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FACULTY OF GRADUATE AND  
POSTDOCTORAL STUDIES

Damiano CONTE

AUTEUR DE LA THÈSE - AUTHOR OF THESIS

Ph.D (Microbiology and Immunology)

GRADE - DEGREE

Department of Biochemistry, Microbiology and Immunology

FACULTÉ, ÉCOLE, DÉPARTEMENT - FACULTY, SCHOOL, DEPARTMENT

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Immune Response

R. Korneluk

DIRECTEUR DE LA THÈSE - THESIS SUPERVISOR

CO-DIRECTEUR DE LA THÈSE - THESIS CO-SUPERVISOR

EXAMINATEURS DE LA THÈSE - THESIS EXAMINERS

D. Bulman

K. Dimock

S. Sad

P. Santamaria

J.-M. De Koninck, Ph.D.

LE DOYEN DE LA FACULTÉ DES ÉTUDES  
SUPÉRIEURES ET POSTDOCTORALES

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The Role of the X-linked Inhibitor of Apoptosis (XIAP) and the Cellular Inhibitor  
of Apoptosis 2 (cIAP2) in T cell Development and in an Innate Immune  
Response

A Thesis Submitted to the School of Graduate Studies

University of Ottawa

In Partial Fulfillment of the Requirement for the Degree of

Doctor of Philosophy

Department of Biochemistry, Microbiology and Immunology

Faculty of Medicine

By

Damiano Conte

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## ***Abstract***

The fundamental physiological process of apoptosis plays a crucial role in maintaining the homeostasis of the mammalian immune system. The major sites of apoptotic influence occur throughout lymphopoiesis, during the deletion of unwanted carcinogenic or virally infected cells and at the waning of an antigen-induced immune response.

The cellular inhibitor of apoptosis 2 (cIAP2) is a potent inhibitor of apoptotic death. In contrast to the other members of the inhibitor of apoptosis (IAP) family cIAP2 is NF- $\kappa$ B-inducible via pro-inflammatory cytokines, such as IL-1 $\beta$  and TNF $\alpha$ , and via bacterial components, such as lipopolysaccharide (LPS). The following studies demonstrate that *cIAP2*<sup>-/-</sup> mice exhibit significant resistance to LPS-induced endotoxic shock, specifically via an attenuated inflammatory response. We show that due to a lack of cIAP2, *cIAP2*<sup>-/-</sup> macrophage cells have a heightened susceptibility to apoptosis in a LPS-induced pro-inflammatory environment and hence are unable to maintain a normal inflammatory response. These results raise the intriguing possibility that pharmacological agents targeting and inhibiting cIAP2 expression may be sufficient to confer protection to the lethal onset of sepsis.

The X-linked inhibitor of apoptosis (XIAP) and other members of the inhibitor of apoptosis (IAP) family can suppress apoptosis induced by a diverse variety of triggers. Functional studies done to date have focused upon tissue culture models and adenovirus over-expression of XIAP and other IAP proteins. These studies report the phenotype of the first engineered transgenic mouse over-

expressing a human IAP, as well as assessing the long-term consequence of IAP over-expression. The relative protein expression levels of the endogenous murine homologue to XIAP within thymocyte and T cell sub-populations are also documented. The consequence of lymphoid-targeted over-expression of XIAP in transgenic mice suggests a physiological role for the endogenous murine XIAP. *Xiap*-transgenic mice accumulated thymocytes and/or T cells in primary and secondary lymphoid tissue, T cell maturation was perturbed, and transgenic thymocytes resisted a variety of apoptotic triggers both *in vitro* and *in vivo*. These observations imply a possible key function for the intrinsic cellular inhibitor XIAP in maintaining the homeostasis of the immune system.

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## **DEDICATION**

To Josie, for her love, support and especially for her healing powers

To Arianna, my little angel, baby girl, who has filled my heart with love

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## ***List of Abbreviations***

a.a. -	Amino Acid
APC	Antigen Presenting Cells
Apaf -	Apoptosis Protease Activating Factor
AIDS	Autoimmune Deficiency Syndrome
Bcl -	B cell lymphoma
BIR -	Baculoviral IAP Repeat
bp -	Base Pair
BIRP -	BIR Containing Protein
BMP -	Bone Morphogenic Protein
°C -	Degree Celsius
Caspase	Cysteine Aspartase
CARD -	Caspase Recruitment Domain
cIAP -	Cellular Inhibitor of Apoptosis
cDNA -	Complementary DNA
JNK -	c-Jun N-terminal Kinase
CpGV -	<i>Cydai pomonella</i> Granulovirus
CrmA -	Cytokine Response Modifier A
CTL -	Cytotoxic T Lymphocyte
dATP -	Deoxyadenosine Triphosphate
dCTP -	Deoxycytidine Triphosphate

DD - Death Domain

DED - Death Effector Domain

DISC - Death Inducing Signaling Complex

DNA - Deoxyribonucleic Acid

DTT - Dithiothreitol

DIAP1 - *Drosophila* IAP

EDTA - Ethylenediaminetetraacetic Acid

FADD - Fas Associated Death Domain

FLIP - FLICE-like inhibitory protein

FITC - Fluorescein IsoThioCyanate

FACS - Fluorescence Activated Cell Sorting

GST - Glutathione S Transferase

h - Hour

HIAP - Human Inhibitor of Apoptosis

Ipafl - ICE-Protease-Activating Factor

IAP - Inhibitor of Apoptosis

ICE - Interleukin-1 $\beta$  Converting Enzyme

kb - Kilobase pairs

LB - Luria-Bertani Medium

MALT - Mucosa Associated Lymphoid Tissue

MHC - Major Histocompatibility Complex

min - Minute

MAPKKK - Mitogen-Activated Protein Kinase Kinase Kinase

MIAP - Mouse Inhibitor of Apoptosis

μg	-	Microgram
μL	-	Microlitre
μM	-	Micromolar
ml	-	Millilitre
mM	-	Millimolar
mRNA	-	Messenger RNA
NK		Natural Killer cells
NAIP	-	Neuronal Apoptosis Inhibitory Protein
NF-κB	-	Nuclear Factor κB
NOD	-	Nucleotide Binding Oligomerization Domain
ORF	-	Open Reading Frame
OpNPV	-	<i>Orgyia pseudotsugata</i> nucleopolyhedrovirus
PBL	-	Peripheral Blood Leukocytes
PE		PhycoErythrin
PCD	-	Programmed Cell Death
PCR	-	Polymerase Chain Reaction
PMSF	-	Phenylmethylsulfonyl Fluoride
RING	-	Really Interesting New Gene
RAIDD	-	RIP Associated Protein with a DD
RIP	-	Receptor Interacting Protein Kinase
RPM	-	Revolutions Per Minute
RT-PCR	-	Reverse Transcription PCR
SMA	-	Spinal Muscular Atrophy
Smac	-	Second Mitochondrial-Derived Activator of Caspases

SMN - Spinal Motor Neuron  
SDS - Sodium Dodecyl Sulfate  
SSC - Standard Saline Citrate  
TCR - T cell Receptor  
TAB1 - TGF- $\beta$  Activated Kinase-1 Binding Protein-1  
TAK1 - TGF- $\beta$  Activated Kinase-1  
Ts-IAP - Testis-Specific IAP  
TRAF - TNF Receptor Associated Factors  
TNF - Tumour Necrosis Factor  
TRADD - TNF Associated Death Domain  
E1 - Ubiquitin-Activating Enzyme  
E2 - Ubiquitin-Conjugating Enzyme  
E3 - Ubiquitin Ligase Enzyme  
UTR - Untranslated Region  
UV - Ultraviolet  
XAF - XIAP Associated Factor  
XIAP - X-linked IAP

# **Chapter 1. Introduction**

This thesis documents the role of the X-linked inhibitor of apoptosis (XIAP) and the cellular inhibitor of apoptosis 2 (cIAP2) in the developmental phase of T cells, known as thymocytes, and during an innate immune response. Given that apoptosis is a key regulator of immune function this introduction will describe the general process of apoptosis as it applies to the family of IAP proteins, on their functions and inhibitors.

## ***Programmed cell death***

### **Historical background**

All cells originating from insects to mammals have the intrinsic capacity to self-terminate, in a process known as apoptosis. This self-destructive process is active in a wide range of tissues, and is an essential feature in both development and day-to-day maintenance of multicellular animals. Apoptosis is an integral component of embryogenesis, differentiation, neural development, tumor regression and in regulating the constituent and targeted cells of the immune system. Moreover, aberrant control of cell death, with either too much or not enough apoptosis, is associated with a number of disorders such as cancer, AIDS, Alzheimer's disease, multiple sclerosis and rheumatoid arthritis (Thompson 1995).

Over 50 years ago, researchers who studied developmental embryology were the first to recognize that controlled cell death is as much a part of embryogenesis as is cell proliferation and differentiation (Saunders 1966). The term programmed cell death (PCD) was first proposed in 1965, by Lockshin and Williams (Lockshin and Williams 1965), to describe cells that seemed to be destined to die. Two widely recognized examples of this type of apoptosis, better known as PCD, is the slow dissolve of the tadpole tail during the development of the frog or the formation of digits whereby apoptosis eliminates the interdigital tissue (Ganan, Macias et al. 1996). In the mammalian system, apoptosis purges countless neurons and lymphoid cells during the process of building and shaping the nervous and immune system, respectively. A consequence of this early work clearly demonstrated a morphological delineation between apoptotic death and accidental cell death, known as necrosis.

Necrotic cell death occurs as an outcome of a severe injury, caused by physical damage, resulting in the loss of osmotic balance, and hence, cell swelling (Kerr 1971). In addition, another hallmark of necrotic death is the induction of a host inflammatory response. Normally, an inflammatory response is beneficial in controlling invading pathogens and clearing debris, however, considerable damage may be inflicted to neighbouring tissue. In contrast, an apoptotic cell shows no signs of swelling and will actually shrink and pull away from adjacent, non-apoptotic cells. In addition, accompanying the cell volume reduction is blebbing of the cell membrane, chromatin condensation, DNA fragmentation, and the formation of apoptotic bodies.

These particulates are then eventually ingested by scavenging cells, such as macrophages, or neighboring cells to rid the body of these dying cells, thus avoiding an inflammatory response (Saraste and Pulkki 2000).

Interestingly, not all apoptotic cells are deemed worthless waste awaiting prompt and efficient removal. Indeed, certain cell-types are, by design, engineered to persist for long periods of time. Two prominent examples are the cells that comprise the eye and the skin. The corpses of both cell-types replace cytoplasmic proteins with either crystallin, in the eye, or keratin in the skin. Keratinocytes are generated in the skin's deep layer and expire during their migration to the surface, where, along with the generation of the protein keratin, develop a water-repellent surface to form the outer, protective skin (Weisfelner and Gottlieb 2003).

The visible features of PCD and its importance to the process of embryogenesis were well documented by the 1950s; however, its role in maintaining the daily homeostasis of a mature organism was not yet established. In a landmark publication, by Kerr et al., (Kerr, Wyllie et al. 1972) the term apoptosis (classical Greek meaning "dropping off") was first coined to describe what was contended to be a similar type of cell death to what was observed during embryogenesis, but featured in a mature organism. The authors asserted that apoptosis was complementary to, but the opposite of, the cell proliferation process known as mitosis. They also stated that the disrupted regulation of cell suicide might contribute to many diseases and pathologies, such as cancer. Despite the fact that this paper has become one the most widely cited documents within the field of apoptosis, a concerted

effort into the investigation of the apoptotic process was started only after work on the genetic study of the nematode *Caenorhabditis elegans* demonstrated that there existed a dedicated and conserved pathway for PCD (Hengartner and Horvitz 1994; Hengartner and Horvitz 1994; Horvitz, Shaham et al. 1994).

### ***General features of apoptosis***

Today we know that the course of apoptosis is a morphologically and biochemically distinct process of cell death in which cells die and are cleared in a controlled manner, in response to specific stimuli, without affecting neighboring cells or eliciting an inflammatory response. PCD plays a role in four crucial areas: a) sculpting of body parts; b) elimination of unwanted structures; c) controlling cell numbers; and d) elimination of non-functional or harmful cells (Jacobson, Weil et al. 1997). Some defining characteristics include cell shrinkage, translocation of phosphatidylserine from the inner to the outer surface of the cell membrane, condensation of nuclear chromatin, aggregation of cytoplasmic organelles, cytoplasmic blebbing, depolarization of the mitochondrial membrane potential, the release of cytochrome c and finally, fragmentation of membrane-enclosed small apoptotic bodies sequestered by macrophages without eliciting an inflammatory response (Schwartzman and Cidlowski 1993; Hale, Smith et al. 1996; Saraste and Pulkki 2000). In addition, regardless of the origin or type of apoptotic stimulus all apoptotic routes converge at a conserved family of proteases known as cysteiny aspartate proteinases (caspases) (Fadeel, Orrenius et al. 2000).

Moreover, several mammalian apoptotic genes are evolutionarily conserved, similar to those found in *C. elegans* (Lamkanfi, Declercq et al. 2002). Accordingly, this organism has become a simple and invaluable genetic model system for the study of PCD during development (Fraser 1999; Horvitz 1999; Liu and Hengartner 1999). Despite the similarities, the mammalian apoptotic pathway is more complex, as multiple mammalian genes seemed to have been derived from similar genes found in single *C. elegans* apoptotic predecessors (Lamkanfi, Declercq et al. 2002). This permits specific groups of cells to undergo apoptosis at appropriate times. From nematodes to vertebrates, the ability of cells to undergo programmed cell death appears to be an inherent, normal, physiological process.

## ***Caspases as mediators of apoptosis***

### **The history of caspases**

In the late 1970's studies of the *C. elegans* cell lineage, by Horvitz and Sulston (Sulston and Horvitz 1977; Horvitz, Brenner et al. 1979; Greenwald and Horvitz 1980; Horvitz and Sulston 1980), led to the discovery that 131 of 1090 somatic cells were pre-destined to die during development. The subsequent genetic study uncovered three central components of PCD in *C. elegans*: *ced-3*, *ced-4* and *ced-9* (Hengartner, Ellis et al. 1992; Yuan and Horvitz 1992; Yuan, Shaham et al. 1993). The *ced-9* gene product was shown to encode an inhibitor of apoptosis, the *ced-4* protein was a pro-apoptotic adaptor molecule and the *ced-3* protein proved to be a cysteine protease central to the implementation of the apoptotic process. More recent findings

have demonstrated that the focal mediators of the apoptotic pathway are a large family of cysteine proteases, known as caspases (Cryns and Yuan 1998). The story of caspases began with the finding that the *C. elegans ced-3* gene (Yuan, Shaham et al. 1993) was shown to encode a human homologue, namely the interleukin-1 $\beta$  processing enzyme (ICE) (Cerretti, Kozlosky et al. 1992; Thornberry, Bull et al. 1992), which was later renamed as caspase-1. Over-expression of caspase-1 or of the *ced-3* gene product caused Rat-1 cells to undergo apoptosis (Miura, Zhu et al. 1993). This suggested that caspases might play a similar role in mammalian apoptosis to that of *ced-3*. Subsequently, it was shown that the cell death caused by caspases-1 could be suppressed by over-expression the *cytokine response modifier A* (*crmA*) gene, a specific inhibitor of caspase-1, to protect neurons from the apoptotic trigger of trophic factor withdrawal (Gagliardini, Fernandez et al. 1994). As well, caspase-3 was proven to be an essential factor necessary for cytotoxic T lymphocyte (CTL)-mediated PCD of immune targeted cells (Darmon, Nicholson et al. 1995), thereby indicating an endogenous functional role for caspases in apoptotic cell death. Finally, the identification of caspases and their homologues in *C. elegans* and in all vertebrates demonstrated a basic commonality of the apoptosis program (Lamkanfi, Declercq et al. 2002).

### **Classification of caspases**

Presently, there have been 11 human, 10 murine, 4 avian, 4 fish, 8 amphibian, 7 insect and 3 nematode caspases identified (Alnemri, Livingston et al. 1996; Lamkanfi, Declercq et al. 2002). The human set of proteases

includes: caspases-1-10 and caspase-14. All caspases have several discrete features in common. One characteristic is the three conserved catalytic amino acid residues that are responsible for the specific substrate cleavage C-terminally of an aspartic acid residue (Rano, Timkey et al. 1997). Second, all caspases are initially synthesized as inactive zymogens, procaspases, that contain a variable sized prodomain followed by the large (p20,  $\alpha$  chain) and small (p17,  $\beta$  chain) subunits (Ramage, Cheneval et al. 1995). Third, as a first step in the activation of all procaspases they are proteolytically cleaved, C-terminally, of a highly conserved aspartic acid residue (Yamin, Ayala et al. 1996; Stennicke and Salvesen 1997).

Apoptosis is a highly complex and regulated process involved in an intricate cascade of events. Discrete groups of caspases function at different points along the PCD pathway, linking distinct upstream signaling events with the downstream execution steps (Miller, Myerson et al. 1997). One method used to classify caspases is based on the site of function along this apoptotic pathway (Nicholson 1999). Caspases that are utilized upstream are designated as initiator caspases. These caspases have a long prodomain that contains either the death effector domain (DED) (Boldin, Mett et al. 1995; Chinnaiyan, O'Rourke et al. 1995) (caspase-8 and -10) or the caspases activation and recruitment domain (CARD) (Qin, Srinivasula et al. 1999; Vaughn, Rodriguez et al. 1999) (caspase-1, -2, -4, -5, -9, -11 and -12). These DED and CARD domains are the conduits that allow for caspase interaction with upstream adaptor molecules. In addition, the initiator caspases may be further subdivided to a group that is involved with cytokine activation and inflammation

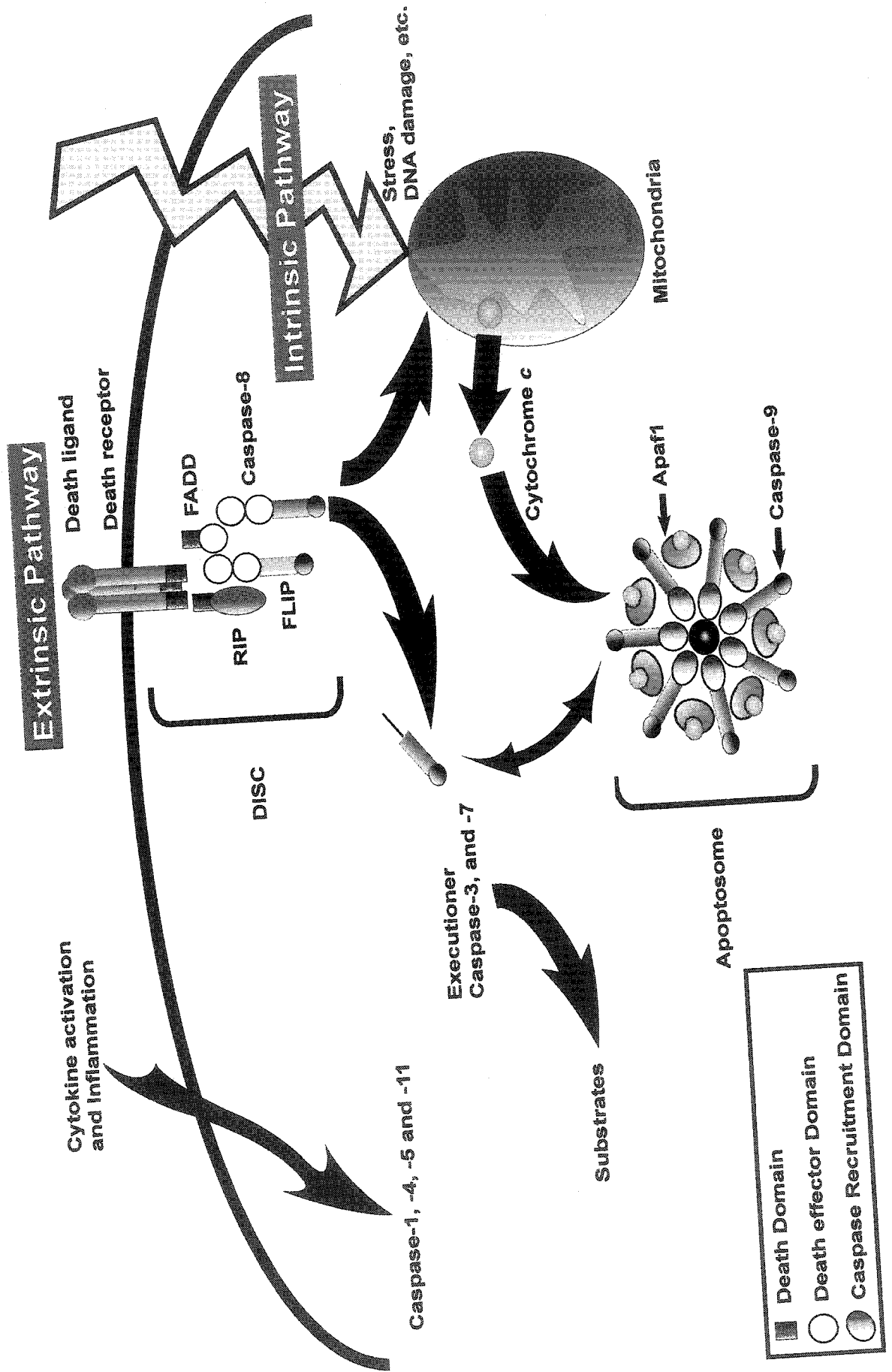
(caspase-1, -4, -5, -11 and -14). The downstream acting caspases are termed the executioner caspases (caspase-3, -6 and -7) (Zimmermann, Bonzon et al. 2001). These caspases contain a short prodomain, relative to that of the initiator caspases, and target multiple cellular substrates for cleavage (Fischer, Janicke et al. 2003).

## **The extrinsic and intrinsic apoptotic pathways**

There are two major apoptotic signaling pathways that incorporate the processing of caspase zymogens (Figure 1-1) (Salvesen and Duckett 2002): a) the extrinsic pathway relies on cell surface ligand-receptor-mediated stimulus; and b) the intrinsic pathway occurs due to intracellular stress-mediated stimulus. The extrinsic pathway signals through the tumor necrosis factor (TNF) super family of death receptors. The death receptors are characterized by the presence of the protein-protein interaction death domain (DD) (Brakebusch, Nophar et al. 1992). Ligand-receptor binding causes receptor multimerization that leads to the formation of the death inducing signaling complex (DISC), which consists of multiple adaptor proteins. The death receptors transmit the apoptotic signal to the interior of the cell through the assembly of this DISC. This formation of the DISC complex includes the following proteins: a) Fas associated DD (FADD) (Chinnaiyan, O'Rourke et al. 1995), b) INF receptor associated DD (TRADD) (Hsu, Xiong et al. 1995), c) receptor interacting protein kinase (RIP) (Stanger, Leder et al. 1995), d) RIP associated protein with a DD (RAIDD) and finally the e) FLICE-like inhibitory protein (FLIP) (Thome, Schneider et al. 1997). The establishment of the DISC

### **Figure 1-1. The intrinsic and extrinsic apoptotic pathways**

This is a schematic representation of the known apoptotic pathways involving the main caspases, as well as the caspases involved with proteolytic activation of inflammatory processes. The extrinsic pathway is initiated via cognate ligand-binding and subsequent trimerization of cell surface death receptors. Consequently, adaptor proteins are recruited forming the DISC complex, which allows for the proximity auto-activation of the initiator caspase-8. Mature caspase-8 proceeds to cleave and activate the executioner caspases-3 and -7 leading to the limited proteolysis of key cellular components. Irreversible DNA damage initiates the intrinsic pathway. This is followed by the release of mitochondrial cytochrome *c* eliciting the formation of the apoptosome, which consists of Apaf1, cytochrome *c* and procaspase-9. The apoptosome allows for the proteolytic activation of the initiator caspase-9 and subsequently it proceeds to cleave and activate the executioner caspases- 3 and -7. In addition, the caspases involved with the cytokine pathway are shown.



then allows for the recruitment and activation of the initiator caspase, caspase-8, through the interaction of the N-terminal DED of FADD to the N-terminal DED of caspase-8 (Muzio, Stockwell et al. 1998). This activated initiator caspase then activates the downstream caspases-3, -6 and -7.

The other major apoptotic signaling pathway, the intrinsic pathway, occurs as a consequence of cellular stress, such as DNA damage or cytotoxic drugs (Kroemer and Reed 2000). This pathway is mediated by the release of cytochrome *c* from the mitochondria. Cytochrome *c*, situated in the cytosol, binds the adapter molecule apoptosis protease-activating factor 1 (Apaf1) and in the presence of ATP/dATP promotes the assembly of a multiple protein complex called the apoptosome that, in turn, binds and ultimately leads to the activation of procaspase-9 (Acehan, Jiang et al. 2002). The apoptosome is the mediator of the mitochondria-dependent apoptosis. The apoptosome consists of seven Apaf1 monomers each bound to one molecule of cytochrome *c*. Apaf1 possesses an N-terminal CARD domain that upon activation interacts with the CARD domain of procaspase-9, which allows for the recruitment of procaspase-9 to the apoptosome (Acehan, Jiang et al. 2002). The adaptor protein, Apaf1, acts as a cofactor, allowing for the activation of the initiator caspase, caspase-9. This activated caspase, in turn, then proteolytically activates the downstream caspases, such as caspase-3 and -7.

### **Formation of the 'inflammasome' complex**

Not all caspases are associated with the apoptosis pathway. There is a subclass of initiator caspases involved in cytokine processing and inflammation

(Figure 1-1) that are part of a unique, but similar type of activation complex as the apoptosome. As with Apaf1, the ICE-protease-activating factor (Ipaf) (Poyet, Srinivasula et al. 2001) forms a large, signal-induced multiprotein complex, the inflammasome, resulting in the recruitment and activation of pro-inflammatory caspases. Ipaf specifically associates with and activates caspase-1. Subsequently, caspase-1 then proceeds to proteolytically process the precursor 'proform' of the secreted inflammatory cytokines IL-1 $\alpha$  and IL-1 $\beta$ .

### **Caspase zymogen activation**

The identification of caspases as proteases, which cleave at critical aspartic acid residues, immediately suggested that proteolytic processing of caspase zymogens might be mediated by the caspases themselves. However, it is easy to envision that mature initiator caspases activate effector caspases, but how are the initiator procaspases activated? Caspase auto-activation was clearly demonstrated when overexpressing procaspase-1 in bacteria, but not an active site mutant, led to the full processing of caspase-1 (Orth, O'Rourke et al. 1996; Stennicke and Salvesen 1997). A more definitive proof came when it was observed that the oligomerization of procaspases-8, caused by the aggregation of the linked Fas-Fas ligand binding, would produce the subsequent zymogen activation (Muzio, Stockwell et al. 1998). It is now known that intramolecular processing of initiator procaspases occurs upon achieving high zymogen concentrations via the formation of the DISC, Apaf1 or Ipaf complexes. Since initiator caspases are activated by oligomerization, it

is not surprising to find that initiator zymogens, such as caspase-1, -8 and -9, display high auto-protease activity when compared to executioner procaspases (Salvesen and Dixit 1999). This propensity to autoactivate may be a function of the initiator caspases to act as signal transducers. Complete processing of procaspases into mature caspases occurs by sequential proteolysis of two Asp cleavage sites (Thornberry, Bull et al. 1992). The cleavage products, the large and small subunits, then associate to form the caspase enzyme active site. Crystallographic studies revealed that the active caspase is a tetramer consisting of two heterodimers.

### ***Apoptosis inhibitors***

All cell types contain the apoptotic machinery and hence have the intrinsic ability to undergo cell death. Therefore, it is essential for a cell to prevent inappropriate activation of apoptosis to ensure survival. Recently there have been many proteins identified that function at various points along the apoptotic pathway to inhibit improper activation of apoptosis. Most mechanisms that act to inhibit cell death are upstream of caspase activation. However, there is one family of cellular proteins, the inhibitors of apoptosis (IAP) proteins, that directly inhibits both caspase activation and activity. In addition, IAPs are the only naturally occurring proteins that inhibit caspase function by directly interacting with the caspase enzyme. IAP family members have been shown to be essential for cell survival. Moreover, many forms of cancers have deregulated expression of IAPs. These observations suggest that IAPs are important apoptotic inhibitors and regulators and that their

deregulation may have profound effects on human disease.

## **Discovery of the IAP family**

Investigation into the direct inhibition of caspases started upon the identification of the baculoviral p35 anti-apoptotic protein (Clem, Fechheimer et al. 1991). Indeed, most of what is known of the mechanisms of direct caspase inhibition came with the study of viruses. Upon viral infection, cells self-destruct by undergoing apoptosis in an altruistic effort to protect the host organism from a viral assault. Several viruses have been able to circumvent this host self-defense mechanism by producing viral anti-apoptotic proteins that directly inhibit caspases. The p35 protein, from the *Autographa californica* nucleopolyhedrovirus was the first viral protein shown to be able to block host (lepidopteran) cell death upon viral infection. Since then, p35 has been demonstrated to also inhibit apoptosis in a variety of organisms, such as insects (Hay, Wolff et al. 1994), nematodes (Xue and Horvitz 1995) and mammals (Izquierdo, Grandien et al. 1999). Moreover, p35 was shown to inhibit apoptotic death by acting as a broad-spectrum caspase inhibitor, by direct interaction with caspases (Bump, Hackett et al. 1995).

Subsequently, a screen for baculoviral-encoded suppressors of apoptosis using a p35-deleted *Autographa californica* nucleopolyhedrosis virus led to the identification of two death-suppressing genes from two other viruses: *Cydia pomonella* granulovirus (CpGV) (Crook, Clem et al. 1993) and *Orgyia pseudotsugata* nucleopolyhedrovirus (OpNPV) (Birnbaum, Clem et al. 1994).

The encoded proteins of Cp-IAP and Op-IAP were unlike p35, but had a high degree of similarity to each other. In fact, these viral proteins defined the first members of the IAP family of proteins. Each protein contained two N-terminal modular domains known as the baculovirus IAP repeat (BIR). The BIR domain consists of approximately 70 amino acids with a conserved spacing of cysteine and histidine residues,  $C_{X2}C_{X6} W_{X3}D_{X5}H_{X6}C$  where X is any amino acid, representing a novel zinc-binding fold. In addition, Cp-IAP and Op-IAP also contain a C-terminal really interesting new gene (RING) finger domain.

Since the original discovery of the baculoviral IAPs, the family of IAPs has expanded to include proteins from a diverse set of organisms, ranging from yeast to humans. Criteria for inclusion in this family of proteins are based on the presence of one or more copies of the BIR motif and the ability to inhibit apoptosis. The inclusion of cell-death inhibition as a criterion is crucial since not all BIR containing proteins (BIRP) block cell death (Miller 1999). Some viral, *C. elegans* and yeast BIRPs do not impede apoptotic death. Therefore, all anti-apoptotic IAP family members contain one or more BIR domains, but not all proteins with BIR motifs are IAPs. BIR domains are known to be critical for the inhibitory activity of IAP proteins by providing an interaction site for caspases.

During a positional cloning search for the causative gene for spinal muscular atrophy (SMA) the first mammalian IAP homologue, the neuronal apoptosis inhibitory protein (NAIP) was identified (Liston, Roy et al. 1996). In contrast to previous IAP members, NAIP possessed 3 BIR domains and in place of a RING finger had a relatively large C-terminus with homology to members of the

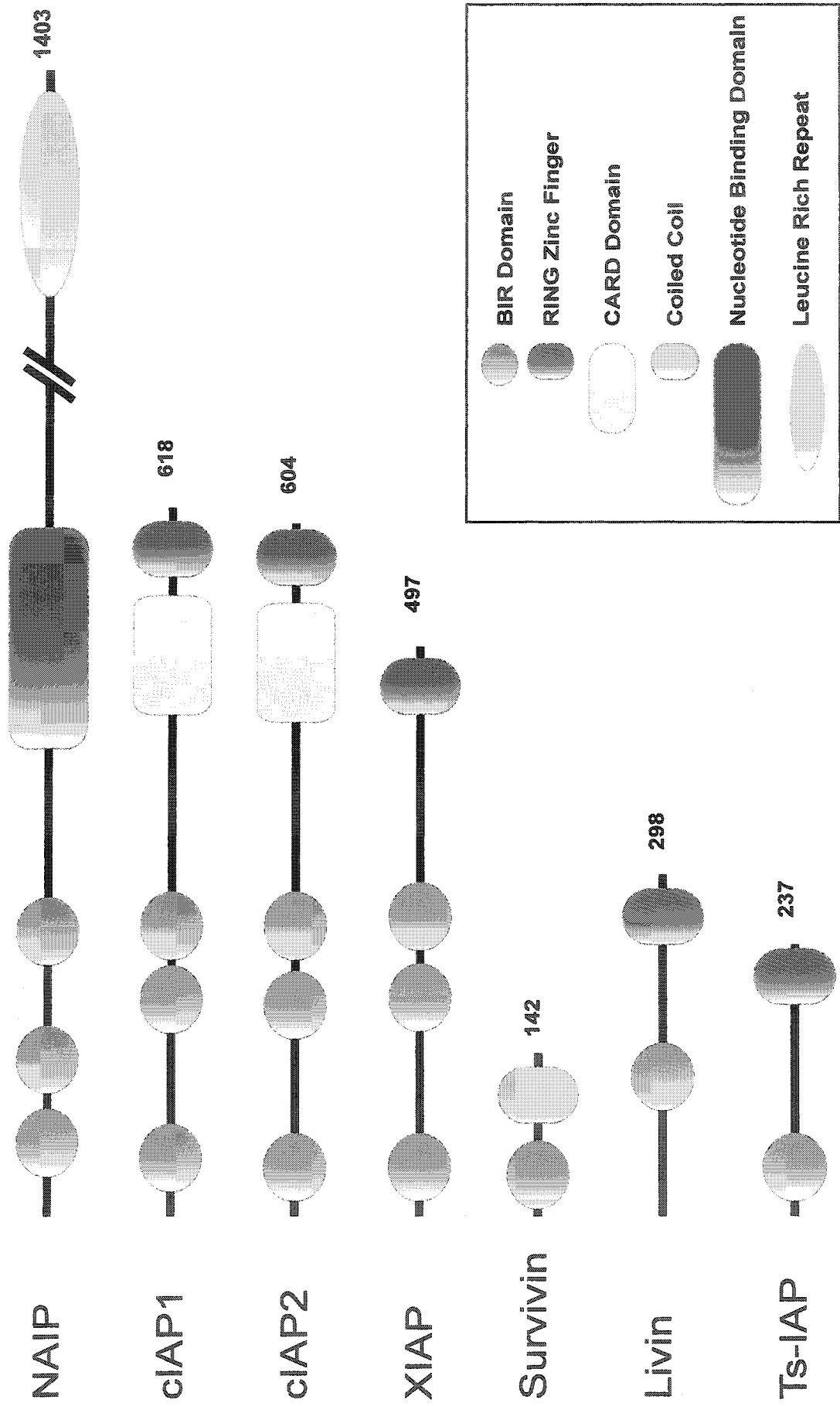
NACHT sub-family of nucleotide binding oligomerization domain (NOD) proteins (Figure 1-2) (Koonin and Aravind 2000). Subsequently, homology to the BIR domain was used to isolate three additional mammalian cellular proteins the X-linked IAP (XIAP), the cellular IAP1 (cIAP1) and the cellular IAP2 (cIAP2) (Liston, Roy et al. 1996; Rothe, Pan et al 1995) that inhibit apoptosis. Each protein contains three N-terminal BIR domains and a C-terminal RING zinc finger (Figure 1-2). The mammalian IAP family now includes additional members: a) Survivin, single BIR motif, C-terminal coiled-coiled domain (Ambrosini, Adida et al. 1997); b) Livin, single BIR motif and C-terminal RING finger (Lin, Deng et al. 2000; Vucic, Stennicke et al. 2000); and c) testis-specific IAP (Ts-IAP), single BIR motif and C-terminal RING finger (Lagace, Xuan et al. 2001; Richter, Mir et al. 2001) (Figure 1-2).

#### **Function of the BIR domain**

The importance of IAPs for cell survival was first clearly demonstrated when it was observed that the *Drosophila* IAP (DIAP1) was required for the survival of multiple cell types of the fly (Hay, Wassarman et al. 1995). In addition, functional studies upon tissue culture models and adenovirus over-expression of IAP proteins showed their ability to suppress or delay apoptosis in varied multiple cell death models (LaCasse, Baird et al. 1998). Despite not knowing the mechanics of this inhibition, the IAPs were shown to block both the intrinsic and extrinsic apoptotic pathways. Mechanistic insight into IAP cell death inhibition was demonstrated when XIAP was shown to physically interact with and block caspase function (Deveraux, Takahashi et al. 1997).

## Figure 1-2. Modular structure of the human IAP family

The organizational arrangement of the human IAP domains is described as follows: baculoviral IAP repeat (BIR), really interesting new gene (RING) zinc finger, caspase recruitment domain (CARD), nucleotide-binding oligomerization domain (NOD) and the leucine-rich repeat (LRR) domains. Amino acid length is indicated to the right of each protein.

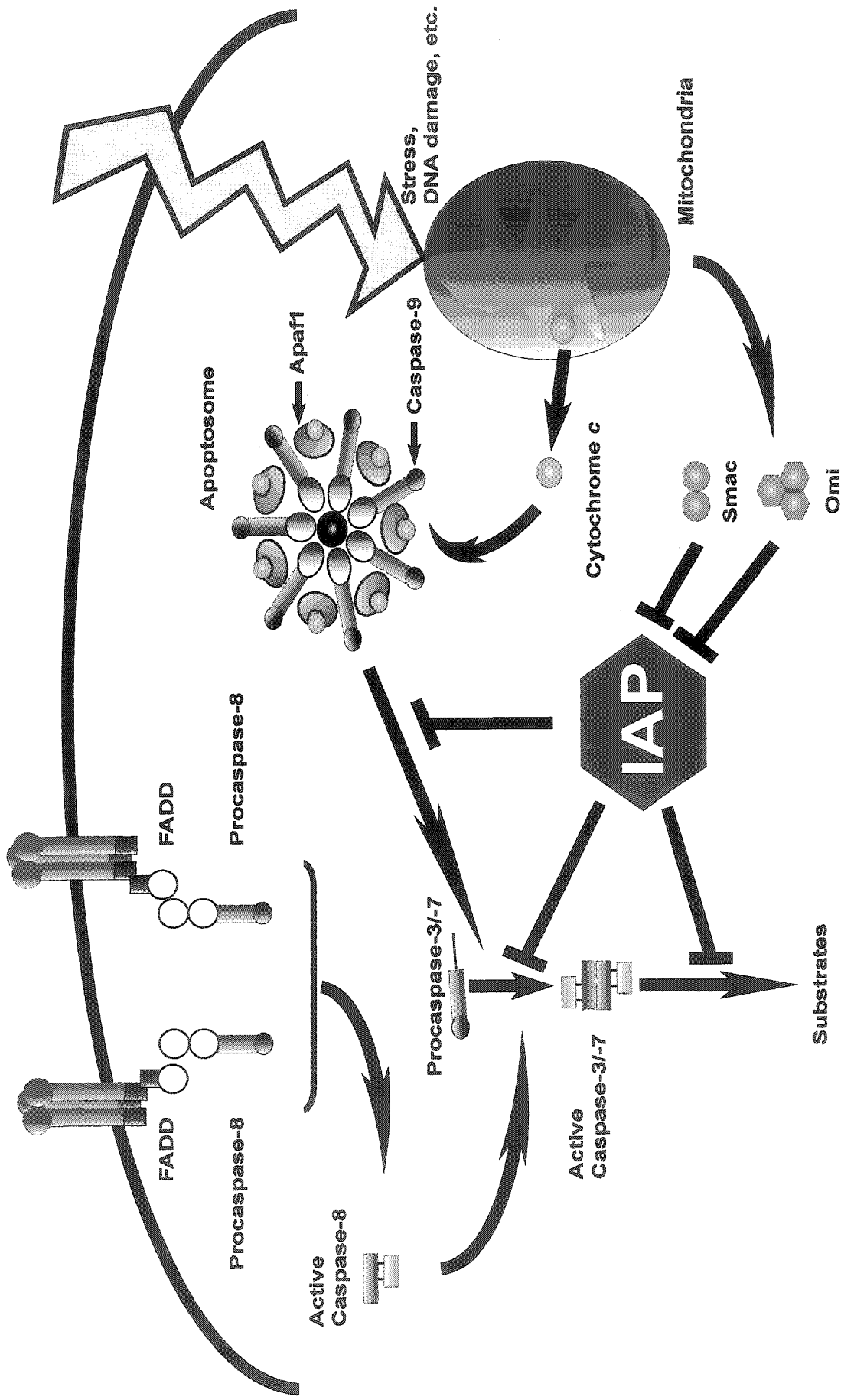


Subsequently, cIAP1 and 2 were also shown to operate under a similar mechanism (Roy, Deveraux et al. 1997) and along with XIAP were demonstrated to specifically inhibit caspase-3, -7 and -9. Indeed, the IAPs are the only naturally occurring cellular proteins that directly rather than indirectly affect caspase activation and/or function. In addition, IAPs are the only cellular proteins that inhibit both initiator (caspase-9) and executioner (caspase-3 and -7) caspases (Figure 1-3).

