP has been shown to suppress cell death induced through either the receptor-mediated or the mitochondrial pathways, and experimental evidence strongly supports a critical role for caspase binding in this suppression (reviewed in (196)). XIAP is itself regulated by several inhibitory proteins, including Smac/DIABLO, a nuclear-encoded protein which is localized mitochondrially
in healthy cells, but which is released into the cytosol during apoptosis. Smac/DIABLO binds to XIAP in the same domain utilized by XIAP to bind caspases, leading to a displacement of the IAP:caspase interaction and the triggering of caspase-dependent cell death (98, 99, 101, 205).

The characterization of genetically modified mice has frequently revealed profound insights into the function of the gene product, and in many cases shed light on the pathogenesis of human diseases in which the orthologous gene is targeted. Interestingly, although XIAP has been well characterized as a potent inhibitor of Caspase-3, Caspase-7 and Caspase-9 in vitro and in human cell lines (196) as described above, primary cells derived from mice lacking Xiap (also known as Miha) have been reported not to show increased sensitivity to apoptotic stimuli (193). Here we examine this apparently paradoxical finding in more detail, using IAP-deficient cells from matched, littermate controls, using defined apoptotic conditions, and find that cells deficient in XIAP and c-IAP2 exhibit heightened sensitivity to pro-death signals.


Materials and Methods

Cells:

All cells were cultured in DMEM (Mediatech) supplemented with 10% fetal bovine serum (FBS) (Mediatech), 2mM glutamine (Gibco) and 1% penicillin/streptomycin (Gibco) at 37°C, 5% CO₂. XIAP (day 12.5), c-IAP1 (day 14.5) and c-IAP2 (day 14) MEFs were isolated from individual embryos from timed matings of XIAP WT male with XIAP heterozygous female or two c-IAP1 heterozyotes according to standard procedures. After two passages, WT and KO embryos were transformed by serial infection with lentiviruses expressing Ras and E1A (199). Experiments were performed with male XIAP WT and KO cells and female c-IAP1 WT and male c-IAP1 KO cells.

Lungs were removed from male littermate XIAP WT and KO mice, minced, and shaken at 37°C in RPMI1640 with 5% FBS, 1mg/ml collagenase A (Roche) and DNase (Sigma). Suspensions were then expelled through an 18G needle 10 times and suspended in HBSS (Mediatech). Red blood cells were lysed and the remaining cells were placed in culture media and allowed to grow. All experiments were performed between passages 2 and 4.

Mice:

XIAP KO mice (193) were backcrossed in the C57BL/6 strain for at least 12 generations. Mice lacking c-IAP1 were generated as described (206). All mice were housed under specific pathogen-free conditions within the animal care facility at the University of Michigan. The University of Michigan Committee on the Use and Care of Animals approved all experiments.
Reconstitution of XIAP MEFs:

Stable reconstitution of Xiap-null MEFs was accomplished by infection with a lentivirus expression system (manuscript in preparation) encoding either full length XIAP or a D148A/W310A double mutant generated by site-directed mutagenesis.

Death assays:

Cells were treated in triplicate as indicated with media, recombinant murine TNF (Roche) and cycloheximide (Sigma) or etoposide (Bristol-Meyers Squibb) for 24 hours. Floating cells were collected and combined with adherent cells lifted with trypsin-EDTA (Mediatech), and all were resuspended in propidium iodide (PI) buffer (2µg/ml PI [Sigma], 1% bovine serum albumin [Sigma] in 1x PBS) for flow cytometry. Data were collected on a Beckman-Coulter Cytomics FC-500 machine and analyzed using FlowJo (Treestar).

Western blotting:

Whole cell lysates were prepared using RIPA lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM DTT, 1mM PMSF in 1x PBS) supplemented with protease inhibitors. Samples were resolved on 4-12% gradient SDS-PAGE gels (Invitrogen), transferred onto nitrocellulose (Invitrogen) and blocked in 5% milk in Tris-buffered saline containing 0.1% Tween (Bio-Rad). Membranes were incubated at room temperature for 1h or overnight at 4°C with the following antibodies: cleaved Caspase-3 (Cell Signaling), XIAP (BD Pharmingen), β-actin (Sigma), or rIAP (Robert Korneluk, University of Ottawa, Canada). Secondary horseradish peroxidase-conjugated anti-
mouse, anti-rabbit or anti-rat (GE Healthcare) were used for 1h at room temperature. Enhanced chemiluminescence (GE Healthcare) and Kodak XAR film were used for visualization purposes.

*siRNA*: Oligos were obtained from Invitrogen with the following sequences: c-IAP2 #1 GAGGCUUGCAAAAGCUCAAAGGCAUG, c-IAP2 #2 UAGAUCAUCUGACUCCUCCUC, control GCGACAAUUGCAAGUAGUCACCAUA. Two serial transfections were performed in 12-well plates using 4ul oligo, 24 hours apart with Lipofectamine 2000 (Invitrogen), and cells were treated as indicated 30 hours after the second transfection.
Results and Discussion

Murine XIAP can modulate apoptosis

To address whether murine XIAP may modulate apoptosis, fibroblasts isolated from lungs of Xiap-null mice and control littermates were treated with tumor necrosis factor (TNF), a ligand commonly used to stimulate the extrinsic apoptotic pathway. In cell culture systems, TNF does not kill without the addition of the protein synthesis inhibitor cycloheximide (CHX), in part because TNF is generally thought to induce transcription of genes that suppress apoptosis (207-209). Interestingly, as shown in Figure 3.1A, lung fibroblasts lacking XIAP were highly sensitive to treatment with TNF and CHX as compared to their littermate counterparts, suggesting that XIAP does in fact suppress apoptosis in murine cells.

Murine XIAP is closely related to the human protein and contains all the critical components for functioning similarly to its human counterpart (210). However, previous studies using Xiap-null murine embryonic-derived fibroblasts (MEFs) did not reveal sensitivity to TNF (193). To further examine the responsiveness of Xiap-deficient pulmonary fibroblasts, we evaluated the role of murine XIAP in receptor-mediated death using matched littermate MEFs. Intriguingly, the concentration of cycloheximide (CHX) previously used to sensitize cells to TNF-induced death was found to induce killing under these experimental conditions, even in the absence of TNF (data not shown). Using a lower concentration of CHX (0.1µg/ml), death was potentiated through the TNF receptor without killing the cells with CHX alone. As shown in Figure 3.1B, Xiap-null MEFs were significantly more sensitive to death than their wildtype counterparts. Reintroduction of wildtype murine XIAP into the deficient line (KO+XIAP) demonstrated that this effect
was wholly dependent on XIAP, since these cells were protected to approximately the same levels as the wildtypes. Furthermore, reintroduction of a mutated form of XIAP which cannot inhibit caspases (D148A/W310A; KO+mutant) was unable to protect the cells to the same degree as the wildtype protein. These studies were also performed in primary MEFs as well as MEFs generated from distinct embryos with essentially the same results (data not shown). These data further support those from the pulmonary fibroblasts, suggesting that murine XIAP does modulate the apoptotic threshold in a similar, caspase-dependent manner to the human protein.

