T cell intrinsic and extrinsic role of XIAP, during CD8 T cell response against intracellular pathogens.

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A thesis submitted to the University of Ottawa in partial fulfillment of requirements for the

Degree of Master of Science in Microbiology and Immunology

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Abstract

The magnitude and effectiveness of CD8 response against intracellular pathogens is directed by survival and apoptotic signals that govern the fate of T cells. XIAP is a bona fide endogenous inhibitor of apoptotic signals. In this thesis, I have investigated the role of XIAP at various stages of CD8 T cell response. I used both *in vivo* and *in vitro* models to show that XIAP acts in a CD8 T cell extrinsic and intrinsic manner to regulate the expansion and contraction phases of the CD8 T cell response, respectively. During the expansion phase, XIAP prevents the cell death of APCs to promote APC-T cell interaction and cytokine release, which facilitates the proliferation and survival of activated T cells. During the contraction phase, XIAP functions in a cell-intrinsic fashion to inhibit the proapoptotic signals in the activated CD8 T cells to prolong the immune response. Finally, I also demonstrate that the expression of XIAP in T cells is critical for their differentiation in to memory subsets. Overall, I present that XIAP plays a critical role in generating an effective CD8 T cell immune response.

Acknowledgments

First and foremost, I would like to thank Dr. Subash Sad, for guiding me throughout my studies. It was a pleasure working in your lab. Most importantly, thank you for being extremely patient.

Next, I would like to thank all and former members of the 'Happy' Sad lab, for making this experience enjoyable. I will definitely miss the witty banter, the bad puns and walk to Timmies. You have been an indispensable source of inspiration and friendship.

Additionally, I would also like to thank my TAC members (Dr. Lee and Dr. Wang) for their valued and much need advice.

Finally, I would like to thank my family for their constant source for support and motivation.

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List of Abbreviations

AP-1	Activator protein-1
Apaf-1	Apoptotic protease-activating factor 1
APC	Antigen-presenting cell
ATP	Adenosine triphosphate
BCL2	B cell lymphoma 2
BIR	Baculovirus Inhibitor of apoptosis Repeats
BMDC	Bone marrow derived dendritic cells
BMDM	Bone marrow derived macrophages
CARD	C-terminal caspase recruitment domain
CFSE	Carboxyfluorescein succinimidyl ester
CFU	Colony forming unit
CSF	Colony-stimulating factors
CTL	Cytotoxic T lymphocyte
DAMP	Damage-associated molecular patterns
DC	Dendritic cell
DD	Death domain
DIABLO	Direct IAP binding protein with low pI
DNase	Deoxyribonuclease
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot assays
ER	Endoplasmic reticulum
GM-CSF	Granulocyte/macrophage CSF
HIV	Human immunodeficiency virus
HRP	Streptavidin-horse radish peroxidase
i.v.	Intravenous injection
IAP	Inhibitor of apoptosis protein
IBD	Inflammatory bowel disease
IBM	IAP binding motif
IFN	Interferons
IKK	Inhibitory IκB kinase
IL	Interleukin
IRAK	IL-1 receptor-associated kinase
JNK	c-Jun N-terminal kinases
LCMV	Lymphocytic Choriomeningitis Virus
LM	*Listeria* monocytogenes
LN	Lymph node
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
M-cell	Microfold cell
M-CSF	Macrophage-CSF
MDP	Muramyldipeptide
MHC	Major Histocompatibility Complex
MLKL	Mixed lineage kinase domain like pseudokinase
MOI	Multiplicity of infection
MPEC	Memory precursor effector cell

MyD88	Myeloid differentiation primary response gene 88
NAIP	NLR apoptosis inhibitory protein
Nec-1	Necrostatin-1
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	Nod-like receptors
NLRC4	NLR family CARD-domain containing protein 4
NLRP3	NLR Family Pyrin Domain Containing 3
NOD	Nucleotide-binding oligomerization domain receptors
OVA	Ovalbumin
PAMP	Pathogen-associated molecular patterns
PIT	Pore-induced intracellular traps
PRR	Pattern-recognition receptors
RipK	Receptor-interacting serine/threonine-protein kinase
rmIL-6	Recombinant murine IL-6
ROS	Reactive oxygen species
RPMI	Rosswell Park Memorial Institute (RPMI) media-1640
SCV	Salmonella containing vacuole
SLEC	Short-lived effector cell
SMAC	Second mitochondria-derived activator of caspase
ST	*Salmonella enterica* serovar Typhimurium
T3SS	Type 3 secretion system
TAB	TAK1 binding proteins
TAK1	Transforming growth factor-β-activated kinase-1
Tcm	Central-memory T cells
TCR	T cells receptor
Teff	Effector T cells
Tem	Effector-memory T cells
TGFβ	Transforming growth factor-β
TICAM1	TIR domain-containing adaptor molecule 1
TLR	Toll-like receptors
TNFR	TNF receptor
TRAF6	TNF receptor–associated factor 6
TRAIL	TNF-related apoptosis-inducing ligand
TRIF	TIR-domain-containing adapter-inducing interferon-β
UBA	Ubiquitin-associated domain
WT	Wildtype
XIAP	X-linking inhibitor of apoptosis protein
YopE	Yersinia outer membrane protein E

1. Introduction

1.1 Immune system

The immune system is a complex network of cellular and molecular components responsible for maintaining tissue homeostasis and protecting the body from invasion of foreign substances such as microbes (organisms such as bacteria, fungi, and parasites) and viruses (1). The immune system acts to eliminate pathogens from the body by cellular and humoral defense strategies. The cellular immune response consists of specialized cells that recognize and remove pathogens, clear cellular debris, or destroy infected cells (1). The humoral immune defense consists of antibodies that prevent pathogens from damaging or infecting host cells. Antibodies may prompt targeted cellular immunity. Together, the humoral and cellular immune defenses control pathogen replication and eliminate infected and altered cells (2, 3).

Infection or physical injury may invoke an inflammatory response, which involves the recruitment of immune cells to the infection site and the elimination of invading pathogens and afflicted cells (1, 3, 4). There are two main pillars of the immune system: innate immunity, a broad and quick response; and adaptive immunity, a specialized and target response (1). An innate immune response is a nonspecific immune defense mechanism, which comes into play immediately or within hours of pathogen encounter. This broad and rapid response prevents the spread of pathogens throughout the body (2). Although considered a 'nonspecific response' due to its inability to distinguish between pathogens; the innate immune cells are crucially able to distinguish between self and foreign cells by using a limited repertoire of germline-encoded pattern-recognition receptors (PRRs) (5). In sharp contrast, the adaptive immune system is characterized by the

vast diversity in receptor reservoirs, generated through a random assembly of gene segments during the development of adaptive immune cells. These receptors specifically target a particular epitope – a small site on an immunogenic protein, known as an antigen. However, an effective adaptive immune response takes onwards of five to seven days to manifest (6).

Due to the differential in the timing of activation, the innate and the adaptive immune responses were initially thought to function at separate levels. With innate immunity responsible for initial control of the infection, until adaptive immunity can act. However, studies have suggested various components of innate immunity supplement, prompt, and control the adaptive response. Critically, innate immune cells are responsible for the induction of an adaptive immune response. Both immune response work in congruence with each other to eliminate the pathogen (1, 4).

Interestingly, programmed cell death is an indispensable mechanism for development, activation, operation, and resolution of the immune response (7). During an active immune response, cell death mechanisms are suppressed to support the survival of immune cells within infectious and cytotoxic microenvironments. Conversely, increased survival of immune cell death can lead to an overactive immune response (8). Many of the symptoms associated with disease/illness, such as fever, swelling and redness, are a result of an active immune response. Therefore, the resolution of the immune response requires the clearance of effector cells by cell death to critically avoid immunopathological disorders, such as autoimmune diseases, allergy/asthma and degenerative diseases (1). A clear understanding of cell death pathways during the immune response is essential for

furthering the understanding of the immune system and the etiology of autoimmune diseases.