Mutational studies showed that the conserved BIR domains were required for IAP anti-apoptotic function (Sun, Cai et al. 1999; Sun, Cai et al. 2000). In fact, most of the functional IAP interactions have been localized to the BIR motifs, specifically: a) caspase inhibition; b) cIAP1 and 2 binding to INF repositor associated factors (TRAFs) and c) XIAP interaction with the TAB1 protein (Figure 1-4). The presence of a single BIR domain in Survivin suggests that one BIR is sufficient for functionality thereby raising the possibility that the 3 BIR domains of XIAP, cIAP1 and 2 were not redundant copies of each other. Indeed, specific interactions with caspase-9 were mapped to the third BIR domain (Deveraux, Roy et al. 1998) while the second BIR domain along with some flanking N-terminal sequence, the linker region, was sufficient to inhibit caspase-3 and -7 (Sun, Cai et al. 1999). The single BIR domain IAPs were shown to inhibit caspase-3 and -7 (Survivin) (Shin, Sung et al. 2001) and caspase-9 (Ts-IAP) (Richter, Mir et al. 2001) while Livin demonstrated a broader spectrum of activity by inhibiting all three caspase-3, -7 and -9 (Vucic, Stennicke et al. 2000).

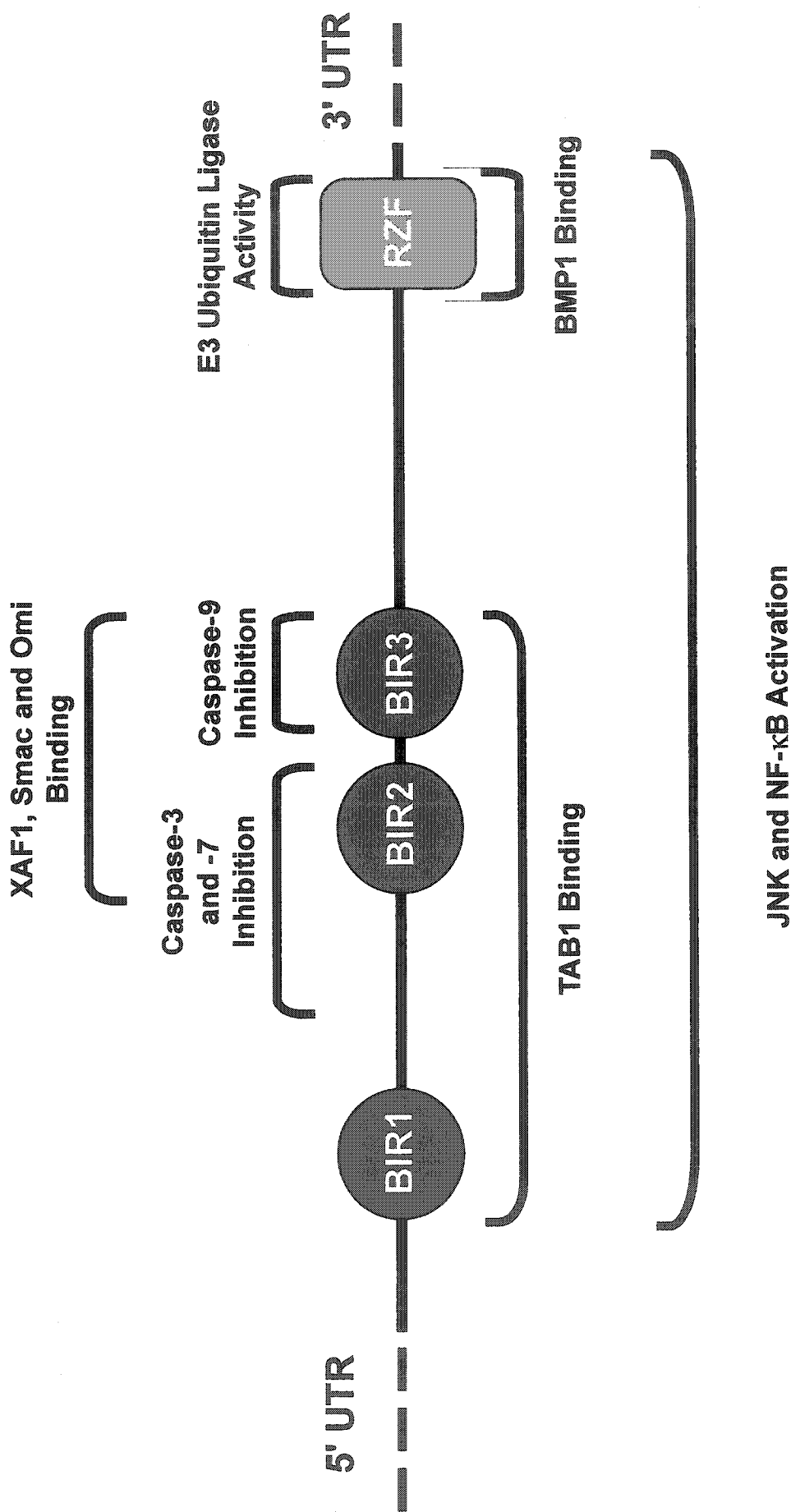
**Figure 1-3. The role of IAPs in the extrinsic and intrinsic apoptotic pathways**

The ability of the IAPs to inhibit both executioner (caspase-3 and -7) and initiator (caspase-9) caspases situates them within both apoptotic pathways. Disruption of the mitochondrial membrane releases the XIAP inhibitors Smac and Omi. These two proteins disrupt XIAP-caspase interactions thereby allowing for the progression of the apoptotic program (see text for details).



#### **Figure 1-4. Functional diagram of XIAP function and interactions**

The sections of XIAP and their corresponding interacting partners are diagramed above and below XIAP. Caspase-3 and -7 interact with BIR2 and a small N-terminal leader sequence, while caspase-9 specifically binds BIR3. The XIAP inhibitor proteins (XAF1, Smac and Omi) associate with BIR2 and 3. TAB1-XIAP interactions have been identified to be BIR domain-dependent. XIAP-JNK and -NF- $\kappa$ B activation has yet to be localized to a specific XIAP modular domain. The 5' and 3' UTRs are not to scale (see text for details).



Mutational and crystallographic studies of XIAP BIR domain 2 complexed with either caspase-3 or -7 demonstrated that the BIR domain was not the major structural determinant of the complex. In fact, the 18 amino acid linker region, N-terminal to BIR domain 2, was shown to be far more critical than the BIR domain itself (Chai, Shiozaki et al. 2001; Huang, Park et al. 2001; Riedl, Renatus et al. 2001). The linker region forms critical hydrogen bonds and hydrophobic interactions with the catalytic groove of caspase-3 and -7. In this way the linker region prevents substrate entry into the active site. The importance of the linker region was clearly demonstrated when a recombinant protein containing the linker fused to GST was able to inhibit caspase function independent of the BIR domain (Chai, Shiozaki et al. 2001; Huang, Park et al. 2001).

The BIR 3 domains of XIAP, cIAP1, cIAP2 (Deveraux, Roy et al. 1998; Bratton, Walker et al. 2001) and the single BIR domains of Livin (Vucic, Stennicke et al. 2000) and Ts-IAP (Richter, Mir et al. 2001) have been shown to directly interact with caspase-9. In contrast to BIR 2 domain binding to caspase-3 and -7, the BIR 3 domain of XIAP was shown to be required for caspase-9 inhibition. However, the flanking linker region was superfluous, suggesting that the mechanism of inhibition is distinct to that of BIR2 to caspase-3 and -7. Moreover, another mechanistic distinction is the prerequisite need for the proteolytic processing of caspase-9, at Asp<sub>315</sub>, prior to being able to bind members of the IAP family. XIAP BIR3 has been shown to bind at this exposed N-terminus and hence inhibit caspase-9 activity. A second cleavage event at

Asp<sub>330</sub> abrogates XIAP BIR3 binding, but not caspase-9 activity, hence abolishing XIAP inhibition (Srinivasula, Hegde et al. 2001).

The BIR 2 and 3 domains of XIAP, cIAP1, cIAP2 and NAIP have shown that distinct mechanisms of caspase inhibition exist for the caspase targets of the IAP family members. However, the BIR 1 domain of these IAP members has yet to demonstrate any caspase inhibition. This, nonetheless, does not preclude the discovery of a yet unknown function of the BIR 1 domain.

### **IAP RING zinc finger function**

Several IAP members contain a highly conserved C-terminal RING zinc fingers. RING fingers are a sub-class of zinc finger domains characterized by seven cysteines and one histidine that chelate two zinc ions in a distinctive cross-brace arrangement. The RING motif has been previously identified in several cellular proteins and shown to possess multiple functions. Recent studies (Joazeiro and Weissman 2000) showed that IAP RING fingers play a crucial role as adapter proteins in targeted-protein degradation via the ubiquitin-proteasome pathway. This system involves the sequential addition of the C-terminus of one free ubiquitin protein which is covalently bonded to an ubiquitin-activating enzyme (E1) (Weissman 2001). The ubiquitin-E1 functional group is then transferred to an ubiquitin-conjugating enzyme (E2) and then subsequently transferred to a target protein. An ubiquitin ligase enzyme (E3) catalyzes this final step. The RING finger is the signature motif of an E3 ligase of many proteins. It acts as an adapter protein that can facilitate the final transfer of ubiquitin to the target protein (Weissman 2001).

Indeed, the RING zinc finger domains of XIAP, cIAP1 and 2 have been shown to have E3 capacity. Recent studies on the RING finger domains of XIAP and cIAP1 suggest the RING finger causes the ubiquitination and degradation of IAP proteins in response to apoptotic stimuli, such as glucocorticoids or etoposide (Yang, Fang et al. 2000). Proteasome inhibitors could block this IAP self-degradation. Interestingly, it was demonstrated that cIAP2 and XIAP could trigger the ubiquitination of caspase-3 and -7, suggesting that targeting of caspases to the proteasome may be one of the anti-apoptotic mechanisms of the IAPs (Huang, Joazeiro et al. 2000; Suzuki, Nakabayashi et al. 2001). Conflicting results have been obtained using RING finger deletion mutants of the IAPs as to their pro- or anti-apoptotic nature (Yang, Fang et al. 2000; Clem, Sheu et al. 2001; Suzuki, Nakabayashi et al. 2001). The necessity of this domain in suppressing apoptosis appears to be dependent on the cellular context and under some circumstances, removal of this RING domain actually enhanced the anti-apoptotic function of these proteins. Further work remains to be done to establish whether the IAP RING finger domain contributes to the apoptotic inhibitory activity of the protein, or whether it in fact antagonizes this activity.

### **Caspase recruitment domain (CARD)**

Of the IAP family members cIAP1 and 2 are unique in that they contain a caspase recruitment domain (CARD) situated between BIR3 and the RING finger. The CARD domain of other proteins typically assist in protein-protein interactions between CARD motifs (Weber and Vincenz 2001). However, deletion of the CARD domains from these proteins does not prevent inhibition

of apoptosis (LaCasse, Baird et al. 1998). This nonetheless does not preclude the discovery of a yet unknown function of the CARD domain.

### **IAP regulatory antagonists**

Cell death induction requires the elimination of IAP family mediated caspase inhibition. Recently, three proteins have been identified that bind to and negatively regulate the caspase-inhibiting activity of IAPs: a) the second mitochondrial activator of caspases (Smac), in humans or direct IAP binding protein with low pI (DIABLO), in mice; b) Omi (a.k.a. HtrA2) and c) XIAP associated factor1 (XAF1) (Figure 1-3). Smac is a nucleus-encoded protein that is localized to the inter-membrane space of the mitochondria of non-apoptotic cells, via a N-terminal mitochondrial-targeting signal (Du, Fang et al. 2000; Verhagen, Ekert et al. 2000). The proteolytic excision of the N-terminal 55 amino acid residues of Smac is necessary, as it exposes an IAP-binding tetrapeptide (alanine-valine-proline-isoleucine). Mature Smac is evidently released along with cytochrome *c*, in response to an apoptotic trigger. Smac binds and apparently displaces IAP family members from caspases, allowing for the apoptotic process to proceed. *In vitro* studies have shown the ability of Smac to bind several IAP proteins including XIAP, cIAP1, cIAP2 (Du, Fang et al. 2000) and Livin (Vucic, Deshayes et al. 2002).

Crystallographic studies have revealed that Smac can bind BIR2 and BIR3 of XIAP, thereby interfering with XIAP interactions with either caspase-3, -7 or caspase-9 (Chai, Du et al. 2000; Liu, Sun et al. 2000; Wu, Chai et al. 2000). The N-terminal residues of mature Smac are homologous to those of the

exposed caspase-9 N-terminus that bind to the BIR3 of XIAP. Smac may then bind to the same pocket of XIAP thus displacing and releasing caspase-9 from IAP inhibition. A synthetic peptide consisting only of the four Smac N-terminal residues 56-59 was able to mimic the IAP-regulatory properties of mature Smac. Notably, Smac links the IAP family members with mitochondrial-mediated cell death.

Interestingly, the resolution of the Smac IAP-inhibition mechanism provided insight into the function of the pro-apoptotic *Drosophila* proteins Reaper, Hid and Grim. These proteins encompass all facets of cell death in *Drosophila* and can all be inhibited by DIAP1 (Song, Guan et al. 2000). In addition, these proteins share little or no sequence conservation. However, the four N-terminal residues of these proteins all share homology with the Smac tetrapeptide motif, and have been shown to bind a similar groove on the surface of DIAP1.

Subsequently, more recent structural studies identified a second mitochondrial IAP binding protein, called Omi (Suzuki, Imai et al. 2001; Hegde, Srinivasula et al. 2002; Martins, Iaccarino et al. 2002; van Loo, van Gurp et al. 2002; Verhagen, Silke et al. 2002). Omi, like Smac, is released from the mitochondria of apoptotic cells and is processed to generate a Smac-like tetrapeptide motif at the N-terminus. Omi directly binds to XIAP and thus inhibits XIAP-caspase interaction in much the same manner as Smac. However, Omi has additional unique pro-apoptotic activities distinct from Smac-like function. The Omi serine protease may also contribute to cell death in a non-caspase dependent manner, although the Omi cellular targets have

not been identified (Suzuki, Imai et al. 2001; Hegde, Srinivasula et al. 2002; Verhagen, Silke et al. 2002). The proteolytic removal of the 155 amino acid mitochondrial targeting peptide of Omi is a self-catalyzed event, thereby raising the possibility that Omi may process other mitochondrial leader sequences, including that of Smac.

A third and discrete mammalian IAP interacting protein, XAF1, was identified through a yeast two-hybrid screen (Liston, Fong et al. 2001). XAF1 is a novel, zinc finger rich protein that was shown to localize to the nucleus. *In vitro* studies using recombinant proteins have shown that XAF1 directly binds and interferes with XIAP-caspase-3 inhibition. Adenoviral co-expression of XAF1 and XIAP in cell lines suggested that XAF1 inhibits XIAP activity by sequestering and localizing XIAP to the nucleus. Notably, XAF1 expression levels are low or non-existent in multiple tumor cell lines, therefore suggesting that XAF1 may function as a tumor suppressor (Fong, Liston et al. 2000).

### **IAP functions independent of caspases**

Evidence is mounting to suggest that members of the IAP family have functions that are independent of caspase inhibition. Gene-disruption studies of Survivin and the *C. elegans* homolog BIR-1 demonstrated that protein expression was restricted to the G2/M point in the cell cycle and that Survivin is the only known IAP to directly interact with chromatin structures (Li, Ackermann et al. 1999). Ablation of the Survivin gene in mice resulted in embryonic lethality, with the main defect being the aberrant formation of the mitotic spindles (Uren, Wong et al. 2000). As with Survivin, Livin has been

shown to be localized to the nucleus, however it does not seem to be associated with any nuclear structures (Kasof and Gomes 2001).

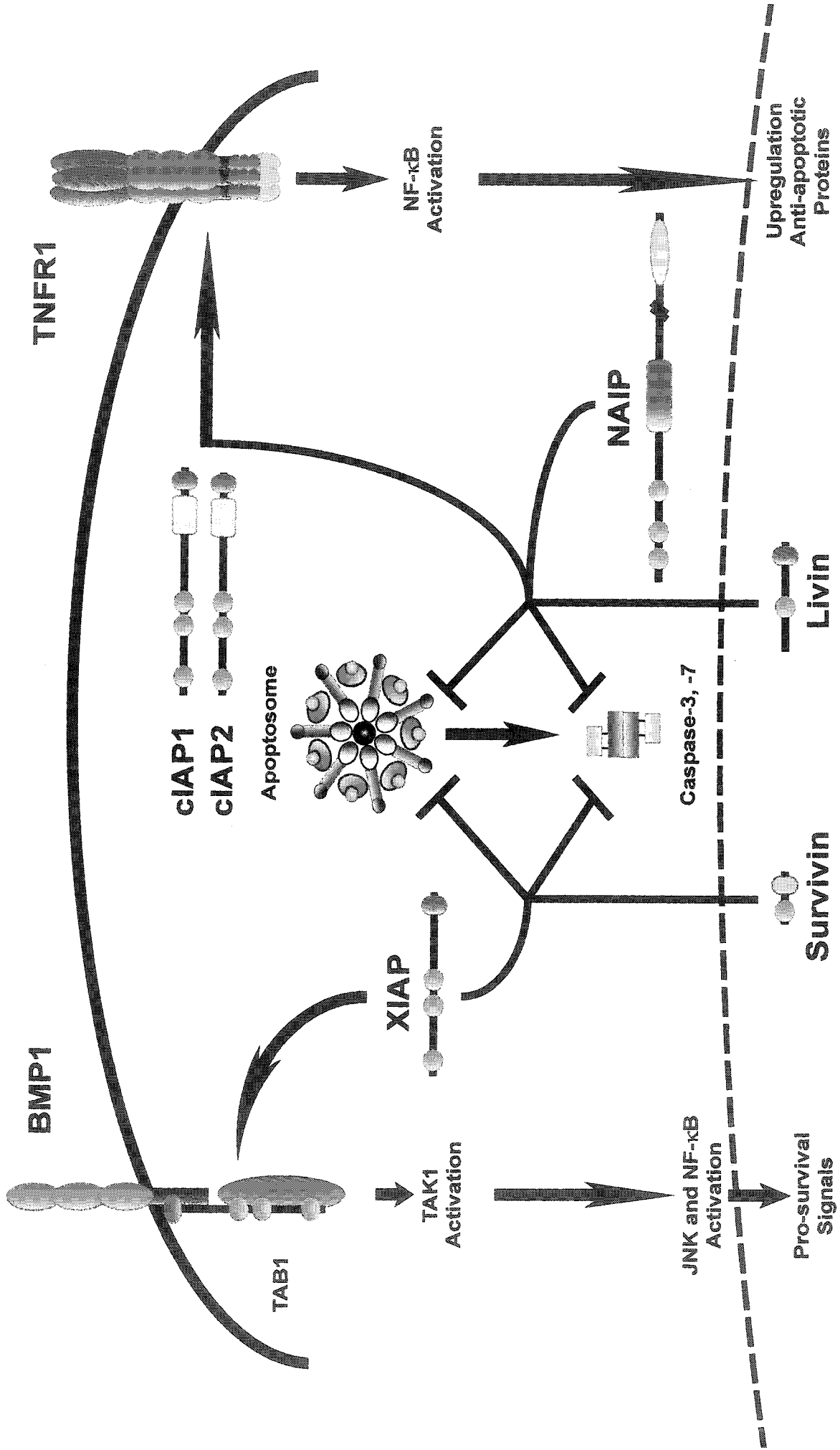
The modular structure of XIAP suggests a possible multifunctional role beyond that of caspase inhibition. Recent work has pointed to XIAP being a signal transduction molecule. In a yeast two-hybrid study the XIAP RING zinc finger domain was found to interact with the bone morphogenic protein (BMP) receptor while the XIAP BIR domain associated with the BMP-associated protein TGF- $\beta$  activated kinase-1 binding protein-1 (TAB1) (Yamaguchi, Nagai et al. 1999). TAB1 can then in turn recruit the TGF- $\beta$ activated kinase-1 (TAK1) to the receptor complex (Massague, Blain et al. 2000). TAK1 is a mitogen-activated protein kinase kinase kinase (MAPKKK) that could activate both NF- $\kappa$ B and the c-Jun N-terminal kinase (JNK) signal pathways. Moreover, XIAP overexpression was demonstrated to augment all three distinct signaling pathways of TGF- $\beta$  and that of BMP responses (Yamaguchi, Nagai et al. 1999). Subsequently, XIAP, but not cIAP1 or 2, was shown to activate JNK1 (Sanna, Duckett et al. 1998). The XIAP BIR domain was demonstrated to be essential for JNK1 activation, however direct interaction between the proteins was not observed. Thus, XIAP may suppress cell death via three major pathways: a) inhibition of caspases, b) ubiquitin-targeted degradation of executioner caspases and c) transcriptional activation of prosurvival pathways via TAK1.

Of the IAP family members cIAP1 and 2 are unique in that they both interact with members of the tumor necrosis factor-receptor (TNFR) family (Rothe, Pan et al. 1995). Figure 1-5 shows that both cIAP1 and cIAP2 can be recruited by and bind to the signaling intermediates TRAF1 and TRAF2 receptor complexes.

This interaction appears to be specific, as other IAPs do not bind to these receptor complexes, and cIAP1 and cIAP2 do not bind to other TRAF receptors. An alternative mechanism of the protection against apoptosis, independent of caspases and mediated by IAP family members in response to TNF signaling has also been attributed to the ability of these receptors to activate the nuclear factor- $\kappa$ B (NF- $\kappa$ B). The activation of the NF- $\kappa$ B family of transcription factors rapidly induces the up-regulation of inflammatory and anti-apoptotic genes, including cIAP2 (Chu, McKinsey et al. 1997). cIAP2, *in vitro*, has been shown to be highly inducible via multiple activating agents mediated through the NF- $\kappa$ B pathway (Lee and Collins 2001). Interestingly, it has been reported that cIAP2 can reciprocate and induce NF- $\kappa$ B (Chu, McKinsey et al. 1997). The cIAPs demonstrate that IAPs can regulate apoptosis at various points in different pathways, via caspase inhibition or at multiple levels of a single pathway, to protect the cell from aberrant cell death.

### **Figure 1-5. IAP functional summary**

IAP function is not exclusive to caspase inhibition. Recently cIAP1 and cIAP2 were shown to be components of the TNF signaling pathway, involved with NF- $\kappa$ B-regulated survival pathways. XIAP can recruit TAB1 and TAK1 to the BMP receptor leading to the activation of JNK and NF- $\kappa$ B.



**BMP1**

**TNFR1**

**TAB1**

**CIAP1**

**CIAP2**

**Apoptosome**

**TAK1**

**Activation**

**XIAP**

**Activation**

**NF-κB**

**Activation**

**NAIP**

**Caspase-3, -7**

**JNK and NF-κB**

**Activation**

**Survivin**

**Livin**

**Upregulation**  
**Anti-apoptotic**  
**Proteins**

**Pro-survival**  
**Signals**

## ***Thesis outline***

The Korneluk group isolated several human IAP family members, which include NAIP, XIAP, cIAP1 and cIAP2, and subsequently demonstrated that when over-expressed, members of the IAP family block a broad range of apoptotic signals in a variety of cell types *in vitro*, including Jurkat cells (a human T cell leukemia line), and *in vivo* (LaCasse, Baird et al. 1998). Moreover, this same laboratory discovered that the human thymus and spleen expressed high levels of *xiap*, *ciap1* and *ciap2* mRNA (Liston, Young et al. 1997). This thesis documents the role of the X-linked inhibitor of apoptosis (XIAP) and the cellular inhibitor of apoptosis 2 (cIAP2) in the developmental phase of T cells, known as thymocytes, and during an innate immune response. The above results combined with the well-recognized fact that apoptosis plays a crucial part in a variety of immune functions suggested that IAPs may have a potential role, either direct or indirect, in normal immune system function.

As an initial starting point, the first general hypothesis was formulated: during the course of normal immune function IAP expression levels may be directly correlated to apoptotic susceptibility of discrete lymphocyte populations and macrophages. The objective was to observe mRNA levels of the IAPs, specifically *xiap*, *ciap1* and *2*, during T cell development and in thymocytes or T cells that have been exposed to a variety of T cell-specific proliferative and apoptotic stimuli. In addition, the *iap* mRNA levels were observed for macrophages activated with lipopolysaccharide (LPS). The results from the

thymocyte protein and message level experiments for XIAP suggested that there was a link between the apoptotic susceptibility of thymocytes to dexamethasone and XIAP protein and mRNA levels. Therefore, a more explicit hypothesis was formulated: specifically altering thymus-XIAP expression may disrupt thymocyte susceptibility and development. The objective was to engineer and characterize a transgenic mouse that specifically over-expressed XIAP in the thymus.

In the course of these experiments it was demonstrated that thymocyte sub-populations of *xiap*-transgenic mice were disrupted and accumulated in the thymus. In addition, *xiap*-transgenic thymocytes resisted a variety of apoptotic triggers both *in vivo* and *in vitro*. This work suggested that altering the endogenous XIAP expression levels disrupts thymocyte development.

In contrast to *xiap* and *ciap1* mRNA levels, *ciap2* message levels of macrophages showed a rapid and dramatic increase in response to LPS treatment. In addition, a modest increase of *ciap2* message levels in T cells exposed to IL-7 was observed. Therefore, a second more specific hypothesis was formulated: apoptotic susceptibility of macrophages and T cells may be altered by a rapid induction of the *ciap2* gene. The objective was to engineer and characterize a *ciap2*-null mouse, thus preventing the ability to up-regulate *ciap2*.

These studies demonstrated that cIAP2-null mice are highly resistant to LPS-induced endotoxic shock and display an attenuated proinflammatory response. In addition, LPS-activated cIAP2-null macrophages are extremely susceptible to apoptotic triggers relative to normal macrophages that have been induced

with LPS. This work suggested that the inability to up-regulate *ciap2* adversely affected the apoptotic susceptibility of macrophages and T cells.

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## Chapter 2: Methods and Materials

### *lck<sup>Pr</sup>-xiap methods and materials*

#### **Resources available at the start of my studies:**

Prepared "in house":

$\alpha$ -XIAP antibody (specificity: mouse and human)

$\alpha$ -cIAP1 antibody (specificity: human)

$\alpha$ -cIAP2 antibody (specificity: human)

XIAP, cIAP1 and cIAP2 adenovirus expression systems

Primers for quantitative RT-PCR of human and mouse mRNA for *xiap*, *ciap1* and *ciap2*

#### **Construction of lck<sup>Pr</sup>-xiap vector and production of transgenic mice**

A 1.5 kb cDNA fragment containing the coding region of human *xiap* was inserted into the lck-human growth hormone (hGH) vector (Chaffin, Beals et al. 1990). Transgenic mice were generated at the Biomedical Research Center at the University of British Columbia.

#### **Southern blot analysis**

Genomic DNA was isolated from mouse tissue by digestion with protease K @ 55° C overnight followed by phenol/chloroform and chloroform extractions. DNA was precipitated and 5  $\mu$ g was digested with BamHI/XhoI, separated on agarose gels and transferred to Biodyne Nylon Paper. <sup>32</sup>P-labeled, full-length *xiap* cDNA probes were prepared using Rediprime (Amersham) and <sup>32</sup>P-dCTP

(Amersham) according to the manufacture's directions. Final washes were carried out in 0.1XSSC/0.1 % SDS at 65° C.

### **Western blot analysis**

Mouse tissue was lysed in 5 volumes homogenization buffer (10 mM Tris:HCl pH 6.8, 150 mM NaCl, 2 mM MgCl<sub>2</sub>, 1 mM phenylmethylsulphonyl fluoride) and then crushed, followed by centrifugation @ 250 *g* for 2 min. The supernatant (S/N) was then adjusted to 2% SDS and boiled for 20 min., followed by centrifugation @ 14K for 10 min. The S/N was transferred to a new microcentrifuge tube and centrifuged again @ 14K for 15 min. Protein content was assayed by BCL kit (Pierce) and equal amounts of protein samples of the S/N lysate were loaded per lane and separated by SDS-PAGE and analyzed by Western blotting using rabbit polyclonal antibodies to XIAP (1:3000 dilution) (Ægera).

### **Flow cytometry and lymphoid cell isolation**

T cells, splenocytes and thymocytes were isolated from mouse lymphoid tissue by first mincing and then pressing the tissue through a 10 mm gapped metal mesh. For splenocytes 3 ml of the minced and pressed tissue (~2X10<sup>7</sup>splenocytes/ml) was layered on 5 ml of room temperature Lympholyte-M (CedarLane, Canada) and centrifuged at 1500Xg/20 min. @ RT. Cells were collected from the interface layer, washed twice with complete DMEM and re-suspended in 1 ml complete DMEM. Total lymphocytes were enumerated using trypan blue exclusion. 1 x 10<sup>6</sup> cells were incubated with the

following conjugated monoclonal antibodies: anti-Thy1.2-fluorescein isothiocyanate (FITC), CD4-phycoerythrin (PE), CD8a-Cy-Chrome, CD45R-FITC (PharMingen). Flow cytometric analyses were performed using a Coulter XL cytometer (Coulter Canada).

### **Primary tissue culture and death assays**

Primary cultures were maintained in RPMI 1640 supplemented with 10 % fetal calf serum, 50 mM  $\beta$ -mercaptoethanol 125 mM L-glutamine, penicillin and streptomycin. Thymocytes ( $5 \times 10^6$ /mL) were cultured in 24-well plates and exposed to different apoptotic triggers at the time periods indicated. Some wells were coated with anti-CD3 antibody (clone 145-2C11, PharMingen, 10  $\mu$ g/mL overnight and then washed with PBS). Cell death/apoptosis was measured by trypan blue exclusion and anti-annexin V-FITC staining of cells using an Annexin V-FITC kit (Immunotech) and analysis using a Coulter XL cytometer.

### ***In vivo* apoptotic death**

For *in vivo* experiments mice were injected intraperitoneally with PBS (anti-Fas antibody control), saline (dexamethasone control), mouse specific anti-Fas antibody (clone Jo2, 100  $\mu$ g) (PharMingen) or dexamethasone (Sigma). Thymocytes from dexamethasone and anti-Fas antibody injected mice were harvested at 48 h and 2 h respectively. All thymocytes were isolated as described above and cell death/apoptosis was measured using the Annexin V-FITC kit. Thymocytes from dexamethasone treated mice were further stained

to assess affected T cell sub-populations (anti-Thy1.2-fluorescein isothiocyanate (FITC), CD4-phycoerythrin (PE), CD8a-Cy-Chrome (PharMingen).

#### **T cell/thymocyte sub-population sorting**

T cells/thymocytes were first isolated as previously described, and sorted according to CD8/CD4 expression using anti-CD4/CD8-antibody coated magnetic beads (Miltenyi Biotec) according to the manufacturer's directions.

## ***cIAP2<sup>-/-</sup> mice methods and materials***

### **Generation of *cIAP2<sup>-/-</sup>* mice**

129/sv genomic clones (Liston, Lefebvre et al. 1997) spanning the mouse *ciap2* gene were used to construct a replacement-type targeting vector in which an IRES-*lacZ* and phosphoglycerate kinase (PGK)-neomycin (neo) cassette (SA-IRES- $\beta$ geo; (Mountford, Zevnik et al. 1994)) replaced exons 2 to 5 in the plasmid pKO (Holcik and Korneluk, unpublished). The resultant targeting vector (pKO.hiap1) was comprised of a 4.1 kb 5' arm and a 5.5 kb 3' arm bracketing the IRES-*lacZ*/PGK-neo insertion. RW4 embryonic stem (ES) cells were electroporated as described (Wurst W. 1993) and DNA from neomycin resistant clones was extracted and analyzed. Disruption of the *ciap2* allele was confirmed by Southern blot analysis of *EcoRV* digested genomic DNA after hybridization with a probe corresponding to exon 1 of the *ciap2* gene. Chimeric mice were produced by morula aggregation (Wood, Allen et al. 1993) with targeted RW-4 cells. Chimeric male progeny were mated with 129/SvJ females and heterozygous progenies were backcrossed to C57BL/6 mice and used as founders for two separate lines of mutant mice. Both the electroporation of ES cells and the generation of chimeric animals were performed at the Genome Systems Inc. facility (St. Louis, MO). Mice were housed in a specific pathogen-free environment and all experiments were performed in accordance with the guidelines of the Canadian Council on Animal Care and protocols approved by the University of Ottawa Animal Care Committee.

### **Generation of germline chimeras and homozygous mice**

Expanded targeted ES clones were injected into C57BL/6 blastocysts. The resulting chimeras were backcrossed to C57BL/6 (Charles River Laboratories, Wilmington, MA). The germline transmission was assayed by Southern blot analysis using the EcoRV fragment. Heterozygous mice were then crossed to produce homozygous cIAP2-negative mice.

### **Assessment of the physiological effects of LPS, LPS/D-galactosamine (DGLN) and $\alpha$ -Fas antibody in cIAP2-deficient mice**

Age-matched C57BL/6 and 129Sv (129Sv mice were only used in the case of the LPS intra-peritoneal (i.p.) injection alone) cIAP2-deficient mice, and control heterozygous and wild-type littermates, were injected i.p. in a total adjusted volume of 0.2 ml of LPS alone (*Escherichia coli* serotype 0127:B8, Sigma-Aldrich, Oakville, ON Canada) (35 mg/Kg, LD<sub>100</sub>), or LPS along with D-galactosamine (0.6 g/kg mouse) (Sigma-Aldrich, Oakville, ON Canada) or  $\alpha$ -Fas antibody alone (clone Jo2, 100  $\mu$ g/mouse) (PharMingen) all suspended in nonpyrogenic saline.

### **Southern blot analysis**

Genomic DNA was isolated by standard methods and digested with EcoRV, separated on agarose gels, and transferred to Biotransfer Nylon Paper (Life Technologies, Rockville, MD). Full-length <sup>32</sup>P-labeled cIAP2 cDNA probes were prepared by using Rediprime (Amersham Pharmacia) and <sup>32</sup>P-dCTP

(Amersham Pharmacia) according to the manufacturer's directions. Membranes were washed with 0.1 X SSC/0.1% SDS at 65°C for 10 minutes.

### **Western blot analysis**

Mouse tissue was weighed and subsequently lysed in five volumes (w/v) of lysis buffer (50 mM Tris•HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM vanadate, 1% (v/v) Nonident P-40, 0.25% (v/v) sodium deoxycholate, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 mM phenylmethylsulphonyl fluoride) and then crushed and well mixed. The samples were then rotated for 45 minutes at 4°C. The samples were then centrifuged for 15 minutes at ~14,000 rpm, in a microcentrifuge. The supernatant was collected and assayed by BCL kit (Pierce) and equal amounts of S/N lysate were loaded per lane. Proteins were separated on a SDS/PAGE gel and analyzed by Western blotting using a rabbit polyclonal  $\alpha$ -cIAP2 antibody (1:2500 dilution) and an enhanced chemiluminescence kit (Pierce)

### **Flow cytometry and cytokine assay**

T cells, B cells and thymocytes were isolated from mouse lymphoid tissue (spleen or thymus) by first mincing and then pressing the tissue through a 10 mm gapped metal mesh, and were then counted using trypan blue exclusion. Macrophages were harvested from mice using an *intra-peritoneal* (i.p.) lavage (3 X 10 ml of media: 5% FCS, 50 µM  $\beta$ -mercaptoethanol, 125 mM L-glutamine, penicillin, and streptomycin, @ 4° C) washed once (centrifuged at 1500Xg/20 min). Peritoneal macrophages were resuspended at  $\sim 2 \times 10^7$

cells/ml. Subsequently, peritoneal macrophages were layered on 5 ml of room temperature Lympholyte-M (CedarLane, Canada) and centrifuged at 1500Xg/20 min. @ RT. For splenocyte macrophages 3 ml of the minced and pressed tissue ( $\sim 2 \times 10^7$  splenocytes/ml) was layered on 5 ml of room temperature Lympholyte-M (CedarLane, Canada) and centrifuged at 1500Xg/20 min. @ RT. Subsequently, either peritoneal- and spleen-derived macrophages were collected from the interface layer, washed twice with complete DMEM and re-suspend in 1 ml complete DMEM. The resulting cells were then counted using trypan blue exclusion and Diff Quik™ Stain Kit (IMEB INC, San Marcos, CA). Cells ( $10^5 - 10^6$ ) were incubated with the following conjugated monoclonal antibodies: anti-CD3e-fluorescein isothiocyanate (FITC), CD4-phycoerythrin (PE), CD8a-Cy-Chrome, CD69-PE, B220-FITC, B220-PE, CD11b-Cy-Chrome, (Pharmingen) F4/80-FITC and F4/80-PE (Cedarlane Laboratories Limited). Flow cytometric analyses were performed on a Coulter XL cytometer (Coulter, Canada). Concentrations of TNF $\alpha$  and IL-1 $\beta$  in the serum of treated mice and in culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) (R&D Systems).

#### **Primary tissue culture, *in vitro* cytokine levels and death assays**

Primary cultures were maintained in DMEM supplemented with: 10% FCS and 10 nM GM-CSF (R&D Systems) (for peritoneal-derived macrophages), 5% FCS (for T cells, B cells and thymocytes) and with 50  $\mu$ M  $\beta$ -mercaptoethanol, 125 mM L-glutamine, penicillin, and streptomycin ( $\sim 85\%$  confirmed using a FITC-conjugated  $\alpha$ -CD3 antibody and flow cytometry).

### **mRNA Isolation and quantitative mRNA analysis.**

mRNA was extracted using Qiagen Rneasy 96 wells extraction kit (Qiagen, Mississauga, ON) and analyzed on a TaqMan® instrument (Perkin-Elmer, Foster City, CA) using specific DNA probes for murine *xiap*, *ciap1* and 2 and the TaqMan® EZ RT-PCR kit (Qiagen, Mississauga, ON).

### **Isolation of thymocyte sub-populations for quantitative mRNA analysis**

Thymocytes were isolated (*see above for details*) and  $10^6$  cells were placed in 0.5 ml of "staining and sorting" PBS medium (pH 7.4) (5 % FCS, 50  $\mu$ M,  $\beta$ -mercaptoethanol, 125 mM L-glutamine, penicillin, streptomycin and 0.01 % sodium azide) at 4 °C and stained with a combination of either  $\alpha$ -CD3-FITC,  $\alpha$ -CD44-PE and  $\alpha$ -CD25-Cy5 or  $\alpha$ -CD3-FITC,  $\alpha$ -CD4-PE and  $\alpha$ -CD8-Cy5 (Amersham Bioscience, Piscataway, New Jersey) for 20 min., protected from light. The samples were washed once with cold "staining and sorting" PBS medium and resuspended in 0.5 ml of the "staining and sorting" PBS media and placed on ice. The cells were sorted (DaykoCytomation, MoFlo® Sorter; Fort Collins, Colorado) into their main developmental stages, delineated by cell surface marker expression and placed at -80 °C.

### **Isolation of IAP mRNA from T lymphocytes exposed to T cell specific stimuli and apoptotic triggers**

T cells from wild-type C57Bl/6 mice (Charles River) were isolated purified (*see*

*above for details*), plated at  $10^5$  cells/ml and exposed to proliferative agents, plate-bound  $\alpha$ -CD3 antibody (10  $\mu$ g/ml) with and without free  $\alpha$ -CD28 antibody (0.02  $\mu$ g/ml), IL-7 (10 ng/ml) and to Fas mediated apoptotic death (plate-bound  $\alpha$ -Fas antibody, 10  $\mu$ g/ml) for 2 hours.