Figure 3.1: XIAP modulates apoptosis in murine fibroblasts.
A) Lung fibroblasts were treated with mouse TNF (200U/ml) and CHX (1µg/ml) or left untreated (UT) for 24 hours. Floating and adherent cells were harvested together, stained with propidium iodide and analyzed by flow cytometry. Data represent 3 mice per group, and error bars represent standard error. One-way ANOVA was used to calculate significance, and p-values of less than 0.01 are indicated with an asterisk (**). B) The indicated transformed MEF lines were treated with 200U/ml mTNF and 0.1µg/ml CHX for 24 hours, and cells were harvested and analyzed as in A. All data represent at least 3 independent experiments, with error bars indicating standard error.

Different effects of c-IAP1 and c-IAP2 on receptor-mediated apoptosis

The lack of XIAP in genetically targeted mice has been suggested to be compensated for by c-IAP1 and c-IAP2 overexpression (193). Therefore, the levels of
these proteins in MEFs from IAP-null mice were examined. As shown in Figure 3.2A, the level of c-IAP1 was found to be higher in transformed fibroblasts lacking XIAP, consistent with initial reports (193). As previously described (206), c-IAP2 was upregulated in c-lap1-null MEFs, the only lysates in which c-IAP2 could be detected. Additionally, XIAP protein levels appeared to be unaffected by the absence of either c-IAP1 or c-IAP2.

Since changes in the expression of c-IAPs were observed, the possible contribution of these proteins to protection from receptor-mediated death was also examined. MEFs from mice deficient in c-lap1 or c-lap2 and corresponding littermates
were treated with TNF and CHX to induce apoptosis. Under these experimental conditions, c-IAP1 deficiency did not affect the amount of death observed in response to TNF/CHX treatment (Figure 3.2B). Interestingly, cells lacking c-IAP2 were significantly more sensitive to TNF and CHX-induced death than their wildtype counterparts, suggesting that unlike c-IAP1, c-IAP2 may play a role in protection from apoptosis under these experimental conditions.

The function of XIAP in inhibiting cell death is to specifically block activation of caspases, leading us to examine caspase activation in IAP-deficient MEFs upon treatment with TNF and CHX. After 8 hours of treatment, a significant amount of cleaved Caspase-3 was observed in wildtype MEFs by immunoblot (Figure 3.2C). This level increased dramatically in TNF/CHX treated Xiap-deficient MEFs, consistent with the notion that XIAP is responsible for blocking cleavage and activation of Caspase-3. Additionally, reconstitution with a wildtype murine XIAP blocked Caspase-3 cleavage even more than the endogenous protein at this timepoint. Supporting the viability studies, no difference in the amount of Caspase-3 cleavage was observed between c-Iap1-null MEFs and littermate control cells. Caspase-3 activation in the c-IAP2 MEFs also corroborated the findings from the viability studies, showing increased cleavage in treated c-Iap2-null MEFs.

**XIAP and c-IAP2 are protective in the intrinsic pathway, c-IAP1 is not**

Ectopic expression of human XIAP has also been shown to be protective against intrinsic or mitochondrial cell death (205), so the intrinsic apoptotic pathway in IAP deficient MEFs was also examined. As shown in Figure 3.3A, Xiap-null cells were more
sensitive to etoposide-induced death than wildtype MEFs, and this phenotype was reversed by reconstitution with wildtype XIAP. Expression of D148A/W310A XIAP was unable to protect against this stimuli in the deficient fibroblasts. These data together indicate not only that murine XIAP modulates the threshold for mitochondrial death, but that similar to human XIAP, the caspase-binding activity is likely to be the primary anti-apoptotic function of the protein. The activity of the c-IAPs in the mitochondrial death pathways was also tested with etoposide. MEFs lacking c-IAP1 were not found to be more sensitive to death, but instead were slightly more resistant than wildtype (Figure 3.3B). However, similar to the effects observed with TNF-induced death, c-IAP2 deficiency resulted in a significant sensitivity to etoposide-induced death (Figure 3.3C),

Figure 3.3: Xiap- and c-Iap2-null MEFs are sensitive to etoposide-induced apoptosis.
The indicated transformed MEF lines were treated with 1µg/ml etoposide for 24 hours, and cells were harvested and analyzed as in B. Error bars = SEM, n≥3.
indicating that c-IAP2 can protect from both mitochondrial and receptor-mediated apoptosis.

*Knockdown of c-IAP2 in c-lap1-null MEFs sensitizes to apoptosis.*

Since c-IAP2 was observed to be upregulated in *c-lap1*-null fibroblasts, we investigated whether c-IAP2 was protecting these cells from death to compensate for the loss of c-IAP1. Using RNAi, we were able to knock down expression of c-IAP2 in *c-lap1*-null MEFs (Figure 3.4, inset) and evaluate the death response to TNF plus CHX or to etoposide. As shown in Figure 3.4, with less c-IAP2 present, *c-lap1*-null MEFs were more sensitive to death induced by both stimuli. However, the sensitization induced in these cells was no greater than that in the *c-lap2*-null cells with intact c-IAP1, which suggested that c-IAP1 did not contribute to apoptotic resistance.

**Figure 3.4: Knockdown of c-IAP2 in c-lap1-null MEFs sensitizes them to apoptosis.**

MEFs lacking c-IAP1 were transiently transfected with siRNA oligos to c-IAP2 followed by treatment with either 200U/ml mTNF and 0.1µg/ml CHX or 0.5µg/ml etoposide for 16 hours. Floating and adherent cells were harvested together, stained with propidium iodide and analyzed by flow cytometry. Inset is a representative immunoblot of c-IAP2 upon transfection. Error bars = SD, n≥2.
Discussion

Previous studies have suggested that murine XIAP, in contrast to the human protein, may be dispensable for protection against apoptosis, since mice deficient in the protein did not display any immediately obvious defects in apoptosis (193). The studies described here demonstrate that murine XIAP is capable of inhibiting caspase activation to modulate apoptosis. As in human cells, Xiap-deficient mouse cells are more sensitive to apoptosis, likely due to an increased activation of the effector Caspase-3. This is supported by the data showing that mutation of the caspase-binding residues results in the same phenotype as complete lack of protein. This was observed for apoptosis induced by both the intrinsic (etoposide) and extrinsic (TNF) pathways.