1.2 Programmed Cell death

Cell death is an indispensable mechanism for tissue development and maintaining homeostasis. Cell death eliminates surplus, irreversibly damaged, or potentially harmful cells. Cell death can be either programmed or accidental (9). Accidental cell death is uncontrollable and instantaneous demise of a cell, corresponding to physical disassembly of the plasma membrane by extreme physical (temperatures or osmotic forces), chemical (pH variations), or mechanical (shear forces) cues. In contrast, programmed cell death is a regulated event that relies on dedicated machinery. Cues from the intracellular or extracellular microenvironment, pharmacological or genetic interventions can modulate (kinetically; accelerate or delay) the demise of cells (9).

Cell survival is also critical for effective clearance of infection. Immune cells must acquire resistance to cell death to function efficiently in an infected and cytotoxic microenvironment. Activation of immune cells, during inflammation, generally corresponds to increased cell survival, as some of the pro-inflammatory pathways that are driven by NFκB (10) and MAPK (11) signaling also induce the expression of various endogenous inhibitors of cell death. Substantial cross-talk between inflammatory signaling and cell-death pathways ensures a balanced response that facilitates the control of pathogens. Cell death of infected cells limits the egress and spread of pathogens. As such, cell death of infected immune cells can also be viewed as a contingency for a weak immune response. This integration of cell death and inflammatory signaling is beneficial for

eliminating active immune cells, during the end of an inflammatory response, to avoid additional tissue damage (7).

From the perspective of a pathogen, controlling cell death would increase their chance of survival and proliferation. Some intracellular pathogens employ strategies to prevent cell death in infected cells during pathogen replication and promote lytic cell death during escape and dissemination. Additionally, pathogens have evolved methods of eliminating immune cells by promoting the induction of cell death pathways (12). For example, Human Immunodeficiency Virus (HIV) (13), *Salmonella* (14), *Listeria* (15) and *Legionella* (16) are able to induce cell death in immune cells.

Regulation of cell death is equally important for both the host and the pathogen. Thus, multicellular organisms and their pathogens can be viewed to be in an evolutionary 'arms race', to control cell death pathways and the subsequent immune response. Consequently, evolution has shaped multiple intricate, multimodal pathways for inducing multiple forms of cell death (17). Cell death mediated by host-pathogen interaction could be of lytic or non-lytic morphology, varying based on the triggered cell death pathway. Lytic cell death, such as necroptosis and pyroptosis, release intracellular components (DAMPs) into the environment, which can drive inflammation. Necroptotic or pyroptotic cell deaths are highly immunogenic. Whereas, non-lytic forms of cell death, such as apoptosis, are not immunogenic. The dynamic interplay between the host, the pathogen, and the environment dictates the form and immunogenicity of cell death (7, 9, 18).

1.2.1 Apoptosis

The term 'apoptosis' was first coined, by Kerr and colleagues in 1972, to describe a specific pattern of cell death is human tissue (19). The morphology associated with this form of cell death was characterized as nuclear condensation, fragmentation of DNA and

membrane blebbing. Cells maintain their membrane integrity as they progress through apoptosis to prevent the release of intracellular DAMPs, which can induce inflammation (19, 20). The apoptotic corpses present an 'eat me' signal on their plasma membrane to recruit phagocytes, which can engulf and remove the apoptotic cells. This process of clearing bodies of apoptotic cells, via phagocytic activity, is commonly known as efferocytosis (21). Phosphatidylserine presentation on the cell surface functions to tether apoptotic cells to phagocytes, via receptor-ligand interaction, to potentiate efferocytosis. In a healthy cell, phosphatidylserines are routinely present on the inner leaflet of the plasma membrane. Upon induction of apoptosis, the membrane asymmetry is lost, and phosphatidylserine is presented on the outer leaflet of the plasma membrane (22). In addition to promoting efferocytosis, 'eat me' signals can also induce an anti-inflammatory response from the phagocytes. Phosphatidylserine-dependent ingestion of apoptotic cells by macrophages induces secretion of anti-inflammatory cytokines such as transforming growth factor-β (TGFβ) and interleukin-10 (IL-10) and simultaneously decrease the secretion of the inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), IL-1β, and IL-12 (23, 24). Interestingly, when the cell debris generated by apoptotic cell death is not cleared in an efficient and timely manner, they can undergo secondary necrosis – a process characterized by disruption of the cell membrane and loss of cell integrity – and release DAMPs to cause inflammation (25).

Cell extrinsic (receptor-mediated) and intrinsic (mitochondrial-stress mediated) signaling pathways induced apoptosis by controlling the activation of a cysteine proteases family known as Caspase. Proapoptotic signaling cumulates to activate initiator caspases (caspase -2, -8, -9, and -10), which are responsible for proteolytic cleavage and activation of executioner caspases (caspase-3, -6 and -7). Executioner caspases are converted into

their active form by cleavage at internal aspartate residue. Activated executioner caspases mediate apoptotic morphology by cleaving both cytoplasmic and nuclear proteins. Cleavage of scaffold proteins of the nuclear envelope, lamins, leads to nuclear shrinkage and fragmentation (26). Additionally, loss of cytoskeletal proteins, due to caspase cleavage, causes plasma blebbing and loss of cell shape (27, 28). Caspase-3 and -7 are also responsible for cleavage of genomic DNA. Active caspases are translocated to the nucleus, where they activate DNA fragmentation factor subunit-β, by cleaving its inhibitor (29). Caspase-3 is also responsible for the externalization of phosphatidylserine, which is an 'eat me' signal essential for efferocytosis (30).

Intrinsic pathways of apoptosis are initiated by the intracellular perturbation of DNA damage, reactive oxygen species (ROS) overload or microtubular alterations, all cumulating to induce irreversible mitochondrial membrane permeabilization. Pro-apoptotic and anti-apoptotic members of the B cell lymphoma 2 (BCL2) family control mitochondrial membrane integrity (9). Permeabilization of the mitochondrial membrane promotes the release of apoptogenic factors, such as cytochrome-C, from the intermembrane space to promote assembly of apoptosome complex. Anti-apoptotic members of the BCL2 protein family, such as BCL-2 and BCL-XL, inhibit mitochondrial cytochrome-C release. While proapoptotic members of the BCL2 family, such as Bax, Bak, and Bid, trigger mitochondrial cytochrome-C release (31). Apoptosome is a heptameric complex, composed of apoptotic protease-activating factor 1 (Apaf-1), pro-caspase 9 and cytochrome-C, which is responsible for the activation of caspase-9 (25). Active caspase-9, in turn, cleaves and activates caspase-3 and -7, which are responsible for the demolition of cellular components and the manifestation of the apoptotic morphology.

Extrinsic pathways of apoptosis are mediated by death receptors. These include Fas receptors, tumor necrosis factor receptors (TNFR1), and TNF-related apoptosis-inducing ligand (TRAIL) receptors. Signaling from ligand binding to their respective receptors is mediated by a shared cytoplasmic domain, known as the 'death domain' (DD) (32). With the aid of adaptor proteins, FADD/TRADD, death receptor signaling will lead to recruitment, dimerization and activation of the initiator caspase-8, which in turn activates caspase-3 and -7 (9).

Extrinsic signaling can also activate the intrinsic apoptotic pathway through caspase-8 mediated cleavage of the Bid, a proapoptotic member of the BCL2 family (33). The cleaved form of Bid can localize to the mitochondrial surface, which results in mitochondrial dysfunction and the subsequent release of cytochrome-C. This will result in the activation of caspase-9 and the subsequent activation of executioner caspases to induce cell death.

1.2.2 Pyroptosis

Pyroptotic cell death is marked by cellular swelling and plasma membrane permeabilization, which results in the release of cytosolic contents into the extracellular space (20). Pyroptosis is initiated by inflammatory caspase 1 or -11 activity, upon activation of inflammasome signaling pathways. Pyroptotic cell death is generally accompanied by the release of pro-inflammatory cytokines, including IL-1β and IL-18 (34). Canonical or non-canonical activation of inflammatory caspases (caspase-1, -4, and -11) results in proteolytic cleavage of gasdermin-D. Mature gasdermin-D localizes to the inner domain of the plasma membrane and oligomerizes with ~16 symmetric subunits to form a pore in the plasma membrane, which causes water influx, cell swelling, and lysis (35).