### **Isolation of IAP mRNA from thymocytes exposed to T cell specific stimuli and apoptotic triggers**

Cultured thymocytes from 3 wild-type C57Bl/6 mice (Charles River) were isolated (*see above for details*) plated at  $10^5$  cells/ml and exposed to concanavalin A (ConA) (2  $\mu$ g/ml), IL-7 (5 ng/ml), plate-bound  $\alpha$ -CD3 antibody (10  $\mu$ g/ml) with and without free  $\alpha$ -CD28 antibody (0.02  $\mu$ g/ml), exposed to Fas mediated apoptotic death (plate-bound  $\alpha$ -Fas antibody, 10  $\mu$ g/ml) and dexamethasone (100 nM).

### **Bio-statistical analysis**

The statistical analysis on the data presented in this thesis was done using GraphPad Prism and GraphPad InStat (GraphPad Software Inc. San Diego, CA, U.S.A.).

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# **Chapter 3: IAP mRNA levels during T cell maturation and during T cell and macrophage function**

*Portions of this chapter have been included in a manuscript to be submitted to a journal: IAP mRNA levels during T cell maturation and function. Conte, D., St-Jean, M. and Korneluk, R. G.*

## ***Introduction***

### **Apoptosis and the immune system**

The fundamental physiological process of apoptosis plays a crucial role in maintaining the homeostasis of the mammalian immune system. The innate status of T and B lymphoid cell numbers in mice and humans is to preserve a constant level, however, the normal daily operations of the hematopoietic system produces a constant stream of excess, unwanted lymphoid cells. Apoptosis is the mechanism responsible for the deletion of these superfluous cells and therefore the guardian responsible for maintaining the normal dynamic equilibrium of the immune system. The major sites of apoptotic influence occur throughout lymphoid maturation, during the deletion of dangerous carcinogenic or virally infected cells and during the waning of an antigen-induced immune response. Apoptosis is such a key element of the immune system that perturbations of the naturally occurring rate of cell death

autoimmunity or lymphocyte transformation.

## **Cell surface receptors mediate immune responses**

An adaptive immune response is a collection of interacting and proliferating cells, such as B cells, natural killer cells (NK), macrophages, antigen presenting cells (APC) and all their various subclasses, whose function is primarily orchestrated by the T lymphoid cell. T cells communicate with and mediate the immune function of these cells by the direct interaction of surface receptors with their cognate surface and soluble ligands and by secreting appropriate cytokines. Ensuring the proper formation of surface receptors during lymphopoiesis is therefore paramount for a comprehensive and functional immune response. Sites of lymphoid maturation include the bone marrow, the fetal liver and the main site of T cell lymphoid development, the thymus.

## **Historical perspective of the thymus**

“We shall come to regard the presence of lymphocytes in the thymus as an evolutionary accident of no very great significance.”

Nobel Laureate Sir Peter Medawar, 1963 (Miller 2001).

This succinctly sums up the widely held view, prior to the 1960s, that through evolution the thymus had become superfluous and only served as a depot for dying thymocytes. This reigning belief was based on mainly three observations: (1) immune response characteristics, such as germinal center formation and the occurrence of plasma cells, never formed in the thymus of

immunized animals; (2) unlike other lymphoid cells from antigen primed animals, thymus lymphocytes could not confer antigen-resistance when transplanted into appropriate recipients; and finally (3) thymectomized adult animals displayed no ill effects to both cellular and humoral responses compared to normal animals (Miller 2002). These observations were cited as evidence against an immunological function for the thymus. Contradictory data were first presented in 1961 when Dr. Jacques F. A. P. Miller observed that thymectomized newly born mice displayed poorly developed lymphoid tissues, a severely impaired immune system and multiple intercurrent infections, reminiscent to symptoms of present day patients with acquired immunodeficiency syndrome (AIDS) (Miller 1961). At that time it was well recognized that circulating lymphocytes were immunologically competent cells able to reject skin grafts. However, Dr. Miller's thymectomized neonate mice were tolerant of skin grafts from a foreign mouse strain, thereby suggesting that the thymus is necessary for the initiation and maintenance of a mammalian immune system. Subsequently, in 1962, Dr. Miller's group clearly demonstrated that the repopulation of lymphoid cells in the blood and tissues and immune function in irradiated mice was thymus-dependent (Miller 1962). Neonatally thymectomized mice or irradiated adult thymectomized mice were unable to generate functional lymphoid cells, however, seeding thymus tissue into these animals completely restored normal immune function and responses. Subsequently, in 1967, it was shown that thymocytes could respond to antigen by proliferating and giving rise to a progeny that enabled other lymphocytes, derived from bone marrow, to differentiate to antibody-

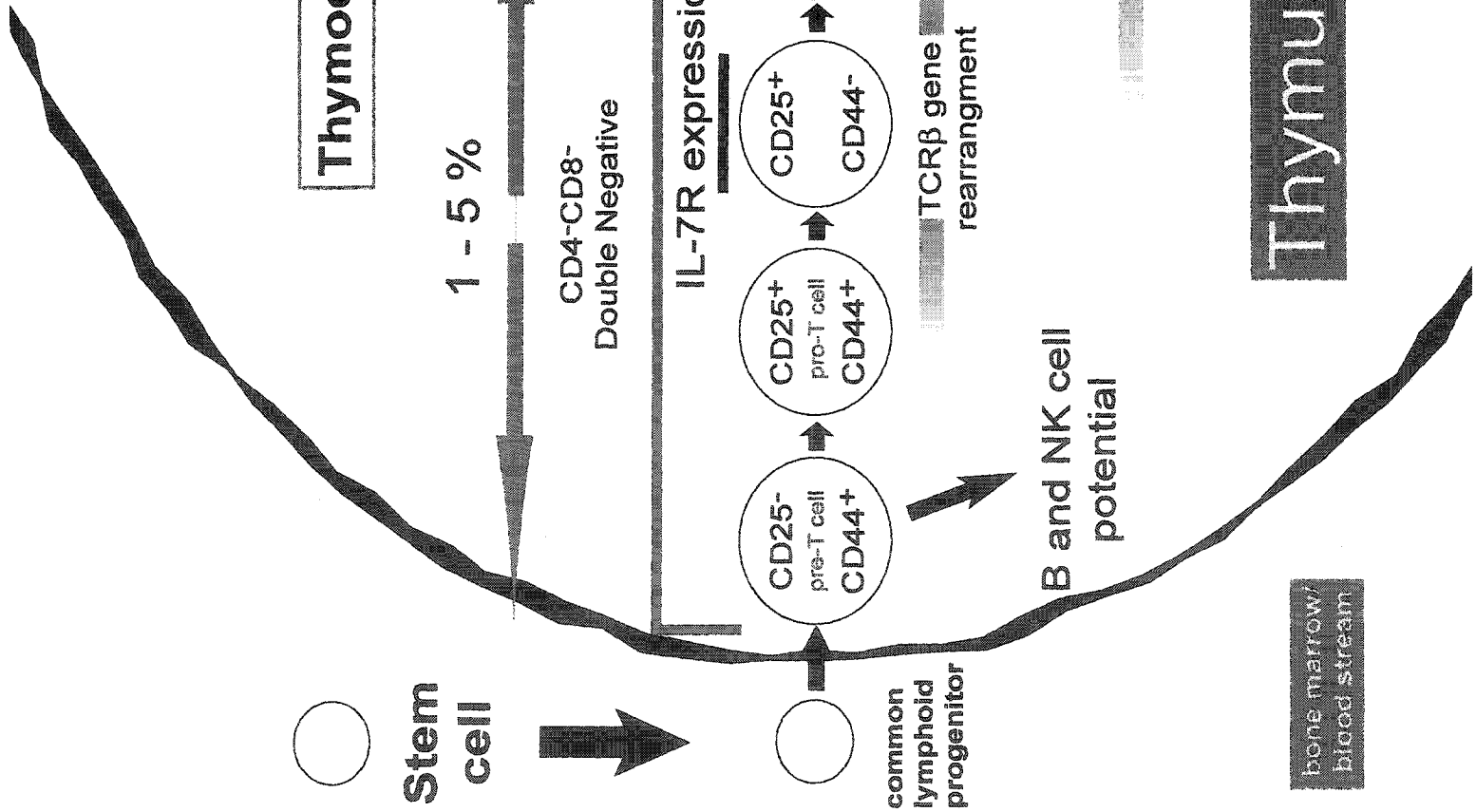
forming cells (Miller 1967). This was the first clear demonstration of the existence of two major interacting subsets of lymphocytes, subsequently named T cells, T for thymus-derived lymphocytes, and B cells, B for bone marrow-derived lymphocytes. Most importantly, this work forced a reassessment of many immunological processes, such as tolerance, memory and autoimmunity, thereby igniting an avalanche of work. These discoveries form the foundation of our present day view of the immune system.

### **T cell lymphopoiesis**

Today we know that T cell maturation and development resides within the thymus. Thymic lymphopoiesis serves to screen and "educate" pre-T cells in preparation for their "graduation" from the thymus to the periphery as competent and functional naïve single positive (SP) CD4<sup>+</sup> or CD8<sup>+</sup> T cells bearing the alpha-beta T cell receptor complex (TCR $\alpha\beta$ ) (Anderson, Moore et al. 1996). Apoptosis is responsible for eliminating thymocytes that fail an "examination", such as inappropriately formed or autoreactive T cells, while progressing past specific check points on their way to the periphery (Surh and Sprent 1994). The progress of thymocyte maturation is marked with discrete stages that are identified by expression of specific cell surface receptors. The initiation of T cell lymphopoiesis begins with the seeding of bone marrow- or fetal liver- derived pre-T lymphoid stem cells (CD4<sup>-</sup>, CD8<sup>-</sup>, CD3<sup>-</sup>, CD44<sup>+</sup>, CD25<sup>-</sup>) within the sub capsular region of the outer cortex of the thymus (Shortman and Wu 1996). At this point these cells maintain the potential to develop into B, NK and of course T cells (Figure 3-1), if placed in the

### **Figure 3-1. Intrathymic thymocyte development**

Thymocyte developmental stages are characterized by the expression of distinct cell-surface markers, including CD4, CD8, CD44, CD25, and especially the TCR. The interactions between thymocytes and thymic stromal cells mediate thymocyte differentiation that eventually results in the generation and exodus of self-tolerant SP CD4<sup>+</sup> helper T cells and CD8<sup>+</sup> cytotoxic T cells from the thymus to the periphery. The points of TCR $\beta$  and TCR $\alpha$  gene rearrangement are indicated. In addition, the time of active thymocyte positive and negative selection are shown.



**Thymocyte subpopulations (%)**

10 - 15 %

1 - 5 %

80 - 85 %

**Stem cell**

CD4-CD8-  
Double Negative

CD4+CD8+  
Double Positive

**IL-7R expression**

common lymphoid progenitor

CD25- pre-T cell CD44+

CD25+ pre-T cell CD44+

CD25- CD44-

CD25+ CD44-

B and NK cell potential

TCRβ gene rearrangement

TCRα gene rearrangement

TCRαβ gene rearrangement

4+ TCRhi

8+ TCRhi

Single Positive

Positive Selection

β Selection

Negative Selection

**Thymus**

bone marrow/  
blood stream

appropriate environment. Up-regulation of CD25 signals a shift from a hematopoietic pluripotent pre-T cell to a robust, proliferating, T cell-committed pro-T cell stage (Penit, Lucas et al. 1995). Concurrently, IL-7-receptor  $\alpha$  gene is also up-regulated and is necessary for recognition of an essential pro-survival, anti-apoptotic IL-7 cytokine signal (Hare, Jenkinson et al. 2000). Down-regulation of CD44, signals the start of the prudently economical V(D)J loci rearrangement, which leads to the formation and expression of the TCR $\beta$  chain (Hoffman, Passoni et al. 1996). This combinatorial heterogeneity is the foundation for the vast T and B lymphocyte antigen repertoire. Expression of the complete pre-TCR components follows. This complex includes TCR $\beta$ , CD3 and the pre-TCR $\alpha$  and expression marks the induction of CD4, CD8 and TCR $\alpha$  expression and the down-regulation of CD25 (Penit, Lucas et al. 1995). The thymocytes then migrate to the inner cortex of the thymus and develop into non-dividing double positive (DP) (CD4<sup>+</sup> and CD8<sup>+</sup>) T lymphocytes (Prockop and Petrie 2000). The final phase of maturation is completed with the migration of the thymocyte to the medulla and the down-regulation of either CD4<sup>+</sup> or CD8<sup>+</sup>, on a DP thymocyte, leading to the formation of a mature single positive (SP) (CD4<sup>-</sup>CD8<sup>+</sup> and CD4<sup>+</sup>CD8<sup>-</sup>) T cell, which is then exported to the periphery.

### **Apoptosis mediated T cell selection during lymphopoiesis**

Since the discovery in the 1980s that two physiological stimuli, glucocorticoids and T cell receptor (TCR) -engagement, induce apoptosis of thymocytes, it has been recognized that regulated cell death plays a crucial role in thymocyte

development and antigen-specific selection. Developing T cells have a rapid turnover whereupon approximately 97 % of the thymocytes produced daily are purged (Tough and Sprent 1994). Differentiating thymocytes undergo selection processes that are mediated by apoptosis during several stages of maturation. First, at the DN stage (CD44<sup>+</sup>CD25<sup>+</sup> stage), thymocytes that fail to express an early functional pre-T cell receptor V $\beta$  (TCR $\beta$ ), or later, the full TCR $\alpha\beta$ , are incapable of receiving a positive signal via the TCR, and therefore die via apoptosis, a process termed death by neglect (Starr, Jameson et al. 2003). Second, thymocytes with correctly rearranged TCR $\alpha\beta$  that cannot bind to self major histocompatibility complex molecules (MHC I/II) also die of neglect (Starr, Jameson et al. 2003). The process of classical positive selection is the rescue of cells bearing the full TCR $\alpha\beta$  with low-to-moderate avidity for self MHC from death by neglect. Last, high affinity interaction of TCR $\alpha\beta$  with MHC I/II proteins associated with self-peptide triggers apoptosis in a process known as negative selection, or clonal deletion. Negative selection within the thymus occurs during the immature thymocyte developmental stage, DP, and the early mature SP phases of growth (Figure 3-1). Only 3 % of the daily seeded thymocytes survive these apoptotic selection "check-points", to emerge from the thymus as either a naïve CD4<sup>+</sup> or CD8<sup>+</sup> T cell. Therefore, the tight regulation of apoptotic functions and processes of the thymic environment is crucial for the proper formation, function and homeostasis of the mammalian immune system.

## **The survival cytokine IL-7, the thymus, and naïve T cells.**

IL-7 is a non-redundant cytokine that is required for murine T and B lymphopoiesis, by promoting survival and proliferation (Kondo, Takeshita et al. 1994). IL-7 binds to a receptor composed of the  $\gamma$  common chain ( $\gamma$ C) complexed to a second chain known as the IL-7R $\alpha$  (Noguchi, Nakamura et al. 1993; Ziegler, Morella et al. 1995). The IL-7R $\alpha$  has been identified on immature B cells through the early pre-B stage, on thymocytes, and on most mature T cells (Armitage, Ziegler et al. 1991; Sudo, Nishikawa et al. 1993).

Initial *in vitro* experiments showed that IL-7 could enhance the viability of thymocytes independent of a proliferative effect (Watson, Morrissey et al. 1989; Suda and Zlotnik 1991). Subsequently, studies using IL-7-null mice showed a 20-fold decrease in the total number of thymocytes (Moore, von Freeden-Jeffry et al. 1996) and any thymocytes that survived to be exported to the periphery as mature naïve T cells were non-functional. Similarly, IL-7 was shown to promote survival of mature T cells by the inhibition of programmed cell death (Gringhuis, de Leij et al. 1997). Primarily, IL-7 promotes thymocyte and T cell survival through the up-regulation of protective apoptotic inhibitory genes, such as members of the Bcl superfamily of anti-apoptotic proteins (Akashi, Kondo et al. 1997; Maraskovsky, O'Reilly et al. 1997). Recently, it has been demonstrated that a member of the IAP family of anti-apoptotic proteins, cIAP2, was the key up-regulated protective factor responsible for the apoptotic resistance shown by IL-7-pretreated T cells that were exposed to the apoptotic trigger, dexamethasone (Sade and Sarin 2003).

Not surprisingly then, IL-7 is considered a crucial anti-apoptotic factor for T-cell and thymocyte viability.

### **Fas receptor, T cell homeostasis and thymocytes**

Fas is a cell surface "death receptor" and a member of the TNFR superfamily that is involved in transducing cellular death signals (Tartaglia, Ayres et al. 1993). Fas and its signaling proteins are highly expressed in DP thymocytes (Sharova, Dzutsev et al. 2001) and appropriately it was demonstrated that thymocytes are highly susceptible to Fas-induced death. Surprisingly, studies involving gene-deleted mice demonstrated that thymocyte-negative and positive selection were not Fas-dependent (Sharova, Dzutsev et al. 2001). However, Fas-null mice did have highly enlarged lymphoid glands that were filled with excess T cells (Watanabe-Fukunaga, Brannan et al. 1992). These studies suggested that in mature T cells, the Fas pathway is essential for the elimination of excess effector cells during immune responses as part of a negative feedback mechanism.

### **Glucocorticoids, thymocytes and T cell function**

Glucocorticoids mediate their biological effects by binding and releasing the glucocorticoid receptor (GR) from inhibitory heat shock proteins- (Hsp-) 70 and 90 (Kanelakis and Pratt 2003). Subsequently, the GR translocates to the nucleus to directly or indirectly suppress or activate gene transcription (Pratt, Sanchez et al. 1989). Glucocorticoids are potent inducers of apoptosis of lymphoid cells, such as T cells and especially DP thymocytes. However, the mechanism(s) of action by which glucocorticoids induce apoptosis is still

principally unknown. What is known is that glucocorticoid-elicited thymocyte apoptosis can be inhibited by over-expressing two members of the Bcl superfamily of apoptotic regulators, the anti-apoptotic proteins Bcl-2 or Bcl-x<sub>L</sub> (Chao, Linette et al. 1995). More recent work using thymocyte primary cultures showed that upon exposing thymocytes to the synthetic glucocorticoid dexamethasone, XIAP (Yang, Fang et al. 2000) was selectively eliminated prior to overt death. Interestingly, this same study showed that both Bcl-2 and Bcl-x<sub>L</sub> protein levels remained unchanged. Therefore, in the context of this paradigm XIAP seems to be the major apoptotic inhibitory factor, rather than Bcl-2 or Bcl-x<sub>L</sub>.

### **Investigating the potential role of the IAPs in modulating immune function**

It is well recognized that regulated cell death plays a crucial role in a variety of immune functions including T and B cell development, function, homeostasis, and in autoimmunity. At the start of my investigations we also knew that members of the IAP family, XIAP, cIAP1 and 2, were potent inhibitors of apoptosis. In particular, work done in the Korneluk laboratory demonstrated that adenovirus over-expression of IAPs protects a variety of tissue cell lines, including the human Jurkat T-lymphoid cell line, from a broad range of *in vitro* apoptotic triggers (LaCasse, Baird et al. 1998). In addition, work previously done in this same laboratory uncovered that the human thymus, spleen and macrophages of the peritoneal cavity expressed high levels of *xiap*, *ciap1* and 2 mRNA. This work suggested a potential role, either direct or indirect, for the

IAPs in normal immune function. Therefore, I "naively" decided to investigate the possible link between IAPs and immune function.

**Resources available at the start of my studies:**

Prepared "in house":

$\alpha$ -XIAP antibody (specificity: mouse and human)

$\alpha$ -cIAP1 antibody (specificity: human)

$\alpha$ -cIAP2 antibody (specificity: human)

XIAP, cIAP1 and cIAP2 adenovirus expression systems

Primers for quantitative RT-PCR of human and mouse mRNA for *xiap*, *ciap1* and *ciap2*

**Results**

**Relative mRNA levels of IAPs of T lymphoid cells exposed to immune-specific stimuli, apoptotic triggers and during T lymphopoiesis.**

**Relative levels of *xiap*, *ciap1* and 2 during T cell maturation**

Clearly, apoptotic susceptibility varies according to the stages of thymocyte development and T cell function suggesting tight regulation of anti-apoptotic proteins. Here the possibility that regulated murine *iap* mRNA and therefore IAP protein expression may determine apoptotic susceptibility of distinct lymphocyte populations was investigated. Therefore, as a starting point it seemed appropriate to do a general survey of *iap* mRNA levels, with the goal to ascertain possible investigative avenues, pending results. Thymus-derived

immature T cells from 3 wild-type C57Bl/6 mice (Charles River) were stained with the appropriate antibodies (Amersham Bioscience, Piscataway, New Jersey) and sorted (DaykoCytomation, MoFlo® Sorter; Fort Collins, Colorado) into their main developmental stages, delineated by cell surface marker expression (CD44<sup>+</sup>CD25<sup>-</sup>, CD44<sup>+</sup>CD25<sup>+</sup>, CD44<sup>-</sup>CD25<sup>+</sup>, CD44<sup>-</sup>CD25<sup>-</sup>, CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>-</sup>, CD8<sup>+</sup>CD4<sup>-</sup>). Relative levels of message for both *xiap* (Figure 3-2B) and *ciap1* (Figure 3-2A) were found to be similar throughout the different stages of thymopoiesis. Interestingly, *ciap2* mRNA levels (Figure 3-2C) drop off ( $p < 0.05$ ) between the transition from a pre-T cell (CD44<sup>+</sup>CD25<sup>-</sup>) to a T cell-committed pro-T cell (CD44<sup>+</sup>CD25<sup>+</sup>) (see Figure 3-1), but increase ( $p < 0.05$ ) once more when the whole thymocyte population is cultured for at least one hour.

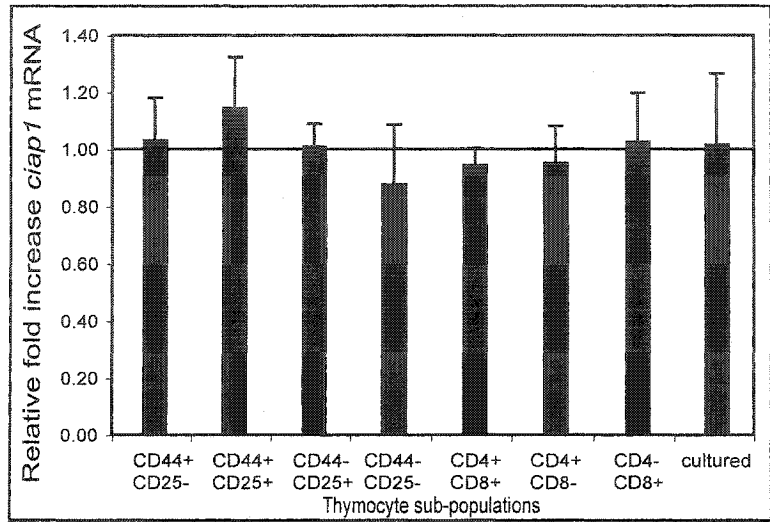
#### **IAP mRNA levels of T lymphocytes exposed to T cell specific stimuli and apoptotic triggers**

Isolated splenic T cells from 3 wild-type C57Bl/6 mice (Charles River) were cultured for 4 hours and exposed to the proliferative agents, plate-bound  $\alpha$ -CD3 antibody (10  $\mu$ g/ml) with and without free  $\alpha$ -CD28 antibody (0.02 mg/ml). The combined CD3 and CD28 cross-linking mimics physiological dual-signal T cell activation and proliferation. T cells were also exposed to the T cell pro-survival cytokine, IL-7 (10 ng/ml), to Fas mediated apoptotic death (plate-bound  $\alpha$ -Fas antibody, 10  $\mu$ g/ml) and dexamethasone (100 nM). The appropriate concentrations were all pre-determined experimentally (data not shown).

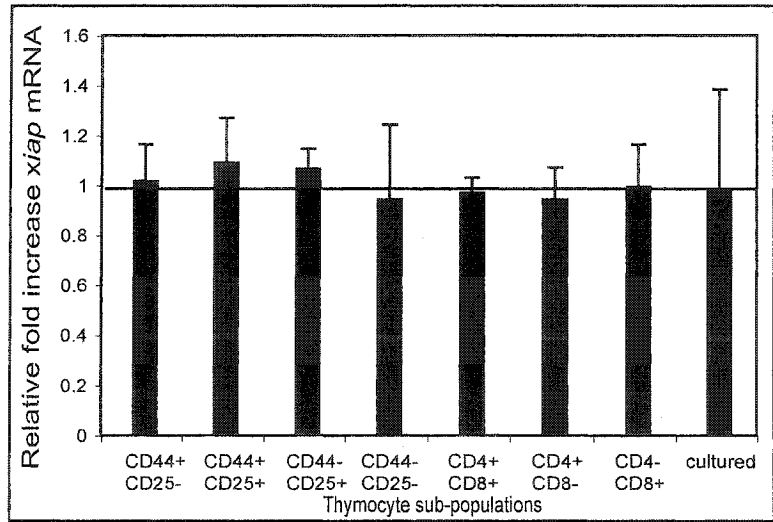
**Figure 3-2. Relative mRNA levels of *xiap*, *ciap1* and *2* during T cell maturation**

Thymocytes from 3 wild-type C57Bl/6 mice (Charles River) (the results are the mean of triplicate experiments, and the error bars represent standard deviation) were stained with a combination of either  $\alpha$ -CD3-FITC,  $\alpha$ -CD44-PE and  $\alpha$ -CD25-Cy5 or  $\alpha$ -CD3-FITC,  $\alpha$ -CD4-PE and  $\alpha$ -CD8-Cy5 (Amersham Bioscience, Piscataway, New Jersey) and sorted (DaycoCytomation, MoFlo® Sorter; Fort Collins, Colorado) into their main developmental stages, delineated by cell surface marker expression. The columns represent the mRNA levels relative to each thymocyte sub-population for each IAP. Relative (A) *ciap1*, (B) *xiap* and (C) *ciap2* mRNA levels for the major thymocyte sub-populations.

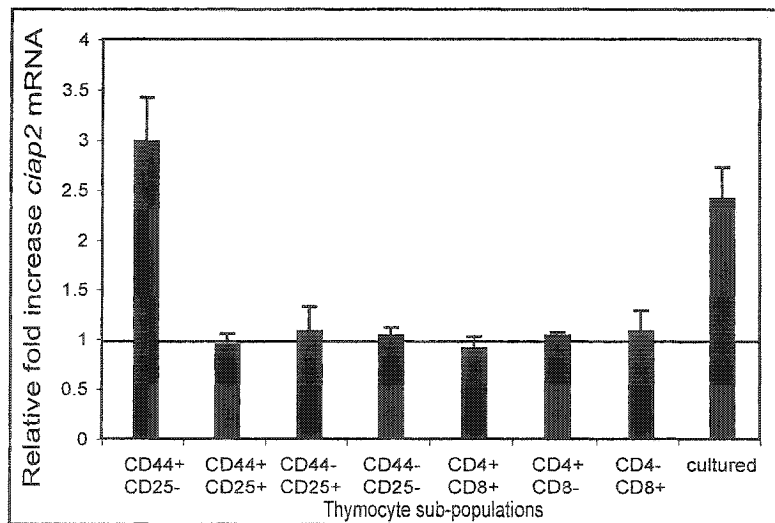
A



B



C



At 2 hours after treatment the *xiap* mRNA levels remain relatively unchanged compared to untreated controls (Figure 3-3C). However, *ciap1* levels show a profound drop upon dual-signal activation, exposure to IL-7 and Fas-induced death (Figure 3-3B,  $p < 0.01$ ). Interestingly, in contrast to the other IAPs and treatments, *ciap2* levels go up upon exposing T cells to IL-7 (Figure 3-3A,  $p < 0.05$ ).

### **IAP mRNA levels of thymocytes exposed to T cell specific stimuli and apoptotic triggers**

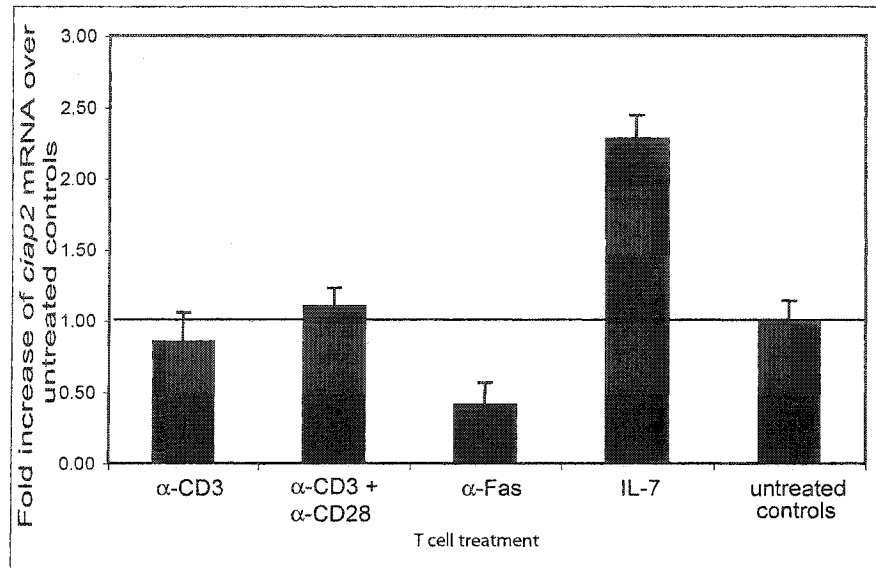
Thymocytes from 3 wild-type C57Bl/6 mice (Charles River) were cultured for 2 hours and subsequently exposed to the proliferative agent concanavalin A (ConA) (2  $\mu\text{g/ml}$ ) and the survival/proliferative cytokine IL-7 (5  $\text{ng/ml}$ ) for two hours. In contrast to mature T cells, plate-bound  $\alpha\text{-CD3}$  antibody (10  $\mu\text{g/ml}$ ) for thymocytes is an apoptotic trigger, moreover, the combined CD3 and CD28 cross-linking increases cell death. Thymocytes were also exposed to Fas mediated apoptotic death (plate-bound  $\alpha\text{-Fas}$  antibody, 10  $\mu\text{g/ml}$ ) and dexamethasone (100 nM). The appropriate concentrations were all pre-determined experimentally (data not shown).

The *ciap2* mRNA levels remained relatively unchanged when exposed to the stimuli compared to untreated controls (Figure 3-4A). However, *ciap1* (Figure 3-4B,  $p < 0.05$ ) and especially *xiap* (Figure 3-4C,  $p < 0.01$ ) levels showed a profound drop upon exposure to dexamethasone-induced death.

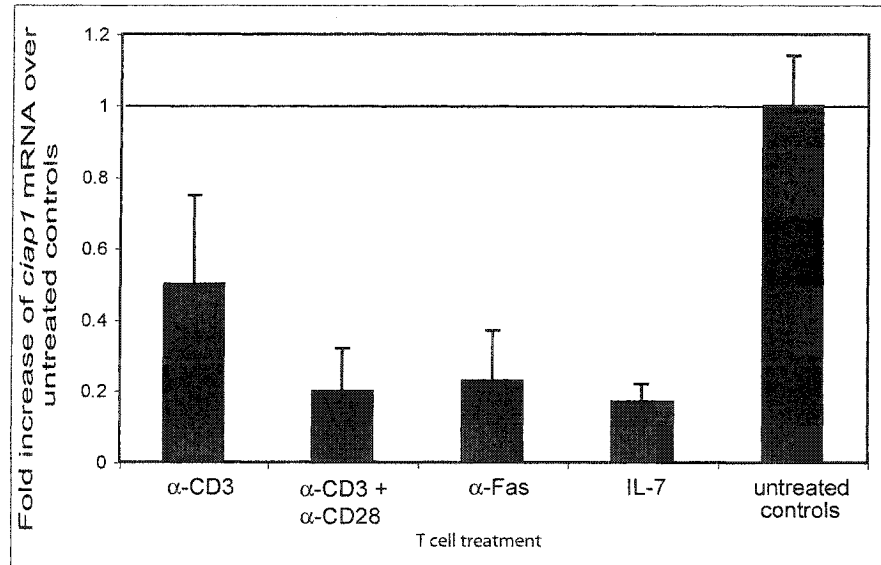
**Figure 3-3. IAP mRNA levels of T lymphocytes exposed to T cell specific stimuli and apoptotic triggers**

Purified T cells from 3 wild-type C57Bl/6 mice (Charles River) (the results are the mean of triplicate experiments, and the error bars represent standard deviation) were exposed to the proliferative agents, plate-bound  $\alpha$ -CD3 antibody (10  $\mu$ g/ml) with and without free  $\alpha$ -CD28 antibody (0.02  $\mu$ g/ml). T cells were also exposed to the T cell pro-survival cytokine, IL-7 (10 ng/ml) and to Fas mediated apoptotic death (plate-bound  $\alpha$ -Fas antibody, 10  $\mu$ g/ml). (A) *ciap2*, (B) *ciap1* and (C) *xiap* mRNA levels relative to untreated controls.

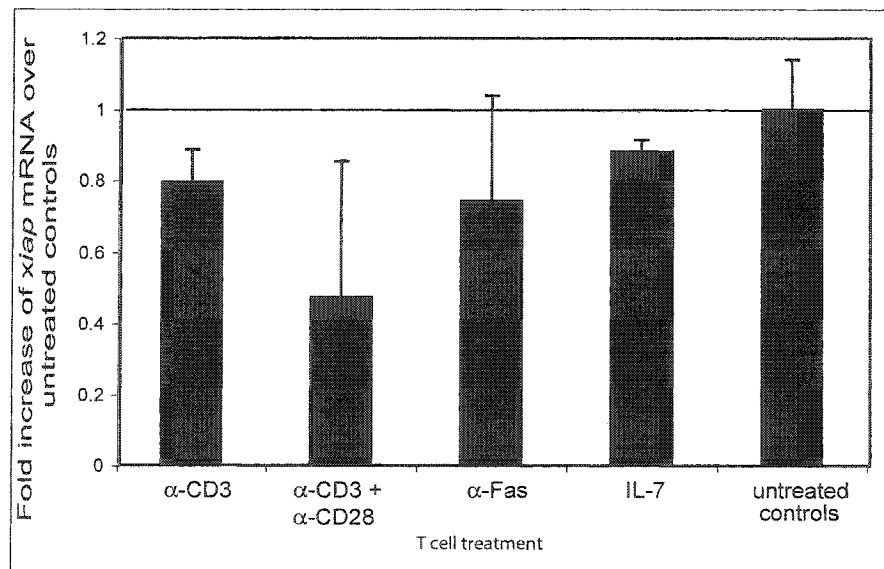
A



B



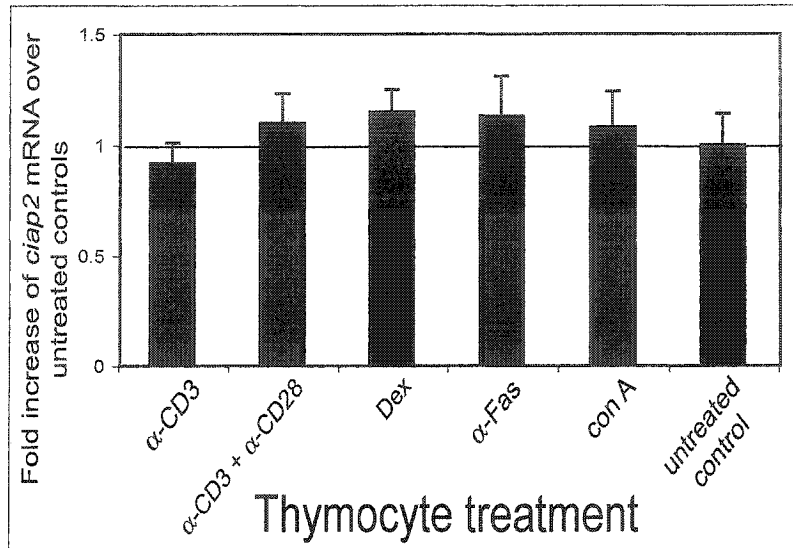
C



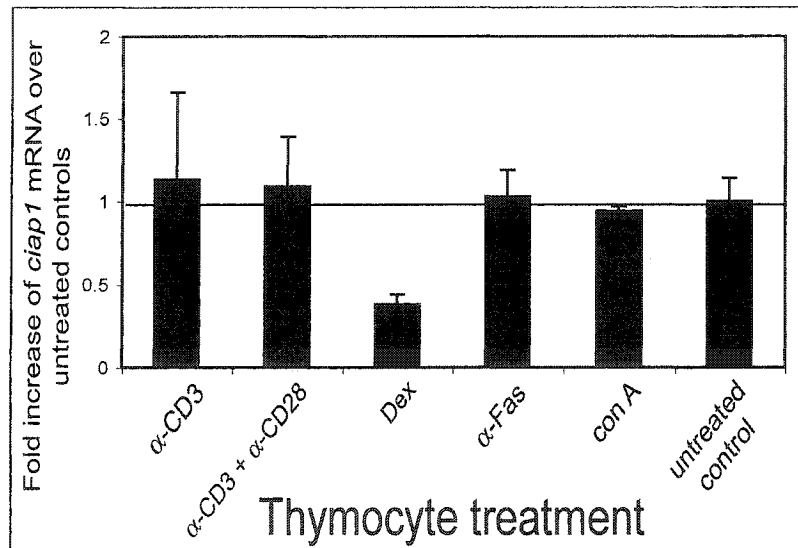
**Figure 3-4. IAP mRNA levels of thymocytes exposed to T cell specific stimuli and apoptotic triggers**

Cultured thymocytes from 3 wild-type C57Bl/6 mice (Charles River) (the results are the mean of triplicate experiments, and the error bars represent standard deviation) were exposed to concanavalin A (ConA) (2  $\mu$ g/ml), IL-7 (5 ng/ml), plate-bound  $\alpha$ -CD3 antibody (10  $\mu$ g/ml) with and without free  $\alpha$ -CD28 antibody (0.02  $\mu$ g/ml), exposed to Fas mediated apoptotic death (plate-bound  $\alpha$ -Fas antibody, 10  $\mu$ g/ml) and dexamethasone (100 nM). (A) *ciap2*, (B) *ciap1* and (C) *xiap* mRNA levels relative to untreated controls.

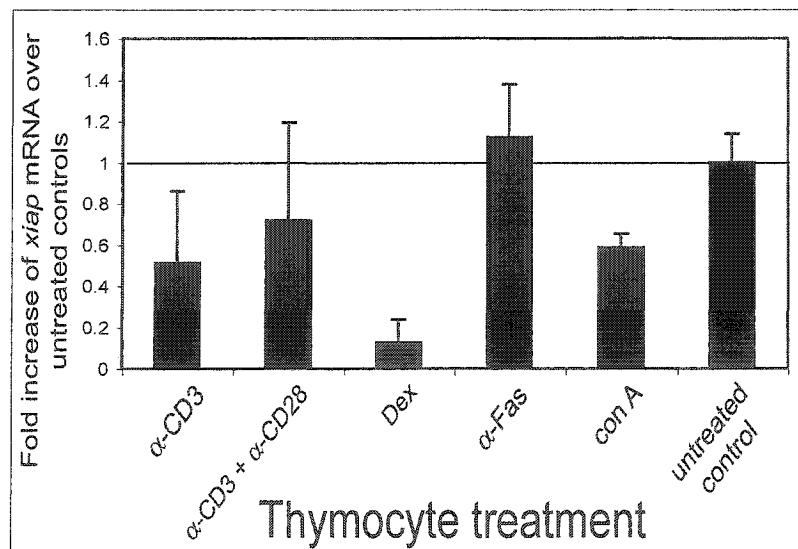
A



B



C



### **Murine XIAP protein levels in thymocyte sub-populations and T cells**

The endogenous murine levels of thymus *xiap* mRNA were observed to be relatively similar between the different thymocyte sub-populations (Figure 3-2B). Therefore, the next stage was to ascertain and compare these values to protein levels. As seen in Figure 3-5A, murine XIAP protein was expressed ubiquitously throughout the total thymocyte and T cell sub-populations. This result was not unexpected, since it was previously demonstrated that relatively high levels of XIAP mRNA were found in all human tissues examined, including the thymus. Interestingly, *xiap* mRNA was shown to be severely depressed upon thymocyte exposure to dexamethasone, suggesting a possible link between the loss of XIAP and thymocyte apoptotic susceptibility. To investigate this potential association a time-course experiment comparing murine XIAP levels of cultured thymocytes to thymocyte susceptibility when exposed to dexamethasone (100 nM), was performed (unsorted thymocytes were cultured for 2 hours prior to exposure to dexamethasone). As seen in Figure 3-5B and C overt thymocyte death was not observed (~4 hours, Figure 3-5C) until after the depletion of XIAP protein (~2 hours, Figure 3-5B). These results suggest that the high susceptibility of cultured thymocytes to dexamethasone-induced apoptosis may be the result of the rapid elimination of murine XIAP protein prior to apoptotic death in combination with the inability to replenish the protein through the removal of *xiap* mRNA (Figure 3-4C).