While a lack of murine XIAP was found to result in sensitivity to apoptosis, c-IAP1 deficiency did not affect apoptosis. Interestingly, cell death was increased in the absence of c-IAP2, in parallel with a rise in Caspase-3 activation in these cells. This suggested that c-IAP2 may be functioning similarly to XIAP, though it is as yet unclear what the mechanism of the inhibition of apoptosis might be. One possibility relates to previous studies showing that a lack of c-IAPs can increase production of and sensitivity to TNF (150-152). This, however, does not account for the sensitivity to etoposide-induced death seen in c-IAP2-deficient MEFs. The similar sensitivity to both intrinsic and extrinsic death signals suggests that c-IAP2 may act at a point where the pathways converge, which could be at the level of Caspase-3 activation. However, it has been shown previously that c-IAP2 lacks the residues required to directly inhibit caspase activation (90). Alternatively, c-IAP2 might affect the function of XIAP in a transcription-independent manner through the IAP inhibitor Smac/DIABLO, for which c-
IAP2 is a ubiquitin ligase (211). Smac/DIABLO is released from the mitochondria upon apoptotic signaling, and binds to XIAP to prevent its association with caspases, allowing apoptosis to proceed (98, 99, 101, 205). It is possible that normally c-IAP2 acts as a homeostatic regulator of spontaneously released Smac/DIABLO, and in the absence of c-IAP2, Smac/DIABLO is better able to neutralize XIAP, abrogating its ability to modulate caspase activation.

In summary, we find that murine XIAP deficiency renders cells more sensitive to apoptosis within a range of concentrations of apoptotic stimuli, both receptor-mediated and mitochondrial, suggesting that it functions similarly to its human homolog. The strength of the apoptotic signal appears to be vital, since modulation of cell death by endogenous XIAP is quickly overwhelmed with greater concentrations of apoptotic stimuli. This may explain the discrepancy with previous studies, and suggests that with further exploration using more sensitive in vivo systems, the function of murine XIAP may be further elucidated. The studies presented here suggest that the Xiap-null mice will be an important tool with which to study human disorders related to cell death deregulation.
Chapter IV

Distinct Functional and Biochemical Properties of XIAP and SAP, Two Factors Targeted in X-linked Lymphoproliferative Syndrome (XLP)

Summary

Mutations in the X-linked inhibitor of apoptosis (XIAP) have recently been identified in patients with the rare genetic disease, X-linked lymphoproliferative syndrome (XLP), which was previously thought to be solely attributable to mutation in another gene, SAP. To further understand the roles of these two factors in the pathogenesis of XLP, we have compared mice deficient in Xiap and Sap. We show here that in contrast to Sap-deficient mice, animals lacking Xiap have apparently normal NKT cell development and no apparent defect in humoral responses to T cell-dependent antigens. However, Xiap-deficient cells were more susceptible to death upon infection with the murine γ-herpesvirus MHV-68 and gave rise to more infectious virus. These differences could be rescued by restoration of XIAP. These data provide insight into the differing roles of XIAP and SAP in the pathogenesis of XLP.
**Introduction**

X-linked lymphoproliferative syndrome (XLP) is a rare immunodeficiency, with the most common manifestations ranging from fatal infectious mononucleosis to B cell lymphomas and hypogammaglobulinemia [reviewed in (170)]. Primary disease often manifests upon Epstein-Barr virus infection, which patients are unable to control, and which frequently results in death. XLP affects approximately 1-3 in 1,000,000 males, though it may be under-diagnosed. Age of onset varies by primary feature of the disease, but 70% of patients die by the age of ten. Bone marrow transplant has emerged as a promising treatment (171).

XLP was first described in the Duncan family in the early 1970s (172) but it was not until the late 1990s that the gene responsible for the majority of diagnosed cases was identified (173-175). The defect was mapped to a single locus within the Xq25 band of the X chromosome, which encodes SLAM-associated protein (SAP/DSP1/SH2D1A), an adaptor molecule for the SLAM family of receptors, a subset of the CD2 subfamily of Ig receptors (179). The majority of patients have a variety of described mutations in the SAP coding region leading to loss of protein expression. A minority, who do not exhibit loss of protein mutations, have been explained primarily by misdiagnosis of XLP or the existence of mutations outside the coding sequence which affect SAP expression levels (181), although the genetic basis of others remained unclear.

Mice lacking *Sh2d1a*, the murine SAP homolog, have been generated and studied in depth (182, 183). *Sap*-null mice are defective in the development of natural killer T (NKT) cells, which echoes the phenotype of XLP patients (178)(184, 185). In addition, T cell help for humoral immunity is severely impaired, which is observed in the mice as an
inability to develop germinal centers (187, 188). Defects in the ability to produce Th2 cytokines in response to TCR stimulation are also observed (189). Natural killer (NK) cells stimulated via the SLAM family receptor 2B4 also have a reduced ability to kill target cells in the absence of SAP (190, 191). Possibly related to one or more of the above defects, Sap-null mice display aberrant immune responses to MHV-68 (192), a murine γ-herpesvirus similar to Epstein-Barr virus (EBV). These defects have been instrumental in describing the function of SAP as well as the functions of the SLAM family of receptors.

Rigaud et al. recently published a report describing three XLP families that expressed normal SAP and share the majority of their symptoms with patients having mutations in SAP (168). Interestingly, these patients had different mutations mapping to the same locus, but the mutated gene was found to be XIAP, which encodes X-linked inhibitor of apoptosis, and the described mutations result in loss of functional XIAP protein. At the immunologic level, this cohort of XLP patients exhibits a loss of NKT cells, similar to patients lacking SAP. Additionally, lymphocytes from these patients demonstrate enhanced apoptosis in response to extrinsic apoptotic stimuli (168). This study was the first to identify patients with XLP to have mutations in a gene other than SAP.

XIAP, a well-characterized member of the IAP family of proteins, has been described to potently inhibit apoptosis. XIAP directly binds and inhibits caspases, aspartate-specific cysteine proteases that carry out the process of cell death (84). Interestingly, the original report describing the murine knockout of XIAP indicated no obvious apoptotic defect (193), which raises the possibility that XIAP may have different
functions in mice. XIAP has additionally been shown to be involved in several signaling pathways, including TGF-β-mediated signaling and activation of NF-κB and JNK (161), as well as in copper trafficking (166). Through its carboxy-terminal RING domain, XIAP can also catalyze the ubiquitination of target proteins (128). Remarkably, though both demonstrate a wide array of functions, SAP and XIAP show no obvious structural or functional similarity beyond a common chromosomal locus and their involvement in XLP. Examination of the commonalities between Xiap- and Sap-deficient mice, as well as exploration of potential interactions between the two molecules, could provide valuable insight into both the function of XIAP and the pathogenesis of XLP.