Pathogen recognition receptors (PRRs) recognize pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) to initiate the formation of an inflammasome complex. Inflammasomes are cytoplasmic platforms composed of PRRs families, such as Nod-like receptors (NLRs), AIM2-like receptors that detect DNA in the cytosol, bacterial flagellin and other virulence factors (34). NLR Family Pyrin Domain Containing 3 (NLRP3) inflammasome has a broad range of activity as it responds to numerous unrelated agonists. As such, NLRP3 inflammasome is activated during bacterial, virus and fungal infections. NLRP3 also responds to various stimuli that represent cellular stress, including ionic flux, mitochondrial dysfunction and the production of ROS (36). Formation of the NLRP3 inflammasome complex results in the activation of caspase-1, which in turn cleaves pro-IL-1β and pro-IL-18 to their mature forms (37).
In contrast, the non-canonical pathway for inducing pyroptosis is mediated by direct recognition of lipopolysaccharide (LPS) by caspase-11. LPS binding with procaspase-11, initiates procaspase-11 oligomerization and maturation. Mature caspase-11 can induce proteolytic cleavage and activation of gasdermin-D. However, they do not directly cleave pro-IL-1β or pro-IL-18 (38, 39).

Both the canonical and non-canonical pathways for the induction of pyroptosis result in pore-formation and lytic cells death. Thus, pyroptosis potentiates inflammatory response in three ways: 1) release of cytosolic DAMPs upon plasma membrane permeabilization, 2) secretion of pro-inflammatory cytokines such as IL-1β/IL-18, and 3) formation of pore-induced intracellular traps (PIT). PITs encompass collapsed organelles and cellular structures that remain within the largely intact plasma membrane. PITs immobilize intracellular pathogens and promote efferocytosis, to kill the pathogen (40).

1.2.3 Necroptosis

Death receptor associated with inducing extrinsic apoptosis signaling also induces necroptosis. There is a plethora of evidence that shows caspase-8 activation drives extrinsic apoptotic signaling towards apoptosis, while caspase-8 inhibition results in necroptosis. Hence, necroptosis is viewed as an alternative mode of cell death, when caspase-8 driven apoptosis is blocked (41, 42). Death receptor signaling induces the formation of RipK1 and RipK3 heterocomplex, known as the necrosome, which promotes the phosphorylation and subsequent oligomerization of Mixed Lineage Kinase Domain Like Pseudokinase (MLKL) (9). MLKL oligomers relocate to the membrane, which results in pore formation, ion influx cellular swelling and rupture (43). Ubiquitin ligase activity of inhibitor of apoptosis proteins (IAPs) is indispensable for blocking RipK3 driven signaling towards cell death(44)

Pore formation by MLKL, during necroptotic cell death, can also induce NLRP3 activation in a cell-intrinsic manner by altering ion homeostasis, which in turn will induce pyroptotic cell death (45, 46). Interestingly, NLRP3 inflammasome can also be induced independent of MLKL and necroptotic cell death, via TNF triggered RipK3 signaling (47, 48) **(Fig 1)**.

Necrosis results in the release of cytosolic content, DAMPs, into extracellular regions and induction of an inflammatory response. In addition to DAMP release, necroptotic cell death has a similar morphology to pyroptosis; as such, it is also associated with the formation of PITs (40) **(Fig 1)**.

1.3 Innate immunity

Innate immunity is the first line of defense against pathogens. It is made up of anatomic and physiologic barriers, antimicrobial peptides and proteins, and specialized

effector cells such as neutrophils, monocytes/macrophages and dendritic cells (DCs) (2).

The innate immune response plays an essential role in preventing infection while tolerating the normal host microbiome. Vertebrates are a complex and thriving ecosystem for thousands of microbial species. These microbes live in close proximity to the host cells and are a part of the normal host flora. They are usually limited to certain areas of the body, including the skin, mouth, respiratory tract, large intestine, and vagina (49). Anatomical (tight junctions in the skin and mucociliary clearance mechanisms) and physiological (antimicrobial peptides and proteins, low pH and chemical mediators) barriers physically and mechanically prevent pathogen entry into the host. Disruption of these barriers due to physical injuries, such as burns and cuts, renders the body susceptible to infection (50).

While microbes that are part of the normal flora require the host to be immunocompromised or injured to cause an infection. Virulent pathogens have developed specialized mechanisms for crossing cellular and chemical barriers. After breaching the barriers, pathogens elicit damage and exploit the biology of the host to support their spread and survival (49, 50).

Breaching of host barriers by pathogens almost always elicits an inflammatory response (50). Stimulation of an inflammatory response is mediated by innate immune cells already present within the infected tissue, such as macrophages, DCs, histiocytes, kupffer cells, and mast cells. Innate immune cells are ubiquitously present in almost all tissues. They continually scan the tissue microenvironment for the presence of pathogens or infected cells. Upon pathogen recognition, tissue-resident innate immune cells can produce cytokines that will promote inflammation. This inflammatory response is characterized by increased permeability of blood vessels and the recruitment of immune cells to the infection site. Rapid recognition of pathogen or tissue damage at the infection site and the resulting

accumulation of innate immune cells leads to the elimination of pathogens and infected cells (2, 51).

Recognition of pathogens and induction of inflammatory response by innate immune cells is mediated by PRRs. These receptors are evolutionarily conserved amongst various species, from plants and fruit flies to mammals. PRRs bind conserved microbial components that are commonly shared amongst pathogens, known as pathogen-associated molecular patterns (PAMPs) (5, 51). Microbial components such as bacterial lipopolysaccharide (LPS), flagellin, peptidoglycans, or viral double-stranded ribonucleic acid are essential for survival and therefore are invariant amongst various pathogens; making them excellent targets for pathogen recognition by PRRs. Most innate immune effectors cells have similar PRR expression profiles, thus facilitating a rapid recognition of a cluster of pathogens(51).

There are multiple families of PRRs. PRRs have distinct localization in various cellular compartments, such as plasma membrane, cytosol, or endosome, to provide adequate screening for pathogens. PRRs such as Toll-like receptors (TLRs) are commonly expressed on the plasma membrane, which allows them to detect extracellular pathogens. Conversely, nucleotide-binding oligomerization domain receptors (NOD)-like receptors (NLRs) are expressed in the cytosol to aid the recognition of intracellular pathogens.

Engagement of PRRs leads to an inflammatory signaling cascade, which induces expression and release of cytokines and chemokines that, in turn, induces anti-microbial activity and rapid recruitment of immune cells to the site of infection, respectively. Signaling cascades induced by PRRs converge on a common set of signaling modules, including nuclear factor kappa B (NFκB) (52), activator protein-1 (AP-1) (53), and

mitogen-activated protein kinase (MAPK) (11). These transcription factors, in turn, induce the expression of genes that promote cell survival, proliferation and inflammation.

Cytokines induce a pleiotropic impact on cell function and have multiple redundancies. Cytokines can be divided based on function into growth factors, immunomodulatory cytokines and chemokines. They function in an autocrine (acts on the same cell) or paracrine (acts on nearby cells) manner (54). Colony-stimulating factors (CSF) are part of the hematopoietin family of cytokines. For example, granulocyte/macrophage CSF (GM-CSF) (55) and macrophage-CSF (M-CSF) (56) induce the proliferation of bone marrow precursors and their differentiation into dendritic cell colonies and macrophage colonies, respectively. Chemokines direct chemotaxis to immune cells into the tissue. Finally, immunomodulatory cytokines can have either pro- or anti-inflammatory effects, and these effects can be contextual (54). They can alter gene expression to affect cellular functions, to further augment the inflammatory response and alter cell survival. Secretion of cytokines, such as interferons (IFNs), IL-1β, IL-6 and TNF-α, is a highly regulated event to limit tissue damage during an inflammatory response against invading pathogens (11). Most conventional cytokines are secreted through exocytosis; newly synthesized cytokines are translocated into the ER and traffic through the ER and Golgi before reaching their extracellular destination. However, IL-1 cytokines lack a signaling sequence necessary for transport through the classical ER-Golgi route. Thus, they rely on cell death pathways, such as pyroptosis, to induce disruption of the plasma membrane for their secretion (57).