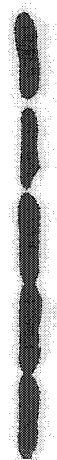
**Figure 3-5. Murine XIAP protein levels in thymocyte sub-populations and T cells**

(A) XIAP protein levels of thymocyte (DP, SP CD4/CD8) and T cell (CD4 and CD8) sub-populations. (B) Murine XIAP levels of whole thymus cultured thymocytes exposed to dexamethasone (100 nM). (C) Thymocyte viability over time upon dexamethasone (100 nM) exposure (the results are the mean of triplicate experiments, and the error bars represent standard deviation).

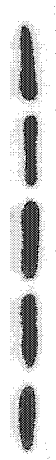
**A**

Thymus    Spleen

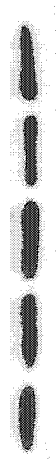
DP   CD4   CD8   CD4   CD8



murine  
XIAP



actin



**B**

Dexamethasone    Untreated

murine  
XIAP



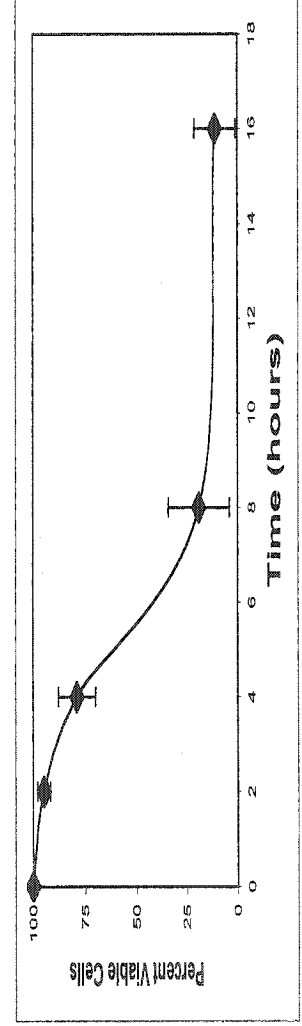
actin



0   1   2   16   16

Time (hours)

**C**

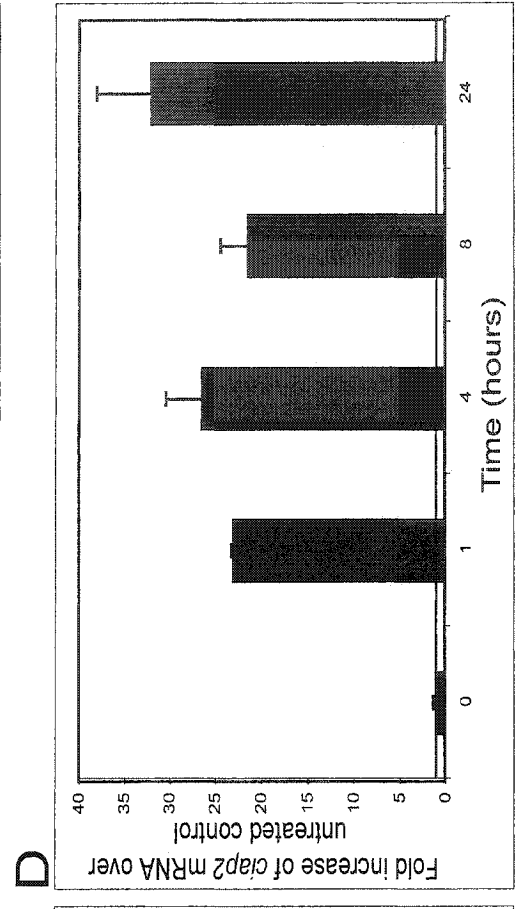
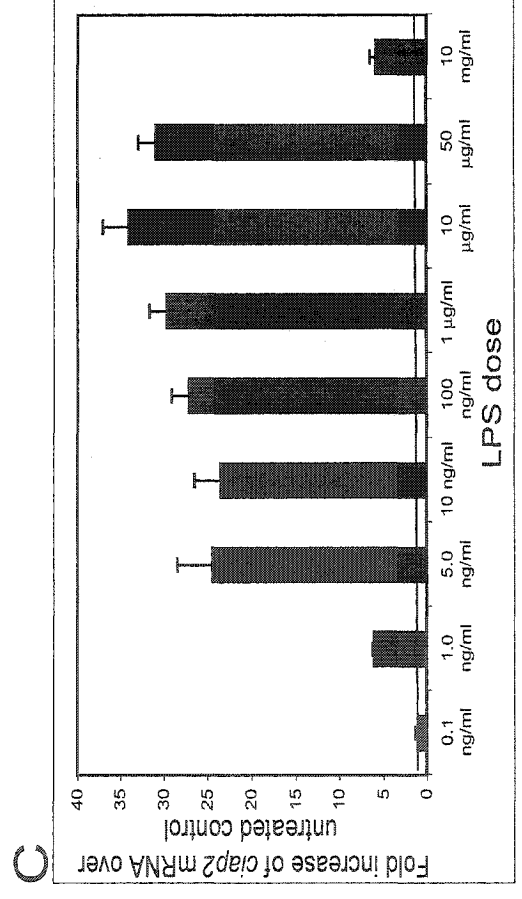
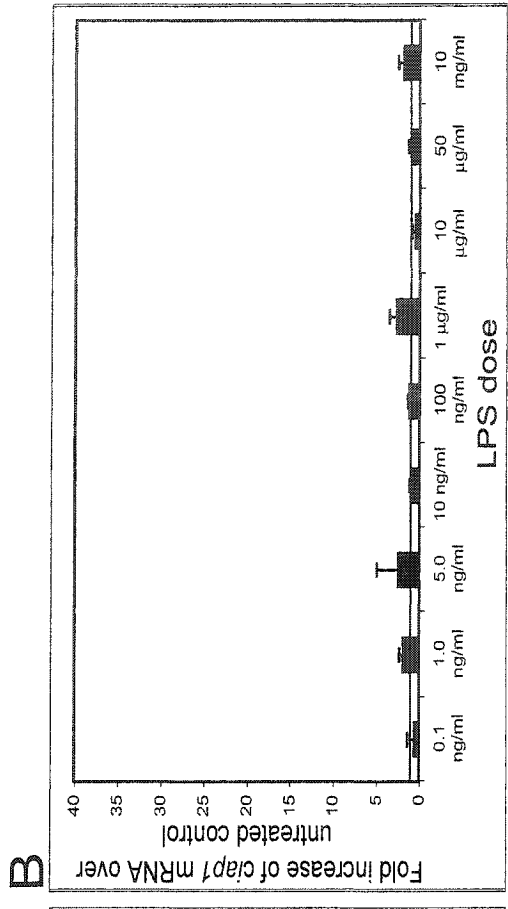
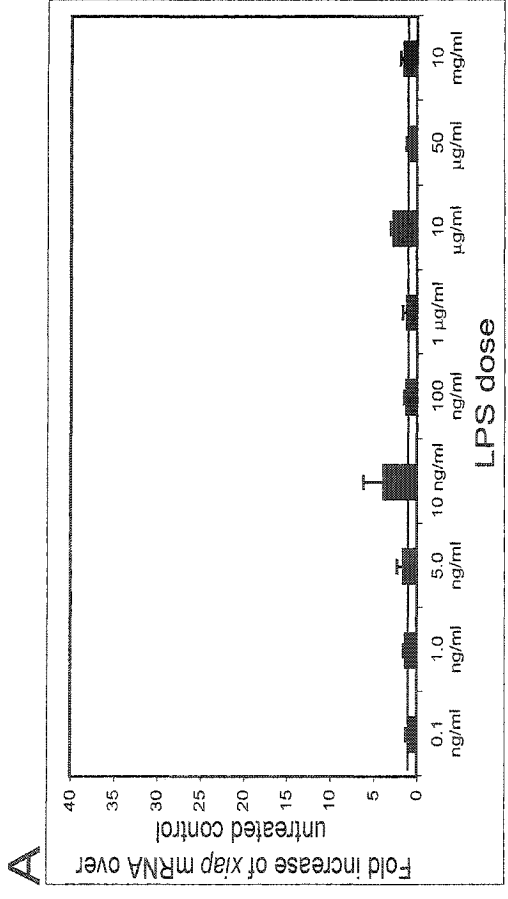


## **IAP mRNA levels of macrophages exposed to bacterial endotoxin**

The immune system can be largely functionally classified under two cooperative, yet distinct branches: adaptive and innate immunity. The T cell is the central adaptive immune system regulator in mounting a humoral and cellular response to a specific pathogen. The macrophage is one of the key mediators in sensing pathogens, mounting an innate immune response and also in shaping the adaptive response (*see Chapter 4 for details*). Lipopolysaccharide (LPS), the biologically active element of the bacterial Gram-negative membrane-component endotoxin, is the archetypal antigen specifically recognized by macrophages. LPS-induced activation of macrophages causes the up-regulation of a multitude of genes, including the production and release of inflammatory mediators, the up-regulation of cell surface receptors and that of cell survival proteins. Therefore, the *iap* mRNA levels of LPS-treated macrophages, derived from the peritoneal cavity, relative to untreated controls, were assayed. Peritoneal macrophages were cultured at  $10^5$  cell/well (96 well, flat bottom plate) and exposed to varying concentrations of LPS for 18 hours. As seen in Figure 3-6A and B, *xiap* and *ciap1* message levels remained relatively unchanged over the range of LPS doses (0.1 ng/ml to 10 mg/ml). In contrast, *ciap2* levels increased dramatically, up to 30 fold above untreated controls (Figure 3-6C) (p value

**Figure 3-6. IAP mRNA levels of macrophages exposed to bacterial endotoxin**

The *iap* mRNA levels of LPS-treated macrophages, derived from the peritoneal cavity of 6 C57Bl/6 mice (Charles River) (the results are the mean of triplicate experiments, and the error bars represent standard deviation), relative to untreated controls were assayed. (A) *xiap* (B) *ciap1* and (C) *ciap2* message levels of macrophages exposed to a range of LPS doses (0.1 ng/ml to 10 mg/ml, 18 hours) relative to untreated controls. (D) *ciap2* mRNA message of macrophages exposed to LPS (10  $\mu$ g/ml) was assayed over a 24 hour period.



was < 0.01 for all LPS doses). The innate immune system provides protection within the first minutes to hours after a pathogenic challenge. Therefore, a LPS-*ciap2* mRNA time-course assay was performed (10  $\mu$ g/ml LPS). As seen in Figure 3-6D, *ciap2* mRNA message is substantially up-regulated, > 20 fold above untreated controls, after only 1 hour and this increased level was maintained for 24 hours. A macrophage functions within an inherently hostile environment. An inflammatory response produces nitric oxide (NO), reactive oxxygen intermediates (ROI) and the up-regulation of Fas ligand on immune-regulating lymphocytes, all of which are detrimental to both invading pathogens and resident cells. Therefore, this data suggests the possibility that the up-regulation of a survival protein, such as cIAP2, may be crucial for macrophage survival and function.

## ***Discussion***

IAPs are potent inhibitors of apoptosis and provide protection against a wide range of death stimuli. At the onset of my studies this statement in effect encapsulated the total knowledge base of what was known about the IAPs. Understanding of the mechanisms of action or functions was still in its infancy. Concurrently, it had been clearly demonstrated that controlled cell death played a central role in all aspects of immune functions. Therefore, the "naïve" hypothesis: that there may be a potential role, either direct or indirect, for the IAPs in normal immune function, was formulated. As a starting point the assumption that murine *iap* mRNA and therefore IAP protein expression may

determine apoptotic susceptibility of distinct immune populations was investigated. Figure 3-5A shows that XIAP is ubiquitously expressed in all T lymphoid cells and that the levels of protein expression are largely equivalent in each sub-population (DP, SP CD4/8, CD4/8). Therefore, the relatively equivalent levels of *xiap* message seen in the thymocyte sub-populations (Figure 3-2B), suggests that in the case of XIAP there is a direct correlation between mRNA and protein levels. The summarized thymocyte *xiap* mRNA data in Table 3-1 shows a general decrease in *xiap* message levels in response to T cell-specific and general stimuli, suggesting a role for XIAP in determining thymocyte sensitivity. In contrast, the *xiap* mRNA levels in T cells treated with a variety of T cell-specific and general stimuli are relatively unchanged, suggesting that in the context of the experiments done XIAP does not play a prominent role in peripheral T cell function. In addition, as seen in Figures 3-5B and C, these data suggest that there is a requirement for the removal of XIAP prior to any observed thymocyte death in response to dexamethasone-induced apoptosis. These results suggest that XIAP expression levels are ubiquitous and relatively equivalent in all thymocyte sub-populations. Given that apoptosis is a key feature of thymopoiesis these results suggest the following hypothesis: XIAP expression levels regulate thymocyte sensitivity to apoptotic triggers and that removal of XIAP is crucial for normal thymocyte development. As an objective, it would seem that the best course of action to study thymocyte-XIAP dependence would be to disrupt the orderly removal of thymocyte-XIAP in response to apoptotic stimuli. Therefore, it was decided to

**Table 3-1. Summary of IAP mRNA levels of thymocytes exposed to T cell specific stimuli and apoptotic triggers**

Treatment	IAP mRNA levels (fold increase over untreated controls)		
	cIAP2	cIAP1	XIAP
$\alpha$ -CD3 (10 $\mu$ g/ml)	n/c	n/c	-
$\alpha$ -CD3 + $\alpha$ -CD28 (10 $\mu$ g/ml, 0.2 $\mu$ g/ml)	n/c	- - -	-
Dexamethasone (100 nM)	n/c	- - -	- - -
$\alpha$ -Fas (10 $\mu$ g/ml)	n/c	n/c	n/c
Con A (2 $\mu$ g/ml)	n/c	n/c	-

T cells ( $2 \times 10^4$ /well, 96 well plate) isolated from 3 C57Bl/6 mice (4-6 weeks of age) were exposed to the indicated agents at the indicated doses. The grading system is as follows: + small, ++ moderate, or +++ high fold increase of IAP mRNA versus untreated controls; - small, - - moderate, or - - - high fold decrease of IAP mRNA versus untreated controls; n/c represents little to no significant change in mRNA levels

try to disrupt thymocyte apoptotic sensitivity and development by engineering a transgenic mouse that specifically over expressed XIAP in the thymus (see Chapter 5).

The levels of *ciap2* mRNA during the transition from a pre-T to a pro-T thymocyte are seen to drop (Figure 3-2C) and increase once again upon the culturing of thymocytes. However, as seen in Tables 3-1 and 3-2, *ciap2* message levels are largely unchanged for thymocytes and T cells treated with a variety of agents. The one significant difference was the moderate increase of *ciap2* mRNA message upon treating T cells with IL-7. This was unlike the mRNA of the other two IAPs where their up-regulation was never observed, under any conditions used. Interestingly, LPS-induced activation of peritoneal macrophages caused a dramatic increase of *ciap2* mRNA levels (Figure 3-6C) and yet, no significant change was observed with the other two *iap* message levels (Figures 3-6A and B). In contrast to XIAP, where a change in the endogenous apoptotic state is dependent on the removal of XIAP and to the reduction of available mRNA, cIAP2 function seems to be dependent on up-regulating message and subsequently resulting in increased protein levels. Given the accumulated data of *ciap2* mRNA levels during thymocyte development (Figure 3-2C), treatment of thymocytes (Table 3-2) and T cells (Table 3-1) with a variety of stimuli including especially the exposure of macrophages to LPS (Figure 3-6C) suggest the following hypothesis: apoptotic susceptibility of macrophages and T cells may be altered by a rapid induction of the *ciap2* gene. Therefore, it was decided that the objective would be to try

**Table 3-2. Summary of IAP mRNA levels of T lymphocytes exposed to T cell specific stimuli and apoptotic triggers**

IAP mRNA levels (fold increase over untreated controls)			
Treatment	CIAP2	CIAP1	XIAP
$\alpha$ -CD3 (10 $\mu$ g/ml)	n/c	-	n/c
$\alpha$ -CD3 + $\alpha$ -CD28 (10 $\mu$ g/ml, 0.2 $\mu$ g/ml)	n/c	- - -	-
IL-7 (10 ng/ml)	+ +	- - -	n/c
$\alpha$ -Fas (10 $\mu$ g/ml)	n/c	- -	n/c

T cells ( $2 \times 10^4$ /well, 96 well plate) isolated from 3 C57Bl/6 mice (4-6 weeks of age) were exposed to the indicated agents at the indicated doses. The grading system is as follows: + small, ++ moderate, or +++ high fold increase of IAP mRNA versus untreated controls; - small, - - moderate, or - - - high fold decrease of IAP mRNA versus untreated controls; n/c represents little to no significant change in mRNA levels

to disrupt thymocyte apoptotic sensitivity, development (see *Chapter 5*) and macrophage LPS-induced function by engineering a *ciap2* gene-ablated mouse (see *Chapter 4*).

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## **Chapter 4: A deficiency of NF- $\kappa$ B-inducible cIAP2 causes a severe impairment of LPS-induced endotoxic shock and the pro-inflammatory response.**

*Portions of this chapter have been included in a paper submitted to a journal: A deficiency of NF- $\kappa$ B-inducible cIAP2 causes a severe impairment of LPS-induced endotoxic shock. Conte, D., Holcik, M. Lacasse, E., Wright, K. E. and R. G. Korneluk*

### ***Introduction***

#### **Sepsis: systemic inflammatory response syndrome (SIRS)**

Despite significant advances in the understanding, treatment and diagnosis of the sepsis cascade, unacceptable morbidity and mortality rates continue due to severe sepsis and septic shock (Martin, Mannino et al. 2003). Sepsis develops as a host response to infection and has been defined as a generalized inflammation response of the entire organism that often manifests itself as the systemic inflammatory response syndrome (SIRS) (Fry, Pearlstein et al. 1980) and can be sub-classified as severe sepsis when patients manifest organ failure. The onset of sepsis is characterized by fever or hypothermia and usually followed by tachycardia and tachypnea. The most common mechanism

by which tissues are damaged during SIRS is probably due to systemic vascular endothelial injury and microthrombosis. This tissue damage ultimately results in myocardial dysfunction, acute renal failure, adult respiratory distress syndrome, hepatic failure, disseminated intravascular coagulation and finally multiple organ failure (MOF) (Beal and Cerra 1994).

### **Pathogenesis of sepsis**

The pathogenesis of septic shock arises from an imbalance of the normal interplay between a host immune system and bacterial components. Normally, an inflammatory response is beneficial in controlling invading pathogens and in clearing debris. The systemic activation of endothelial cells and macrophages predominately by lipopolysaccharide (LPS), which is the biologically active element of the bacterial Gram-negative membrane-component endotoxin, can, however, induce a hyper-inflammatory response resulting in pathogenic endotoxic shock (Sriskandan and Cohen 1995). Under normal conditions LPS and other bacterial constituents stimulate a response that involves an innate and adaptive immune response generating competing pro- and anti-inflammatory mediators such as: cytokines, coagulation factors, adhesion molecules and heat shock proteins (Dofferhoff, Bom et al. 1992; Gutierrez-Ramos and Bluethmann 1997; Klosterhalfen, Hauptmann et al. 1997). Sepsis can cause a disruption of this balance that manifests itself as an extreme variation in the relative production of mediators. Therefore, the pathogenesis of septic shock can be defined as a disequilibrium syndrome of pro- and anti-inflammatory mediators (Pinsky 2001).

## **The role of LPS and other bacterial components in sepsis**

Paradoxically, the chief bacterial agent responsible for the septic cascade, LPS (Baumgartner, Glauser et al. 1985), is often liberated into the blood stream after bacterial lysis by antibiotic treatment (van Langevelde, Kwappenberg et al. 1998).

LPS can be divided into three structural domains that include: O-antigen specific sugar chain, core region and the membrane anchor lipid A component. The biological activity and hence the interaction of LPS with mammalian cells is mediated by a specialized hepatocyte-generated protein, the LPS-binding protein (LBP) (Figure 4-1) (Schumann, Leong et al. 1990). The high affinity LPS-LBP binding complex is able to specifically ligate to mammalian cells coated with the CD14 receptor. CD14 expression is restricted predominately to monocytes/macrophages (Haziot, Chen et al. 1988), and neutrophils (Haziot, Tsuberi et al. 1993), but to a lower extent. CD14 contains 10 copies of a leucine-rich repeat (LRR) motif and 7 of 10 LRRs can be deleted without affecting LPS binding (Juan, Hailman et al. 1995). In addition, mapping studies showed that additional residues other than those of the LRR region of CD14 are necessary for LPS binding (Juan, Hailman et al. 1995). Despite the lack of a signaling transmembrane and intracellular domain of CD14, mouse CD14 ablation studies clearly demonstrated the essential role of CD14 in mediating the biological activity of LPS (Haziot, Ferrero et al. 1996). The presence of CD14 lowers the concentration of LPS needed to activate a macrophage by a factor of 100 – 1000 compared to LPS alone. Endothelial cells are also activated by LPS, however whether this interaction is CD14-

dependent is still not clear (Pugin, Schurer-Maly et al. 1993; Jersmann, Hii et al. 2001).

Other bacterial components derived from Gram-positive bacteria, which lack LPS, have recently been shown to be potent inducers of SIRS. The bacterial components peptidoglycan and lipoteichoic acid (LTA) were shown to synergistically induce septic shock and MOF (De Kimpe, Kengatharan et al. 1995). Interestingly, the biological activity of LTA was also dependent on LBP and CD14 (Fan, Stelter et al. 1999).

### **Pathogen-associated molecular pattern (PAMP) receptors**

Receptors that recognized LPS and other highly conserved pathogenic components are termed pathogen-associated molecular pattern (PAMP) receptors. While each bacterial product, such as LPS, peptidoglycan and LTA, has several strain- and species-specific variations, it always has a highly conserved and invariant core structure among microbes, which is recognized by the PAMP receptors of the innate immune cells (Janeway 1989). In the case of LPS the lipid A portion represents the invariant pattern and is the biologically active region, while the variable O-antigen portion differs from species to species.

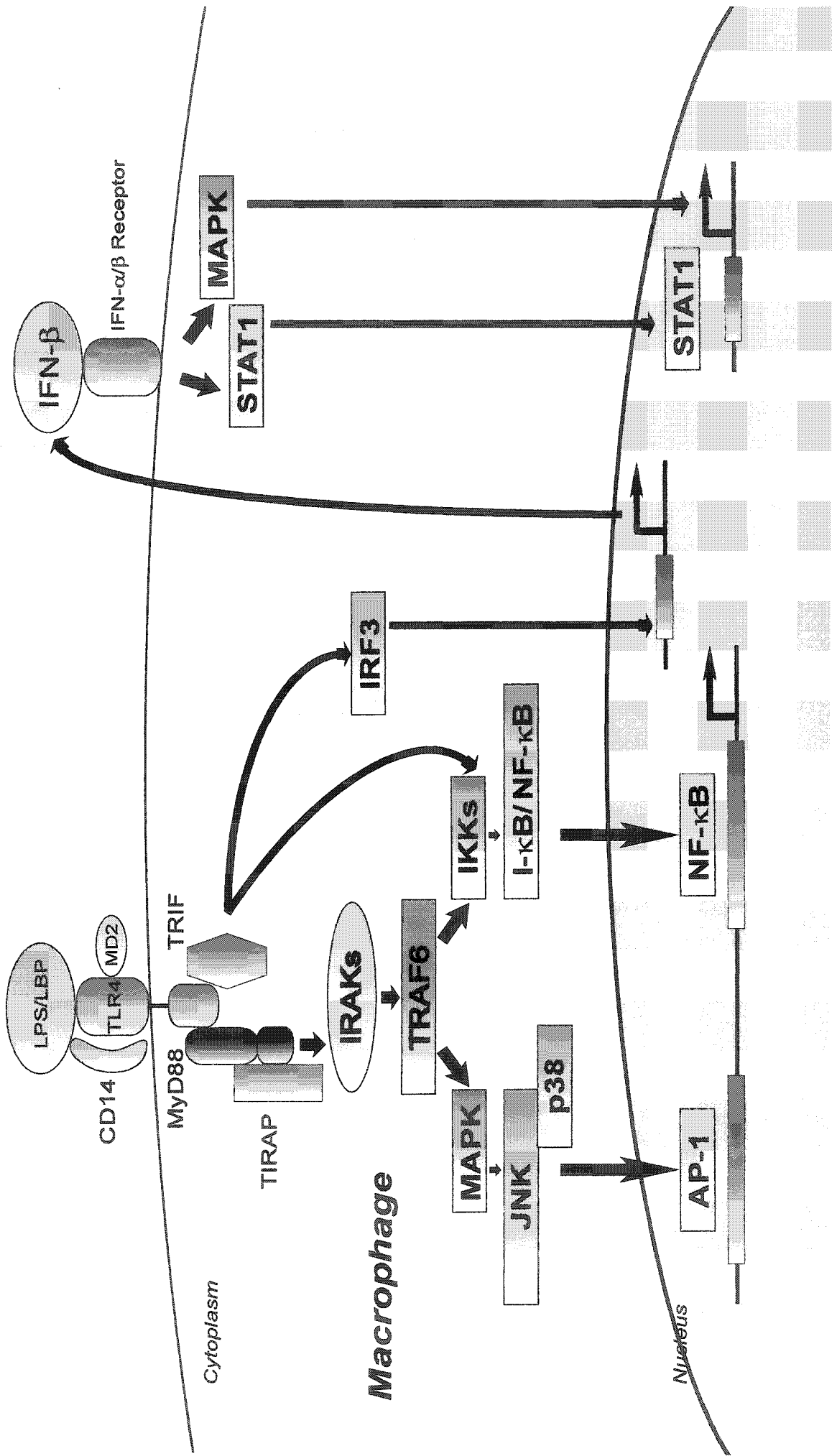
LPS-activation signaling commences after the LPS-CD14 complex interacts with an ancient PAMP signaling receptor of the innate immune system, known as the toll-like receptor (TLR) (Figure 4-1). Studies of the *Drosophila* innate immune system identified a series of Toll receptors responsible for anti-

#### **Figure 4-1. LPS-activated macrophage pathways**

A schematic diagram of LPS-induced macrophage activation mediated by TLR4 signaling through MyD88-dependent and independent pathways. LPS-mediated TLR4 activation initiates an intracellular signaling cascade by first recruiting MyD88, whereby IRAKs can transiently associate with MyD88. The activated IRAKs subsequently activate TRAF6, which in turn activates the IKK complex and MAPK kinase. MAPK kinase activates the transcription factor AP-1 through JNK and p38 MAPK. The IKK complex activation results in the phosphorylation of I $\kappa$ B and to its subsequent degradation releasing NF- $\kappa$ B to transcribe inflammatory cytokine genes. TLR4 induction of the translocation of IRF3 to the nucleus is not MyD88-dependent. IRF3 induces the up-regulation of IFN- $\beta$ , which results in the activation of STAT1.

MyD88-dependent pathway

MyD88-independent pathway



pathogenic immunity (Hoffmann, Kafatos et al. 1999). Subsequently, 10 subtype homologues of the fly Toll receptors were identified in mammals, known as the TLR (Medzhitov and Janeway 1997). To date only the functions of 6 TLR are known: TLR4 recognizes LPS (Beutler 2000); TLR2 recognizes toxins from Gram-positive bacteria and yeast (Underhill, Ozinsky et al. 1999); TLR3 recognizes double stranded RNA (Alexopoulou, Holt et al. 2001); TLR5 recognizes bacterial flagellin (Hayashi, Smith et al. 2001) and TLR9 recognizes conserved bacterial DNA patterns (Hemmi, Takeuchi et al. 2000).

## **LPS TLR4 signaling pathways**

### **LPS CD14-MyD88 dependent pathway**

Intracellular signals initiated by interaction between Toll receptors and specific PAMPs result in an inflammatory response (Figure 4-1). LPS is transported by the carrier LBP to specifically bind the macrophage cell-surface receptor, CD14 (Haziot, Chen et al. 1988), which subsequently interacts with TLR4 (Beutler 2000) along with MD2, the TLR4-helper accessory 20-30 kDa glycoprotein, that is required for efficient response to LPS (Shimazu, Akashi et al. 1999). These three cell surface components unite to form the LPS receptor complex. The TLR4 intracellular region, the TLR IL-1 receptor (TIR) domain, recruits the toll-adaptor proteins, myeloid differentiation factor 88 (MyD88) (Weighardt, Kaiser-Moore et al. 2002), along with another adaptor protein, TIR domain containing adaptor protein (TIRAP) (Horng, Barton et al. 2001), to elicit activator protein-1 (AP-1) and NF- $\kappa$ B-induced up-regulation of pro-inflammatory cytokines. A family of IL-1 receptor-associated kinases (IRAK),

and the adaptor protein, TNF receptor-activated factor 6 (TRAF6), regulate the downstream LPS signaling events. An N-terminal death domain and a C-terminal TIR domain mediate MyD88 protein-protein interactions. The TIR domains of MyD88 and TLR4 interact, whereas the N terminal death domain sequesters the N-terminal death domain of IRAK (Muzio, Ni et al. 1997) (Medzhitov, Preston-Hurlburt et al. 1998) (Wesche, Henzel et al. 1997) (Burns 1998). Upon activation the IRAKs are released from the receptor complex to interact with TRAF6 (Cao, Henzel et al. 1996) (Muzio, Natoli et al. 1998). Subsequently, TRAF6 induces AP-1 and I $\kappa$ B kinase (IKK) complex activation. Following activation the IKK complex induces phosphorylation of I $\kappa$ B, resulting in its degradation, thereby allowing NF- $\kappa$ B to translocate into the nucleus and activate the transcription of many NF- $\kappa$ B-dependent genes, including TNF- $\alpha$  and IL-1 $\beta$ .

Gene ablation studies demonstrated that LPS-activated macrophages derived from MyD88-null mice were unable to activate IRAK-1 and did not produce the inflammatory cytokines TNF- $\alpha$  or IL-1 $\beta$  (Kawai, Adachi et al. 1999). Interestingly, the nuclear translocation of NF- $\kappa$ B could still occur, upon LPS-induction in MyD88-ablated macrophages. Therefore, this work suggested that TLR4 uses at least 2 signal transduction pathways for the activation of NF- $\kappa$ B, one MyD88-dependent and the other MyD88-independent.

LPS-activation of a macrophage results in enhanced phagocytosis of bacteria and the release of cytokines prompting other macrophages, phagocytes and T cells to the site of infection. This initiates a pro-inflammatory response and thereby influences the nature of the adaptive immune response. Macrophages

are now well recognized to be the primary mediators for the lethal effects caused by bacterial- or LPS-induced septic shock (Koay, Gao et al. 2002).

### **LPS CD14-MyD88 independent pathway**

Studies using MyD88-deficient macrophages demonstrated that LPS-induced stimulation of TLR4 was still able to activate IFN regulatory factor 3 (IRF3) protein and the expression of IFN- $\beta$ , resulting in the phosphorylation of the signal transducer and activator of transcription 1 (STAT1) and subsequently inducing IFN-inducible genes, all in a MyD88-independent manner (Figure 4-1) (Toshchakov, Jones et al. 2002). In addition, LPS activation of caspase-1 (Seki, Tsutsui et al. 2001), and LPS-induced maturation of dendritic cells were shown to be MyD88-independent. Recently, a novel TIR containing protein was identified, named TIR domain-containing adaptor inducing IFN- $\beta$  (TRIF) (Yamamoto, Sato et al. 2002)/TIR domain-containing adaptor molecule-1 (TICAM-1) (Oshiumi, Matsumoto et al. 2003), respectively. Investigations using a dominant negative form of TRIF demonstrated a strong inhibition of TLR4-induced activation of NF- $\kappa$ B (Yamamoto, Sato et al. 2002). Subsequent gene ablation studies clearly demonstrated that TRIF was crucial for TLR4-mediated IFN- $\beta$  expression, IRF3 activation, and IFN-inducible gene expression in response to LPS stimulation (Yamamoto, Sato et al. 2003). LPS activated TRIF-null cells show normal activation of NF- $\kappa$ B and MAPK, whereas double TRIF- and MyD88- null cells show a complete loss of activation of NF- $\kappa$ B and MAPK (Yamamoto, Sato et al. 2003).

## **Alternative pathways of LPS recognition and signaling**

### **LPS Integrins CD11/CD18 and LPS recognition and signaling**

A secondary set of predominately macrophage cell surface proteins involved in LPS recognition and signaling are the CD11/CD18  $\beta_2$  integrins. The integrins are a family of heterodimeric glycoproteins, whereby the CD18  $\beta$  subunit noncovalently complexes with CD11b/Mac-1 or CD11c/CR4. Upon binding LPS these heterodimers activate signaling cascades leading to NF- $\kappa$ B activation (Ingalls and Golenbock 1995) (Ingalls, Arnaout et al. 1997) (Ingalls, Arnaout et al. 1997) and (Ingalls, Monks et al. 1998) and the induction of a panel of inflammatory genes, such as cyclooxygenase 2, IL-12 p35, IL-12 p40, TNF $\alpha$ , and IP-10. Optimal LPS induction of cyclooxygenase 2, IL-12 p35, IL-12 p40, was CD14-and TLR4-dependent in concert with the integrin heterodimers. However, optimal induction of TNF $\alpha$  and IP-10 was observed in CD11b/CD18-deficient macrophages (Perera, Mayadas et al. 2001). Therefore, CD14, TLR4 and the integrins are required, but contribute differentially to the LPS-induced activation of signaling inflammatory pathways.

### **Cytosolic PAMP receptors**

Recently, a family of cytosolic proteins, nucleotide binding oligomerization domain (Nod) proteins, has been shown to act as PAMP receptors able to initiate an innate immune response (Inohara, Ogura et al. 2002). *In vitro* studies showed Nod1 imparted NF- $\kappa$ B responsiveness to LPS in HEK293T cells independent of TLR4, MyD88, and TRAF6 (Inohara, Ogura et al. 2001). Gene ablation studies using receptor-interacting protein 2 (Rip2)-deficient cells demonstrated that Rip2 is essential for the activation of NF- $\kappa$ B by Nod1 (Chin,

Dempsey et al. 2002) (Kobayashi, Inohara et al. 2002). In fact, a more thorough investigation uncovered that Nod1 actually binds specifically to peptidoglycan instead of LPS (Girardin, Boneca et al. 2003). Therefore, Nod1 acts as a cytoplasmic PAMP sensor.

### **LPS mediated induction of septic shock cascade cytokines**

Cytokines are the immune system's "signaling" messengers. They are highly potent small peptides that are primarily synthesized by cells of the immune system to elicit and regulate immune function. Normally, the serum levels of proinflammatory cytokines are low or nonexistent, but are rapidly generated/released in response to pathogens. The main proinflammatory mediators produced during a localized infection are tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), interleukin- $1\alpha$  (IL- $1\alpha$ ), IL- $1\beta$ , IL-12 and IL-18. Release of other cytokines and chemokines, such as interferon- $\gamma$  (IFN- $\gamma$ ), lymphotoxin  $\alpha$  (LT $\alpha$ ) and IL-2, activates the microbicidal function of neutrophils and macrophages, mainly through the production of reactive oxygen and nitrogen species. Production of these cytokines and other mediators by monocytes/macrophages regulates the efficient dissemination of pathogens.

The production of the two key inflammatory mediator cytokines, TNF $\alpha$  and IL- $1\beta$  are principally synthesized by LPS-activated macrophages (Kumar, Thota et al. 1996). These cytokines act synergistically in the initiation of the inflammatory cascade of septic shock (Cannon, Tompkins et al. 1990). This leads to a wide range of proinflammatory signaling events within neutrophils (Varani and Ward 1994) and endothelial cells (Madge and Pober 2001) which

eventually leads to cell damage (Varani and Ward 1994). Other mediators of the inflammatory response are IL-6, bioactive lipids, including the eicosanoids and platelet activating factor (PAF), toxic oxygen metabolites, and neutrophil-derived tissue proteases.

### **TNF $\alpha$ and septic shock**

The binding of TNF $\alpha$  to its cognate cell surface receptors, either to the TNF receptor 1 (TNFR1) or the TNFR2 (both are members of a large superfamily of TNF receptors (Baud and Karin 2001)), initiates most of its biological effects via NF- $\kappa$ B activation (Sun and Andersson 2002). In response to LPS-activation a macrophage releases TNF $\alpha$  very rapidly. TNF $\alpha$  serum levels are seen to increase markedly within minutes of endotoxin exposure (van Deventer, Buller et al. 1990). TNF $\alpha$  serves to amplify the inflammatory response by stimulating the release of a variety of other proinflammatory cytokines and lipids that participate in the pathogenesis of MOF, including IL-1 $\beta$ , IL-6, eicosanoids, and PAF. In addition, TNF $\alpha$  recruits neutrophils to the site of inflammation by up-regulating the expression of adhesion molecules on the surface of endothelial cells and augments neutrophil antimicrobial functions, such as phagocytosis, degranulation and free radical generation. Moreover, TNF $\alpha$  elicits the production of inflammatory enzymes, such as phospholipase A2, cyclooxygenase, and nitric oxide synthase (Levi, ten Cate et al. 1993). Ultimately, LPS-induced TNF $\alpha$  release results in endothelial cell injury leading to increased permeability, and edema formation resulting in systemic effects, such as an imbalance of fluid dynamics, acute lung injury, and finally MOF. Administering TNF $\alpha$  to animal models eventually leads to the development of a

similar pathology to a systemic LPS-induced release of TNF $\alpha$ . Therefore, TNF $\alpha$  is a primary mediator of SIRS resulting in MOF.

### **IL-1 $\beta$ and septic shock**

The primary producer of IL-1 $\beta$  is the macrophage. Endotoxin-activation causes IL-1 $\beta$  reach their peak values several hours post LPS administration. IL-1 $\beta$  causes the release of other proinflammatory cytokines and lipids (TNF $\alpha$ , IL-6, and PAF), endothelial cell activation, and increased adhesion molecule expression. IL-1 $\beta$  and TNF $\alpha$  act synergistically to cause the fever, hypotension and MOF in response to a systemic dose of LPS.

### **The role of NF- $\kappa$ B in septic shock**

The extent of cytokine response to inflammatory agents, such as LPS, is regulated by NF- $\kappa$ B. The activation of the NF- $\kappa$ B family of transcription factors rapidly induces the up-regulation of inflammatory and anti-apoptotic genes. In addition to cytokines, NF- $\kappa$ B mediates the transcription of adhesion molecules, immunoreceptors and procoagulatory factors (Moll, Czyz et al. 1995; Wagner, Klein et al. 1998). NF- $\kappa$ B activation and function is tightly regulated by the inhibitor of NF- $\kappa$ B (I $\kappa$ B) (Whiteside and Israel 1997). NF- $\kappa$ B activation is accomplished by the release I $\kappa$ B allowing NF- $\kappa$ B to then translocate to the nucleus and to activate specific gene transcription (Figure 4-1). NF- $\kappa$ B initiates a wide-ranging network of both extracellular and intracellular regulatory events that in turn modulate the inflammatory cascade through the regulation of NF- $\kappa$ B activation (Blackwell and Christman 1997). Repeated non-lethal dose exposure to LPS results in a hyporesponsive cellular inflammatory response,

with a lower output from macrophages and endothelial cells of key inflammatory mediators such as TNF- $\alpha$  (Haas, Meyer et al. 1990; Lush, Cepinskas et al. 2000). This LPS-tolerance is believed to be NF- $\kappa$ B dependent to prevent aberrant production of proinflammatory cytokines (Yoza, LaRue et al. 1998).