In this study, we compare the functions and interactions of SAP and XIAP in the context of known phenotypes of Sap-deficient mice. We do not find evidence that XIAP and SAP interact, nor are they observed to act similarly in the pathways controlling NKT development and humoral immune responses. Instead, it was shown that Xiap-null cells are different from control cells in response to a murine γ-herpesvirus analogous to EBV, raising the possibility of an alternate mechanism by which patients lacking XIAP may demonstrate an XLP-like disease when infected with a γ-herpesvirus.
Materials and Methods

Immunoprecipitations:
All cells were cultured in DMEM (Mediatech) supplemented with 10% fetal bovine serum (FBS) (Mediatech), 2mM glutamine (Gibco) and 1% penicillin/streptomycin (Gibco) at 37°C, 5% CO₂. HEK293 cells were transfected using a standard calcium phosphate procedure with plasmids that have been previously described (179). Whole cell lysates were prepared using RIPA lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM DTT, 1mM PMSF in 1x PBS) or Triton X-100 lysis buffer (1% Triton X-100, 10% glycerol, 25mM Hepes, 100mM NaCl, 1mM EDTA, 1mM DTT, 1mM PMSF, 1mM NaF, and 1mM NaOV₄) supplemented with complete protease inhibitor tablets (Roche). For immunoprecipitations, lysates were incubated with glutathione sepharose beads or antibody to FLAG followed by protein A agarose beads. Centrifugation was performed to recover agarose beads, followed by washing in the appropriate lysis buffer. Precipitated proteins were eluted by adding LDS sample buffer (Invitrogen) and heating the samples for 5 min at 95°C. Recovered proteins were subsequently separated by electrophoresis, and immunoblot analysis was performed as described below.

Immunoblotting:
Samples were resolved on 4-12% gradient SDS-PAGE gels (Invitrogen), transferred onto nitrocellulose (Invitrogen) and blocked in 5% milk in Tris-buffered saline containing 0.1% Tween (Bio-Rad). Membranes were incubated at room temperature for 1h or overnight at 4°C with the following antibodies: GST (Santa Cruz), XIAP (BD
Transduction Labs), HA-HRP (Sigma), FLAG-HRP (Sigma), SAP (182), and β-actin (Sigma). Secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit (GE Healthcare) were used for 1h at room temperature. Enhanced chemiluminescence (GE Healthcare) and Kodak XAR film were used for visualization purposes.

*Mice:*
XIAP KO mice (193) were backcrossed in the C57BL/6 strain for at least 12 generations. All mice were housed under specific pathogen-free conditions within the animal care facility at the University of Michigan. The University of Michigan Committee on the Use and Care of Animals approved all experiments.

*NKT cell quantification:*
Single-cell suspensions were generated from XIAP wildtype and knockout thymi and spleens and immediately stained with the following antibodies purchased from BD Pharmingen: CD24-FITC (M1/69); NK1.1-PE-CY7 (PK136); TCR-beta-PE-Cy5 (H57-597). Thymocytes were also stained with PE-conjugated, αGC-loaded CD1d tetramer. Expression data was collected by flow cytometry on a Cytomics FC500 from Beckman Coulter and analyzed using FlowJo (TreeStar Inc).

*SRBC treatment:*
Mice were injected intraperitoneally with sheep red blood cells (SRBC; Colorado Serum Co.) diluted 1:10 in PBS, 200ul per mouse. Control mice were injected similarly with PBS alone. Six days later, spleens were harvested and split for two procedures. Frozen
sections were made and stained according to standard procedures with peanut agglutinin (PNA)-biotin (Vector Labs) and B220 (BD Pharmingen), with streptavidin-Alexa488(Invitrogen) and anti-rat-594 (Invitrogen), and imaged on an Olympus BX-51 microscope with an Olympus DP-70 high resolution digital camera. The remainder of the spleen was made into a single-cell suspension and stained with the following antibodies: B220-PE-Cy7, IgD-biotin, Fas-PE, GL7-FITC, and CD38-FITC (BD Pharmingen) and PNA-FITC (Biomeda; secondary: streptavidin-PE-alexa610[Invitrogen]). Data was collected by flow cytometry as above.

MHV-68 infection of MEFs:

XIAP WT and littermate KO MEFs were generated by standard procedures and reconstituted with either full length murine XIAP or a D148A/W310A double mutant generated by site-directed mutagenesis, by infection with lentivirus as described (manuscript submitted). MEFs were infected with MHV-68 (ATCC, WUMS strain) at 0.1 pfu/cell and washed once with PBS 24 hours later. 72 hours after infection, cells were visualized with a Nikon Eclipse TS100 microscope with A CoolSnap-Pro cf camera (Media Cybernetics). Floating cells were then collected and combined with adherent cells lifted with trypsin-EDTA (Mediatech), and all were resuspended in propidium iodide (PI) buffer (2µg/ml PI [Sigma], 1% bovine serum albumin [Sigma] in 1x PBS) for flow cytometry, performed as above. Supernatant was saved, filtered through 0.45 um PVDF (Millipore) and serially diluted 1:2 in media, starting with 1:1000. 3T12 cells were washed in the viral supernatant for 1 hour at 37°C, and carboxymethylcellulose (CMC, Sigma) mixture (CMC, culture media, 2x MEM [BioWhittaker], FCS,
penicillin/streptomycin, glutamine, Hepes, NEAA [HyClone], fungizone [Gibco]) was added for 1 week. Plaques were visualized by fixing and staining with 70% methanol plus 0.35% methylene blue (Fisher).
Results

No detectable interactions between XIAP and SAP

The discovery that human X-linked lymphoproliferative syndrome was a possible link between SAP and XIAP led us to examine the possibility that the two proteins interact. We have previously demonstrated the association of SAP with the cytoplasmic tails of several members of the CD2 family, including SLAM and 2B4 (179). Using this system, the cytoplasmic signaling domain of SLAM fused in-frame with glutathione-S-transferase (SLAM-GST) was expressed with FLAG-epitope-tagged SAP (SAP-FLAG) and XIAP. Upon precipitation with glutathione sepharose beads, SLAM was observed to interact with SAP, but not with XIAP (Figure 4.1A). In co-immunoprecipitation using FLAG antibody, SAP-FLAG was expressed with SLAM-GST and XIAP (data not shown). Here again, an association between SAP and SLAM was observed, but no XIAP was detectable in the complex. XIAP does not coprecipitate with either SLAM or SAP, and notably, it was also not observed to disrupt the association between these two proteins.

While interactions between SAP and SLAM are phosphorylation-independent, another CD2 family member, the 2B4 receptor, requires phosphorylation to associate with SAP (179). We examined the possibility of a phosphorylation-dependent interaction of XIAP with 2B4 by expression of a GST-2B4 chimera along with the tyrosine kinase Lck, SAP-FLAG, and XIAP. As demonstrated previously, 2B4 was capable of precipitating SAP in the presence of Lck, but XIAP was not detected (Figure 4.1B). Additionally, a point mutant of XIAP was introduced, H467A, which is incapable of ubiquitinating target proteins (128). Similar to the wildtype protein, this point mutant
also not found to coprecipitate with SAP and 2B4. Thus, we found no evidence of a
physical interaction between XIAP and SAP.