In addition to cytokine production, PPR engagement also promotes the direct elimination of pathogens through phagocytosis. PRR-ligand signaling promotes the phagocytic activity in innate immune cells such as neutrophils, macrophages and DCs. The

phagocytic action promotes the clearance of dead cells and foreign substances present in the tissue. Phagocytosis is a form of endocytosis, in which a large particle ($\geq$ 0.5 μm) is engulfed. Opsonin receptors, surface PRRs such as TLRs and Fc receptors, on the surface of phagocytic cells, recognize the microbes or apoptotic/infected host cells and induce their phagocytosis (11). Plasma membrane moves to encapsulate the particle, such that the phagocytosed particle is completely enclosed in a bubble-like structure, called a phagosome, within the cytoplasm. After internalization, the phagosome matures (decrease in pH) and fuses with intracellular vesicles called the lysosome. The enzymes present in the lysosomes destroy ingested pathogens/proteins. Degradation of the engulfed pathogen is mediated by a reduction in pH, and expression of ROS and antimicrobial proteins (11). Beyond the neutralization of pathogen, phagocytosis is critical for activation of the adaptive immune response. Some innate immune cells, such as the DCs, are known as the professional antigen-presenting cells (APCs). They continually scan the body for pathogens, via phagocytosis or endocytosis. Microbial peptides generated from phagocytized pathogen or debris from infected cells are engulfed, degraded, processed and presented to activate an antigen-specific adaptive immune response (11).

1.3.1 Dendritic cells (DCs)

Immature DCs are widely distributed in all tissues, especially those that interface with the external environment (e.g. skin and mucosal surfaces) and lymphoid organs (e.g spleen and lymph nodes). Additionally, the establishment of inflammation at the infection site can also induce the recruitment of immature DCs or monocytes, which may differentiate into DCs (93).

In their immature state, DCs are not capable of inducing activation of T cells. Immature dendritic cells screen for antigens, pathogens by phagocytosis, macropinocytosis,

or via cell surface receptor-mediated endocytosis. Various mechanisms associated with antigen uptake and processing can induce DCs activation and maturation. DC maturation is associated with the upregulation of MHC molecules, co-stimulatory molecules and cytokine expression, along with the down-regulation of antigen internalization mechanisms (93, 97). Additionally, DC maturation also induces expression of their chemokine receptor repertoire, such as CCR7 (102, 103) and CXCR4 (104), that favors responsiveness towards LN-tropic chemokines.

DCs take up large volumes of surrounding fluid via macropinocytosis, which is a nonspecific screening mechanism for extracellular antigens. Some of the processed antigens also include self/host antigens. Similarly, receptor-mediated efferocytosis of cellular debris also results in the processing of self and non-self antigens. Therefore, PAMP ligation to PRRs and inflammatory cytokines during infection are critical for the acquisition of strong antigen-presenting capability and providing tolerance to self-antigens.

Cell surface receptors on mature DCs also aid in maintaining physical contact with T cells. Given the mass diversity in the TCRs, the ratio of naïve T cell expressing a 'cognate' TCR specific for any individual MHC-peptide complex is only 1 in 10^5-10^7 (105, 106). During initial stages, where the antigen carrying DCs is screening for an appropriately matched TCR, DC-SIGN expression promotes the transient clustering of multiple T cells to allow the DCs to screen numerous T cells (107). Stable interaction between DC and T cells can promote T cell activation. DC-T cell interaction is bidirectional, as such, it also induces the activation of DCs to promote the expression of adhesion molecules, co-stimulatory molecules and secretion of cytokines to regulate and support T cell differentiation and survival (**Fig 2 B)**.

1.3.2 Macrophage

Macrophages, like DCs, are ubiquitously present innate immune cells in the peripheral tissues. Macrophages are large specialized cells that primarily induce receptor-mediated phagocytosis of pathogens and damaged tissue. They can also initiate an inflammatory response by secreting cytokines (4). Finally, they also present antigens to stimulate a T cell immune response (93, 97).

The tissue-resident population of macrophages arises from circulating monocytes that, upon localization to the peripheral tissues, differentiate into macrophages. Macrophages also acquire specialized function based on the metabolic and growth factors signaling within the tissue microenvironment. In different tissues, macrophages have special names, such as alveolar macrophages in the lungs, kupffer cells in the liver (108), microglial cells in the brain (109), and osteoclasts in bones (108).

1.3.3 Pro-inflammatory and survival signaling

Recognition of PAMPs and DAMPs by TLRs located on the cell surface or endosomal/lysosomal compartment induces TLR signaling to promote an inflammatory response (5, 58). TLRs and pro-inflammatory cytokines such as IL-1 and TNFα promote the activation of the canonical NFκB signaling. TLR4-signaling is an example of a canonical NFκB signaling pathway. TLR4 recognizes bacterial LPS and viral envelope proteins, and induces signaling in a myeloid differentiation primary response gene 88 (MyD88) or TIR-containing adaptor inducing interferon-β (IFN-β)/TIR domain-containing adaptor molecule 1 (TRIF/TICAM1) dependent fashion (59). Conformational changes of the cytoplasmic domain of TLR4 upon ligand interaction promotes the recruitment of MyD88, which subsequently recruits members of the IL-1 receptor-associated kinase

(IRAK) family. IRAK (1, -2 and -4) phosphorylation catalyzes the autoubiquitination of TNF receptor–associated factor 6 (TRAF6). TRAF6 is a RING-domain E3 ubiquitin ligase, which is responsible for the polyubiquitination of NEMO. Ubiquitinated NEMO and TRAF6 recruit transforming growth factor-β-activated kinase-1 (TAK1) and TAK1 binding proteins (TAB -1, -2 and -3) to form an NFκB activating TAB2/TAK1/NEMO complex. TAK1 kinase activity as part of the TAB2/TAK1/NEMO complex induces phosphorylation and formation of the inhibitory IκB kinase (IKK) complex. The IKK complex triggers the NFκB activation through the phosphorylation and subsequent degradation of inhibitory IκB proteins. Additionally, the TAB2/TAK1/NEMO complex can also induce activation of MAPK cascade (ERK, JNK, p38) pathways. IL-1 family of cytokines also function in the same manner as TLRs, due to the common cytoplasmic TIR domain on IL1 receptors (52, 59, 60) **(Fig 1)**.

Nucleotide-binding oligomerization domain (NOD) protein, NOD2, which is an intracellular PRR, detects motifs in bacterial peptidoglycan. Upon engagement NOD2 signaling also results in IKK complex formation and NFκB activation. Upon recognition of bacterial cell wall domains, NOD2 undergoes self-oligomerization and recruits receptor-interacting serine/threonine-protein kinase 2 (RipK2). Activation of RipK2 requires the autophosphorylation of RipK2 at Y474 and XIAP mediated ubiquitination. The active form of RipK2 promotes the formation of TAB2/TAK1/NEMO complex and subsequent MAPK and NFκB pathway activation (61).

Interestingly, death receptor signaling, which is commonly understood to induce apoptosis or necroptosis can also mediate NFκB driven survival and inflammatory gene expression. Ubiquitination-mediated regulation of RipK1 directs TNFα stimulation towards

survival and inflammation, rather than death or necroptosis (62). Polyubiquitinated RipK1 serves as a platform to recruit the TAB2/TAK1/NEMO and IKK complexes, which can activate NFκB and MAPK signaling pathways. Further, the IKK complex directly phosphorylates RipK1 to stabilize RipK1 mediated NFκB signaling, upon TNFα stimulation (63). Disrupting ubiquitination on RipK1 by CYLD, converts RipK1 from survival-signaling molecule to a death-signaling molecule (64); leading to TNFR mediated cell death signaling via Caspase-8 mediated apoptosis or RipK3-MLKL driven necroptosis (41) **(Fig 1)**.