### **NF- $\kappa$ B-regulated anti-apoptotic protein cIAP2**

As stated previously, the activation of the NF- $\kappa$ B family of transcription factors rapidly induces the up-regulation of many anti-apoptotic genes. One such protein is the cellular inhibitor of apoptosis 2 (cIAP2) (Wang, Mayo et al. 1998). The *ciap2* gene was first identified as a member of the evolutionarily conserved inhibitor of apoptosis (IAP) family of proteins (Liston, Roy et al. 1996) that have recently emerged as critical repressors of apoptosis. Subsequently, cIAP2, along with cIAP1, were found to be components of the TNFR2 (Rothe, Pan et al. 1995) complex and were, therefore, constituents of the TNF $\alpha$  signaling pathway. As seen from Figures 3-6C and D of Chapter 3 LPS dramatically and rapidly induces the up-regulation of *ciap2* mRNA (> 30 fold, in less than an hour) in macrophages. LPS has also been demonstrated to potently elicit the up-regulation of cIAP2, via the NF- $\kappa$ B pathway, specifically within macrophages (Boldrick, Alizadeh et al. 2002; Nau, Richmond et al. 2002; Hashimoto, Morohoshi et al. 2003). Notably, LPS-activation also imparts a macrophage cell with an increased resistance against apoptotic triggers. An inflammatory response produces nitric oxide (NO), reactive oxygen intermediates (ROI) and the up-regulation of Fas ligand on immune-regulating

lymphocytes, all of which are detrimental to both invading pathogens and resident cells. Therefore, this LPS-induced increased resistance to apoptosis is essential for a macrophage to function within an inherently hostile, anti-microbial pro-inflammatory environment.

Considerable interest in the function of cIAP2 has arisen from its role as a major NF- $\kappa$ B-regulated survival factor. cIAP2 has been shown to be the essential component chiefly responsible for protecting rat hepatocytes from a LPS-induced lethal assault (Schoemaker, Ros et al. 2002). In addition, cIAP2 was the key protective factor responsible for the apoptotic resistance shown by IL-7-pretreated T cells that were exposed to the apoptotic trigger, dexamethasone (Sade and Sarin 2003). Significantly, the induction of cIAP2 was also found to be necessary for macrophage survival and function against the intrinsically produced anti-bacterial NO (Hortelano, Traves et al. 2003). Moreover, recent *in vitro* studies suggested that the increased vigor and apoptotic resistance displayed by LPS-induced macrophages was directly correlated to cIAP2 induction (Cui, Imaizumi et al. 2000). Given that cIAP2 is a key targeted survival factor induced via NF- $\kappa$ B activation in regulatory immune cells, I investigated whether cIAP2 could be an essential component during an innate pro-inflammatory response.

## **Results**

### **Establishment of cIAP2-null mice**

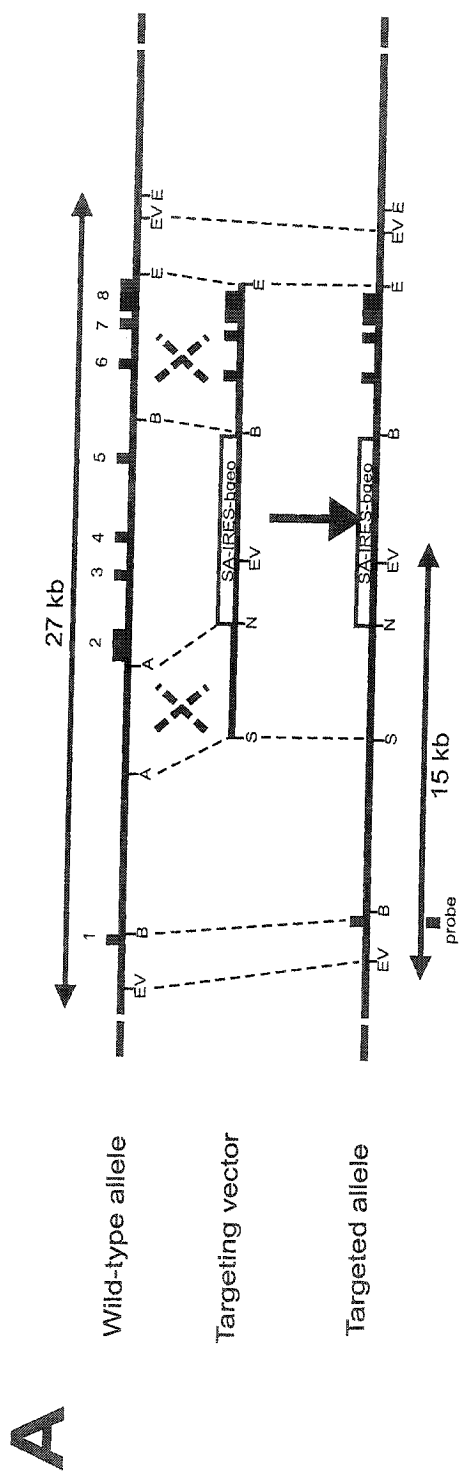
To study the function of cIAP2 the murine *ciap2* gene was disrupted by homologous recombination in embryonic stem cells (Figure 4-2). *cIAP2*<sup>-/-</sup> mice provided by M. Holcik showed no overt phenotype, were fertile, followed Mendelian frequency of inheritance and appeared healthy up to 52 weeks of age. Whole mouse and organ weights as well as primary and secondary lymphoid compartment, thymus and spleen, cell count numbers for *cIAP2*<sup>-/-</sup> and control littermates, up to the age of 36 weeks (Figure 4-3), showed no significant difference. Western analysis (Figure 4-2D) and quantitative RT-PCR (Figure 4-4) showed no compensatory increase of IAP family members in *cIAP2*-ablated tissue.

### ***cIAP2*<sup>-/-</sup> mice are resistant to LPS-induced endotoxic shock**

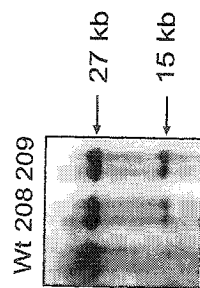
The apoptotic inhibitor cIAP2 has been suggested to be the key NF- $\kappa$ B-inducible survival factor against LPS-elicited apoptotic death of liver cells. Therefore, to investigate the role of cIAP2 in an innate immune response the *cIAP2*<sup>-/-</sup> mice were treated with LPS. Administering various doses of intraperitoneal (IP) injected LPS proved fatal to both wild-type (Figure 4-5A and B) and *cIAP2*-heterozygous (data not shown) mice, but not to *cIAP2*<sup>-/-</sup> mice (Figure 4-5A and B). As seen in Figure 4-5A *cIAP2*<sup>-/-</sup> mice (solid shapes) at 40 mg LPS/kg or less all survive, while littermate controls (open shapes) succumb in a dose dependent manner. In fact, the LD<sub>100</sub> dose for *cIAP2*<sup>-/-</sup> mice

**Figure 4-2. The disruption of the *ciap2* gene by homologous recombination**

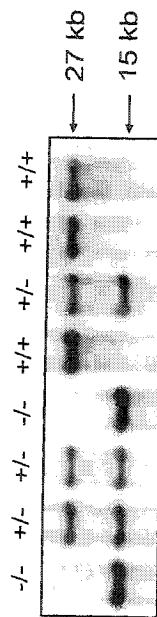
The structure of the 5' end of the mouse *ciap2* locus, targeting vector, and the targeted *ciap2* allele are shown (constructed by Dr. M. Holcik) (**A**). The closed boxes denote the *ciap2* exons; the vertical black lines indicate restriction sites. The opened black box shows the position of the probe used for the genomic Southern blot analysis. Southern blot analysis of EcoRV-digested genomic DNA from embryonic stem cells (**B**) reveals the presence of the targeted (15 kb) and the wild type (27 kb) alleles. Southern blot analysis is shown for EcoRV-digested genomic DNA from homozygous, *cIAP2*<sup>-/-</sup>; heterozygous, *cIAP2*<sup>+/-</sup>; and wild-type, *cIAP2*<sup>+/+</sup>; *cIAP2* mice (line 208) (**C**). Western blot analysis of lung protein extracts indicates absence of the 66 kDa *cIAP2* full length polypeptide (**D**).



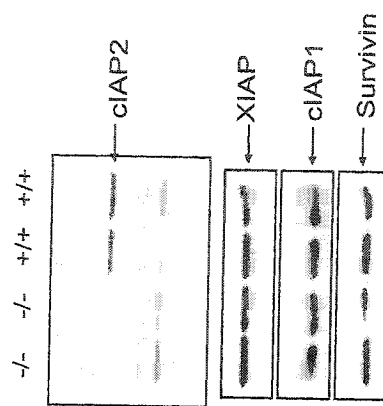
**B** Southern Blot (ES cells)



**C** Southern Blot (mouse tails, line 208)

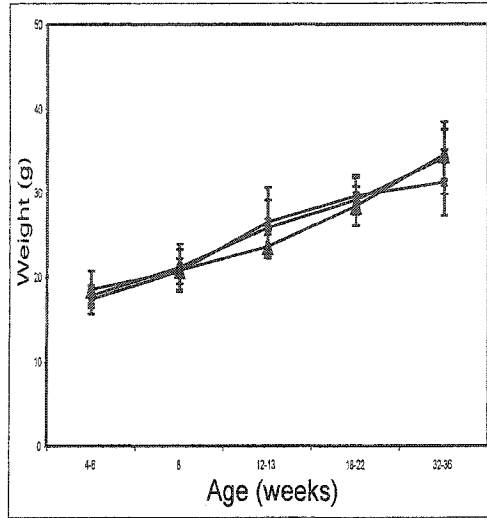
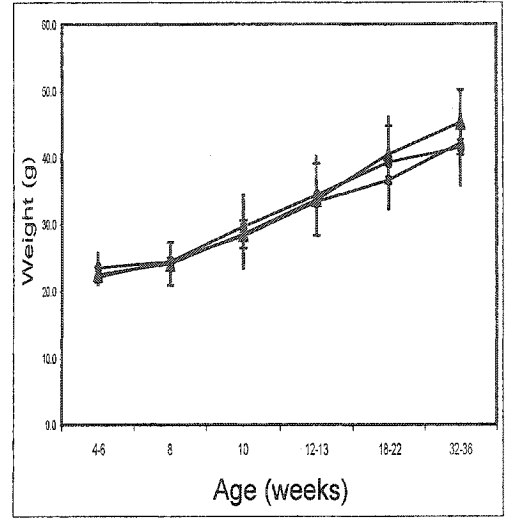
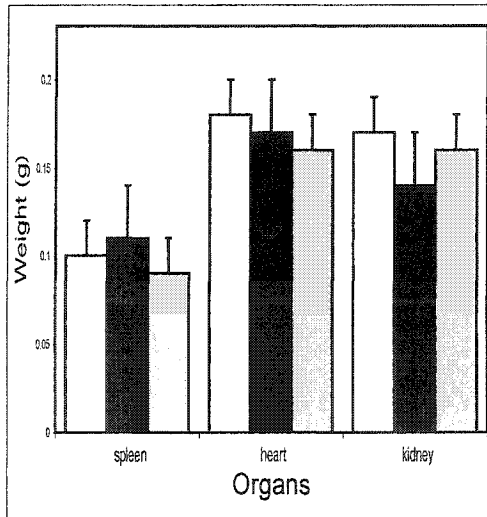
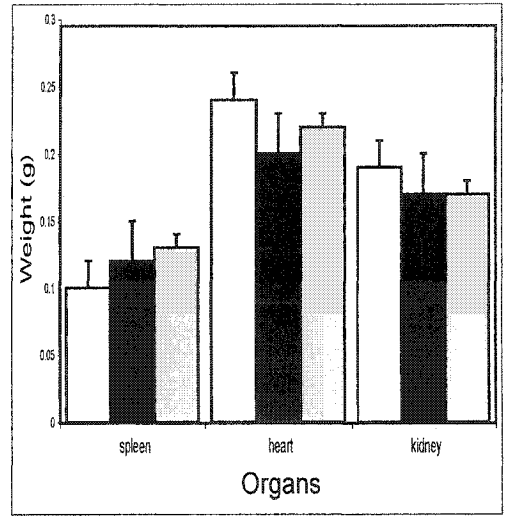
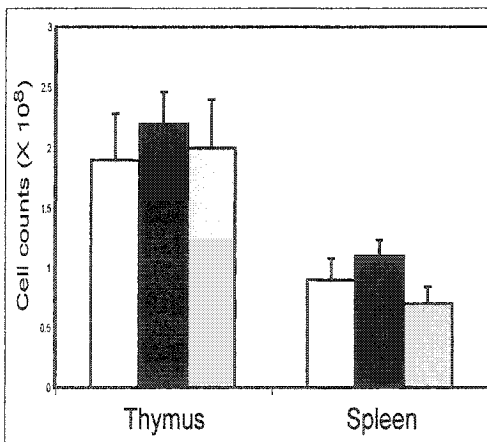
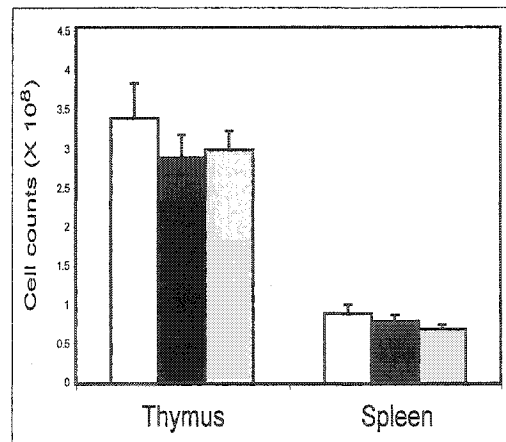


**D** Western Blot (lung)



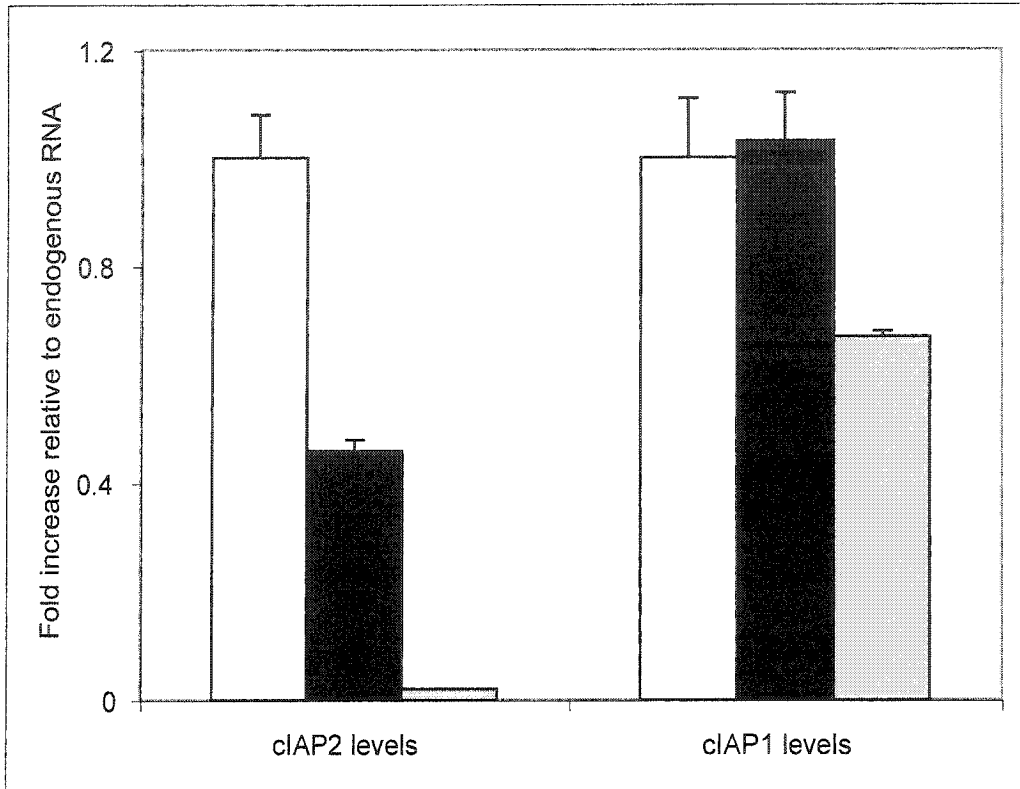
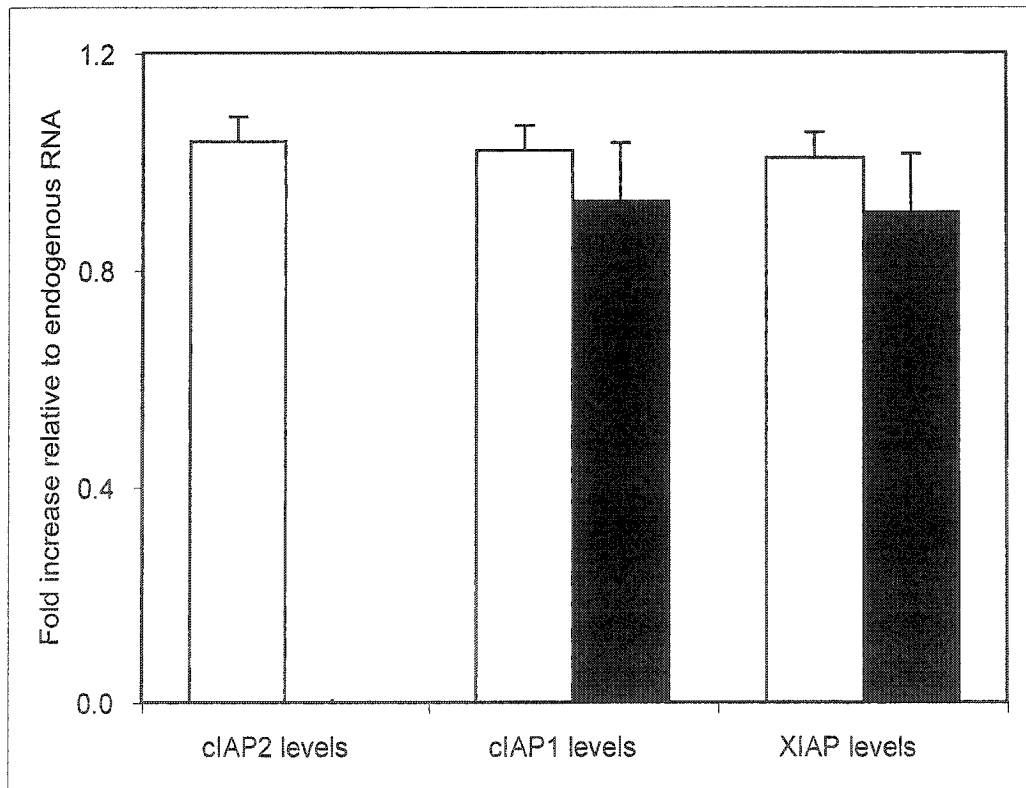
**Figure 4-3. Mouse, organ weights and cell count numbers of *cIAP2*<sup>-/-</sup> mice**

The mean weights of *cIAP2*<sup>-/-</sup> (▲) mice and littermate controls, heterozygous (■) and wild-type (●) mice (at least 15 per group), for both sets of female (A), and male (B) mice over a range of ages (4 to 36 weeks) are shown. The mean weights of spleen, heart and kidney from *cIAP2*<sup>-/-</sup> (□) mice and littermate controls, heterozygous (▨) and wild-type (■) mice (at least 10 per group), for both female (C), and male (D) mice aged 12 weeks old, are shown. The mean cell count numbers of thymocytes and splenocytes for *cIAP2*<sup>-/-</sup> (□) mice and littermate controls, heterozygous (▨) and wild-type (■) mice (at least 10 per group), for both female (E), and male (F) mice aged 4-6 weeks old, are shown.

**A****B****C****D****E****F**

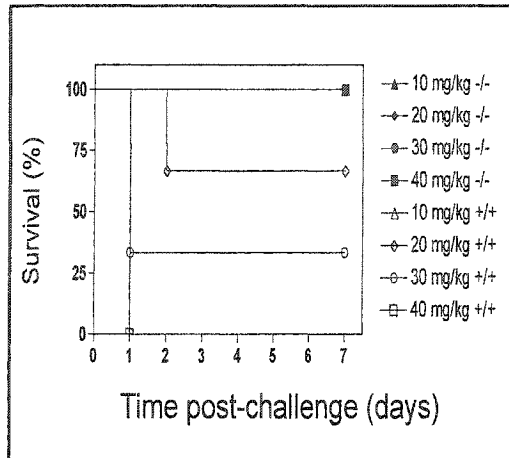
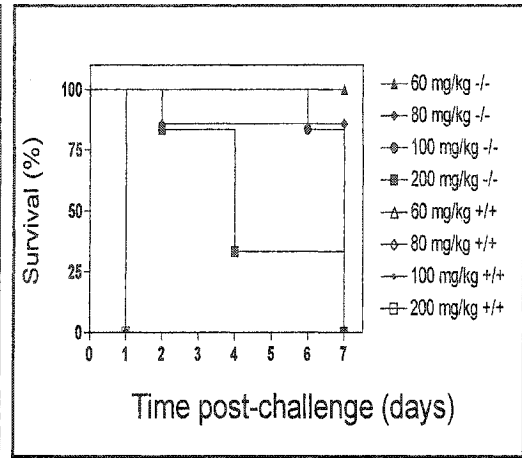
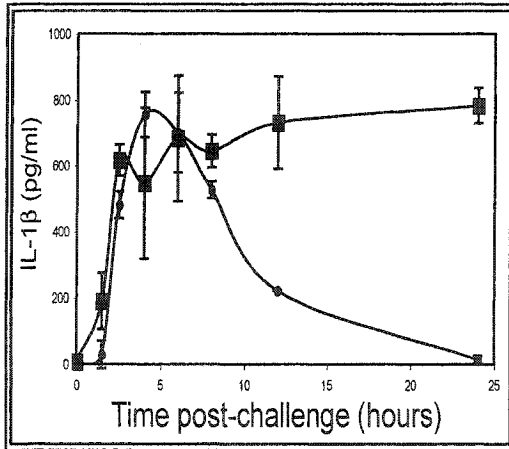
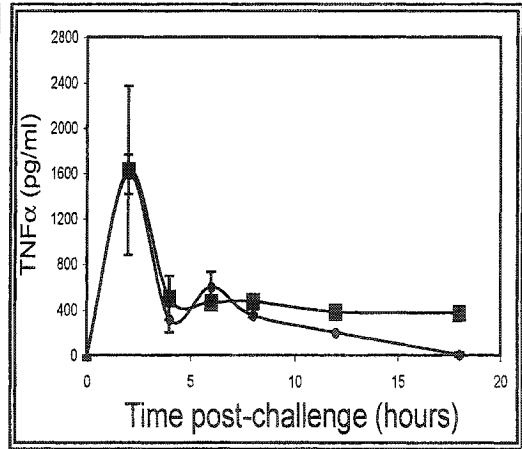
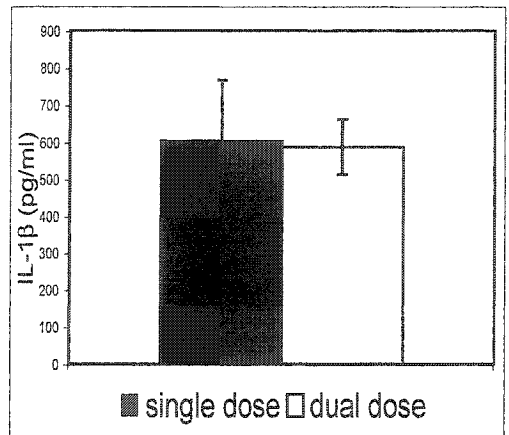
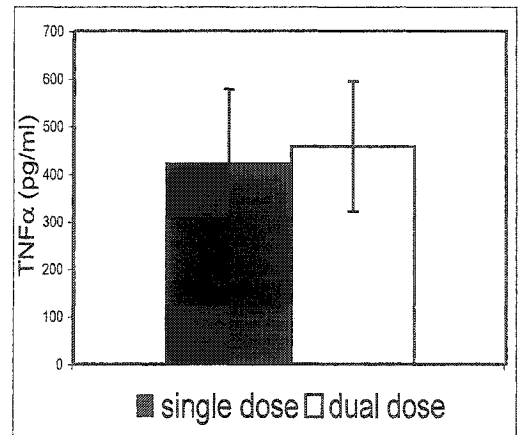
**Figure 4-4. Disruption of *cIAP2* mRNA levels in *cIAP2*<sup>-/-</sup> mice**

The levels of *cIAP2* and *cIAP1* mRNA, which was isolated from the livers of *cIAP2*<sup>-/-</sup> mice (▨), heterozygous (■) and wild-type (□) littermate mice (3 per group, in triplicate, bars represent standard deviation), are shown in (A). The levels of *cIAP2*, *cIAP1* and *xiap* mRNA, which was derived from mouse embryonic fibroblasts of *cIAP2*<sup>-/-</sup> (■) and wild-type (□) littermate mice (3 per group, in triplicate, bars represent standard deviation), are shown in (B).

**A****B**

**Figure 4-5. *cIAP2*<sup>-/-</sup> mice are resistant to LPS-induced endotoxic shock.**

Mice (6 to 10) (male to female ratio ~1:1) were injected intraperitoneally with a range of LPS doses (indicated below) in a total volume of 0.2 ml nonpyrogenic saline. (A) *cIAP2*<sup>-/-</sup> mice (solid shapes) and littermate controls (open shapes) were treated with a range of LPS doses (▲ 10, ◆ 20, ● 30 and ■ 40 mg LPS/kg, p value < 0.01 for 40 mg LPS/kg). (B) *cIAP2*<sup>-/-</sup> mice (solid shapes) and littermate controls (open shapes) were given a higher range of LPS doses (▲ 60, ◆ 80, ● 100 and ■ 200 mg LPS/kg, p values were all < 0.05). (C) Serum IL-1 $\beta$  levels in mice (3 per group), *cIAP2*<sup>-/-</sup> (●) and littermate control (■) mice, injected intraperitoneally with a LD<sub>100</sub> dose of LPS for normal mice (35 mg LPS/kg) and the serum concentrations of IL-1 $\beta$  (C) and TNF $\alpha$  (D) determined by ELISA values represent means plus or minus standard deviation of results with 3 animals. LD<sub>100</sub> doses of LPS were predetermined in separate experiments. Two sets of similar experiments, of (C) and (D), gave similar results. IL-1 $\beta$  (E) and TNF- $\alpha$  (F) serum levels were determined from *cIAP2*<sup>-/-</sup> mice, either treated with a single IP dose of LPS (serum collected 6 h post injection) or a second dose administered 4h post the initial injection and then harvested (serum harvested 2 h later). Error bars represent the standard deviation of the mean of values from 6 mice in each group.

**A****B****C****D****E****F**

was found to be approximately 3X greater (100 mg LPS/kg) compared to that of control littermates. Moreover, *cIAP2*<sup>-/-</sup> mice survived 2-7 days even at a LPS dose of 200 mg LPS/kg (Figure 4-5B), in contrast to control littermates that all died within 24 h, even at the lower dose of 40 mg LPS/kg (Figure 4-5A).

***cIAP2*-null mice display an attenuated inflammatory response, lower serum levels of IL-1 $\beta$  and TNF $\alpha$ , to LPS**

LPS directly activates macrophage cells to produce large amounts of IL-1 $\beta$  and TNF- $\alpha$  and to mediate a cascade of events leading to endotoxic shock. Therefore, the levels of these pro-inflammatory cytokines in serum from *cIAP2*<sup>-/-</sup> mice treated with LPS (35 mg LPS/kg) were assayed. In *cIAP2*<sup>-/-</sup> mice, IL-1 $\beta$  serum levels peaked at 4 h (Figure 4-5C) and then markedly dropped off. This was in stark contrast to littermate controls where IL-1 $\beta$  serum levels continued to rise and remained high until death. Likewise, comparable initial TNF- $\alpha$  serum levels (at 0 to 6 h) were observed in both groups, however, the TNF- $\alpha$  levels dropped off to approximately 10 pg/ml (Figure 4-5D) in *cIAP2*<sup>-/-</sup> mice, while in littermate controls TNF- $\alpha$  serum levels stabilized at approximately 400 pg/ml and were maintained until death. Nevertheless, *cIAP2*<sup>-/-</sup> mice did display early outward signs of septic shock, such as eye exudates and ruffled fur; however, their condition quickly ameliorated, corresponding to the observed waning of the LPS-induced inflammatory cytokines seen within the *cIAP2*<sup>-/-</sup> mice. Administration of a second LPS dose 4 h after the initial IP injection in *cIAP2*<sup>-/-</sup> mice did not result in increased levels of cytokines in serum harvested 2 h later (Figure 4-5E and 5F) compared to levels in serum of mice receiving a single-LPS injection. Therefore, the

attenuation of the serum levels of IL-1 $\beta$  and TNF- $\alpha$ , in *cIAP2*<sup>-/-</sup> mice was not due to the rapid turnover of LPS.

#### ***cIAP2*-null mice resistance to LPS-induced endotoxic shock is unique**

In order to determine the sensitivity of *cIAP2*<sup>-/-</sup> mice to other lethal insults and inflammatory mediators, additional triggers were tested. The response of these animals to an IP injection of  $\alpha$ -Fas antibodies (100  $\mu$ g/mouse) (Table 4-1) and to a second model of endotoxic shock that involved sensitizing mice to the effects of LPS (0.5, to 20  $\mu$ g/kg) with D-galactosamine (0.6 g/kg) (Table 4-2) was examined. In addition, the effect of treatment with platelet-activating factor (PAF), an inflammatory mediator that acts down stream to the LPS activation of macrophages (Sun and Hsueh 1991) (Table 4-3) was examined. In all three cases, both *cIAP2*<sup>-/-</sup> and control littermates demonstrated similar sensitivity and died at identical rates. The response to LPS, therefore, is quite distinct compared to these other insults.

#### **Macrophage cell counts and function is not impaired in *cIAP2*<sup>-/-</sup> mice**

The inability of the *cIAP2*-null mice to sustain IL-1 $\beta$  and TNF- $\alpha$  serum levels in response to LPS suggests a dysfunction of the macrophages. This dysfunction may be correlated to a reduced initial number of macrophages, to a block in the LPS-induced signaling pathway, or to an increased apoptotic susceptibility of the *cIAP2*-deficient macrophages. However, *cIAP2*<sup>-/-</sup> mice have comparable initial numbers of peritoneal- and splenic-derived macrophages relative to control littermates as assessed by trypan blue exclusion and by Diff Quik™ staining and flow cytometry (Figure 4-6A). The

**Table 4-1. Survival of cIAP2<sup>-/-</sup> and control mice after treatment with  $\alpha$ -fas antibody**

Mice	Survival	Average survival time (hours)
CIAP2 <sup>-/-</sup> mice	0/6	3.3 $\pm$ 0.9
CIAP2 <sup>+/-</sup> mice	0/6	3.7 $\pm$ 0.9
CIAP2 <sup>+/+</sup> mice	0/6	3.1 $\pm$ 0.8

Mice (4-6 weeks of age) were injected intraperitoneally with  $\alpha$ -fas antibody (100  $\mu$ g, 0.2 ml, clone Jo2).

**Table 4-2. Survival of cIAP2<sup>-/-</sup> and control mice after treatment with D-galactosamine and LPS**

Mice	LPS concentration					
	0 μg/kg	0.5 μg/kg	1 μg/kg	5 μg/kg	10 μg/kg	20 μg/kg
CIAP2 <sup>-/-</sup> mice	6/6	6/6	6/6	2/6	0/6	0/6
CIAP2 <sup>+/+</sup> mice	6/6	6/6	6/6	2/6	0/6	0/6

Mice (4-6 weeks of age) were injected intraperitoneally with the indicated doses of LPS (*E. coli* K235) with D-galactosamine (0.6 g/kg) in 0.2 ml nonpyrogenic saline. All deaths occurred within 24 h.

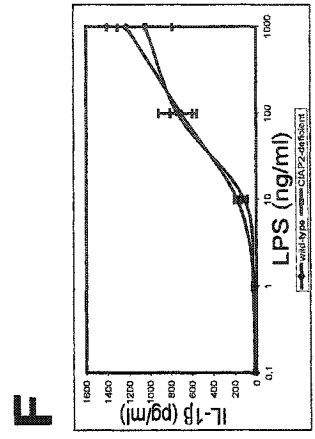
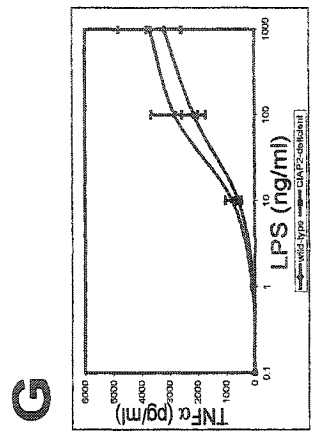
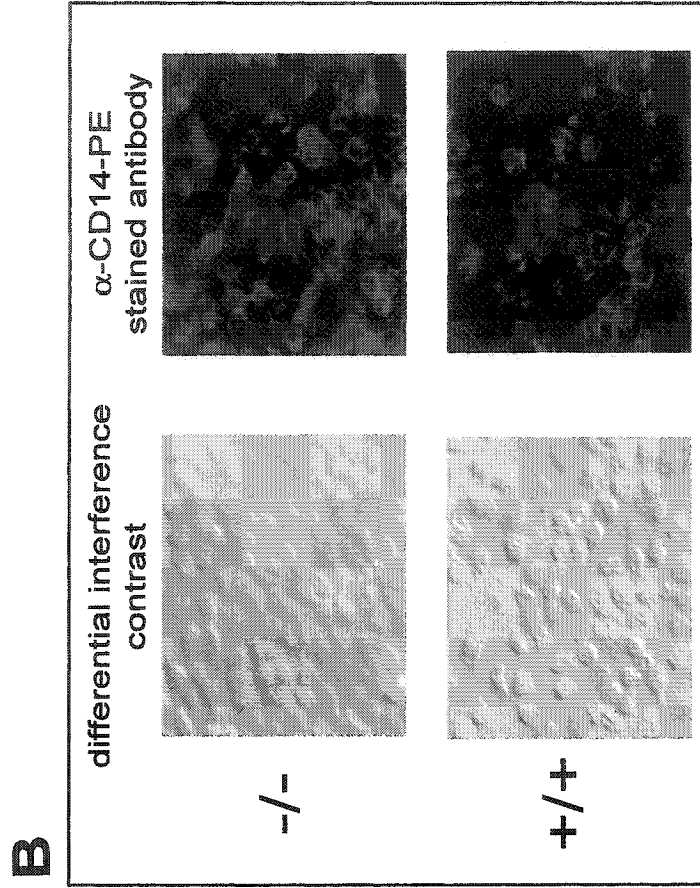
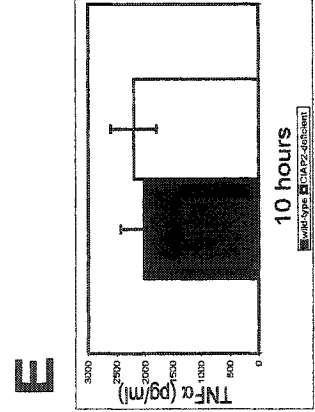
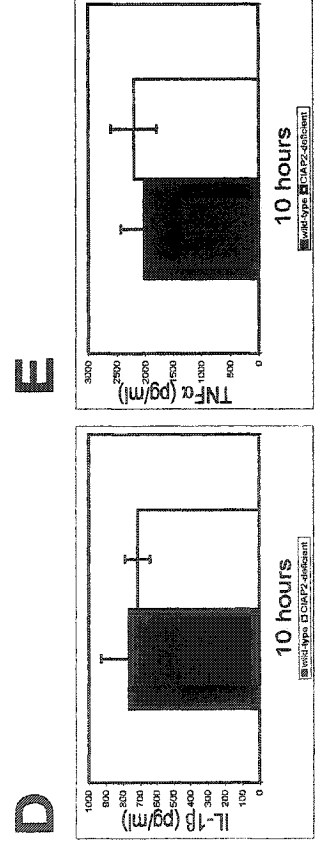
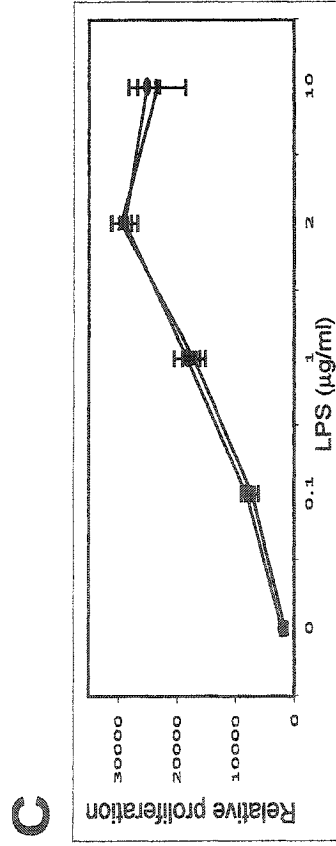
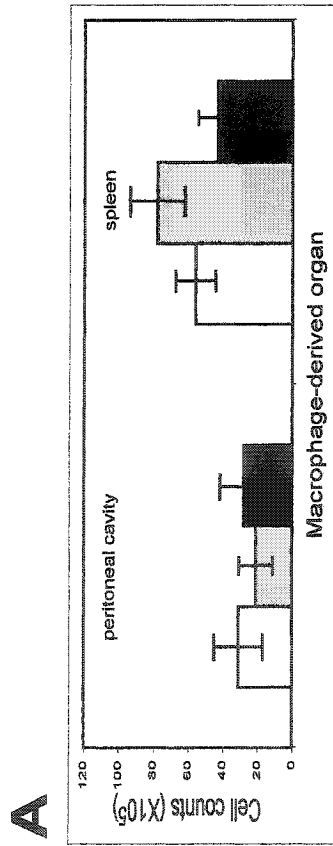
**Table 4-3. Survival of cIAP2<sup>-/-</sup> and control mice after treatment with platelet-activating factor (PAF)**

Mice	PAF concentration					
	0 µg/kg	1 µg/kg	2 µg/kg	2.5 µg/kg	3 µg/kg	5 µg/kg
CIAP2 <sup>-/-</sup> mice	6/6	6/6	6/6	6/6	0/6	0/6
CIAP2 <sup>+/+</sup> mice	6/6	6/6	6/6	6/6	0/6	0/6

Mice (4-6 weeks of age) were injected intravenously with the indicated doses of PAF in 0.2 ml nonpyrogenic saline. All deaths occurred within 24 h.

**Figure 4-6. Macrophage cell counts and function is not impaired in *cIAP2*<sup>-/-</sup> mice**

Spleen- and peritoneal-derived macrophage cell count numbers are shown (A) for *cIAP2*<sup>-/-</sup> (□), *cIAP2*<sup>+/-</sup> (▨) and *cIAP2*<sup>+/+</sup> (■) mice (10 – 20 per group) for male mice aged 8-10 weeks old. Both *cIAP2*<sup>-/-</sup> and littermate control cells stain normally for the LPS-binding CD14 of macrophages (immunostain CD14-PE, 40 X objective, representative of one field of 3 per mouse, 3 mice each) (B). The proliferation of B cells from *cIAP2*<sup>-/-</sup> mice (■) and littermate controls *cIAP2*<sup>+/+</sup> (●) after culturing with varying range of LPS doses (0.1, 1, 2 and 10 μg LPS/ml) is comparable (C). Cultured macrophages from *cIAP2*<sup>-/-</sup> or littermate control *cIAP2*<sup>+/+</sup> mice (4 per group, experiments done in triplicate, bars represent standard deviation) were exposed to 10 μg/ml of LPS for 10 h and subsequently IL-1β (D) or TNFα (E) levels measured by ELISA. Additionally, macrophages were exposed to a range of LPS doses for 24 h and IL-1β (F) or TNFα (G) levels determined (4 mice per group, experiments done in triplicate, bars represent standard deviation).