Figure 4.1: No detectable interaction between XIAP and SAP
A) XIAP and FLAG-tagged SAP were coexpressed with the cytoplasmic tail of SLAM-
GST or GST alone in HEK293 cells. Glutathione-sepharose beads were added to lysates
and bead-associated proteins were separated by SDS-PAGE and immunoblotted for
FLAG, GST and XIAP. Additionally, the last panel shows immunoprecipitation using
an anti-FLAG monoclonal antibody and IgA beads to assess binding of SLAM and
XIAP to SAP. B) HA-XIAP (both wildtype and a H467A point mutant) and FLAG-SAP
were expressed in HEK293 cells in the presence of the tyrosine kinase Lck and either a
GST-tagged cytoplasmic tail construct of the 2B4 receptor or GST alone. As in A, GST
coprecipitations and immunoblots were performed assessing the ability of wildtype
(WT) or D148A/W310A double mutant (MT) XIAP to bind SAP or 2B4. All samples
include Lck.
Similar expression of murine proteins

Although we found no evidence of a direct interaction between XIAP and SAP, the possibility remained that XIAP or SAP could regulate the other’s expression. To test this, we examined whether loss of XIAP or SAP changes expression of the other through mechanisms such as epigenetic silencing or posttranslational modifications including ubiquitination. To address this possibility, SAP expression was examined by immunoblot in thymocytes from several Xiap-null mice and littermate controls, as shown in Figure 4.2A. No gross difference in SAP protein levels was detected between cells from Xiap-deficient mice and control littermates. Similarly, lysates from thymocytes from Sap-null mice were separated by electrophoresis and immunoblotted with an antibody to XIAP (Figure 4.2B). XIAP levels were observed to be similar, regardless of the presence or absence of SAP. These findings suggest that XIAP and SAP do not affect each other either directly or epigenetically and that loss of XIAP does not contribute to XLP by altering SAP expression.

Figure 4.2: Murine expression of XIAP and SAP
Thymocytes were harvested from XIAP (A) and SAP (B) WT and KO mice, lysed and immunoblotted for SAP, XIAP and beta-actin. Asterisk (*) indicates a non-specific band.
**Murine NKT cells are not affected by loss of XIAP**

Since the findings described above suggest that XIAP does not interact with SAP or affect SAP protein, the mechanism by which XIAP is involved in the pathogenesis of XLP remained unclear. One potential explanation is that XIAP might be involved in

![Figure 4.3: NKT cells normal in XIAP KO mice](image)

Splenocytes and thymocytes were isolated from 3 XIAP WT and 3 XIAP KO mice and stained with anti-CD24, NK1.1+ and TCR-β+ (A and B), as well as PE-conjugated α-galactosylceramide-loaded CD1d tetramer, specific for NKT cells (C and D). NKT cells are defined as CD24\text{low}, NK1.1+, TCR-β+, and tetramer+. A and C show total results of at least 3 individual mice, error bars shown are standard error of the mean. B and D are representative FACS plots of the CD24\text{low} subpopulation.
similar molecular pathways as SAP, but at different levels, a possibility that could be
addressed by examining XIAP mice for phenotypes that SAP mice display. One
hallmark of SAP deficiency is impaired development of NKT cells, resulting in a severe
lack of NKT cells in SAP<sup>-/-</sup> mice (184, 185). NKT cells have diverse
immunomodulatory functions, and so their loss likely contributes directly to the
pathogenesis of XLP. Therefore, NKT cell populations were analyzed by flow cytometry
in Xiap-deficient mice and littermate controls, as shown in Figure 3. Surprisingly, both
cohorts contained similar populations of classical NKT cells, defined by expression of
NK1.1 and the T cell receptor (TCR) when gated on the CD24<sup>low</sup> population. In the
thymus, Xiap-deficient littermates contained 33±2% NK1.1+, TCRβ+ cells, and 30±5%
of cells were NK1.1+, TCRβ+ in controls. In the spleen, these cells comprised 1.9±0.3%
and 1.9±0.2% of the Xiap-null and control CD24<sub>low</sub> populations, respectively (Figure
4.3A and B). We verified these results using a CD1d tetramer that recognizes NKT cells,
which confirmed that control thymocytes (16±3%) were comprised of similar numbers of
NKT cells as Xiap-deficient mice (18±1%) (Figure 4.3C and D). NKT cells were
confirmed to function normally in XIAP-null mice by activation marker expression
(Bauler et al). The lack of a difference between NKT cell populations in mice with or
without XIAP indicates that XIAP is not involved in NKT cell development in mice.

*Normal humoral responses in the absence of XIAP*

Several reports have shown that SAP-null mice are defective in their ability to
form humoral responses to helper T cell-dependent antigens, which reflects the
dysgammaglobulinemia found in human XLP patients (187, 188). Since germinal center
Figure 4.4: Normal germinal center formation in XIAP KO mice

A) XIAP WT and KO mice were injected with either SRBC or saline and spleens were harvested 6 days later. Frozen sections were stained with PNA-FITC and anti-B220 and viewed on an Olympus microscope. B) Splenocytes were harvested from mice treated in A and stained with antibodies to B220, IgD and Fas. Subsets were also stained with PNA or antibodies to GL7 or CD38 to specifically identify germinal center B cells. Data represent at least 3 individual mice, and error bars shown are standard error of the mean.
formation is a primary indicator of an intact humoral response, the ability of Xiap-deficient animals to form germinal centers was tested. Xiap-null mice and controls were injected intraperitoneally with sheep red blood cells (SRBC), and splenic germinal center formation was analyzed six days later. Splenic sections were stained with antibody to B220 to detect B cells, and with peanut agglutinin (PNA), a lectin that binds germinal center lymphoid cells. While germinal centers (B220+, PNA+) were rare in saline-injected mice, they were clearly visible in both Xiap-null and -replete SRBC-injected mice (Figure 4.4A), indicating that mice lacking XIAP have normal responses to T cell-dependent antigens.

Additionally, germinal center formation was analyzed by flow cytometry. Whole splenocytes were gated for B220+, IgDlow cells and tested for Fas expression in conjunction with other germinal center markers (GL7+, PNA+ and CD38low). All three markers indicated no difference in germinal center formation between Xiap-null and littermate spleens (Figure 4.4B). These data indicate that, unlike SAP (187, 188), XIAP is not involved in signaling for CD4 T cell-mediated B cell help.