1.3.4 Salmonella

Salmonella enterica serovar Typhimurium (*S. Typhimurium/*ST) is a gram-negative bacterium that causes systemic infection in mice and self-limiting gastroenteritis in humans. Interestingly, infection of mice with *S. Typhimurium* mimics the typhoid fever in humans, which is caused by *Salmonella enterica* serovar Typhi (65, 66).

Following oral ingestion of ST, microfold (M) cells and phagocytic cells such as DCs or macrophages promote the establishment and systemic dissemination of infection. M-cells reside over lymphoid follicles, known as Peyer's patches, and function to transcytose microbes and macromolecules from the gut lumen to the monocytes, macrophages and neutrophils within the Peyer's patches (67). Following the successful crossing of the epithelial barrier, ST facilitates its internalization by tissue-resident macrophages and DCs. ST is a facultative intracellular pathogen that can invade, survive and replicate in a variety of phagocytic cells. Additionally, ST utilizes the innate immune cells within the intestinal mucosa to facilitate its transport to the draining mesenteric lymph

nodes (MLNs), spleen and liver. Dissemination of ST through the lymphatic vessels can result in systemic and fatal infection in immunocompromised hosts (68, 69).

To gain entry into the intracellular niche of host macrophages, ST utilizes two mechanisms: 1) by receptor-mediated phagocytosis 2) or by triggering its own engulfment. ST employs Type 3 secretion system-1 (T3SS-1) to inject virulence proteins, which hijacks macrophage actin cytoskeleton machinery and induces outwards extension of the cell membrane to engulf the bacterium. The internalized ST remains within a modified phagosome known as the Salmonella containing vacuole (SCV) (70–72).

Similar to other phagosomal pathogens such as *Mycobacteria* and *Leishmania*, ST also engages an array of virulence factors to support its survival and replication within the host. T3SS-2 delivers ST virulence factors through the SCV membrane into the host cytosol. These effectors redirect phagosomal maturation and fusion with bactericidal vacuoles (72, 73). SifA and PipB2 released through the T3SS, inhibit vacuolar lysis by interfering with kinesin-mediated vacuolar dynamics and, hence, ensuring intravacuolar replication (74, 75). Finally, ST facilitates its egress from the infected cells by inducing caspase-1-dependent pyroptosis.

Internalized ST introduces various PAMPs such as LPS, flagella, and bacterial DNA, which can trigger PRR mediated activation of NFκB signaling to induce expression of pro-IL-1β and other inflammatory cytokines. Additionally, these PAMPs also induce inflammasome-mediated activation of caspase-1. Mouse NLR apoptosis inhibitory protein (NAIP) recognizes ST virulence factors, such as T3SS components and flagellin, and induces NLR family CARD-domain containing protein 4 (NLRC4) phosphorylation and formation of NLRC4 inflammasome complex. NLRC4 inflammasome consists of the PAMP (cytosolic flagellin), sensor (NAIP), nucleator (NLRC4), adaptor (ASC), and

effector (Caspase-1). NLRC4 inflammasome complex induces proteolytic activation of caspase-1. Additionally, the cytosolic release of ST upon vacuole lysis can also mediate non-canonical induction of pyroptosis through LPS detection by caspase-11 (76, 77). Inflammasome activation and the release of IL-18 and IL-1β, correlates with an inflammatory cell death known as pyroptosis (78).

Inflammatory cell death mediated by inflammasome activation, resulting in the release of IL-18, IL-1β and IL-1α cytokines, and DAMPs, is critical for early inflammatory response to ST and invasion through the gut epithelium. During the early stages of infection, pyroptotic cell death removes ST from their replication niche within the host cells and promotes bacterial clearance via recruited neutrophils and macrophages. Mice deficient in IL-18 and IL-1β show slightly enhanced susceptibility to ST infection (78). Additionally, defective clearance of ST infection has been observed in Caspase-1 (78), NLRP3 or NLRC4 deficient mice (79). Thus, the activation of inflammasomes is an essential factor in generating an efficient innate immune response to ST infection.

1.3.5 Listeria

Listeria monocytogenes (LM) is another facultative intracellular pathogen that is transmitted through the oral-fecal route. It is a gram-positive bacterium that leads to life-threatening bacteremia or meningitis in the immunocompromised patients; and self-limiting, febrile gastroenteritis in healthy individuals (80, 81).

Initial LM infection relies on the survival and colonization within the gastrointestinal tract, followed by transversion of the intestinal epithelium through two principle mechanisms:1) direct invasion of the enterocytes lining the epithelium and 2) translocation across the M-cells in Peyer's patches (82). Following translocation, LM can disseminate through the lymphatics to the lymph nodes, liver and spleen. An uncontrolled

spread of LM can lead to infection at the secondary sites, such as the central nervous system and the placenta (81).

The fate of infection relies on the capacity of the host to mount an effective cellular immune response, in particular the activation of macrophages, and on the ability of LM to counteract bactericidal mechanisms of the host. Internalized LM can escape the phagosome and replicate within the cytosol via the expression of a pore-forming toxin called Listeriolysin O (83). Within the cytosol, *Listeria* is exposed to detection via various cytosolic PRRs that, in turn, induce the expression of pro-inflammatory cytokines, such as TNFα, IFNγ, IL-12, and IL-14 (84). Additionally, the presence of cytosolic DNA during LM infection stimulates the formation of AIM2 inflammasome-induced caspase-1 activity, which promotes the processing of pro-IL-1β into its active form (85).

Although the innate immune response is important for the initial control of LM infection, CD8 T cell response against LM is most effective at mediating protective immunity. Vacuolar escape of LM is necessary for the generation of LM-specific CD8 T cell responses. Bacterial peptide expressed by cytosolic LM can be presented on major histocompatibility Complex-1 (MHC-1) proteins, for the generation of an LM-specific CD8 T cell response (86, 87). LM infection also generates an effective memory response, making it an efficient model for vaccine-related studies regarding the generation, maintenance and function of memory CD8 T cells (84, 87, 88). Vacuolar escape of LM is necessary for the generation of LM-specific CD8 T cell responses.

1.4 Adaptive immunity

Cells of the adaptive immune system recognize a small site on an antigen, called an epitope, and induce a response against that antigen while avoiding other antigens. Proteins

have a complex three-dimensional shape; as such, they also have many distinct epitopes. Pathogens carry a variety of proteins, each with its own variety of own unique epitopes. Therefore, adaptive immune responses can be directed against many different epitopes on a variety of antigens, all belonging to the same pathogen; as such, each cell of the adaptive immune system has unique receptors that are capable of binding specifically with only one specific epitope (1, 4). This diversity between receptors is achieved during lymphocyte development, through the genetic rearrangement of receptor genes, by a process called VDJ recombination (89, 90). However, having a handful of cells that can recognize the pathogen is not enough to clear the infection. Therefore, upon initial activation of naïve lymphocytes, via receptor-ligand binding, results in the proliferation of those cells. Thus, generating an army of cells that recognize the same epitope. This proliferation of specific lymphocytes is known as clonal expansion (91) **(Fig 2 A)**.

B-lymphocytes and T-lymphocytes are the mediators of the adaptive immune response. They express epitope-specific receptors that rely on shape-complementary to bind epitopes. B cell receptors directly bind to epitopes on peptide, protein, polysaccharide, nucleic acid, and lipid antigens. T cells receptors (TCRs) recognize peptide epitopes that are loaded onto MHC-1 and -2 molecules on the cell surface of antigen-presenting cells. Along with TCR and MHC interaction, T cells also require co-receptors for efficient clonal expansion. T cells are classified by surface expression of either the CD4 or CD8 co-receptor. Co-receptors facilitate TCR interaction with antigen-MHC complexes. CD4 co-receptors are present on T helper cells and only binds to MHC-2 complexes, and CD8 co-receptors are present on cytotoxic T cells and binds to MHC-1 complexes (91).