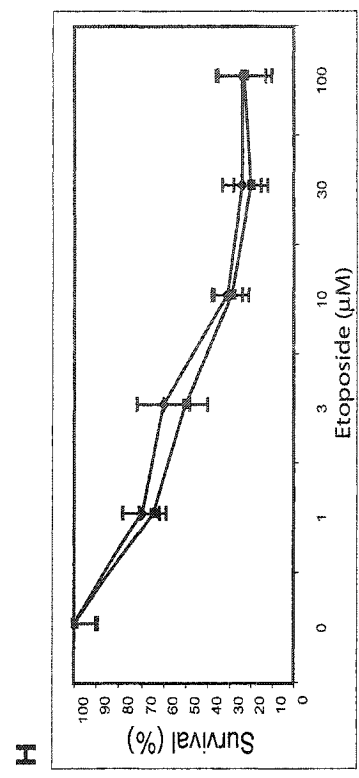
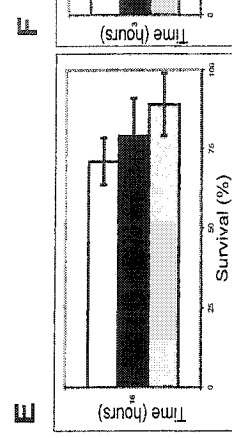
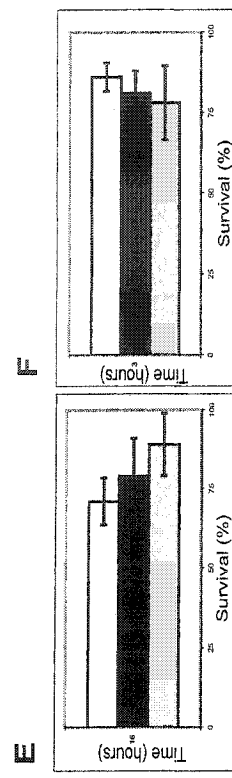
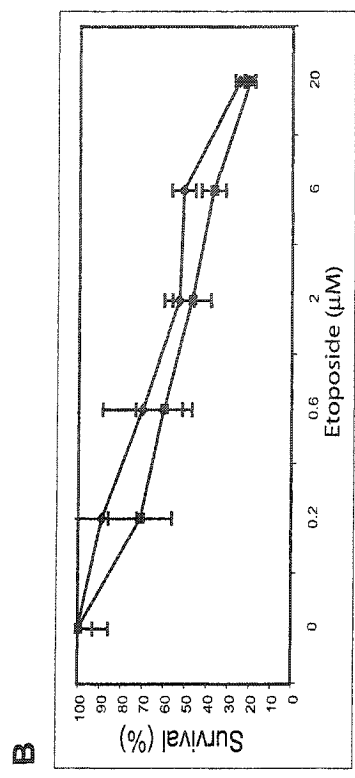
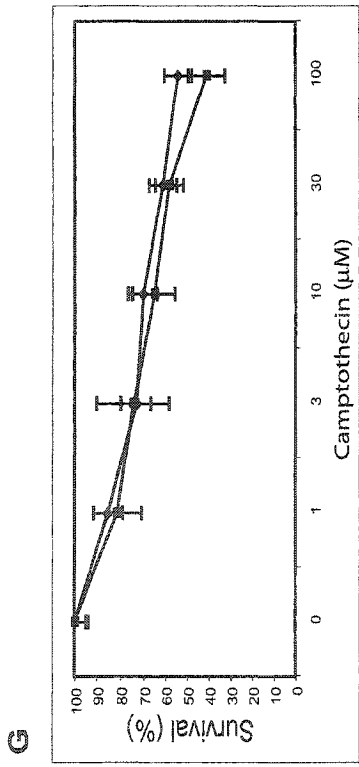
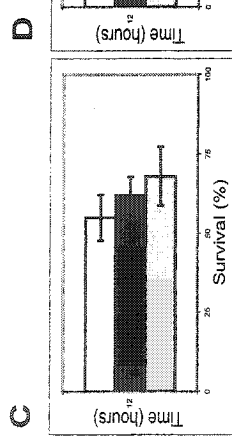
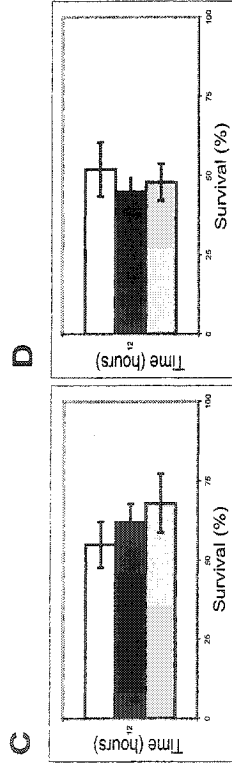
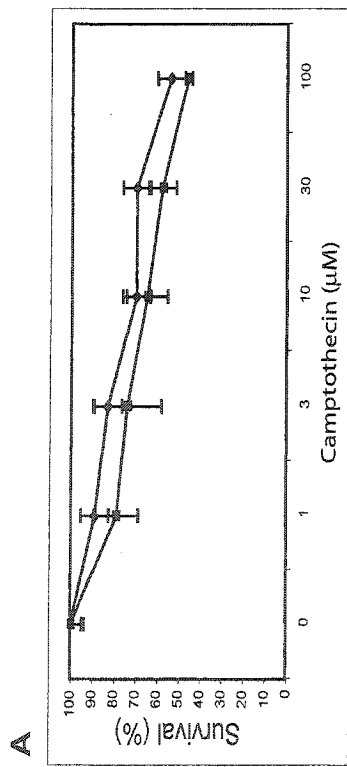
cIAP2-null mice and wild-type macrophages also stained similarly for the LPS-binding receptor, CD14 (Figure 4-6B). Moreover, isolated splenic B cells derived from *cIAP2*<sup>-/-</sup> mice proliferated normally in response to varying concentrations of LPS (0.1 to 100 µg/ml) (Figure 4-6C). In addition, primary cultures of *cIAP2*<sup>-/-</sup> peritoneal macrophages generated comparable levels of TNF-α and IL-1β as littermate controls when exposed to varying doses of LPS (0.1 to 1000 ng/ml) over several time points (10 and 24 h) (Figure 4-6D – G).

**cIAP2-null macrophages and T cells are unable to respond to the protective signals from LPS and IL-7, respectively, versus apoptotic signals**

Despite the demonstrated anti-apoptotic properties of cIAP2, purified B cells, T cells, and mouse embryonic fibroblasts from *cIAP2*<sup>-/-</sup> mice displayed no significant differences in susceptibility to a variety of apoptotic triggers (such as α-Fas antibody, C2-ceramide and dexamethasone) *in vitro*, compared to littermate controls (Figure 4-7). However, since cIAP2 is strongly inducible by NF-κB activation, the anti-apoptotic properties of this apoptotic inhibitor could be potentially observed only under appropriate conditions. Macrophage cells exposed to LPS normally show an increased vigor and resistance towards various apoptotic triggers (Cui, Imaizumi et al. 2000). Specifically, macrophages derived from control littermates and pretreated with LPS showed a remarkable resistance to Fas-induced death when compared to wild-type-derived macrophages that were not pre-exposed to LPS (Figure 4-8C). In contrast, macrophages derived from *cIAP2*<sup>-/-</sup> mice displayed no difference in

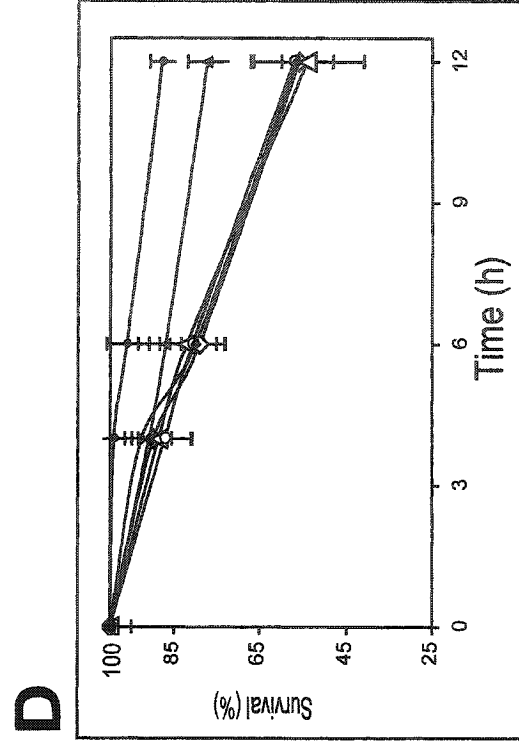
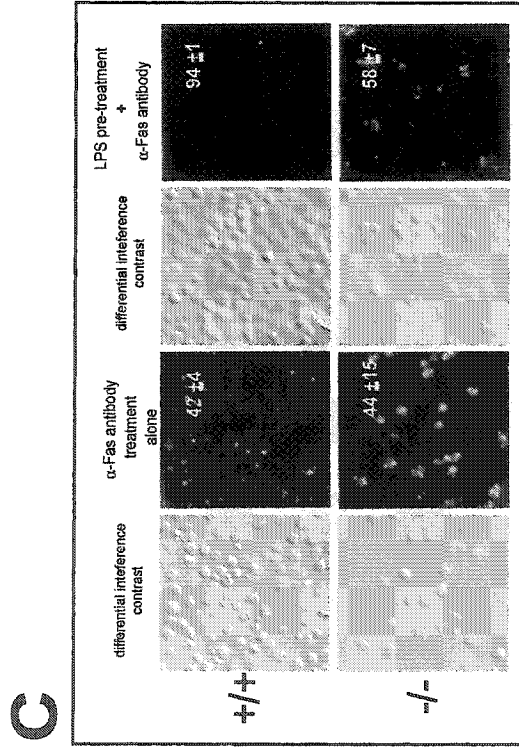
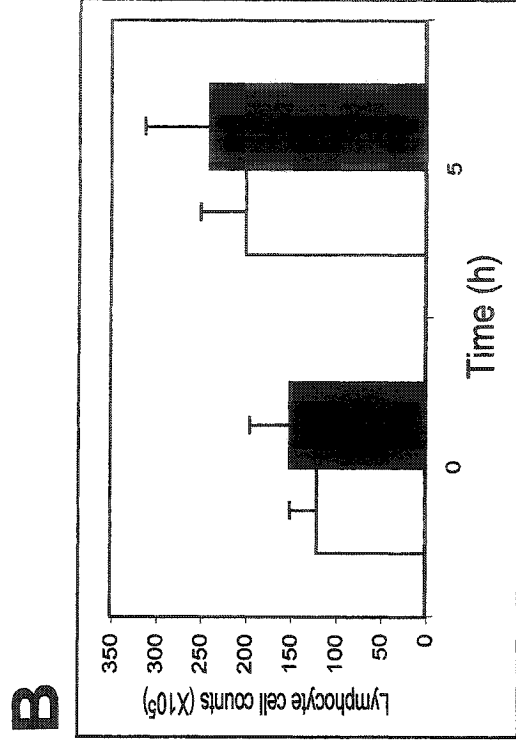
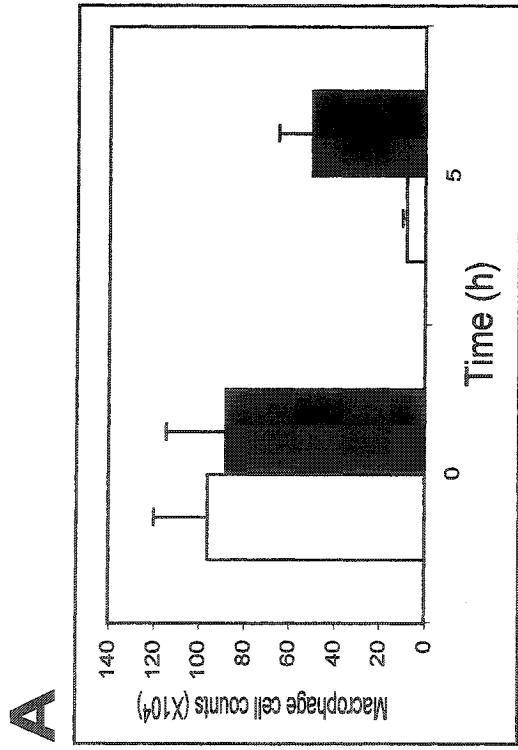
**Figure 4-7. *cIAP2*<sup>-/-</sup>-derived mouse embryonic fibroblasts (MEFs), T and B cells do not display an increased sensitivity to apoptosis**

The relative viability of mouse embryonic fibroblasts (MEFs) from *cIAP2*<sup>-/-</sup> mice (■) and littermate controls *cIAP2*<sup>+/+</sup> (◆) after culturing with varying doses of camptothecin (0, 1, 3, 10, 30 and 100 μM) (A) and etoposide (0, 0.2, 0.6, 2, 6 and 20 μM) (B) was comparable. Spleen-isolated T cell survival from *cIAP2*<sup>-/-</sup> (□), *cIAP2*<sup>+/-</sup> (●) and *cIAP2*<sup>+/+</sup> (■) mice (5 per group) and exposed to the apoptotic triggers of α-Fas antibody (20 μg/ml combined with 30 mM cycloheximide, Jo2) (Bioscience) (C), dexamethasone (500 nM) (Sigma) (D), C2-ceramide (10 μM) (Sigma) (E) and serum withdrawal (F). The relative viability of spleen-isolated B cells from *cIAP2*<sup>-/-</sup> mice (■) and littermate controls *cIAP2*<sup>+/+</sup> (◆) after culturing with varying doses of camptothecin (0, 1, 3, 10, 30 and 100 μM) (G) and etoposide (0, 0.2, 0.6, 2, 6 and 20 μM) (H) was comparable. The bars represent the standard deviation of triplicate experiments from 5 mice aged 6 – 10 weeks. Cell viability was determined by both trypan blue exclusion and by using an Annexin V-FITC kit (Immunotech).



**Figure 4-8. *cIAP2*<sup>-/-</sup>-derived macrophages but not T and B cells display an increased sensitivity to apoptosis**

(A) Mean numbers of peritoneal macrophages from *cIAP2*<sup>-/-</sup> (□) mice and littermate control *cIAP2*<sup>+/+</sup> (■) mice (6 per group) harvested at 5 hours after IP injection with LPS (35 mg LPS/kg). (B) The combined number of T and B cells of *cIAP2*<sup>-/-</sup> (□) mice and littermate control (■) mice (6 per group) that had been IP injected with LPS (35 mg LPS/kg). (C) Peritoneal-derived macrophages from *cIAP2*<sup>-/-</sup> (lower panels) and *cIAP2*<sup>+/+</sup> mice (upper panels) were either pre-treated with LPS (10 μg/ml, 4 h) or not pre-treated prior to exposure to α-Fas antibody (20 μg/ml, clone Jo2) for 4 hours and then TUNEL stained to assess cell viability. Percentages of viable cells (n = 5, average of triplicate wells per mouse, three fields of ~200 cells per well, P values were < 0.01) are shown within each TUNEL stained panel along with the standard deviation. (D) *cIAP2*<sup>-/-</sup>-derived (open shapes) and *cIAP2*<sup>+/+</sup>-derived (solid shapes) T cells were pre-incubated with a range of IL-7 concentrations (◆ 0, ▲ 5 and ● 10 μg/ml) and then exposed to dexamethasone (100 nM) and T cell survival was monitored over a 12 h period (n = 3, average of triplicate wells per mouse, bars represent standard deviation, P values at the 12 h point were < 0.05). Only macrophages capable of up-regulating *cIAP2* in response to IL-7 (*cIAP2*<sup>+/+</sup> derived macrophages at ▲ 5 and ● 10 μg/ml) demonstrated an increased resistance to dexamethasone.



their ability to resist Fas-mediated killing with or without pre-exposure to LPS (Figure 4-8C). This suggested that LPS-induced up-regulation of cIAP2, within a macrophage, was the essential protective component against Fas mediated death. Moreover, it was demonstrated *in vitro* that cIAP2 up-regulation was at least in part responsible for the general anti-apoptotic resistance display by an LPS-induced macrophage (Cui, Imaizumi et al. 2000). A similar dependency on cIAP2 was observed with another key regulatory immune cell, the T cell (Sade and Sarin 2003). T cells derived from wild-type littermates and pre-exposed to IL-7 (5 or 10 ng/ml) showed a dose dependent resistance against dexamethasone-induced death (Figure 4-8D). In contrast, T cells derived from *cIAP2*<sup>-/-</sup> mice with or without IL-7 pretreatment displayed no difference in the resistance to apoptosis (Figure 4-8D).

***cIAP2*<sup>-/-</sup>-derived macrophages, but not T and B cells, display an increased sensitivity to apoptosis**

It was observed that macrophages derived from *cIAP2*<sup>-/-</sup> mice: (1) had typical initial cell count numbers compared to control littermates; (2) produced normal levels of pro-inflammatory cytokine in response to LPS, *in vitro*; and (3) were highly susceptible to apoptotic triggers relative to control littermates when activated by LPS. It was also found that both *cIAP2*<sup>-/-</sup> and (4) control littermates were equally sensitive to PAF, an inflammatory mediator that acts downstream of the LPS-induced activation of macrophages. Given these observations, it was predicted that *cIAP2*<sup>-/-</sup> mice were resistant to endotoxic shock due to the inability of the *cIAP2*<sup>-/-</sup>-derived macrophages to up-regulate cIAP2; thus, leading to a loss of viability and hence a loss of the ability of the

*cIAP2*-null to produce a lethal inflammatory response. Therefore, in *cIAP2*<sup>-/-</sup> mice injected with a normal lethal dose of LPS (35 mg/kg) the expected results would be either a rapid loss of the macrophage populations and/or an increased apoptotic state of macrophages from *cIAP2*<sup>-/-</sup> mice relative to control littermates.

To determine the apoptotic sensitivity of *cIAP2*<sup>-/-</sup> macrophages within an LPS-induced pro-inflammatory environment, the peritoneal macrophage cell numbers before and during an endotoxin-elicited response were assessed by trypan blue exclusion, Diff Quik™ staining and flow cytometry (using the macrophage marker PE conjugated  $\alpha$ -F4/80 antibody). In addition, the apoptotic status of the peritoneal and splenic macrophages derived from LPS-injected *cIAP2*<sup>-/-</sup> and littermate control mice were also assessed via flow cytometry (using the macrophage marker PE conjugated  $\alpha$ -F4/80 antibody and FITC conjugated Annexin V). *cIAP2*<sup>-/-</sup> mice demonstrated a markedly reduced number of peritoneal-derived macrophages at 5 h post-LPS injection relative to littermate controls (Figure 4-8A). However, total lymphocyte cell count numbers (B and T cells) were comparatively unaffected in both animal types (Figure 4-8B). In addition, at 5 hours post LPS injection both the peritoneal and splenic macrophages from *cIAP2*<sup>-/-</sup> mice stained ~100 % positive for Annexin V (Table 4-4). Therefore, peritoneal and splenic macrophages from *cIAP2*<sup>-/-</sup> mice undergoing LPS-induced endotoxic shock are highly sensitive to apoptotic stimuli compared to control littermates, *in vivo*.

**Table 4-4. Peritoneal and splenic macrophages from cIAP2<sup>-/-</sup> mice are highly sensitive to apoptotic stimuli during LPS-induced endotoxic shock, *in vivo***

Mice	cIAP2 <sup>-/-</sup> mice		cIAP2 <sup>+/+</sup> mice	
	Percent macrophage	Percent apoptotic	Percent Macrophage	Percent apoptotic
<b>Peritoneal cavity</b> (At Time: <b>0 hrs.</b> post-LPS)	41 ±24	15 ±3	54 ±29	10 ±2
<b>Peritoneal cavity</b> (At Time: <b>5 hrs.</b> post-LPS)	8 ±11	<b>100 ±3</b>	31 ±17	66 ±8
<b>Spleen</b> (At Time: <b>0 hrs.</b> post-LPS)	24 ±11	2 ±5	33 ±15	5 ±3
<b>Spleen</b> (At Time: <b>5 hrs.</b> post-LPS)	24 ±8	<b>98 ±4</b>	28 ±5	46 ±8

The percentage of apoptotic peritoneal- and splenic-derived macrophages from either cIAP2<sup>-/-</sup> or cIAP2<sup>+/+</sup> mice (4-6 weeks of age; 6 per group; standard deviation shown) that were injected IP with LPS (35 mg/kg) were identified using a FITC conjugated  $\alpha$ -Annexin V antibody with flow cytometry. Macrophage percentages from the peritoneal cavity and spleen were identified using a PE conjugated  $\alpha$ -F4/80 antibody with flow cytometry.

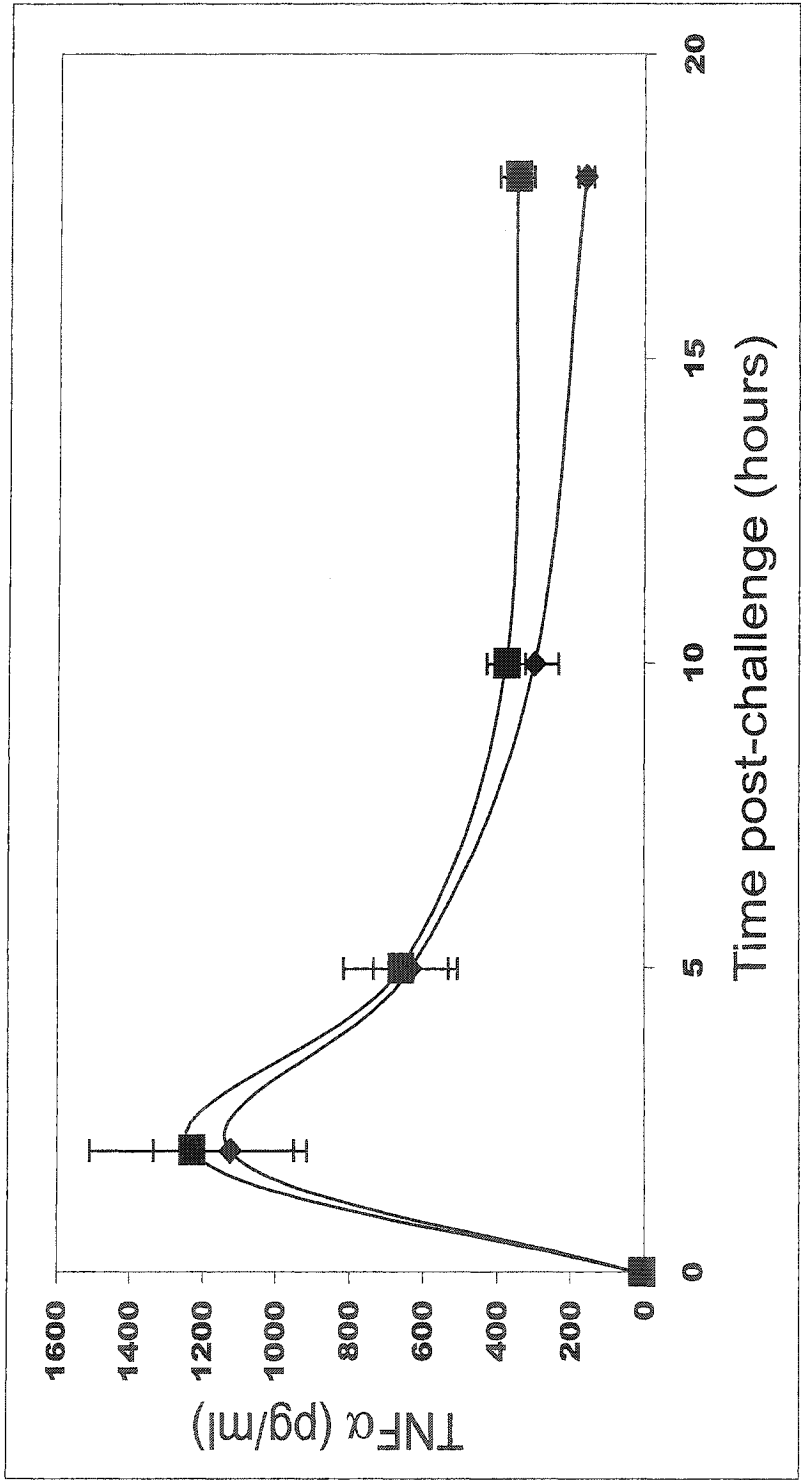
***cIAP2*<sup>-/-</sup> mice are susceptible to endotoxic shock but only at a high dose of LPS**

Host cells other than macrophages, such as endothelial cells and neutrophils, produce inflammatory cytokines in response to LPS-activation, but to a lesser extent (Haziot, Tsuberi et al. 1993). At a high enough dose of LPS these cells, independent of macrophages, generate sufficient amounts of cytokines that are lethal to mice (Haziot, Ferrero et al. 1996). *cIAP2*<sup>-/-</sup> mice survived 2-7 days even at a LPS dose of 200 mg LPS/kg (Figure 4-5B) but ultimately died.

In order to assess whether these other cells might be contributing to cytokine production at high LPS doses *cIAP2*<sup>-/-</sup> mice were injected with a LPS dose of 200 mg/kg. Control littermates synthesized approximately equivalent serum levels of TNF- $\alpha$  at 200 mg/kg of LPS (Figure 4-9) as at 35 mg/kg (Figure 4-5). However, *cIAP2*<sup>-/-</sup> mice at the high dose of LPS produced much higher levels of cytokines ~ 150 pg/ml of TNF- $\alpha$ , (Figure 4-9) compared to those of *cIAP2*<sup>-/-</sup> mice at 35 mg/kg LPS (Figure 4-5). At 200 mg/kg of LPS *cIAP2*<sup>-/-</sup> mice cytokine serum levels were approximately one third that of control littermates. Therefore, cytokines produced by alternative cells, such as endothelial cell and neutrophils, may explain why *cIAP2*<sup>-/-</sup> mice succumbed at high doses of LPS but survived for 2-7 days before death.

**Figure 4-9. TNF $\alpha$  serum levels of *cIAP2*<sup>-/-</sup> exposed to 200 mg LPS/kg**

Mice (5 per group), *cIAP2*<sup>-/-</sup> (●) and littermate control (■) mice, were injected intraperitoneally with a dose of LPS (200 mg LPS/kg) and the serum concentrations of TNF $\alpha$  determined by ELISA (one sample per mouse, error bars represent standard deviation of results with 5 animals). At a high dose of LPS (200 mg LPS/kg) *cIAP2*<sup>-/-</sup> produce TNF $\alpha$  at approximately 1/3 the levels of that for control littermates.



## ***Discussion***

### **cIAP2 is a highly inducible gene**

Clearly, as was demonstrated in Chapters 3 and 4, cIAP2-regulated apoptotic susceptibility, at least in the case of T cells and especially for macrophages, is dependent upon rapid up-regulation of the cIAP2 protein. LPS activation of peritoneal-derived macrophages induced a rapid and dramatic increase of *ciap2* message at 30 times above untreated controls in less than one hour (Chapter 3). Moreover, XIAP and cIAP1 mRNA levels remained constant. Recent *in vitro* studies using human macrophage cell lines observed similar results (Cui, Imaizumi et al. 2000; Hortelano, Traves et al. 2003). cIAP2 message was observed within half an hour of LPS-induction and protein was detected within one hour of activation. Yet, both XIAP and cIAP1 message and protein expression levels remained constant.

### **LPS confers apoptotic resistance to macrophages via induction of cIAP2 protein**

The observed rapid induction of the anti-apoptotic protein cIAP2 mRNA in macrophages in response to LPS activation suggested that, at least in part, cIAP2 might be a key resistance component for maintaining macrophage viability versus apoptotic stimuli. Recent *in vitro* studies suggested that cIAP2 is the essential anti-apoptotic element for LPS-activated macrophages (Cui, Imaizumi et al. 2000; Hortelano, Traves et al. 2003). The anti-apoptotic protein, Bcl-x<sub>L</sub> is a key survival factor of monocytes (Brockhaus and Brune 1999). However, the relatively high expression levels of Bcl-x<sub>L</sub> diminish upon

differentiation to macrophages (Hortelano, Traves et al. 2003). The IAPs have been shown to fill this protective survival role, especially through agent-induced up-regulation of cIAP2 in the macrophage cell (Hortelano, Traves et al. 2003). The work presented here clearly supports these previous studies by demonstrating the critical anti-apoptotic role of cIAP2 for macrophage viability. Peritoneal macrophages derived from either cIAP2-null or control littermates displayed similar sensitivity to Fas-induced death (Figure 4-8C) however, when pretreated with LPS only macrophages from *cIAP2*<sup>+/+</sup> mice displayed an increase resistance to Fas-induced apoptosis.

**Ablation of cIAP2 renders peritoneal and splenic macrophages susceptible to apoptosis during LPS-induced endotoxic shock, in vivo**

Given that LPS-activation of macrophages induces a prompt and considerable induction of cIAP2; and this cIAP2 up-regulation renders macrophages more readily resistant to apoptotic stimuli, the ablation of cIAP2 may impact macrophage viability during LPS-induced septic shock, thereby precluding the ability to elicit pro-inflammatory cytokines and preventing acute endotoxic shock. As seen from Figure 4-8A peritoneal macrophage numbers from *cIAP2*<sup>-/-</sup> mice are reduced considerably by the 5 hour time point post LPS injection, relative to *cIAP2*<sup>-/-</sup> mice at time zero hours and to littermate controls at 5 hours post LPS injection. In addition, ~100% of peritoneal and splenic macrophages from *cIAP2*<sup>-/-</sup> mice 5 hours post LPS injection were apoptotic (Table 4-4).

**LPS-induced production and release of pro-inflammatory cytokines from *cIAP2*<sup>-/-</sup>-derived macrophages function normally, *in vitro***

The loss of cytokine production in *cIAP2*<sup>-/-</sup> mice treated with a lethal dose of LPS may be due to a signaling dysfunction of the macrophages from these *cIAP2*<sup>-/-</sup> mice. Similar to the results obtained for *cIAP2*<sup>-/-</sup> mice, TLR4- (Hoshino, Takeuchi et al. 1999) and MyD88-deficient mice (Kawai, Adachi et al. 1999) have also been found to be resistant to LPS-induced endotoxic shock. However, B cells isolated from these animals failed to proliferate in response to LPS, whereas B cells from *cIAP2*-deficient mice responded normally to LPS. Furthermore, cultured macrophages derived from either TLR4- or MyD88-null mice were unable to produce pro-inflammatory cytokines. Clearly, in the case of TLR4 and MyD88 deficiency, the observed resistance to endotoxic shock is due to a block of the LPS-induced activation pathway of the macrophage. Again, by comparison, *cIAP2*-deficient macrophages exposed to LPS generate normal levels of TNF- $\alpha$  and IL-1 $\beta$  suggesting that the classical LPS-induced NF- $\kappa$ B pathway is intact in macrophages lacking *cIAP2*.

**Legionnaire's disease arises from the increased apoptotic vulnerability of macrophages that are unable to express the IAP family member NAIP**

The attenuated pro-inflammatory response shown in *cIAP2*<sup>-/-</sup> mice protects these animals from exposure to LPS, however, because of this diminished immune response these mice are expected to be more susceptible to a bacterial assault compared to control littermates. The *cIAP2*<sup>-/-</sup> phenotype is also consistent with the findings that mice naturally deficient in another IAP

family member, the neuronal apoptosis inhibitory protein (NAIP), showed an increased susceptibility to Legionnaire's disease (Diez, Yaraghi et al. 2000). NAIP is also up-regulated in activated macrophages and it is known that the pathogenic determinate of Legionella bacteria is mediated through the apoptotic elimination of the macrophage (Diez, Yaraghi et al. 2000). Hence, this susceptibility was deemed to stem from the increased vulnerability to apoptotic stress of the macrophage, due to its inability to up-regulate NAIP. Therefore, as with the *NAIP2*<sup>-/-</sup> mice, due to the increased importance of the IAPs on the survival of macrophages, the up-regulation of cIAP2 is required to maintain macrophage viability under physiological and pathological stresses.

**Proposed mechanism of action: cIAP2-null mice resist LPS-induced endotoxic shock**

LPS challenge of cIAP2-null mice causes the activation of multiple types of genes, including the up-regulation of the survival genes. The pro-inflammatory response generates an inherently hostile environment that can be lethal to both pathogen and host immune cell. Therefore, expression of pro-survival genes might be vital to maintain macrophage viability during an immune response. The inability to up-regulate cIAP2 renders LPS-activated macrophages highly susceptible to apoptotic triggers, thereby quickly eliminating the resident macrophage population soon after the initiation of a systemic inflammatory response. This leads to the loss of the principal source of pro-inflammatory cytokines and subsequently to the attenuation of the immune response, preventing the development of MOF.

### **Possible therapeutic applications**

The work presented here suggests that antagonizing cIAP2 expression and/or function may have therapeutic benefit in patients with septic shock. Despite extensive efforts to formulate an effective treatment for septic shock, mortality rates remain exceptionally high (Martin, Mannino et al. 2003). Many therapies to date have proven to be neurotoxic or have been shown to block only one of the two main inflammatory cytokines, IL-1 $\beta$  (Peake, Pierides et al. 2002) or TNF- $\alpha$  (Eskandari, Bolgos et al. 1992). However, neither IL-1 $\beta$ - (Fantuzzi, Zheng et al. 1996) nor TNF- $\alpha$ - (Marino, Dunn et al. 1997) deficient mice alone are resistant to LPS-induced endotoxic shock. The ablation of cIAP2 expression results not only in a loss of sustained IL-1 $\beta$  production but also that of TNF- $\alpha$ . Therefore, potentially a pharmacological ablation of cIAP2 will limit the severity of inflammatory diseases by transiently abolishing IL-1 $\beta$ - and TNF- $\alpha$ -producing macrophages. These findings may be extended to many other inflammatory or autoimmune disorders

### **Summary**

These results suggest that cIAP2 is a highly regulated protein whereby its apoptotic inhibitory properties can be observed only under a suitable situation. In addition, the cIAP2-inducing agents, LPS and IL-7, impart their target cells, macrophages and T cells, respectively, with an increased apoptotic resistance. More importantly, cIAP2-null macrophages and T cells are unable to respond to these protective signals indicating that cIAP2 is the crucial protective component. One such situation occurs when a systemic LPS-activation of the host macrophage population specifically up-regulates cIAP2 via NF- $\kappa$ B

activation. This response is required in order to resist the intrinsic apoptotic stress initiated by pro-inflammatory cytokines, thereby allowing for the survival of the resident macrophage population and conserving functionality. Consequently, resident macrophages are able to initiate a septic shock cascade. A lack of cIAP2 sensitizes macrophages to apoptotic stresses, thereby eliminating most of the resident population. This would leave the host animal incapable of inducing septic shock and more importantly, the host animal may not be able to efficiently eliminate a localized infection.

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## **Chapter 5: The effect of over-expressing XIAP or ablating cIAP2 on T cell maturation and function**

*Thymocyte-targeted overexpression of xiap transgene disrupts T lymphoid apoptosis and maturation. Conte, D., Liston, P., Wong, J. W., Wright, K. E. and R. G. Korneluk. Proc. Natl. Acad. Sci. April 2001 98(9):5049-5054*

### **Introduction**

Apoptosis or programmed cell death (PCD) is a key component of normal development and tissue homeostasis, especially of the immune system (Vaux and Korsmeyer 1999). Dysregulation of PCD is acknowledged as being an essential component for the development of cancer and autoimmunity (Nagata 1997). A family of cysteine proteases called caspases is the central component orchestrating the orderly break down of cells during the apoptotic process (Salvesen and Dixit 1997). Both the Bcl-2 and IAP gene families encode anti-apoptotic proteins that regulate PCD pathways. An important example of this regulatory process is shown by the fact that XIAP and cIAP2 directly interact with and inhibit caspase-3, caspase-7 and caspase-9 (Deveraux, Takahashi et al. 1997); where caspase-3 is an integral component of the proteolytic caspase cascade during the activation of the classical apoptotic pathway.

PCD shapes thymocyte development through the elimination of aberrantly developed T cells and potentially harmful self-reactive T-lymphocytes. Apoptosis also plays a role in eliminating activated effector T cells in the periphery once an immune response has started to wane. Thymocyte sub-

populations represent different stages of T cell differentiation and development. Progression of T cell maturation within the thymus primarily commences with a double negative (DN) T cell stage ( $CD4^-CD8^-$ ) and then proceeds to a double positive (DP)  $CD4^+CD8^+$  stage. The final phase of maturation is completed with the down regulation of either  $CD4^+$  or  $CD8^+$ , on a DP thymocyte, leading to the formation of a mature single positive (SP) ( $CD4^-CD8^+$  and  $CD4^+CD8^-$ ) T cell, which is then exported to the periphery. Differentiating thymocytes undergo selection processes that are mediated by apoptosis during several stages of maturation. First, at the DN stage, thymocytes that fail to express a functional early T cell receptor  $V\beta$  ( $TCR\beta$ ), or later, the full  $TCR_{\alpha\beta}$ , are incapable of receiving a positive signal via the TCR, and therefore die via apoptosis, a process termed death by neglect (von Boehmer 1994). Second, thymocytes with correctly rearranged  $TCR_{\alpha\beta}$  that cannot bind to major histocompatibility complex molecules (MHC I/II) also die of neglect, the process of classical positive selection. Last, high affinity interaction of  $TCR_{\alpha\beta}$  with MHC I/II proteins associated with self-peptide triggers PCD in a process known as negative selection, or clonal deletion (Nossal 1994). Negative selection within the thymus occurs throughout the immature thymocyte developmental stage, DP and the early mature SP phases of growth.

The over-expression of XIAP and cIAP2 has been demonstrated to inhibit apoptosis in tissue culture cells challenged with a wide variety of apoptotic triggers, such as glucocorticoids,  $TNF\alpha$  or serum withdrawal (LaCasse, Baird et

al. 1998). Recently, in the mouse, T cell and thymocyte-triggered PCD as well as T cell function/activation have been demonstrated to be partially dependent on activation of caspases through their proteolytic processing (Alam, Cohen et al. 1999; Kennedy, Kataoka et al. 1999). Interestingly, high levels of human *xiap* and *ciap2* mRNA expression have been shown within developing T cells of the thymus and peripheral lymph nodes (LaCasse, Baird et al. 1998). In addition, preliminary work presented in Chapter 3 suggests that XIAP expression is ubiquitous and relatively equivalent in all murine thymocyte sub-populations. Moreover, there was a correlation between XIAP expression levels and thymocyte apoptotic susceptibility. Therefore, these results suggest that XIAP expression levels regulate thymocyte sensitivity, and that removal of XIAP is paramount for normal thymocyte development. Therefore, it would seem that the best course of action to study thymocyte-XIAP dependence would be to disrupt the orderly removal of thymocyte-XIAP in response to apoptotic stimuli. Therefore, it was decided to try to examine thymocyte apoptotic sensitivity and development in a transgenic mouse that specifically over expressed XIAP in the thymus.

Here the effect of XIAP over-expression in T cell development and function was investigated by engineering a novel transgenic mouse over-expressing a human *xiap* transgene under the control of a T cell specific promoter, *lck*. In addition, the effect of ablating cIAP2 on thymocyte maturation by developing a cIAP2-null mouse was studied. It was observed that thymocyte total cell numbers were comparatively similar in cIAP2<sup>-/-</sup>, cIAP2<sup>-/+</sup> and cIAP2<sup>+/+</sup> mice. However, thymocyte sub-populations were perturbed and it was demonstrated

that thymocytes derived from *cIAP2*<sup>-/-</sup> mice were not viable when cultured. In *xiap*-transgenic mice it was observed that thymocytes and/or T cells accumulate in primary (thymus) and secondary (spleen) lymphoid tissues relative to wild-type littermates. Elevated DN thymocytes and SP CD4<sup>-</sup>CD8<sup>+</sup> levels suggest that T cell maturation was perturbed. Both *in vitro* and *in vivo* experiments demonstrate that thymocytes derived from *xiap*-transgenic animals display an increased resistance to a variety of apoptotic triggers. However, these results suggest that the perturbation of T cell development in the transgenic mice was due to a corresponding *in vivo* resistance to apoptotic triggers.