Different response to murine γ-herpesvirus-68 in Xiap-null mice

Symptoms of XLP are commonly first triggered or exacerbated by EBV infection, which often results in fulminant infectious mononucleosis (170). Since EBV cannot infect mouse cells, a related virus, murine γ-herpesvirus-68 (MHV-68), is routinely used as a mouse model for human EBV infection [reviewed in (212)]. To evaluate how XIAP affects responses to MHV-68, cells isolated from Xiap-null mice were infected with
Figure 4.5: Xiap-null cells are sensitive to virus-induced death.
A) The indicated MEF cell lines were infected with 0.1 pfu/cell MHV-68 and cultured for 72 hours, after which they were visualized with light microscope. B) Cells were treated as in A, then floating and adherent cells were harvested and PI stained for viability by flow cytometry. Error bars = SEM, n ≥ 3, and significance (<0.001 indicated with an asterisk [*]) was calculated using a one-way ANOVA. C) Supernatants from cells treated as in A were serially diluted and plated on 3T12 cells for a plaque assay. Three wells were counted from the 1:32,000 dilution of supernatant in each of two experiments.
MHV-68 in culture. Upon MHV-68 infection, murine embryonic fibroblasts (MEFs) deficient for XIAP appeared by contrast microscopy to be dying after 72 hours, while control cells were not (Figure 4.5A). To evaluate this quantitatively, we performed propidium iodide exclusion assays on infected cells after 72 hours of infection. As shown in Figure 4.5B, this method demonstrated that cells lacking XIAP (XIAP KO) were significantly more sensitive to death than their wildtype counterparts (XIAP WT), and reconstitution with wildtype XIAP (KO+XIAP) restored the wildtype, resistant, phenotype. Reintroduction of XIAP with both point mutations, D148A and W310A (KO+mutant) affecting the ability to bind caspases (213), was unable to render Xiap-null cells resistant to death. Thus, the sensitivity to death during viral infection was a specific result of the loss of XIAP and further, its caspase-binding activity, implying that the cell death was apoptotic.

Additionally, we examined how the loss of XIAP would affect the production of intact virus particles in embryonic fibroblasts, assessing viral titer in the supernatant by plaque assay. XIAP-null cells were able to produce more virus than wildtype counterparts, which was reversed by reintroduction of XIAP (Figure 4.5C). Viral titers were higher when the reintroduced XIAP was incapable of binding caspases. This suggests a caspase-dependence for the production or release of MHV-68. Taken together, these results suggest that enhanced viral replication in the Xiap-null cells is associated with increased apoptotic cell death.
Discussion

In these studies, we investigated the potential for XIAP and SAP to interact or participate in similar signaling pathways, due to their corresponding involvement in XLP. Our results indicate that XIAP and SAP do not directly interact or affect each other’s expression levels, which led us to pursue the contribution of XIAP to biological processes in which SAP is known to be involved. Unlike SAP, we have shown that in mice, XIAP does not play a role in NKT cell development or in generating T cell help for humoral immunity. Thus, it appears that XIAP is not necessary for the same physiological processes as SAP, even though it is implicated in the same disease. It is intriguing that the mice appear so different, given that studies of Sap-null mice have been extremely informative in understanding the pathogenesis of XLP in patients lacking SAP. The results presented here suggest that the pathogenesis of the disease in patients lacking XIAP is likely different from SAP-null patients, even if the outcome is the same.

The immune response to EBV has been an important aspect in the study of the pathogenesis of XLP, as a major hallmark of the disease is fulminant infectious mononucleosis. It has been proposed that the lack of NKT cells, whose numbers are greatly impaired in the absence of SAP, contributes to the phenotypes of XLP (184, 185). Xiap-null mice, however, have normal development of NKT cells, even though humans lacking XIAP have few NKT cells. This discrepancy suggests that there may be a difference between mice and humans with respect to the function of XIAP and illustrates a caveat to the direct application of conclusions from mouse models to human disease. Interestingly, recent unpublished data suggest that humans with type II (XIAP-null) XLP
may have relatively normal NKT cell numbers, which may explain the normal numbers of NKT cells in the *Xiap*-null mice.

Despite the potential difference in NKT cell development between mice and humans, cells from *Xiap*-null mice exhibit greater death than controls in response to MHV-68, which suggests that XIAP may yet play a role in the response to γ-herpesviruses such as EBV. We postulate that the defect in individuals lacking XIAP is cell-intrinsic, at the level of the infected cell itself, rather than the immune response to it, as seen in *SAP*-deficient patients. Cells lacking XIAP are more sensitive to death, presumably apoptotic, than cells with intact XIAP, and release more virus into the surrounding milieu. Intriguingly, Latour and colleagues also saw increased apoptosis in lymphocytes from patients lacking XLP (168). However, it was not clear how this would lead to an XLP-type phenotype which is associated with hyperproliferation of T lymphocytes (170). If XIAP affects the infected cells in a cell-autonomous manner, leading to increased cell death and increased viral release, it may provide a mechanism by which increased immune activation may occur. If the immune system is overwhelmed by this excess of viral particles, this would potentially lead to a similarly uncontrolled infection as in the absence of *SAP*. Thus, by a completely different mechanism as that involved in SAP deficiency, a similar result is seen, uncontrolled virus replication, and similar disease state.

In summary, XIAP may play a role in the pathogenesis of XLP, without directly influencing SAP or participating in common pathways. The function may be related to its primary role as a modulator of apoptosis, by slowing death of infected cells, or perhaps by inhibiting viral replication in an as yet undefined way. Usually, viruses are
thought to prevent apoptosis, as many, including γ-herpesviruses, encode antiapoptotic proteins. However, our results suggest that viruses may engage the apoptotic machinery of the host cell for lysis and release of viral particles. Cells that lack XIAP, then, are more susceptible to death, releasing more virus, and disseminating the infection more quickly. It also remains a formal possibility that XIAP limits viral replication and that in its absence, viral replication is enhanced, leading to increased lytic viral production. In this case, the enhanced viral replication may lead to apoptosis via increases in ER stress. Distinguishing between these possibilities will be the focus of future work. Regardless of the mechanism, these findings have profound implications for the study of viral infections in relation to apoptosis/replication and the pathogenesis of XLP.
Chapter V
Conclusions

Aims

Although XIAP has been proposed to participate in a variety of signaling pathways and cellular processes, it has been unclear how XIAP functions in physiological systems. While the most well-demonstrated function of XIAP is inhibition of apoptosis, the Xiap-null mouse has previously been described to not have any global defects in apoptotic induction (193). Additional studies have attributed to XIAP roles in promoting NF-κB and JNK-dependent transcription (158-161), as well as TGF-β signaling (161, 167) and copper homeostasis (163). As in apoptotic inhibition, no major defects in mice lacking XIAP have been observed in the context of any of these signaling pathways. When XLP patients were described who had causative loss-of-protein mutations in XIAP (168), the question arose as to whether XIAP might interact with SAP or, alternatively,
the XLP phenotype might arise from one or more of the previously described functions of XIAP. Therefore in this dissertation, I have attempted to elucidate the physiological functions of XIAP, primarily using the model system of mice genetically targeted for XIAP. Despite the variety of functions attributed to XIAP, I have found that likely the most important physiologically is its ability to inhibit apoptosis.