MHC-1 molecules are expressed by all nucleated cells and present antigenic peptides from intracellular proteins. Thus, almost every infected cell can process proteins,

from the pathogen, into peptides and load them on MHC-1 complexes. The MHC-peptide complex is then translocated to the cell surface, where it mediates CD8 T cells dependent elimination of the infected cell before the pathogen can replicate and escape. Therefore, antigen processing and presentation onto MHC complexes is critical for inducing activation and function of an adaptive immune response (1, 4).

1.4.1 Antigen presentation

Antigen-presenting cells (APCs) are specialized innate immune cells that bridge innate and adaptive immunity. While all nucleated can present MHC-1 antigen complexes during infection with intracellular pathogens, APCs such as DCs and macrophage function as sentinels within the tissue to sense invading pathogens. They also express MHC-2 molecules; as such, they can present both -intracellular and extracellular antigens. These cells sample the tissue environment by multiple antigen uptake mechanisms, including receptor-mediated endocytosis, macropinocytosis and phagocytosis of pathogens or cell debris. Critically, APCs also can support the clonal expansion of T cells (1, 4).

There are three pathways for antigen processing and presentation: 1) peptides from intracellular/endogenous antigens on MHC-1 molecules, 2) cross-presentation of extracellular/exogenous antigens on MHC-1 complexes and 3) extracellular antigens presented on MHC-2 complexes. Endogenous viral or microbial proteins in the cytosol are degraded into a variety of peptide epitopes by cylindrical organelles called proteasomes. Peptides are then translocated into the endoplasmic reticulum (ER) lumen by the transporter associated with antigen processing (TAP) protein. In the ER, the peptides are loaded onto MHC-1 complexes, and the complex is then transported through the Golgi-apparatus to the plasma membrane. This is the principal pathway of antigen presentation to CD8 T cells. For exogenous antigens, the particles internalized via phagocytosis are

degraded in the phagosome upon lysosome fusion. MHC-2 complexes are translocated to the antigen carrying phagosome. Here, the MHC-2 molecules are loaded with exogenous peptide antigen and transported to the cells surface. Exogenous antigens can also activate CD8 T cells through a pathway of 'cross-presentation'. During cross-presentation of exogenous peptides are loaded onto MHC-1, exogenous antigens are transported out of the endosomes into the cytosol for proteasomal degradation and processing similar to any cytosolic antigen (92, 93).

MHC-antigen complex engagement with TCR is not sufficient for the generation of an effective T cell response. Triggering of the TCR alone usually leads to T cell anergy, that is, limited expansion followed by unresponsiveness to antigen exposure. Additional co-stimulatory signals mediated by surface receptors on APCs are required for effective T cell activation. A wide panel of co-stimulatory receptors expressed by APCs provide positive or negative stimulation, to drive naïve T cells towards activation or anergy, respectively. Additionally, some co-stimulatory molecules are also crucial for the generation of a memory response (93). For example, co-stimulation with 4-1BB, OX40 and CD27 are equally important for promoting memory T cell responses (94) **(Fig 2 C)**.

Finally, cytokine secretion by APCs provides an additional signal, which is integral for T cell differentiation, proliferation and acquisition of effector functions. Cytokine stimulation is also important for programming the memory response. CD8 T cells stimulated in the presence of IL-15 and IL-7 rich environment could rapidly transition to a memory phenotype and persist long term (95). The presence of TCR and co-stimulatory signaling is sufficient to induce proliferation. However, in the absence of cytokine signaling, the activated T cells die rapidly and fail to mount an effective clearance of pathogens (96). Thus, the combination of three signal stimulations will determine the

effectiveness and magnitude of CD8 T cell response (93, 97). In the case of CD4 T cells, the presence of cytokines during their activation results in a phenotypic switch in their differentiation into pro-inflammatory Th1 cells or the anti-inflammatory Th2 cells.
The processes involved in antigen presentation also facilitate PRR mediated activation of the APCs. Endocytosis and proteasomal/endosomal degradation of the internalized pathogen or proteins exposes PRRs to PAMPs, which induces pro-inflammatory signaling and activation of APCs. Resting APCs have few MHC molecules on their surface and do not express co-stimulatory molecules or cytokines necessary for effective stimulation of naïve T cells. PRRs signaling, due to the internalized antigen, DAMPs and cytokines in the infected microenvironment, induce expression of MHC complexes, cytokines and co-stimulatory molecules (93). Finally, cell-to-cell interaction between APC and T cells allows for bidirectional co-stimulatory signals. Thus, both the APCs presenting the processed antigen and the T cell receiving the signal are stimulated by the APC-T cell interaction (98, 99). One such co-stimulatory interaction is the CD40 ligand, expressed by T cell, binding to CD40 on APCs (100). This interaction transmits activating signals to the T cell and also induces expression of B7 molecules by APCs, which promotes T cell proliferation. Similar cell dialogue has been observed during interactions of 4-1BB (CD137) on T cells and its ligand 4-1BBL on APCs; with both the T cell and the APC receiving activating signals (101) **(Fig 2 B)**.

1.4.2 CD8 T cell differentiation and memory

An effective primary T cell response to an intracellular pathogen consists of three distinct phases: 1) potent antigen presentation that results in expansion of antigen-specific T cells, 2) contraction of the activated T cells via apoptosis, and 3) survival of activated T cells to become long-term memory cells **(Fig 2 A)**. Antigen-presenting cells provide TCR

stimulation and engagement of co-stimulatory ligands to activate T cells and induce their proliferation. Additionally, APCs secrete cytokines to support T cell survival and proliferation, acquisition of effector function and memory formation. Activation of naïve T cells by APCs triggers the entry of the naïve T cells into the G1 phase of the cell cycle. Additionally, activation signaling also induces the synthesis of IL-2 and its receptor. Autocrine binding of IL-2 to the high-affinity receptor triggers progression through the rest of the cell cycle. Activated T cells can divide multiple times a day for several days, allowing one cell to give rise to thousands of progeny that all carry the same receptor for a peptide/MHC complex. After several days, activated T cells differentiate into effector T cells that can synthesize effector molecules required for their specialized function as cytotoxic T lymphocytes (CTLs). These effector cells critically respond to their specific antigen without the need for co-stimulation (110).

The secreted cytokines, combined with the co-stimulatory signaling, by APCs, determines the fate of activated T cells. Activate T cells differentiate into different effector or memory subsets. These subsets differ in their ability to proliferate, survive and mediate effector function. Primed T cells can be broadly segregated between effector and memory cells relative to surface expression of IL-7 receptor alpha chain (CD127) and killer cell lectin like receptor G1 (KLRG1). Short-lived effector cells (SLEC) are $CD127^{low}KLRG1^{high}$, and memory precursor effector cells (MPEC) are $CD127^{high}KLRG1^{low}$. IL-7R mediates survival signaling, which is crucial for the generation and maintenance of T memory cells. Additionally, expression of CD62L, a cell adhesion molecule essential for entry of T cells into lymphoid organs, in conjunction with CD127 expression can be used to differentiate primed cells into effector T cells (Teff), $CD127^{low}CD62L^{low}$, effector-memory T cells (Tem), $CD127^{high}CD62L^{low}$ and central-

memory T cells (Tcm), CD127^{high}CD62L^{high} (111). Mechanistic studies in mice have shown that Tem cells are more prevalent in tissues, while Tcm cells are more prevalent in lymph nodes. Additionally, Tem cells are associated with rapid effector response to infection in the peripheral tissue, whereas Tcm cells are viewed as early differentiated progenitors with greater self-renewal capabilities and long-survival (112, 113). Finally, CTLs, Teff, or SLECs are CD8 T cells that differentiated to acquire effector function. They are short-lived cells that are recruited to the site of infection to mediate the elimination of infected cells. Antigen recognition, by way of their TCRs and CD8 molecules, prompts activation and killing of target cells by three mechanisms. First is the secretion of cytokines, primarily TNF-α and IFN-γ, which can induce cell death and have anti-viral microbial effects (112). The second major function is the production and release of cytotoxic granules, containing two families of proteins, perforin, and granzymes. Perforin oligomerizes to forms a pore (16-22 nm) in the plasma membrane of the target cell. The perforin pores enable granzymes to diffuse across the plasma membrane into the cytosol of the target cell. Granzymes are proteases that cleave both extracellular and intracellular proteins, and activate cell death morphology similar to apoptosis (114). Finally, CTLs may also induce apoptotic signaling through Fas receptors on targeted cells. Fas binding triggers extrinsic apoptotic signaling in target cells (115).