## Results

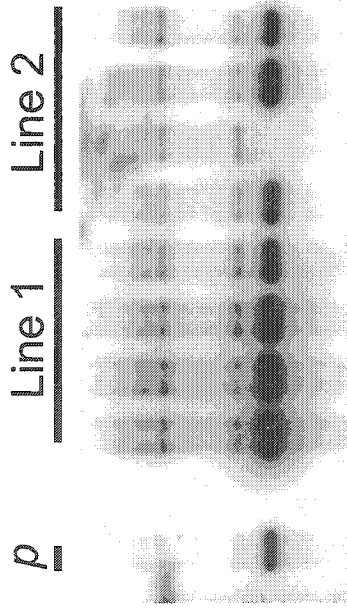
### Establishment of p56<sup>lck</sup>-*xiap* transgenic mice

To assess the effect of XIAP on T cell development, a transgenic mouse model was generated. The transgene construct contained a human cDNA fragment containing the *xiap* open reading frame downstream of the proximal promoter of the p56 *lck* tyrosine kinase gene (*lck*<sup>PR</sup>). The proximal promoter limits transgene expression primarily to thymocytes (Reynolds, Lesley et al. 1990). Two C57BL/6 founder lines were identified that contained the *lck*<sup>PR</sup>-*xiap* construct (Figure 5-1A). Expression of human XIAP protein in the thymus was

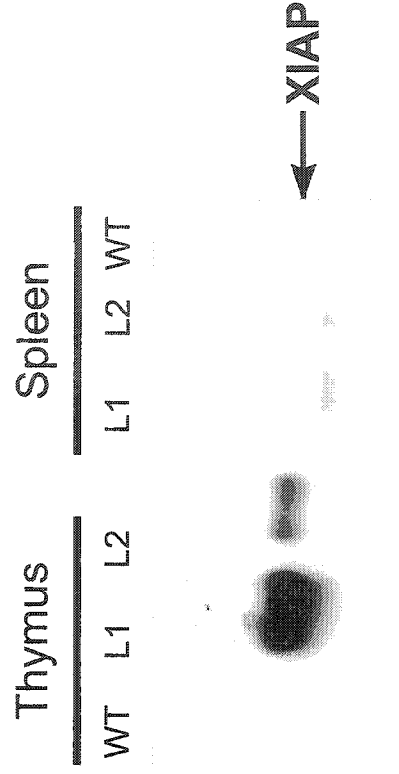
**Figure 5-1. Characterization of *lck<sup>Pr</sup>-xiap* transgenic mice.**

(A) Southern analysis of offspring from founder lines 1 and 2. Lane one plasmid (*p*) is the control lane that contains BamHI/XhoI digested plasmid DNA which contains the human cDNA fragment of *lck<sup>Pr</sup>-xiap*. (B) Western analysis of XIAP expression in the thymus and spleen of founder lines 1 (L1), 2 (L2) and wild-type (WT) mice.

**A**



**B**



demonstrated by Western blot analysis (Figure 5-1B). Offspring of the line 1 founder expressed the XIAP transgene at approximately twice the level of line 2 offspring relative to actin loading control levels. Both founder lines were used to characterize the outcome of transgene expression.

**Over-expression inhibits *in vitro* apoptosis of thymocytes via both general and T cell-specific apoptotic triggers**

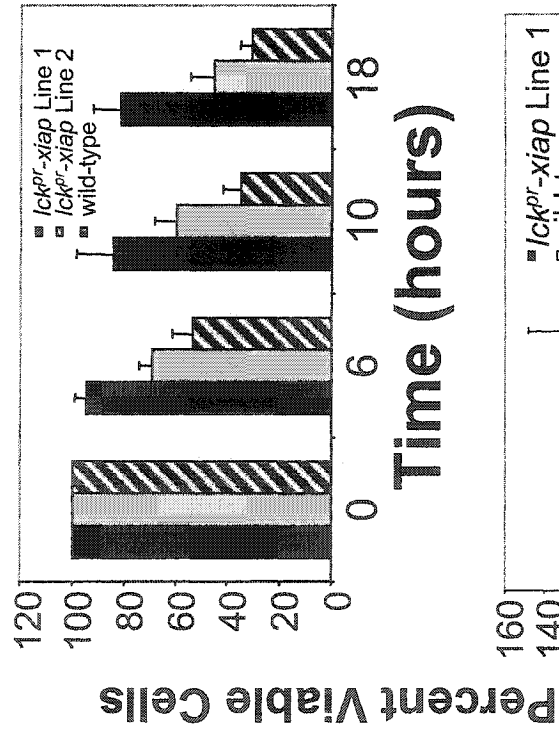
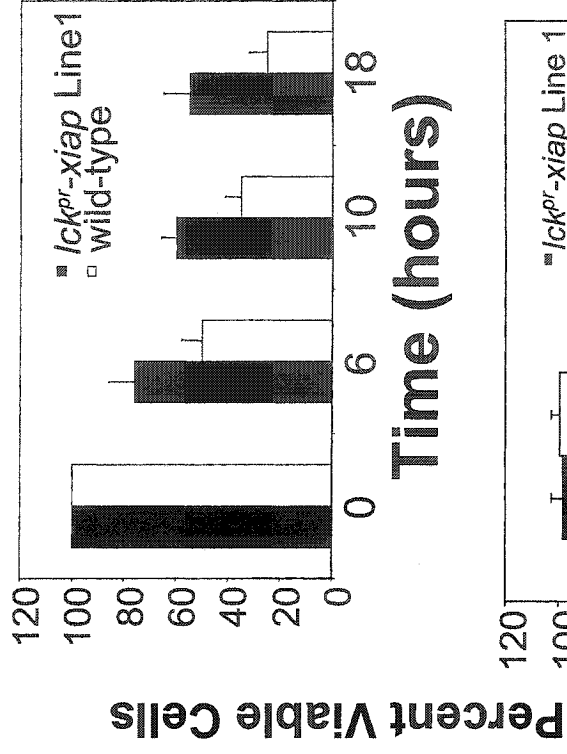
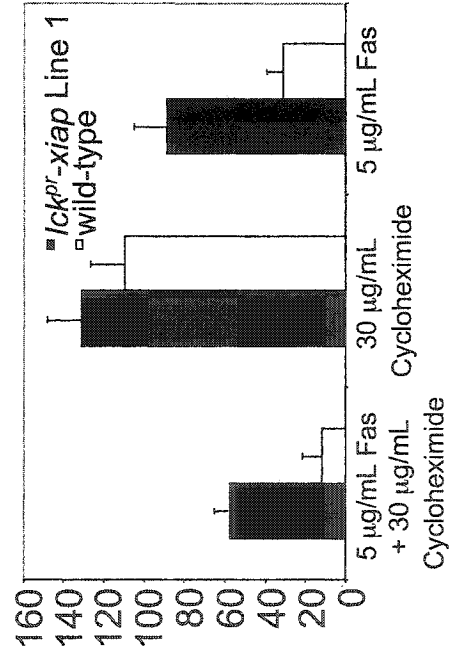
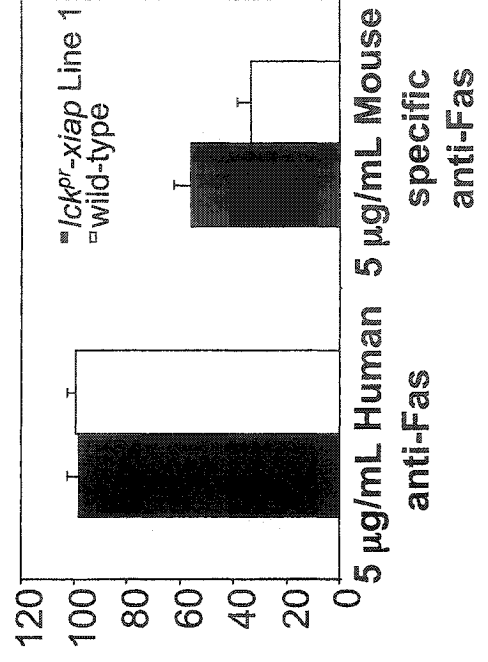
Pre-T cells and thymocytes are highly susceptible to apoptosis, relative to SP and naïve T cells. The fate of the vast majority of these developing T cells is to die during the maturation process in the thymus. Adenovirus over-expression of XIAP protects a variety of tissue cell lines, including the human Jurkat T-lymphoid cell line, from a broad range of *in vitro* apoptotic triggers (LaCasse, Baird et al. 1998). Therefore, the ability of constitutive XIAP to rescue apoptotic-sensitive thymocytes from general apoptotic triggers, such as C2 ceramide, UV radiation and  $\alpha$ -Fas antibody was evaluated. The mechanism of anti-apoptotic function of XIAP is primarily via inhibition of caspase 3 function (Jurgensmeier, Xie et al. 1998). The pro-apoptotic compound, ceramide, triggers apoptosis by directly activating caspase 3 to initiate cell death. Therefore the viability of wild-type and *lck<sup>Pr</sup>-xiap* thymocytes following C2 ceramide exposure (10  $\mu$ M) *in vitro* was compared. For these experiments, results obtained by trypan blue exclusion were verified by quantifying apoptotic cells using an Annexin V-FITC kit (Immunotech). As expected, *lck<sup>Pr</sup>-xiap* thymocytes demonstrated reduced *in vitro* apoptosis, with only 20 % cell death (n=16, p<0.01) relative to untreated *lck<sup>Pr</sup>-xiap* thymocytes over an 18 h time course. In contrast, viable thymocytes from wild-type littermates were

reduced by 70 % over the same time span (Figure 5-2A). PCD proceeds via two major intracellular pathways: (i) initiation via engagement of death receptors, such as Fas and TNF receptors or (ii) initiation via release of apoptotic factors, such as cytochrome *c*, as triggered by agents like glucocorticoids or DNA-damage by UV radiation. The ability of XIAP to inhibit both major apoptotic pathways by comparing wild-type and *lck<sup>Pr</sup>-xiap* thymocyte viability following exposure to UV radiation (1000 mJ/cm<sup>2</sup>) and after engagement of the Fas death receptor using a mouse-specific  $\alpha$ -Fas antibody, clone Jo2 (5  $\mu$ g/ml) was then assessed. *Lck<sup>Pr</sup>-xiap* thymocytes proved resistant to apoptosis triggered by both pathways. UV radiation-induced apoptosis was reduced compared to wild-type thymocytes (Figure 5-2B). There was an approximately 50 % greater level of thymocyte viability of *lck<sup>Pr</sup>-xiap* thymocytes (n=8, p<0.01) compared to those of wild-type littermates at 18 h post-irradiation. *Lck<sup>Pr</sup>-xiap* thymocytes were also able to withstand PCD activation signaled via a mouse specific  $\alpha$ -Fas antibody, both in combination with cycloheximide, (60 % viability compared to 10 % for wild-type littermates at 12 h post-treatment (n=24, p<0.01)), and without cycloheximide, (90 % viability compared to 30 % for wild-type littermates (n=24, p<0.01)) (Figure 5-2C). As a control, a human specific anti-Fas antibody had no effect on either the transgenic or wild-type thymocytes (Figure 5-2D).

Next the ability of the XIAP to interfere with T cell-specific apoptotic events by using the apoptotic triggers  $\alpha$ -CD3 antibody and dexamethasone was investigated. Elimination of pernicious self-reactive T cells, believed to be the

**Figure 5-2. XIAP inhibits general apoptotic triggers, in vitro**

(A) Thymocytes from *lck<sup>Pr</sup>-xiap* lines 1 (n=16, p<0.01) and 2 (n=12, p<0.01), and wild-type thymocytes ( $5 \times 10^6$  cells/ml) were exposed to C2 ceramide (10  $\mu$ M) for the time periods indicated. (B) *Lck<sup>Pr</sup>-xiap* line 1 and wild-type thymocytes ( $5 \times 10^6$  cells/ml) were exposed to UV radiation (1000 mJ/cm<sup>2</sup>) (n=8, p<0.01) for the time periods indicated. (C) *Lck<sup>Pr</sup>-xiap* line 1 and wild-type thymocytes ( $5 \times 10^6$  cells/ml) were treated with either mouse specific  $\alpha$ -Fas antibody clone Jo2 (5  $\mu$ g/ml) in combination with cycloheximide (30  $\mu$ g/ml) (n=24, p<0.01), with cycloheximide (30  $\mu$ g/ml) alone or antibody Jo2 (n=24, p<0.01) alone for 12 h. (D) *Lck<sup>Pr</sup>-xiap* line 1 and wild-type thymocytes ( $5 \times 10^6$  cells/ml) were treated with either mouse specific  $\alpha$ -Fas antibody clone Jo2 (5  $\mu$ g/ml) (n=20, p<0.01) or human specific  $\alpha$ -Fas antibody (5  $\mu$ g/ml) (n=20, p<0.01) for 18 h. All mice used were between 4-5 weeks old.

**A****B****C****D**

primary mediators of auto-immunity, occurs via a thymus-specific negative-selective apoptotic process known as clonal deletion. *In vitro* treatment of thymocytes with  $\alpha$ -CD3 antibody is widely accepted as a model for *in vivo* negative selection whereas the elimination of thymic endogenous glucocorticoids has been shown to accelerate the thymocyte maturation process and disrupt thymocyte positive selection (Vacchio and Ashwell 1997). Both  $\alpha$ -CD3 antibody and dexamethasone trigger *in vivo* and *in vitro* apoptosis of thymocytes (Sentman, Shutter et al. 1991). *Lck<sup>Pr</sup>-xiap* thymocytes treated with dexamethasone (100 nM) or  $\alpha$ -CD3 antibody *in vitro*, at a concentration optimal for inducing death in wild-type cells (10  $\mu$ g/ml), demonstrated enhanced resistance to apoptosis. The number of viable *Lck<sup>Pr</sup>-xiap* thymocytes was approximately double at each time point for both dexamethasone (n=30, p<0.01) and  $\alpha$ -CD3 (n=16, p<0.01) relative to thymocytes from wild-type littermates (Figure 5-3A and 3B respectively).

The broad resistance to apoptosis demonstrated by *Lck<sup>Pr</sup>-xiap* -derived thymocytes can be directly attributed to the over-expression of XIAP for two reasons. Analogous results were obtained with thymocytes from the two independent transgenic lines, and suppression of apoptosis was expression-level dependent, as was demonstrated by comparing the apoptosis-inhibition and corresponding transgene-expression levels of transgenic lines 1 and 2. Both transgene expression levels (Figure 5-1A) and suppression of apoptosis of line 2 (Figure 5-2A and 3B) *Lck<sup>Pr</sup>-xiap* mice were less those of line 1.

### **cIAP2-deficient thymocytes are highly susceptible to apoptotic death, *in vitro***

It was observed that over-expressing an apoptotic inhibitor, XIAP, protects thymocytes from apoptotic stimuli, therefore, ablating an anti-apoptotic protein, cIAP2, could increase thymocyte susceptibility. Prior to exposing cIAP2-deficient thymocytes to a variety of different apoptotic agents the viability of these thymocytes in culture was determined. cIAP2<sup>-/-</sup> thymocytes, not exposed to any apoptotic stimuli, were generally not viable in culture (Figure 5-4A). In contrast to cultured cIAP2<sup>-/+</sup> and cIAP2<sup>+/+</sup> thymocytes the cIAP2<sup>-/-</sup> thymocyte survival has dropped by 33 % (Figure 5-4A) after only 8 hours. Cultured thymocytes derived from wild-type C57BL/6, xiap-transgenic, cIAP2<sup>-/+</sup> and cIAP2<sup>+/+</sup> mice were 85 to 95 % viable after one week, while cIAP2<sup>-/-</sup> thymocytes were < 25 % viable at the same time point (Figure 5-4B). Due to this lack of viability, further apoptotic paradigms with cIAP2<sup>-/-</sup> thymocytes, *in vitro*, were not possible.

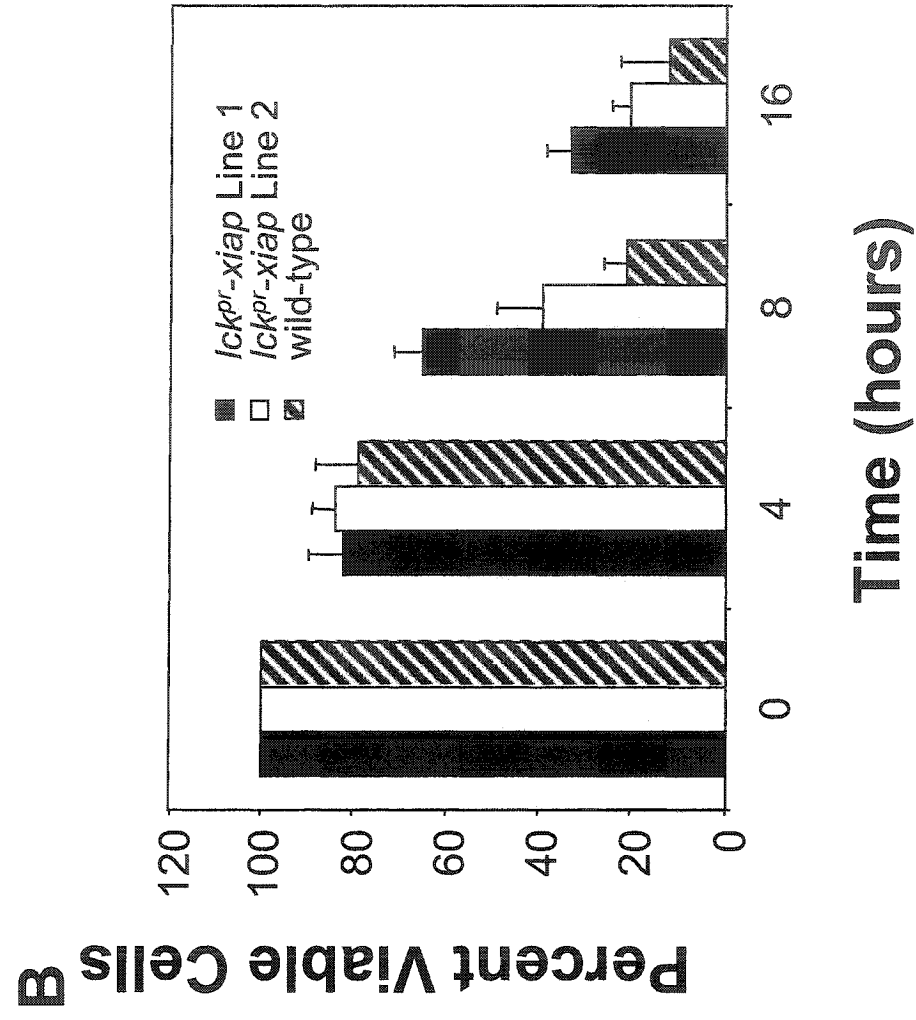
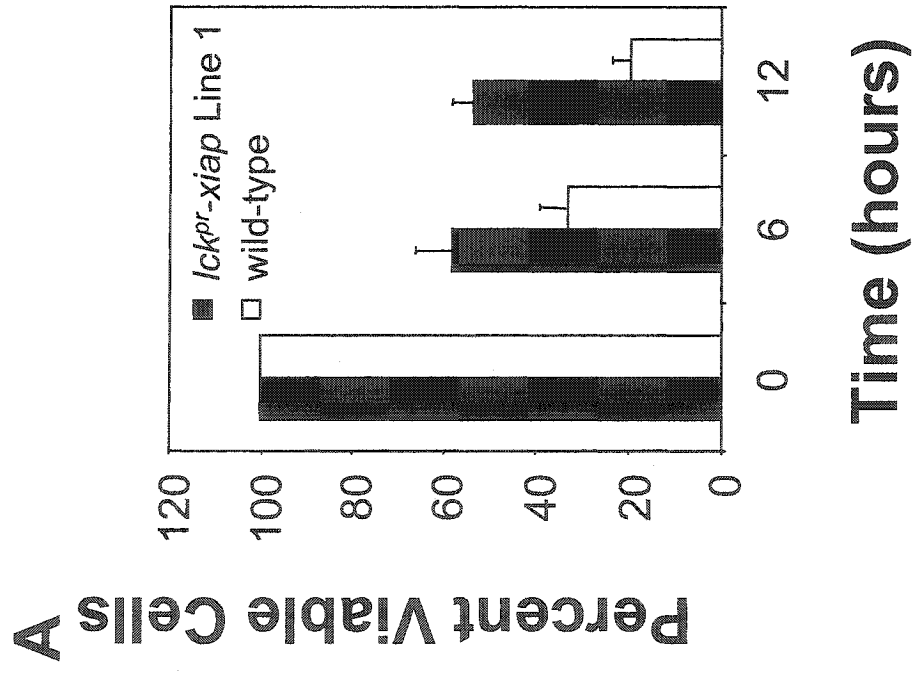
### **Murine endogenous XIAP expression**

Apoptosis susceptibility is known to vary during the life span of thymocytes/T cells, specifically during certain stages of maturation (up to and including the T cell effector stage) suggesting tight regulation of anti-apoptotic proteins. Here the possibility that regulated expression of murine XIAP may determine apoptotic susceptibility of distinct T lymphocyte populations was investigated. Murine XIAP protein expression levels within thymocyte and T cell populations

**Figure 5-3. XIAP inhibits T cell specific apoptotic triggers, in vitro**

(A) *Lck<sup>Pr</sup>-xiap* line 1 and wild-type thymocytes ( $5 \times 10^6$  cells/ml) were exposed to  $\alpha$ -CD3 antibody (10  $\mu$ g/ml) (n=16, p<0.01) for the time periods indicated.

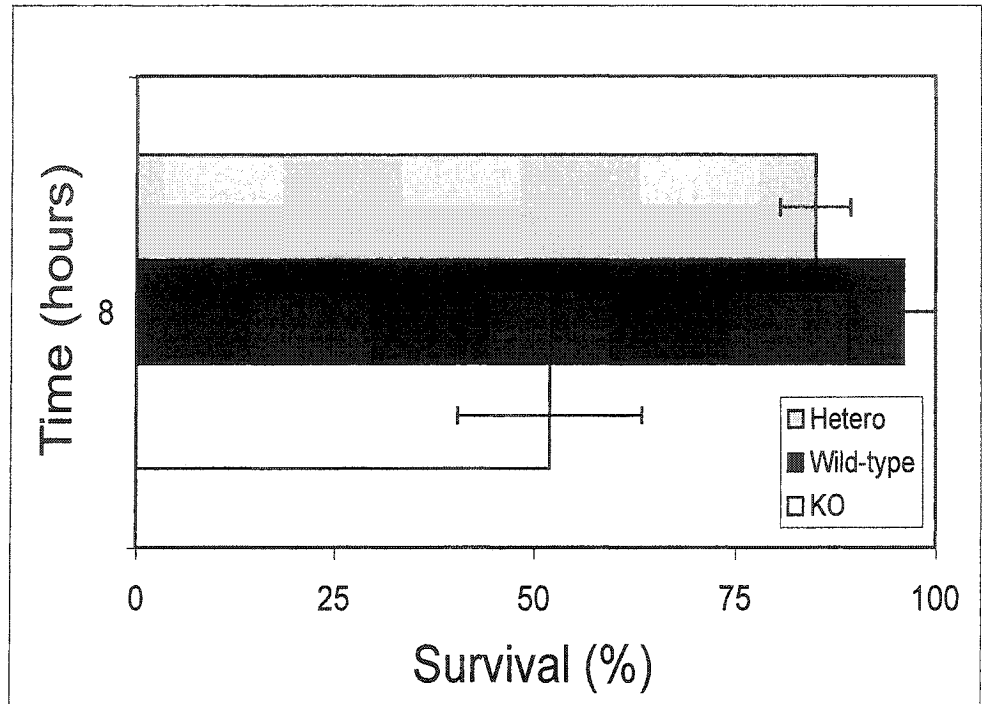
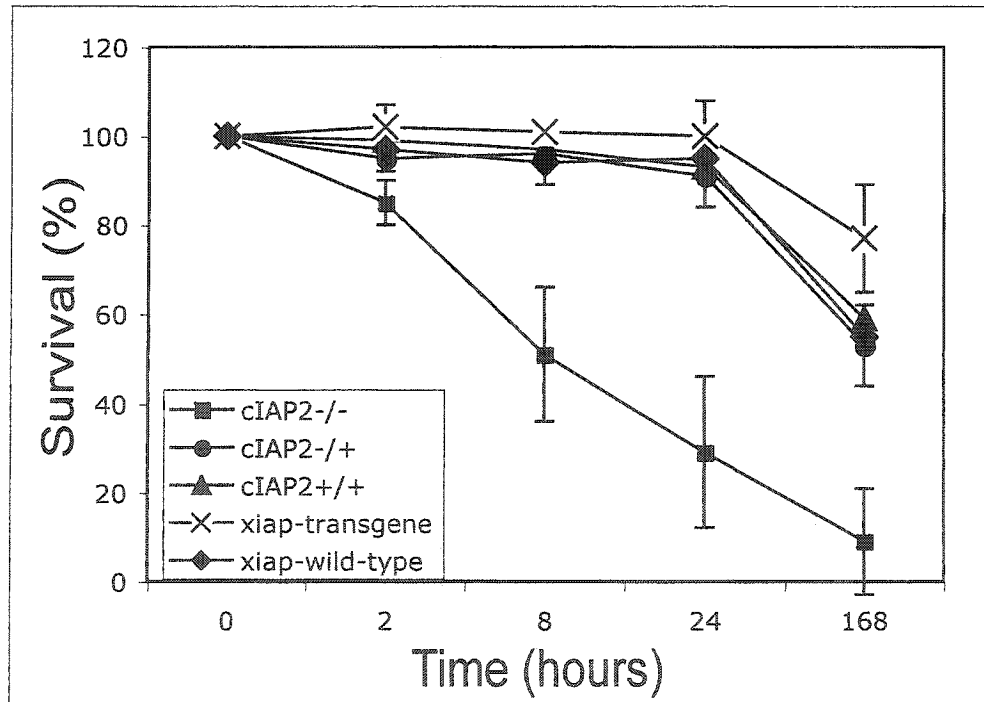
(B) *Lck<sup>Pr</sup>-xiap* lines 1 (n=30, p<0.01) and 2 (n=18, p< 0.01) and wild-type thymocytes ( $5 \times 10^6$  cells/ml) were exposed to dexamethasone (100 nM) for the time periods indicated. All mice used were between 4-5 weeks old.



**Figure 5-4. *cIAP2*<sup>-/-</sup> thymocytes are not viable in culture**

(A) *cIAP2*<sup>-/-</sup>, *cIAP2*<sup>-/+</sup> and *cIAP2*<sup>+/+</sup> thymocytes (n=5, error bars represent mean of triplicate experiments, p<0.05, 5x10<sup>6</sup> cells/ml) were cultured for 8 hours and viability was measured using trypan blue and Annexin-V staining.

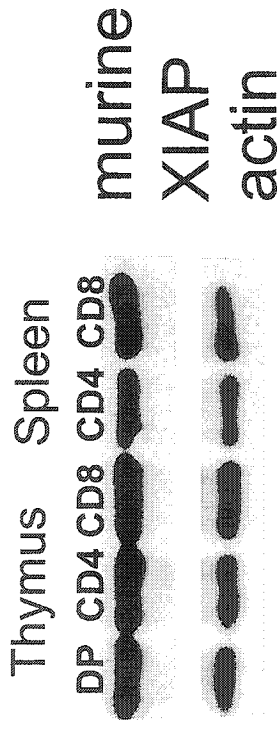
(B) *cIAP2*<sup>-/-</sup>, *cIAP2*<sup>-/+</sup>, *cIAP2*<sup>+/+</sup>, *xiap*-transgene and *xiap*-wild-type thymocytes (n=5, error bars represent mean of triplicate experiments, p<0.01, 5x10<sup>6</sup> cells/ml) were cultured over a range of 1 week and viability was measured using trypan blue and Annexin-V staining. All mice used were between 4-5 weeks old.

**A****B**

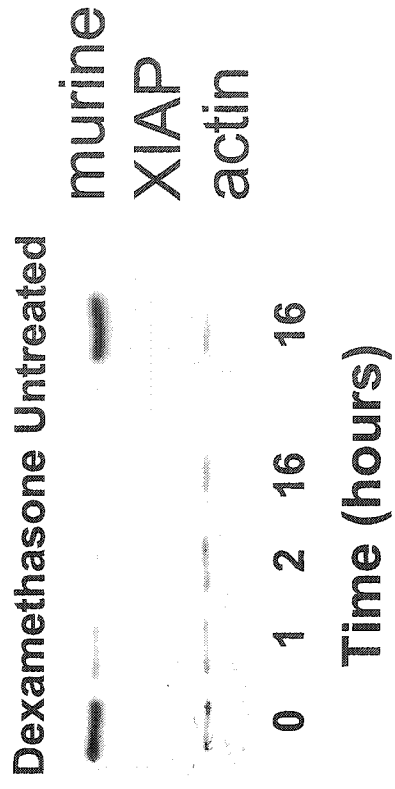
**Figure 5-5. Thymic murine XIAP protein is eliminated upon treatment of wild-type DP thymocytes with dexamethasone, *in vitro***

(A) Western analysis of normal murine endogenous XIAP expression in thymus/T cell sub-populations. (B) Murine endogenous XIAP protein levels of isolated wild-type thymocytes that had been treated with dexamethasone (100 nM) *in vitro*. (C) XIAP transgene protein levels of isolated *Ick<sup>Pr</sup>-xiap* thymocytes (Line 1 mice) that had been treated with dexamethasone (100 nM) *in vitro*. All mice were 4 weeks old.

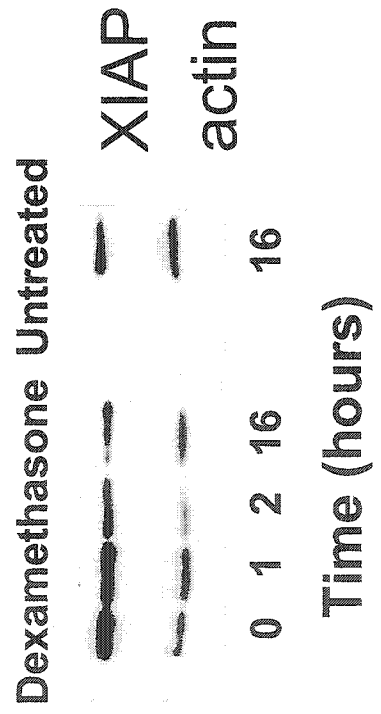
**A**



**B**



**C**



have not been previously reported. As seen in Figure 5-5A and from preliminary work (Chapter 3: Figure 3-5A), murine XIAP is expressed at "high" levels and ubiquitously throughout the total thymocyte and T cell subpopulations. This result was not unexpected, since it was previously demonstrated that relatively high levels of XIAP mRNA were found in all human tissues examined (Liston, Roy et al. 1996). Given that XIAP and murine XIAP are key anti-apoptotic proteins, then why are pre-T cells, DP and effector T cells so highly sensitive to apoptosis? Previously it was shown that upon exposing thymocytes to the apoptotic trigger dexamethasone, XIAP was selectively eliminated prior to overt death (Yang and Ashwell 1999). To assess the potential fate of the XIAP transgene encoded protein, and that of endogenous murine XIAP in apoptotic-sensitive DP thymocytes, the transgene and murine XIAP protein levels, were monitored by Western Blot, during thymocyte exposure to dexamethasone (100 nM) *in vitro*. As seen in Figure 5-5B, in wild-type thymocytes treated with dexamethasone, endogenous murine XIAP protein was eliminated by the 2 h interval while apoptosis was not observed until 4 h post exposure (Figure 5-3B). However, transgene encoded protein levels were reduced but still detectable at 2 h in *lck<sup>pr</sup>-xiap* thymocytes treated dexamethasone at the 2 h interval. Transgene encoded XIAP protein was not eliminated possibly due to an excess of both endogenous murine XIAP and the transgene XIAP proteins present in the *lck<sup>pr</sup>-xiap* thymocytes. These results suggest that the high susceptibility of DP thymocytes to apoptosis may be the result of the rapid elimination of endogenous murine XIAP prior to apoptotic death. More importantly, these results also suggest that the

apoptotic-resistance of *lck<sup>Pr</sup>-xiap* thymocytes compared to wild-type littermates is directly attributable to the continued presence of the transgene protein XIAP.

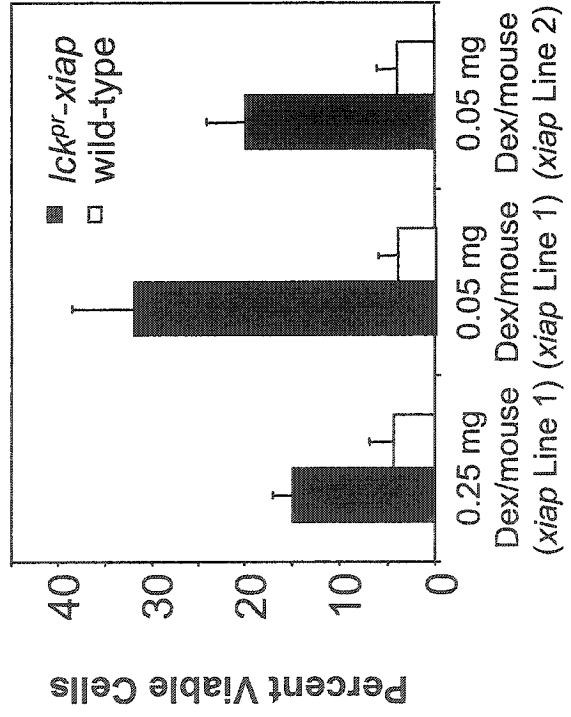
**Over-expression of XIAP transgene inhibits dexamethasone and  $\alpha$ -Fas initiated apoptosis, *in vivo***

Since *lck<sup>Pr</sup>-xiap* thymocytes resisted *in vitro* apoptotic signals, it was then questioned whether the transgene might also inhibit apoptosis *in vivo*. Wild-type thymocytes are quickly depleted when mice are treated with either dexamethasone or  $\alpha$ -Fas antibody. Specifically, treating mice with dexamethasone (from 0.1 to 0.5 mg/mouse, 48 h), results in  $\geq 95$  % depletion of thymocytes, mostly of the double positive (DP) CD4<sup>+</sup>CD8<sup>+</sup> thymocyte population (Blomgren and Svedmyr 1971; Sentman, Shutter et al. 1991). Here the *lck<sup>Pr</sup>-xiap* or wild-type C57BL/6 mice were treated with either saline, 0.05 or 0.25 mg of dexamethasone intraperitoneally and examined thymocytes 48 h later. Dexamethasone at 0.05 or 0.25 mg reduced thymocyte numbers by 94 and 96 % respectively in control mice, relative to saline treatment alone (Figure 5-6A). However, thymocytes derived from *lck<sup>Pr</sup>-xiap* line 1 mice were depleted by only 72 - 82 % with either dose. Flow cytometric analysis of the surviving thymocytes shows that all the DP population had been eliminated in the control animals (Figure 5-6B), while  $\sim 23$  % survived in the *lck<sup>Pr</sup>-xiap* mice (n=20, p<0.01) (Figure 5-6B). Again, it was found that this effect was dose-dependent, since lower transgene expression levels in line 2 mice correlated with decreased protection against dexamethasone (Figure 5-6A).

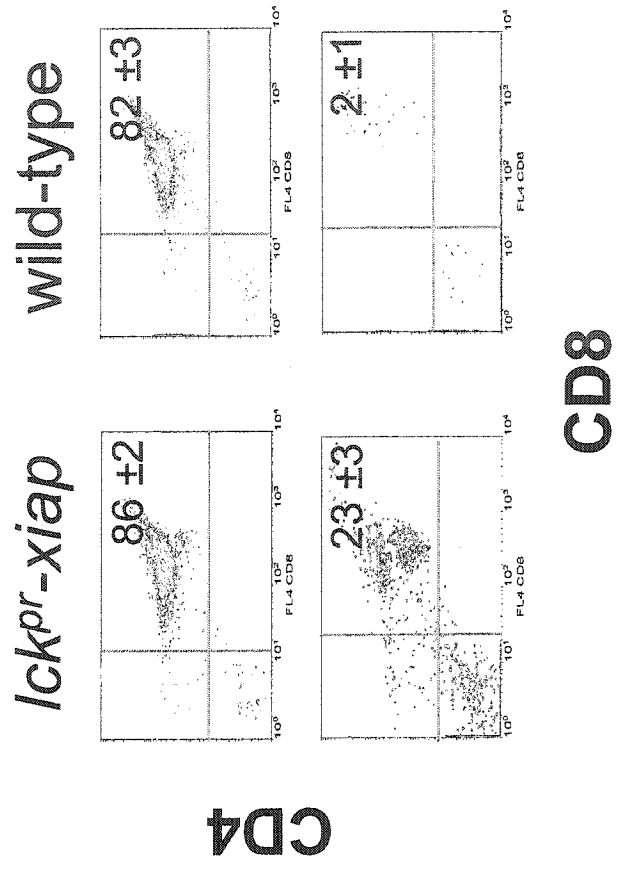
**Figure 5-6. XIAP inhibits the apoptotic trigger dexamethasone, *in vivo***

(A) Total thymocytes recovered 48 h after treatment with 0.05 mg [*lck<sup>pr</sup>-xiap* lines 1 (n=20, p<0.01) and line 2 (n=18, p<0.01)] or 0.25 mg [*lck<sup>pr</sup>-xiap* lines 1 (n=20, p<0.01)] of dexamethasone intraperitoneally. All mice used were between 4-5 weeks old. (B) Two-colour flow cytometry contour plots of CD4 and CD8 expression on surviving thymocytes from *lck<sup>pr</sup>-xiap* line 1 and wild-type mice. The surviving 0.25 mg dexamethasone or saline treated thymocytes were doubly stained with  $\alpha$ -CD4-phycoerythrin (PE) and CD8a-Cy-Chrome. The percentages of the T cell sub-populations given are the average from all the mice used in the corresponding experiment.

**A**



**B**

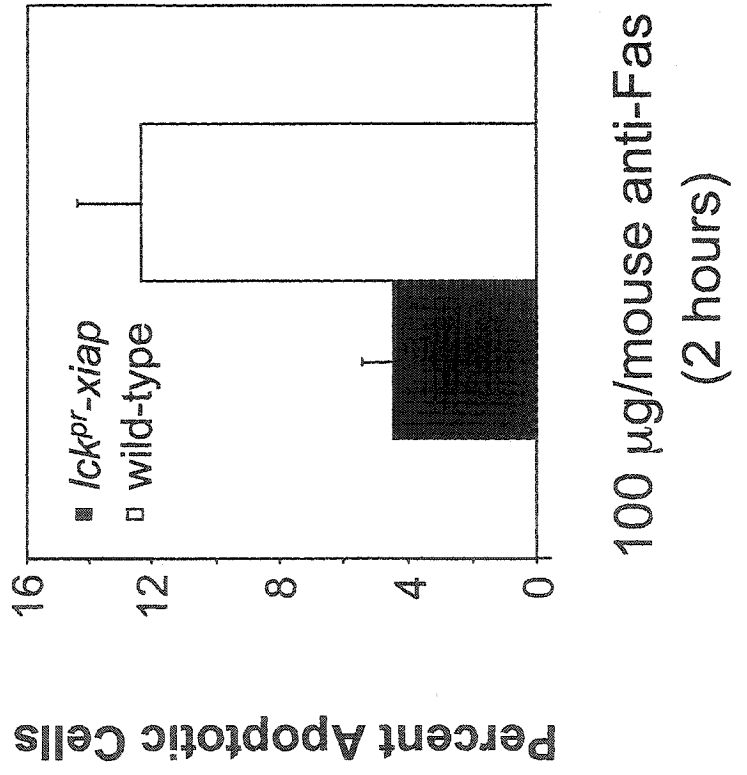


Studies of the mutant mice *lymphoproliferation* (*lpr*) and *generalized lymphoproliferative disease* (*gld*) (Watanabe-Fukunaga, Brannan et al. 1992; Takahashi, Tanaka et al. 1994), which carry loss-of-function mutations in the Fas and FasL genes, have helped to define an essential role for Fas in development, homeostasis and self-tolerance of lymphocytes in both mice and humans (Nagata and Suda 1995). Although both DP and SP thymocytes express Fas in the mouse thymus, the DP population is most sensitive to Fas triggered apoptosis. In order to study whether *lck<sup>PR</sup>-xiap* thymocytes resist Fas-induced apoptosis *in vivo*, as we previously demonstrated *in vitro*, the effect of  $\alpha$ -Fas antibody in *lck<sup>PR</sup>-xiap* mice was analyzed. Mouse specific  $\alpha$ -Fas antibody (clone Jo2) (100  $\mu$ g/mouse) was injected intraperitoneally into control and *lck<sup>PR</sup>-xiap* mice, and levels of apoptosis were compared in liver and thymus 2 h later. Annexin V staining of thymocytes, followed by flow cytometry analysis, assessed the extent of apoptosis. The thymocytes of mice treated with Jo2 antibody were shown to undergo extensive apoptotic death. As well, intraperitoneal inoculation of this antibody has been previously shown to cause hepatic failure and death of BALB/c and C57BL/6 mice within hours, due to Fas-mediated hepatocyte apoptosis (Ogasawara, Watanabe-Fukunaga et al. 1993; Bulfone-Paus, Ungureanu et al. 1997). Significantly fewer thymocytes from *lck<sup>PR</sup>-xiap* mice were undergoing apoptosis than in control mice after treatment with Jo2; 4.5 % compared to 12 % (Figure 5-7A) (n=24, p<0.01). The DNA laddering pattern obtained from DNA isolated from wild-

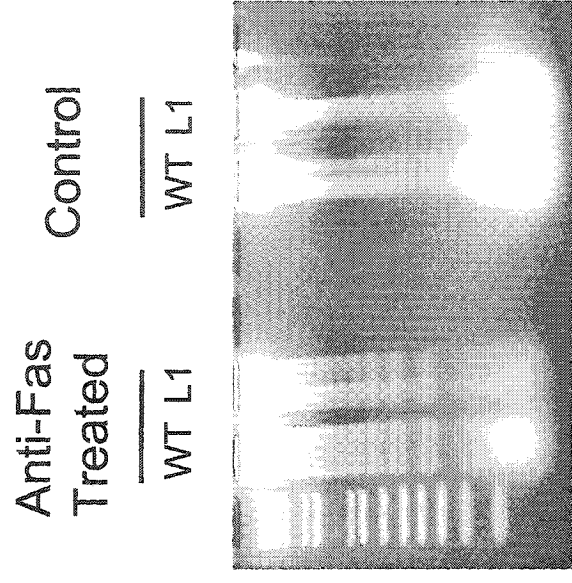
**Figure 5-7. XIAP inhibits  $\alpha$ -Fas antibody triggered apoptosis, *in vivo***

Total *Ick<sup>Pr</sup>-xiap* lines 1 (n=24, p<0.01) and wild-type thymocytes undergoing apoptosis 2 h after treatment with 100  $\mu$ g of clone Jo2 anti-Fas antibody intraperitoneally (a). All mice used were between 4-5 weeks old. (b) Agarose gel electrophoresis of DNA extracted from the livers of  $\alpha$ -Fas treated wild-type (WT) and *Ick<sup>Pr</sup>-xiap* line 1 mice (100  $\mu$ g, 2 h) and non-treated saline injected control mice.