**Signaling by XIAP**

The contribution of XIAP to TGF-β signaling and NF-κB transcription was investigated. Under several different types of NF-κB-inducing stimuli, loss of XIAP made no difference to the transcription of classical NF-κB-dependent genes. XIAP also was not required under the experimental conditions examined as an adaptor molecule for TGF-β signaling, as transcription of traditionally TGF-β responsive genes was unaffected by loss of XIAP. It remains possible that XIAP participates in a subset of signaling events for each of these pathways, as this was not an exhaustive study of cell types or responsive genes. However, classically responsive systems were used and showed that XIAP was not absolutely required for signaling through TGF-β receptors or to NF-κB-dependent transcription. Previous studies were mostly performed using overexpression of XIAP and in some cases with luciferase reporter systems, in which it is easy to observe false positives. This may be especially true for a RING-containing protein like XIAP, since ubiquitination can be a very powerful tool for inducing large-scale changes in cell behavior. As an E3 ubiquitin ligase, XIAP likely has the ability to interact with many proteins in the cell when overexpressed that it does not when expressed at endogenous levels and in the appropriate compartment. When overexpressed, XIAP may be able to
ubiquitinate and thus profoundly affect cellular components in ways it normally would not.

A further limitation of luciferase systems with XIAP is also related to its function as an E3 ligase. XIAP is known to interact with mitochondrial proteins such as Smac and apoptosis inducing factor (AIF) (98, 99, 214). Overexpression of XIAP could have unexpected effects on these proteins, including ubiquitination, which could alter availability of ATP for luciferase function and elicit false positives in the luciferase assay. Interestingly, in most of the previously described luciferase reporter assays with XIAP, an XIAP mutant lacking RING function abrogated the transcriptional activity of the reporter (161). The RING domain was thus concluded to be involved in signaling, while the possibility remains that this was a side effect of altered cell processes. Our system, observing real-time gene transcription along with the knockdown or total loss of XIAP protein is likely to produce fewer artifacts, and thus the conclusions have fewer caveats. Therefore, it is possible to conclude based on these studies that XIAP has less of a role in TGF-β signaling and NF-κB-dependent transcription than was previously thought.

It should be noted, however, that these data do not address the role of XIAP in JNK-dependent transcription. Recent studies have shown a role for XIAP in the innate response to pathogens, primarily through activation of JNK. Like NF-κB, this signaling pathway also proceeds through activation of TAK1, with which XIAP has been associated. This pathway may be the link for XIAP to non-apoptotic signal transduction, as endogenous XIAP does appear to play a role. Additionally, this may explain how overexpression studies with XIAP appear to activate NF-κB transcription and TGF-β
signaling, as all of these pathways can activate TAK1, and excessive expression of XIAP may induce TAK1 to signal inappropriately. Further studies of the mechanism of JNK activation by XIAP will elucidate the function of this protein in innate immunity as well as generally defining its non-apoptotic roles.

Apoptosis Inhibition in the IAP Family

The best-described function for XIAP is as an inhibitor of apoptosis, while the inhibitory role of other members of the IAP family, such as c-IAP1 and c-IAP2, remains controversial. Since mice genetically targeted for XIAP have not been found to display significant defects in apoptosis (193), while c-IAP2 knockout mice are suggested to have apoptotic defects in certain cell types (215), we investigated apoptotic responses in cells derived from IAP-knockout mice. Interestingly, we found that cells lacking either XIAP or c-IAP2 exhibited greater sensitivity to apoptotic stimuli than their wildtype counterparts. As expected, in the case of XIAP, this was dependent on the ability to bind caspases (Figure 5.1). This represented the first time that murine XIAP was found to be protective from apoptotic stimuli induced through either the receptor-mediated or mitochondrial pathway. Thus, the mouse had previously not been considered a good model for XIAP-related studies, but these studies suggest that it presents an excellent model. The mouse protein appears to function in the same way as the human, because these studies show a modulatory role for XIAP in apoptosis, rather than as an absolute inhibitor of apoptotic signaling. This suggests that XIAP is a very important switch in the life-death balance in a cell – a small shift in the pro-survival-pro-apoptotic axis can be sensed by XIAP and alter the outcome of signaling. These results further suggest that the
XIAP mouse model may be very useful in future studies to continue to elucidate XIAP function. As XIAP has been shown to be overexpressed in some neoplasms (104-106), it has been an attractive target for chemotherapeutics (107, 109, 110, 216), and the consequences of the loss of XIAP should be understood for these therapies to go forward safely. Additionally, the recent studies showing a loss of XIAP in XLP (168) would be greatly enhanced by using a mouse model in which the murine XIAP behaves as its human homolog does.

Figure 5.1: IAPs in apoptotic inhibition.
XIAP prevents apoptosis by direct inhibition of caspases. c-IAP2 may block apoptosis by either directly inhibiting caspases or by sequestering Smac from XIAP.
The additional results indicating that c-IAP2 can also play a protective role in murine cells were intriguing, as the anti-apoptotic role of c-IAP2 remains controversial. Conte et al demonstrated increased macrophage death in the absence of c-IAP2 during highly proinflammatory conditions (215), yet the fact remains that c-IAP2 lacks the critical structure required by XIAP for caspase inhibition (90). As shown in Figure 5.1, possible explanations for c-IAP2 involvement in apoptotic inhibition include a completely different mechanism of cell death prevention (1) or a slightly different mechanism of c-IAP2 inhibition of effector caspases (2). Either way, it is evident from our studies that c-IAP2 prevents apoptosis at the point where the mitochondrial and receptor pathways converge, at Caspase-3 activation.

Perhaps the most likely mechanism for the inhibition of apoptosis by c-IAP2 is through sequestration of Smac, since Smac binds in the groove of the BIR that is intact in c-IAP2 (101), which in XIAP allows binding of caspases. In this model, the absence of c-IAP2 would cause greater Smac binding to XIAP, and prevention of its inhibitory activity. One caveat to this model is the presence of the same Smac-binding groove in c-IAP1, by which it would be expected that c-IAP1 would have the same activity as c-IAP2. However, our results clearly demonstrate that c-IAP1 does not have any protective effect against apoptotic stimuli, even when c-IAP2 expression is suppressed by RNA interference. In addition, data not shown here suggest that the loss of Smac does not render cells more resistant to apoptosis, as might be predicted by this model. Future experiments could further evaluate this by examining cells deficient in both c-IAP2 and
Smac. Here, if c-IAP2 works through sequestration of Smac, the sensitization resulting from loss of c-IAP2 would be abrogated in the absence of Smac.

It is possible that c-IAP2 directly inhibits effector caspases, though in a slightly different way from XIAP. It appears that caspases can still bind via the highly conserved groove region within the BIR2 of c-IAP2 (Figure 5.2), but the amino-terminal region that in XIAP forms the fold that blocks caspase activation is altered in c-IAP2. In fact, c-IAP2 also differs from c-IAP1 significantly in this region, and the studies describing the binding but lack of caspase inhibition by the c-IAPs actually focus more on c-IAP1 than c-IAP2 (90). To evaluate the veracity of this model, the structure of c-IAP2 binding to Caspase-3 bears further examination for its potential caspase-inhibitory function.