The peak response is generated within the first week of infection, mainly at day 7-8, which is followed by a rapid phase of T cell contraction, wherein >90% of activated cells are culled. Immediately after the generation of peak T cell response, usually at day 7 post-infection, most of the activated cells undergo apoptosis, leaving behind a small population of memory cells. Such a response ensures robust control of invading pathogens and lifelong immunity to future invasions. Elimination of CTLs ensures the availability of space,

cytokines, and nutrients for other cells, and limit the tissue damage, in case of reactivation. Cell survival signals are presumably dominant during the expansion phase of the response to allow >1000-fold expansion in the numbers of specific T cells that differentiate to effectors to eliminate the pathogen swiftly. Conversely, cell death/apoptotic signals are dominant during the contraction phase of the response, which leads to the culling of activated cells and return to homeostasis. Thus, cell death and survival signaling are inherently linked to T cell differentiation and memory development (91).

Cell death during T cell contraction is, for the most part, facilitated by Bim-mediated Bax/Bak-dependent intrinsic apoptosis (116). Extrinsic cell death signaling mediated by caspase-8 or RipK3 induced apoptosis and necroptosis, respectively, were dispensable during contraction. However, inhibition of caspase-8 has been shown to result in hyper-accumulation of CD8 T cells (117). Furthermore, chemical inhibitors of caspases limit T cell activation. Our lab has previously shown that caspase-3 activation plays a role in promoting T cell proliferation(118). The non-apoptotic function of caspase-3 activation has been associated with the differentiation of skeletal muscle cells, macrophages, and erythroblasts (118). This adds to a growing paradigm whereby apoptotic mechanisms, normally associated with cell death, also play an important role in cell proliferation and differentiation.

1.5 XIAP

X-linking inhibitor of apoptosis protein (XIAP) is an endogenous inhibitor of cell death, which belongs to the inhibitor of apoptosis protein (IAP) family. XIAP shares important functional and structural features with two other cellular IAPs, cIAP-1 and cIAP-2. XIAP consists of three BIR domains (Baculovirus Inhibitor of apoptosis Repeats), a

ubiquitin-associated domain (UBA), and a RING domain E3 ubiquitin ligase. XIAP, cIAP1, and cIAP2 all share a conserved BIR2 for binding and subsequent inhibition of both executioner caspases, caspase-3, and -7 (119). XIAP is the most potent caspase inhibitor in the IAP family and is the only direct inhibitor of protease activity. Additionally, XIAP also binds and inhibits processed caspase-9, via its BIR3 domain (120). Based on the affinity and potency of inhibiting apoptotic caspases, targeted inhibition of XIAP has been studied as a therapeutic approach for inducing cell death in cancerous cell and autoimmune cells. Second mitochondria-derived activator of caspase (SMAC), also known as direct IAP binding protein with low pI (DIABLO), antagonizes IAPs, hence restoring caspase activity. Upon apoptotic stimulation, SMAC/DIABLO is released from the mitochondria into the cytosol. They bind IAPs at the BIR domains, thus freeing caspases to potentiate apoptotic cell death (120). XIAP-deficiency in proliferating T cells reduces the threshold for apoptotic cell death. Similarly, inhibition of all IAPs during infection with Lymphocytic Choriomeningitis Virus (LCMV) resulted in reduced numbers of antigen-specific CD8 cells (121).

However, IAP function is not limited to caspase inhibition; they also play a protective role against inflammatory forms of cell death. This is mediated by their C-terminal RING domain, which has an E3 ubiquitin ligase activity. IAPs regulate the ubiquitination of RipK1 to prevent downstream association with RipK3, upon TNF stimulation (47, 48) (**Fig 1)**.

In addition to inhibiting cell death processes, the C-terminal E3 ubiquitin ligase, also facilitates a large variety of signaling pathways. In the innate immune compartment, XIAP has been shown to promote the activation of NFκB (122), and various MAPK pathways such as p38 and ERK (123). Additionally, XIAP promotes K63-linked

ubiquitination of RipK2 to induce NFκB signaling, upon NOD2 receptor binding of bacterial muramyldipeptide (MDP) (124). Due to the pleiotropic functions of XIAP in inducing pro-inflammatory signaling and preventing cell death in innate immune cells, XIAP-deficient mice succumb to infections with intracellular gram-negative and -positive bacteria, such as *Listeria* monocytogenes (125), *Chlamydophilia* pneumonia (126) and *Shigella* flexneri (127). Additionally, XIAP-deficient mice also have impaired control over viral infection with MHV-68, a murine γ-herpes virus (47). Finally, XIAP deficiency in humans is characterized by clinical manifestations of a triad of key autoinflammatory disorders, including hemophagocytic lymphohistiocytosis, splenomegaly and inflammatory bowel disease (IBD) with the features of a Crohn's disease (128, 129).

1.6 Rationale

The magnitude and effectiveness of CTL response against intracellular pathogens is directed by survival and apoptotic signals that govern the fate of T cells. A small imbalance in favor of survival or death can result in disastrous pathological outcomes, such as cancer, autoimmunity, or immune deficiency. Cell death may be influenced by both cell-intrinsic and cell-extrinsic variables, such as the presence of antigen, co-stimulatory factors, and cytokines in the tissue environment. XIAP expression promotes the survival of immune cells by inhibiting caspase-driven apoptotic and RipK1/RipK3 mediated inflammatory cell death signaling. Additionally, it also facilitates the activation of various cell survival and pro-inflammatory signaling in both innate and adaptive cells. Therefore, understanding the precise molecular mechanisms by which XIAP regulates the inflammatory response and cell death in the innate and acquired immune compartment could reveal new therapeutic avenues for treating various inflammatory diseases. For my thesis, I wanted to test the role of XIAP in regulating CD8 T cell response. In this present work, I evaluated T cell expansion, differentiation, and memory development in XIAP-deficient mice upon infection with intracellular pathogens.

1.7 Hypothesis

XIAP plays a key role in restricting the contraction of the CD8 T cell response

1.8 Objective

My overall goal was to evaluate whether XIAP impacts the development and maintenance of CD8 T cell response to intracellular pathogens, such as *Salmonella enterica serovar* Typhimurium (ST) and *Listeria monocytogenes* (LM). Since innate immune cells, APCs, also play a major role in regulating T cell response, I segregated my objectives to individually study the impact of XIAP-deficiency in either innate or adaptive immune compartments in regulating T cell response.