**A**



**B**



type and *lck<sup>Pr</sup>-xiap* hepatocytes demonstrates that both sets of Jo2 injected mice were under going extensive apoptosis in other tissues (Figure 5-7B).

### **T Cell Homeostasis is disrupted in *lck<sup>Pr</sup>-xiap* mice, but not in *cIAP2<sup>-/-</sup>* mice**

#### Thymus

##### ***xiap-transgenic thymocyte cell counts***

The above results clearly demonstrated that *lck<sup>Pr</sup>-xiap* thymocytes resist a variety of general and T cell-specific apoptotic signals, both *in vitro* and *in vivo*. Therefore it may be expected that over-expression of the xiap transgene may have an adverse effect on T cell ontogeny and cell numbers. Therefore the thymocyte numbers in mice of varying ages, ranging from 1 week to 4 months were enumerated. As seen in Table 5-1, the total number of thymocytes was increased by 13 - 37 % at all time points from line 1 mice, while only marginally so (5 -15%) for line 2 mice. The greatest difference was found in four-week-old mice, where line 1 *lck<sup>Pr</sup>-xiap* mice had 37 % more thymocytes (n=80, p<0.01) than wild-type littermates.

##### ***cIAP2-null mice thymocyte cell counts***

Since *cIAP2<sup>-/-</sup>* thymocytes are not viable in culture, it would be expected that

**Table 5-1. Thymocyte counts in normal and *lck<sup>pr</sup>-xiap* mice<sup>a,b</sup>**

<b>Mouse Strain</b>		<b>Age</b>		
		<b>1 week</b>	<b>4 weeks</b>	<b>4 months</b>
T cells/ thymus	<i>Lck<sup>pr-</sup> xiap line 1</i>	9.6x10 <sup>7</sup> ±0.3	1.60x10 <sup>8</sup> ±0.4	1.0x10 <sup>8</sup> ±0.2
	<b>Wild-type control</b>	8.2x10 <sup>7</sup> ±0.2	1.17x10 <sup>8</sup> ±0.3	8.8x10 <sup>7</sup> ±0.2
<b>n value</b>		26	80	46
T cells/ thymus	<i>Lck<sup>pr-</sup> xiap line 2</i>	8.6x10 <sup>7</sup> ±0.2	1.21x10 <sup>8</sup> ±0.4	9.1x10 <sup>7</sup> ±0.3
	<b>Wild-type control</b>	8.1x10 <sup>7</sup> ±0.3	1.06x10 <sup>8</sup> ±0.4	8.9x10 <sup>7</sup> ±0.4
<b>n value</b>		12	25	10

<sup>a</sup>Total cell numbers were determined by trypan blue exclusion

<sup>b</sup>P values for all ages were less than 0.01

total cIAP2<sup>-/-</sup> thymocyte numbers could be lower than normal littermates. However, as seen from Chapter 4 Figure 4-3E thymocyte counts of cIAP2<sup>-/-</sup> (1.9 X 10<sup>8</sup> cells, ±0.5), cIAP2<sup>+/+</sup> (2.2 X 10<sup>8</sup> cells, ±0.4) and cIAP2<sup>+/-</sup> (2.0 X 10<sup>8</sup> cells, ±0.6) were comparable for female mice 4-6 weeks of age.

***Increased Double Negative (CD4<sup>-</sup>CD8<sup>-</sup>) Population of xiap-transgene thymus***

Since XIAP over-expression altered total thymocyte numbers, the effect on sub-populations of maturing thymocytes was determined. When analyzed by flow cytometry the percentage of DN CD4<sup>-</sup>CD8<sup>-</sup> pro-T cells was 4.5 fold greater (n=36, p<0.01) than wild-type littermates (Table 5-2). The percentage of double positive CD4<sup>+</sup>CD8<sup>+</sup> cells was only slightly elevated, while the percentage of single positive (SP) population, which represents mature T cells, was normal. However, the CD4<sup>+</sup>/CD8<sup>+</sup> ratio of single positive thymocytes in *lck<sup>pr</sup>-xiap* transgenic mice was inverted with respect to wild-type littermates (Table 5-2). Western blot analysis revealed no difference in transgene xiap expression in SP CD4<sup>+</sup> versus CD8<sup>+</sup> thymocytes (data not shown). The same inversion had been noted previously in transgenic mice expressing Bcl-2 under the control of the *lck<sup>pr</sup>* (Sentman, Shutter et al. 1991). These results demonstrate that *lck<sup>pr</sup>-xiap* transgenic mice have a moderate increase in the number of thymocytes and a skewing of the T cell population in the thymus, suggesting that over-expression of XIAP does interfere with T cell maturation and/or development.

**Table 5-2. Flow cytometry of triple stained thymocytes from normal and *lck<sup>Pr</sup>-xiap* mice<sup>a,b</sup>**

<b>Mouse Strain</b>	<b>Thymic T cell Sub-Populations at age 4-5 weeks</b>			
	<b>Thy1.2</b>	<b>DN</b>	<b>DP</b>	<b>CD4/CD8 Ratio</b>
<b><i>Lck<sup>Pr</sup>-xiap</i></b> (Line 1)	96% ±3	2.3% ±0.5	88% ±2	0.44 ±0.1
<b>Wild-type control</b>	91% ± 4	0.6% ±0.2	83% ±5	2.3 ±0.7

<sup>a</sup>Flow cytometry of cells containing CD4, CD8 or Thy1.2 were analyzed using three-colour immunofluorescence on 10, 000 cells per sample

<sup>b</sup>P values for all ages were less than 0.01 and n=46 for each T cell sub-population.

### ***Increased Double Positive (CD4<sup>+</sup>CD8<sup>+</sup>) Population of cIAP2<sup>-/-</sup> thymus***

There was no overall decrease in the number of cIAP2<sup>-/-</sup> thymocytes. However, there was a distinct upward skew of the DP population at the expense of the other main thymocyte sub-populations. cIAP2<sup>-/-</sup> thymic DP population was  $88.3 \pm 0.57$ , n = 35, and wild-type littermate DP population was  $83.6 \pm 0.68$ , n = 28 (females aged 4-6 weeks old, p < 0.01).

### Spleen

#### Spleen T cell counts

The *lck<sup>PR</sup>-xiap* transgenic mice have a moderate elevation in thymocytes compared to wild-type littermates, but the number of mature T cells in the SP thymocyte pool was no different than in wild-type. When the spleen was examined, there was a more dramatic increase in cell numbers, which increased with the age of the animal. In all cases the total number of isolated splenic lymphocytes were determined by trypan blue exclusion, and the T cell percentage was then subsequently ascertained using flow cytometry. The results from Table 5-3 suggest that there is an ongoing accumulation of T cells in the spleen. At the 4 month time point there were  $4.5 \times 10^7$  splenic T cells from spleens of mice from line 1 more than double those of control spleens  $1.9 \times 10^7$  (n=56, p<0.01).

As observed in the thymus, the inverted thymic SP CD4<sup>+</sup>/CD8<sup>+</sup> ratio was also observed within the spleen. It was discovered that splenocytes from four

**Table 5-3. Spleen counts in normal and *lck<sup>pr</sup>-xiap* mice<sup>a,b</sup>**

Mouse Strain		Age		
		1 week	4 weeks	4 months
T cells/ spleen	<i>Lck<sup>pr</sup>- xiap line 1</i>	6.6x10 <sup>6</sup> ±0.1	3.1x10 <sup>7</sup> ±0.1	4.5x10 <sup>7</sup> ±0.2
	<b>Wild- type control</b>	4.0x10 <sup>6</sup> ±0.2	1.1x10 <sup>7</sup> ±0.3	1.9x10 <sup>7</sup> ±0.5
<b>n value</b>		22	40	56

<sup>a</sup>Total cell numbers were determined by trypan blue exclusion

<sup>b</sup>P values for all ages were less than 0.01

month old *lck<sup>Pr</sup>-xiap* mice had a CD4<sup>+</sup>/CD8<sup>+</sup> ratio of 0.65 ±0.5 (n=30) compared to 1.7 ±0.3 (n=26) for the wild-type controls. This phenomenon is comparable to what was found with *lck<sup>Pr</sup>-bcl-2* transgenics (Sentman, Shutter et al. 1991), where SP CD8<sup>+</sup> population was elevated relative to SP CD4<sup>+</sup>.

## ***Discussion***

Previous *in vivo* XIAP anti-apoptotic functional studies have employed adenovirus over-expression models (LaCasse, Baird et al. 1998), raising concerns about possible secondary adenovirus mediated effects, such as inflammation. Furthermore, adenovirus expression levels are sufficiently high to raise the issue of the physiological relevance of the effects observed. The data presented here are the first demonstrations of the effects of XIAP over-expression and cIAP2 ablation on the viability of thymocytes and T cells derived from a novel IAP-engineered transgenic model, a *lck<sup>Pr</sup>-xiap* transgenic mouse and a cIAP2-null mouse, after challenge with various apoptotic triggers.

When over-expressed, members of the IAP family have been shown to block a broad range of apoptotic signals (LaCasse, Baird et al. 1998; Deveraux and Reed 1999) via the inhibition of caspases. XIAP inhibits more activators of PCD than the two well-known anti-apoptotic proteins of the Bcl family, Bcl-2 and Bcl-x<sub>L</sub>. The ability of XIAP and to resist a wide spectrum of apoptotic signals and triggers is illustrated with the use of dexamethasone and α-Fas antibody as apoptotic promoters. Both are capable of triggering apoptosis *in vivo* and *in*

*vitro*, although the pathways are distinct (Strasser, Harris et al. 1995). Thymic over-expression of Bcl-2 and Bcl-x<sub>L</sub> transgenes provided resistance against dexamethasone induced PCD *in vivo* and *in vitro*, but did not affect Fas induced apoptosis (Strasser, Harris et al. 1995). In contrast, this data demonstrated that over-expression of XIAP in thymocytes was able to inhibit both (Figures 5-2C and 3B), consistent with XIAP functioning at the convergence of these discrete apoptotic pathways.

Beginning with a maturing pre-T cell through to the effector T cell stage, a developing T lymphocyte proceeds through different intervals of relative high and low apoptotic susceptibility. These results suggest a correlation between murine XIAP or XIAP transgene levels with thymocyte sub-population sensitivity or resistance to PCD. The proteolytic removal of murine XIAP has been previously demonstrated to occur in thymocytes exposed to dexamethasone. Moreover, murine XIAP removal was specific, while the protein levels of Bcl-x<sub>L</sub>, which is maximally expressed in DP thymocytes (Grillot, Merino et al. 1995), were unaffected (Yang and Ashwell 1999). In contrast, Bcl-2 protein has been shown to be down regulated during the most apoptotic sensitive stage in thymocyte development, DP thymocytes, and at the effector T cell stage (Strasser, Harris et al. 1991). However, over-expression of Bcl-2 specifically in the mouse thymus did not result in any accumulation of thymocytes or peripheral T cells (Sentman, Shutter et al. 1991; Strasser, Harris et al. 1991). In contrast, these data establish that endogenous XIAP, murine XIAP, was uniformly expressed throughout the mouse thymocyte and T cell sub-populations (Figure 5-5A.), and that murine

XIAP was eliminated by 2 h post dexamethasone exposure prior to apparent apoptotic death (Figure 5-5B). Most importantly, the data demonstrate that apoptotic-resistant *lck<sup>PR</sup>-xiap* thymocytes, at the same time point, still contain visible levels of transgene encoded XIAP protein (Figure 5-5C).

Increased resistance to apoptotic triggers in thymocytes of transgenic mice has resulted in some perturbations of thymocyte maturation as well as increased numbers of cells in both the thymus and spleen. In particular, the proportion of DN pro-T cells was elevated, suggesting that some cells that would normally die by neglect are surviving but are not able to progress to the DP stage. Consequently, maturation of cells that are competent is not disrupted and the proportion of SP mature cells is unchanged compared to controls. However, *lck<sup>PR</sup>-xiap* mice demonstrated a preferential maturation of SP CD4<sup>-</sup>CD8<sup>+</sup> over SP CD4<sup>+</sup>CD8<sup>-</sup> (Table 5-2), which was comparable to what had been observed in *lck<sup>PR</sup>-bcl-2* transgenic mice (Sentman, Shutter et al. 1991), and in contrast to T cell development in normal mice. It has been speculated that the observed increase in the number of SP CD4<sup>-</sup>CD8<sup>+</sup> cells is directly attributable to the prolonged survival of the *lck<sup>PR</sup>-bcl-2/-xiap* thymocytes compared to wild-type mice (Tao, Teh et al. 1994). The cytoplasmic tail of the CD4 receptor interacts with the signal transduction molecule p56<sup>lck</sup> with a much higher affinity compared to CD8 (Turner, Brodsky et al. 1990), which may lead to a reduction in the efficiency of positive selection of SP CD4<sup>-</sup>CD8<sup>+</sup> thymocytes relative to SP CD4<sup>+</sup>CD8<sup>-</sup>, in normal mice. The protracted survival of the *lck<sup>PR</sup>-bcl-2/xiap* thymocytes compared to thymocytes of wild-type mice may compensate for the relatively inefficient

selection of SP CD4<sup>-</sup>CD8<sup>+</sup>, therefore, leading to a relative increase in the number of SP CD4<sup>-</sup>CD8<sup>+</sup> thymocytes found in normal mice, as was observed in the *lck<sup>PR</sup>-bcl-2/-xiap* transgenic mice (Tao, Teh et al. 1994). Although no increase in SP mature cells was noted in the thymus, T cell numbers in the spleen increased with the age of the mouse. Future work will examine the basis of this perturbation in T cell development.

Over-expression of an anti-apoptotic protein results in an outcome analogous to knocking-out a pro-apoptotic gene. Disruption of the Bim gene, a pro-apoptotic member of the Bcl-2 family, also resulted in phenotypic changes similar to what was observed in the *lck<sup>PR</sup>-xiap* transgenic mice (Bouillet, Metcalf et al. 1999). Both Bim knock-out mice and *lck<sup>PR</sup>-xiap* animals displayed a significant increase in DN pro-T cells (Table 5-1) as well as an ever increasing splenic T lymphocyte population (Table 5-3) (Bouillet, Metcalf et al. 1999). Knocking out the Bim gene resulted in a disruption in T cell development and eventually the Bim animals succumbed to autoimmune kidney disease. As a future study it would be interesting to investigate whether the over-expression of a transgene encoded XIAP protein in T cells would eventually lead to autoimmunity in aged mice.

#### **Significance of cIAP2<sup>-/-</sup> thymocyte data and collaborative work done**

In contrast to *xiap*-transgene thymocytes cIAP2<sup>-/-</sup> thymocytes were highly sensitive when removed from the thymic environment compared to littermate controls (Figure 5-4B). As seen in Figure 5-2C in Chapter 3 there was observed an up-regulation of cIAP2 message in wild-type thymocytes that were freshly cultured. This suggests that a loss of some key survival factor,

either some agent or thymic epithelial surface receptor, is compensated for by the up-regulation of cIAP2 in wild-type, but not in cIAP2-deficient thymocytes. This scenario is analogous to work presented in Chapter 4, where, only in situations of cIAP2-induction, such as with macrophages and T cells stimulated with LPS or IL-7, respectively, are the protective properties of cIAP2 observed. To investigate the role of cIAP2 in T cell development in a more appropriate mouse model, the mice were back-crossed onto transgenic mice models that are used to study negative and positive selection in the thymus, in the laboratory of Dr. Pere Santamaria, University of Calgary, Alberta, Canada. The preliminary results suggest that the lack of cIAP2 enhances negative selection, however, cIAP2 ablation has the opposite effect on positive selection (personal communication). The data presented here suggest that cIAP2 does have a role in thymocyte maturation however, at this point the exact nature is unknown and more work needs to be done.

The data presented here suggest that the perturbation of T cell development in the transgenic mice was due to a corresponding *in vivo* resistance to apoptotic triggers which was directly related to the over-expression of the anti-apoptotic protein, XIAP.

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## **Chapter 6: Discussion**

### **Initial thesis “working” hypothesis**

At the onset of my studies the following statement: “IAPs are potent inhibitors of apoptosis and provide protection against a wide range of death stimuli”, encapsulated the extent of IAP investigative work. The mechanisms of inhibitory action, specifically the sequestering and repression of both initiator and executioner caspases, were not known. Concurrently, it had been clearly demonstrated that controlled cell death played a central role in all aspects of immune functions (see Figure 6-1 for a summary of T cell development, function and apoptotic check-points) (Scaffidi, Kirchhoff et al. 1999). Therefore, it seemed reasonable to formulate the following hypothesis: that there may be a potential role, either direct or indirect, for the IAPs in normal immune function.

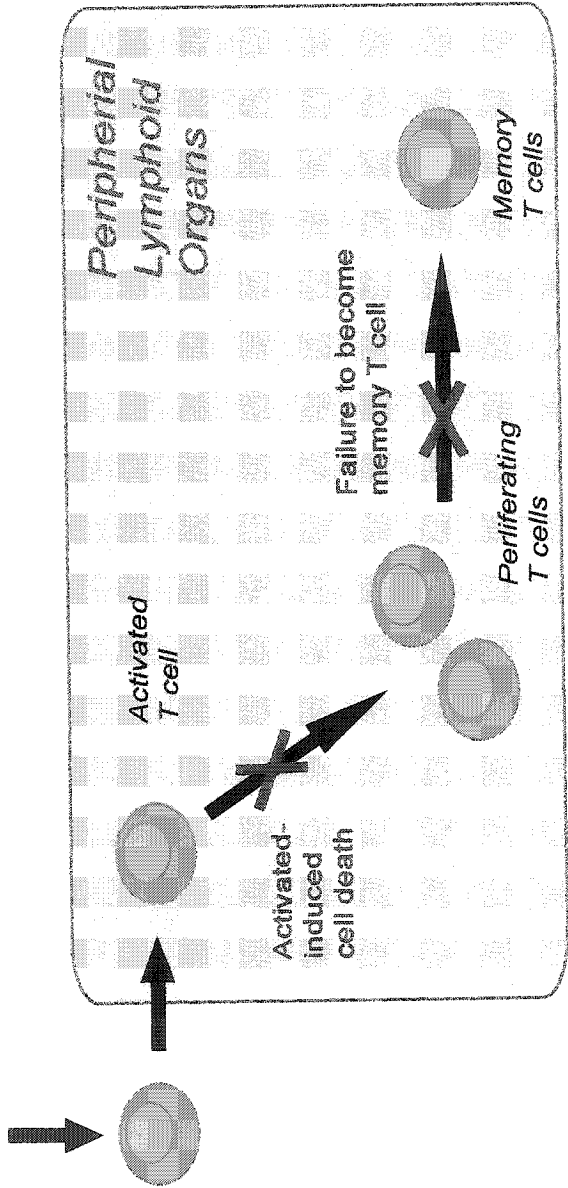
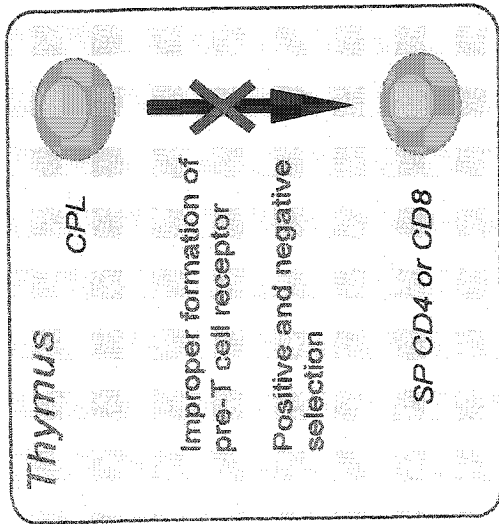
### **IAPs and the immune system: present knowledge and relevance of thesis work**

#### **IAPs and immune disorders**

Animal models are well recognized for their value in the study of mechanisms of human disease. The initial models involved spontaneous diseases that mimicked human disorders. One such study correlated the loss of NAIP in the inbred mouse strain A/J to the human disorder Legionnaires (Diez, Yaraghi et al. 2000). The gene ablation of *ciap2* created a similar scenario as with the

**Figure 6-1. T cell apoptotic “check-points”**

A schematic diagram of apoptotic “check-points” during T cell development and function. Thymocyte development includes multiple stages of apoptotic regulation: (1) pre-T cell receptor formation; and (2) negative and positive selection. Two other stages of apoptotic regulation are during T cell activation and during the waning of an immune response.



**Periphery**

A/J *NAIP*<sup>-/-</sup> mice, that the up-regulation of cIAP2 is required to maintain macrophage viability under physiological and pathological stresses. The diminished immune response of the *cIAP2*<sup>-/-</sup> mice to systemic LPS protects these mice from endotoxic shock, however, and more importantly, this is also expected to render *cIAP2*<sup>-/-</sup> mice more susceptible to a localized or systemic live bacterial assault compared to *cIAP2*<sup>+/+</sup> littermates. Therefore, this work suggests that pathogenic-elicited cIAP2 up-regulation in macrophages is crucial for a proper innate immune response. Previous studies employing a XIAP-null mouse model failed to provide any evidence for a physiological function and claimed compensatory function for cIAP1 and 2 in the absence of XIAP (Harlin, Reffey et al. 2001). These, most importantly, are the first studies that utilize an IAP-null mouse model that clearly demonstrate a non-redundant function of cIAP2 and that clearly display no compensatory up-regulation of XIAP or cIAP1 in the absence of cIAP2.

IAP dysregulation demonstrated via either over-expression, such as with the *lck*<sup>DT</sup> *xiap* transgenic mice, or loss, as with the *ciap2*-null mice, results in lymphoid hyperplasia or immune repression, respectively. These results were also observed in human disorders with corresponding disrupted IAP regulation. The pathogenesis of multiple sclerosis (MS) is thought to be mediated by the inability to eliminate auto-reactive T lymphocytes via apoptosis (Segal and Cross 2000). Recently it was demonstrated that IAP, XIAP and cIAP2 expression levels in mitogen stimulated T lymphocytes from patients with clinically active MS correlated with the severity of the disease

activity, and with T lymphocyte resistance to apoptosis (Semra, Seidi et al. 2002). However, protein levels of members of the Bcl-2 family did not differ between active and stable MS, and were relatively similar between MS patients and controls. Two recent clinical studies of lymphocytes from the aged or Cartilage-Hair Hypoplasia Syndrome patients correlated the loss of IAP expression, specifically cIAP2, to cell-mediated immunodeficiency (Yel, Aggarwal et al. 1999; Gupta 2004). Therefore, these studies suggest a correlation between IAP dysregulation and disease states.

### **Regulation of IAP immune function**

#### XAIP and cIAP1

Lymphoid quantitative *iap* mRNA analysis for *xiap* and *ciap1* (Chapter 3: Table 1) and thymocyte-specific over-expression of XIAP suggest Chapter 3: Figure 5B) that there is a requirement for the removal of XIAP protein and a reduction of mRNA levels prior to any observed thymocyte death in response to dexamethasone-induced apoptosis. These results also suggest that XIAP expression levels regulate thymocyte sensitivity, and that removal of XIAP is necessary for normal thymocyte development. Previous studies showed that protein degradation is important in T lymphoid apoptosis (Orlowski 1999) and that blocking protein degradation inhibited apoptosis. *In vitro* studies utilizing primary thymocyte cultures have recently demonstrated that upon exposure to either dexamethasone or the apoptotic stimulus etoposide that XIAP and cIAP1 are degraded prior to overt death (Yang, Fang et al. 2000). In addition, it was shown that phytohemagglutinin-induced apoptotic sensitivity in a T cell line, Jurkat cells, was associated with a corresponding

decrease of XIAP protein levels (Vitte-Mony, Korneluk et al. 1997). Therefore, these studies corroborated that IAP immune function in the case of thymocyte-XIAP might be regulated via protein degradation. Notably, the lymphoid *iap* mRNA results from Chapter 3, Table 1 suggest that a corresponding decrease of *xiap* and *ciap1* message accompanies protein degradation. The accompanying loss of *iap* message may be necessary to prevent further de novo protein synthesis. However, the nature of the *iap* mRNA-ablation mechanism and if it is either a translational block or mRNA degradation, or a combination of the two, is unknown.

#### cIAP2

Unlike cIAP1 and XIAP, the results presented here indicate that cIAP2 regulation is signal induced-dependent. An up-regulation of *cIAP2* mRNA was elicited in T cells upon exposure to IL-7 (Chapter 3: Table 2) and a dramatic increase was observed in macrophages treated with LPS (Chapter 3: Figure 6C). Subsequently, several studies, utilizing a variety of cell types and agents, have demonstrated that cIAP2 is highly inducible (LaCasse, Baird et al. 1998; Dong, Wang et al. 2003; Hasegawa, Suzuki et al. 2003). There is a possibility that the cIAPs and XIAP may be able to functional "stand-in" for one another. The caspases inhibited by the IAPs coincide, however, cIAP1 and 2 bind with significantly lower affinities compared to XIAP. In addition, each IAP has unique properties and cellular localizations. XIAP is involved in the TAK1/JNK1 signaling cascade (Sanna, Duckett et al. 1998) while the cIAPs associate with TRAFs (Rothe, Pan et al. 1995). In addition, and more importantly, the observed differences in IAP regulation, demonstrated here,

serve to underscore the non-redundant functions of the IAPs and indicate that these proteins cannot completely functionally substitute for each other.

### **Thymocyte murine IAP protein and mRNA levels**

As a point of reference it was assumed that murine *iap* mRNA and therefore IAP protein expression may determine apoptotic susceptibility of distinct immune populations. XIAP and cIAP1 mRNA levels are largely equivalent in each thymocyte sub-population (Chapter 3: Figure 2A and B). However, the levels of *ciap2* mRNA during the transition from a pre-T to a pro-T thymocyte are seen to drop (Chapter 3: Figure 2C) and increase once again upon the culturing of thymocytes. This is the first report of IAP mRNA levels and XIAP protein levels of key thymocyte developmental sub-populations.

### ***Summary and Relevance: concluding remarks***

Figure 6-2 summarizes the apoptotic “check points” of this thesis work and shows the suggested areas where the experimental results fit within the innate and adaptive immune system.

#### The role of IAPs in the immune system: Good Samaritans or mischief-makers?

At a recently attended Keystone Symposia: ‘Apoptosis and Development’, Keystone Resort, Colorado (2004), the following facetious question/statement was asked of a presenter: “Do XIAP, cIAP1 and 2 only exist to facilitate autoimmune disorders and cancers?”. This speaker was one of a long list of many presenters demonstrating IAP involvement in promoting some type of cancer or immune disorder, prompting the ‘mock’

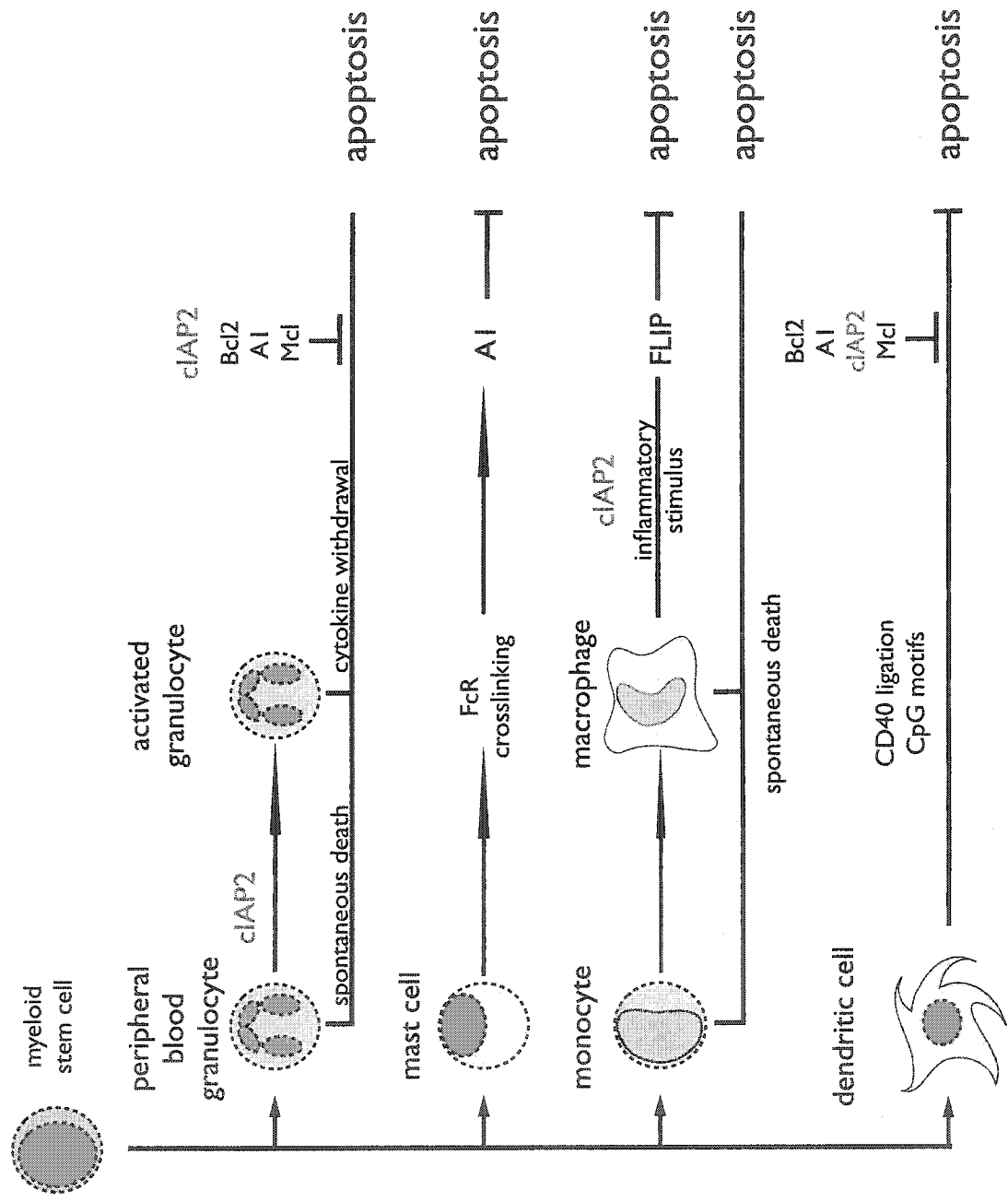
question. Interestingly, this statement does summarize the current direction of IAP research. Indeed, the major body of investigative work involving the IAPs and the immune system centers on IAP ablation as a therapeutic treatment in autoimmune diseases and cancers. In fact, the body of work presented in this thesis does suggest that the ablation of cIAP2 in septic shock and possibly other immune disorders that are mediated by inflammation, such as Crohn's disease, may be of therapeutic value. In addition, the demonstrated anti-apoptotic function of cIAP2 in IL-7 treated T cells suggests that the selected removal of T cell-cIAP2 may be of therapeutic value for MS patients.

However, I believe the thesis work presented here also begins to address the notion that IAPs have a function beyond facilitating the onset of tumors and immune diseases. Specifically, it was demonstrated that cIAP2 is a non-redundant key factor necessary for macrophage survival and function, *in vivo*. Moreover, this work may provide the incentive for additional studies into the possible role of IAPs in the normal 'machinations' of the immune system.

**Figure 6-2. Summary of IAP thesis work**

Increased macrophage-ciap2 mRNA levels in response to LPS-activation suggested a regulatory role for cIAP2 in macrophage innate immunity.

Disrupting cIAP2 endogenous expression and function by engineering a cIAP2-ablated mouse showed that cIAP2 KO macrophages had a heightened susceptibility to apoptosis in a LPS-induced pro-inflammatory environment and hence were unable to maintain a normal inflammatory response.





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Immunol **19**(6): 428-34.

## *Curriculum Vitae*

Damiano Conte

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### **EDUCATION**

- 1997-2004      Ph.D. - BIOCHEMISTRY, MICROBIOLOGY AND IMMUNOLOGY  
University of Ottawa  
Ottawa, Ontario  
Supervisor: Dr. R.G. Korneluk
- 1992-1994      MASTER OF SCIENCE - BIOCHEMISTRY  
University of Toronto  
Toronto, Ontario  
Thesis title: "Iron-substituted estrogen receptor binding  
domain causes DNA damage to cognate response element"  
Supervisor: Dr. B. Sarkar  
Studied the redox potential of iron complexed to the estrogen  
receptor.
- 1981-1983/1985-1987      BACHELOR OF SCIENCE, HONOURS BIOCHEMISTRY  
University of Waterloo  
Waterloo, Ontario

### **PUBLICATIONS**

**Conte, D**, Liston, P, Wong, JW, Wright, KE and RG Korneluk. Thymocyte-targeted over-expression of *xiap* transgene disrupts T lymphoid apoptosis and maturation *Proc. Natl. Acad. Sci. USA* 2001 April 24; 98(9):5049-5054.

Liston, P, Fong, WG, Kelly, NL, Toji, S, Miyazaki, T, **Conte, D**, Tamai, K,

Craig, CG, McBurney, MW, and RG Korneluk. XAF1 regulates the anti-caspase activity of XIAP and is underexpressed in cancer cells. *Nature Cell Bio.* 2001 Feb 1;3(2):128-33

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### **POSTERS, ABSTRACTS AND CONFERENCES ATTENDED**

Pathogenesis and Functional Genomics Meeting (Canadian Genetic Disease Network), Talisman Mountain Resort in Kimberley, ON, Canada (2004)

*A deficiency of NF- $\kappa$ B-inducible cIAP2 causes a severe impairment of LPS-induced endotoxic shock.* Conte, D, Holcik, M, Lefebvre, C, LaCasse, E, Wright, KE and RG Korneluk

Keystone Symposia 'Apoptosis in Development', Keystone Resort, Keystone, Colorado (2004)

*A deficiency of NF- $\kappa$ B-inducible cIAP2 causes a severe impairment of LPS-induced endotoxic shock.* Conte, D, Holcik, M, Lefebvre, C, LaCasse, E, Wright, KE and RG Korneluk

Keystone Symposia 'Molecular Mechanisms of Apoptosis', Banff, Alberta, Canada (2003)

*Xiap over-expression and Hiap1 ablation disrupt T cell maturation.* Conte, D, Holcik, M, Lefebvre, C, LaCasse, E, Wright, KE and RG Korneluk

Pathogenesis and Functional Genomics Meeting (Canadian Genetic Disease Network), St. Sauveur, QC, Canada (2002)

*HIAP1 is essential for thymocyte survival during early stage T cell development.* Conte, D, Liston, P, Wong, JW, Wright, KE and RG Korneluk

The Chronic Anemia Conference, Montreal, QC, Canada (2002)

Attended as a Canadian Thalassemia Foundation representative

Canadian Hematology Society - Anemia Joint Meeting, Montreal, QC, Canada  
(2002)

Attended as a Canadian Thalassemia Foundation representative

1st Canadian Conference on Hepatitis C, Montreal, QC, Canada (2001)

Attended as a CIHR Joint Advisor Committee member

Pathogenesis and Functional Genomics Meeting (Canadian Genetic Disease  
Network), St. Sauveur, QC, Canada (2001)

*Xiap influences T cell function and maturation.* Conte, D, Liston, P, Wong,  
JW, Wright, KE and RG Korneluk

Keystone Symposia 'Signaling in normal and cancer cells', Banff, Alberta,  
Canada (2001)

*Xiap influences T cell function and maturation.* Conte, D, Liston, P, Wong,  
JW, Wright, KE and RG Korneluk

Human Genome Meeting, Vancouver, BC, Canada (2000)

*Xiap over-expression disrupts T cell function and maturation.* Conte, D,  
Liston, P, Wong, JW, Wright, KE and RG Korneluk

Canadian Federation of Biological Sciences, Vancouver, BC, Canada (2000)

*Xiap overexpression disrupts T cell function and maturation.* Conte, D,  
Liston, P, Wong, JW, Wright, KE and RG Korneluk

Variants of Creutzfeldt Jakob Disease and Issues for the Blood System  
(1999). Vancouver, BC, Canada (1999)

National Blood Safety Council Open Forum

Attended as a Canadian Thalassemia Foundation representative

Methods of Reducing the Risk of Transmitting Viruses by Blood Components,  
Toronto, ON Canada (1998)

National Blood Safety Council Open Forum

Attended as a Canadian Thalassemia Foundation representative

Ottawa Life Sciences Conference, Ottawa, ON, Canada (1998)

*XIAP, HIAP1 and 2 protein and mRNA levels in key lymphoid populations.*

Conte, D, Liston, P, Wong, JW, Wright, KE and RG Korneluk

### **COMMUNITY PARTICIPATION AND EXPERIENCE**

1999-present      CIHR Committee member, Joint Advisory Committee  
(JAC)

Health Canada Research Initiative on Hepatitis C

- JAC's mission is to initiate and expand all aspects of current research on the subject of the *Hepatitis C virus* in Canada
- attended and participated in several JAC conferences as a Canadian Thalassemia Foundation representative
- reviewed and judged the relevance of CIHR grant applications on the subject of *Hepatitis C* for funding from the Hepatitis C Prevention, Support & Research Program of Health Canada

1996-1999      Medical Advisory Board Coordinator

Thalassemia Foundation of Canada

- prepared and distributed guidelines for the Thalassemia Foundation of Canada Fellowship Grant Awards
- facilitated and coordinated the grant application review process via the Medical Advisory Board (MAB) which consisted of qualified physicians and researchers (MAB Chair Person: Alan Bernstein - President, Canadian Institutes of Health Research)
- initiated partnership with CIHR

2000-2002      Coordinator & Supervisor of the "Reach-out Canada" program

The "Reach-out Canada" program was an initiative to contact all Thalassemia Major patients/parents throughout Canada to provide them with medical, financial and emotional support, via the Thalassemia Foundation of Canada.

- awarded \$2500.00 grant to complete the "Reach-out Canada" program
- supervised and coordinated the activities of one summer student to complete the project

## **AWARDS**

2000-2002	Ontario Graduate Scholarships in Science and Technology
2000	Thalassemia Foundation of Canada Projects Award
1992-1996	University of Toronto Open Fellowship