**Figure 5.2 Alignment of BIR2 in XIAP and c-IAPs.** Stars indicate key residues in XIAP that inhibit Caspase-3 activation. Line indicates IBM-binding groove.

**XIAP in X-linked Lymphoproliferative Disorder**

Despite the multifunctionality of XIAP, until recently, no pathogenesis had been described to result from a change in XIAP expression or function. Some work had suggested a correlation between IAP overexpression and cancer prognosis (217-219), but no causal role has been demonstrated. When it was discovered that the causative mutation in X-linked lymphoproliferative disorder was localized to Xq25, the locus containing XIAP, it was thought that a physiological function for XIAP would be
elucidated related to the disease. However, shortly thereafter, SAP was found to be the primary target in XLP patients, and energies were focused elsewhere. A minority of patients, mostly those who have no family history of the disorder, have never been found to have mutations in SAP, though it has long been speculated that these would have mutations outside the coding region that affect protein expression or function.

Interestingly, at least some of these patients appear to have loss-of-protein mutations in XIAP that are responsible for the disease (168), which is now being referred to as type II XLP. These patients share the majority of symptoms with type I patients, though given the small size of the type II cohort, the possibility remains that these could be separate diseases. The mouse model for loss of SAP expression has proven to be very informative regarding the mechanisms of disease pathogenesis, therefore we examined the Xiap-null mice for phenotypes that the Sap-null mice have been shown to exhibit.

As described in Chapter 4, we found that Xiap-null mice do not share any of the same phenotypes as Sap-null mice, which should perhaps not be surprising, since XIAP and SAP exhibit no similarities beyond chromosomal locus and association with XLP. We found no evidence of interaction between XIAP and SAP at the protein level or the transcriptional level. In addition, loss of XIAP does not appear to result in impairment of NKT cell development or of humoral responses to T cell-dependent antigens. The most surprising result of these studies is that loss of XIAP causes a different defect in response to γ-herpesvirus infection than does loss of SAP. Xiap-deficient cells are more sensitive to apoptosis upon infection and release more active viral particles than their wildtype counterparts. Given that this is the only defect described thus far that can possibly
account for the phenotype in the XIAP subset of XLP patients, it is intriguing that the mechanism of disease appears so different from that in patients lacking SAP.

It remains possible, however, that other defects caused by a loss of XIAP will prove to play a role in the pathogenesis of type II XLP. XIAP has been shown to play a role in innate immunity through the activation of JNK signaling and cytokine expression by innate receptors. Some of these innate receptors can recognize viruses, so XIAP may also play a role in the innate response to EBV, the loss of which could cause the XLP phenotype. Examination of cytokine responses to γ-herpesviruses in the presence or absence of XIAP would address the role of the innate immune system and XIAP in XLP. Also, both XIAP and SAP have been implicated in NF-κB signaling; SAP specifically in response to a Th2-polarizing T cell receptor signal, and XIAP much more generally. While the data presented here suggest that XIAP does not universally participate in NF-κB signaling, XIAP may play a role in very specific NF-κB signaling pathways similar to those SAP works in. Much has also been made of the NKT cell defect in the absence of SAP and its involvement in the XLP disease, along with the apparent curiosity that NKT cells are absent in type II patients but not in Xiap-null mice. No connection has been made, however, between the loss of NKT cells and disease progression. In fact, genes besides SAP have been shown to exert similar control over NKT cell development, and do not seem to cause XLP-like phenotypes. NKT cell number appears to vary greatly between individuals, and some XLP type II patients have normal NKT cell numbers, similar to Xiap-null mice. These possibilities certainly bear further scrutiny, however, the results presented here, show that the strongest possibility for a role for XIAP in XLP remains in its anti-apoptotic activity.
The contribution of apoptosis to viral pathogenesis is an area of study that remains controversial. Much study has been conducted concerning the mechanisms by which viruses block apoptosis in the host cell, thus allowing the virus time to replicate. Many viruses, including herpesviruses, are known to encode anti-apoptotic genes, some even closely related to cellular anti-apoptotic genes, such as Bcl-2. It should even be noted that the first member of the IAP family was identified in baculoviral genomes. However, a few studies have been put forth suggesting that some viruses can employ the apoptotic machinery for egress (reviewed in (220)). A key component of this theory is that apoptosis is immunologically silent, meaning that if virions are released during cellular apoptosis, an immune response will not be generated to the dying cell and its components (e.g. viral progeny). This allows the virus to disseminate relatively unchallenged and infect further host cells.

The observation that loss of XIAP can sensitize cells to death in the presence of viral infection with γ-herpesvirus, which was recapitulated by mutation of the caspase-binding sites within XIAP, suggests that the virus is initiating caspase-dependent cell death in its host cells. This apoptosis is below the threshold normally blocked by XIAP, and is thus not observed until XIAP is removed from the system. The subsequent observation that Xiap-null apoptotic cells are releasing more virulent viral progeny than the non-apoptotic cells with intact XIAP further suggests that the virus is using apoptosis to disseminate its progeny. These data do not address whether XIAP somehow also controls viral replication, or simply controls escape from the host cell. This could be addressed by evaluating the total amount of virus present in Xiap-null or wildtype cells and the viral supernatant at the point at which Xiap-null cells are undergoing apoptosis.
Though γ-herpesviruses such as MHV-68 and EBV do contain anti-apoptotic genes within their genomes, these data suggest that throughout the life cycle of these viruses, their relationship with apoptosis is variable. In the early stages of viral infection, the virus must counteract the cell’s attempts to eliminate it by apoptosis. Once the virus has reproduced sufficiently, however, it can release the brakes on apoptosis and allow the cell to commit suicide, by which the viral progeny is released without stimulation of an immune response. It is likely that if this is the case for any virus, it would be true for γ-herpesviruses, which have relatively large DNA genomes and complex life cycles involving long-lived latency. XIAP, then, with its position at the crossroads in the decision between life and death, could play an important role in controlling viral dissemination and resulting disease.

The model resulting from these data for how loss of XIAP participates in the pathogenesis of XLP, to produce the same disease as SAP deficiency, is depicted in Figure 5.3. EBV infection in an XIAP-deficient individual is affected at the cellular level, in that XIAP-deficient cells undergo apoptosis at a lower threshold, releasing virions in an immunologically silent manner, allowing the virus to quickly get out of control and cause fulminant infectious mononucleosis. Though this is a completely different mechanism from that in the absence of SAP, in which a rapid, overwhelming, but ineffective immune response results from infection with EBV, they ultimately result in the same clinical outcome, fulminant infectious mononucleosis. Considerable work still needs to be performed with both mice and humans to fully understand this disease and the role of XIAP in it, but these data begin to present an explanation for both the pathogenesis of XLP and the greater function of XIAP.
Figure 5.3: Model of how loss of XIAP or SAP could result in the same phenotype of XLP.
References


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