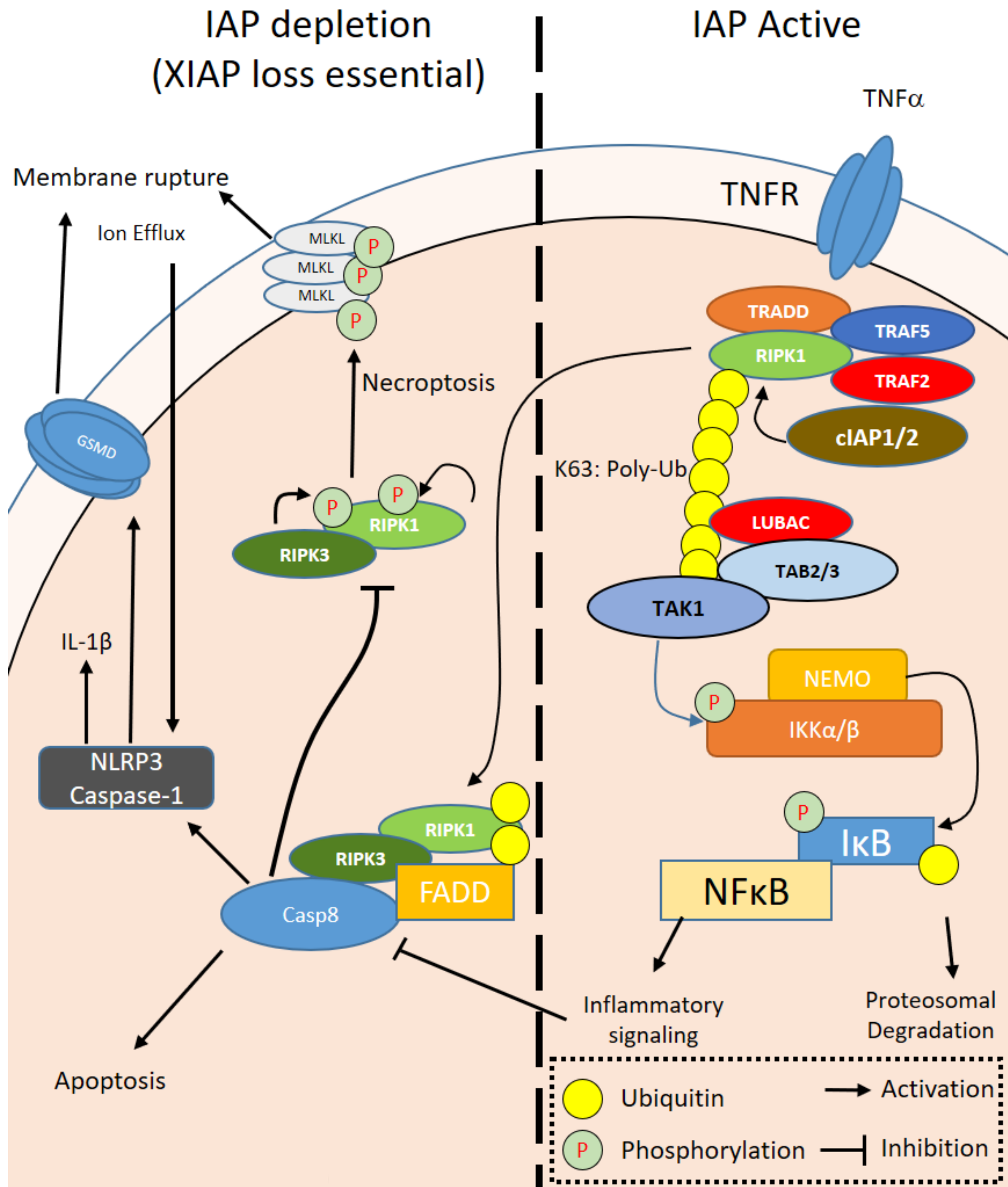
Objective 1: To assess the impact of XIAP on mounting an antigen-specific CD8 T cell response.

Objective 2: To assess the impact of T cell intrinsic XIAP during an immune response

Objective 3: To assess the impact of XIAP-deficient APCs on CD8 T cell response.

Figure 1 Impact of IAPs on TNFR signaling.

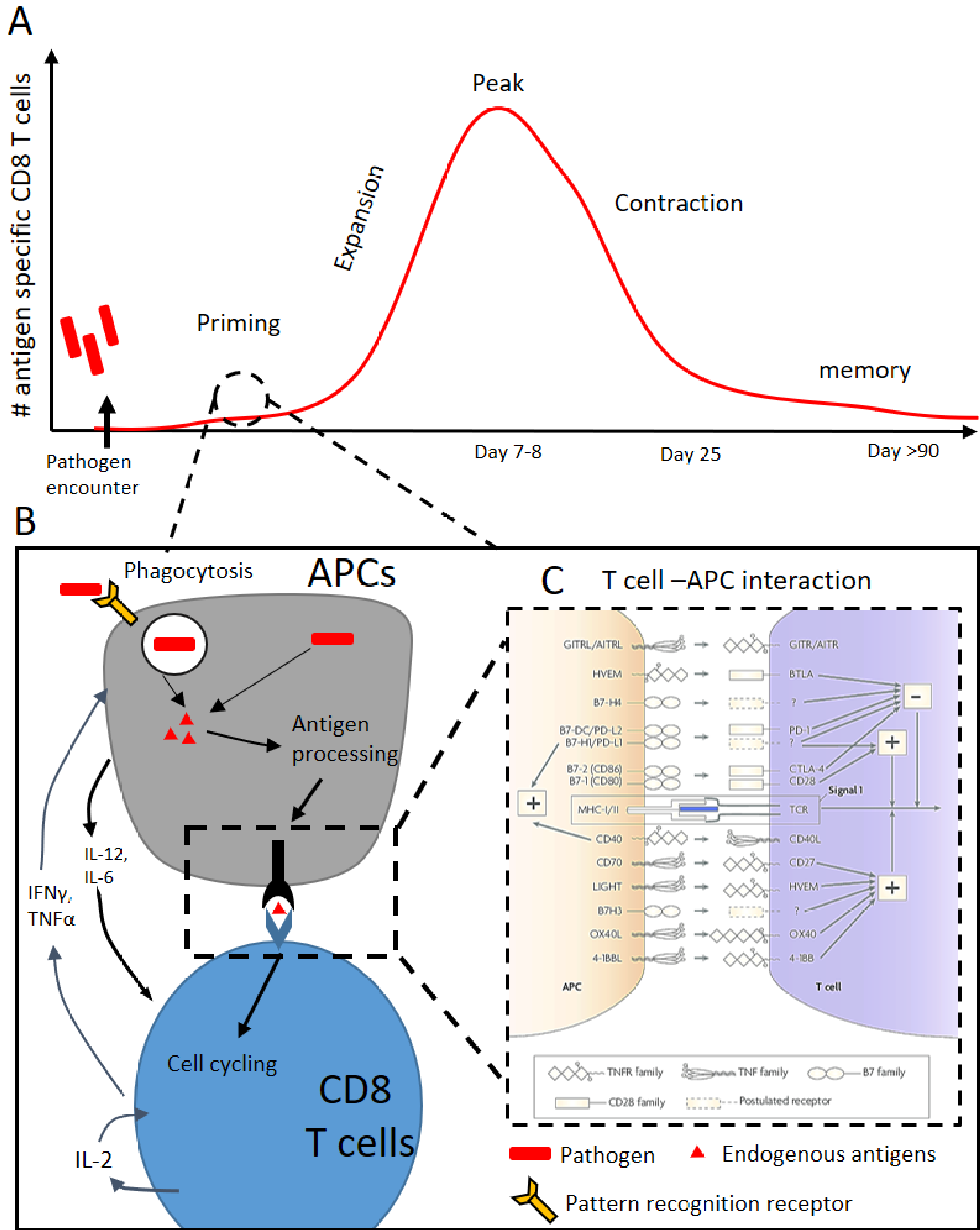
After the binding of TNF to its receptor, TNFR undergoes a conformational change and recruits multiple proteins to form complex-1, consisting of TRADD, TRAF2/5, RipK1 and cIAP1/2. The K63-linked ubiquitination of RipK1 by cIAP1/2 promotes the formation and activation of the TAK1/TAB complex and the IKKα/IKKβ/NEMO complex, which induces the NFκB pathway and cell survival. When cIAPs are blocked, RipK1 is deubiquitylated, and a complex consisting of RIP1, RIP3, FADD and caspase-8 is assembled. This complex initiates apoptosis by caspase-8 or pyroptosis through the NLRP3-dependent caspase-1 activation. Caspase-1 activation results in proteolytic cleavage of gasdermin-D. Mature gasdermin-D localizes to the inner domain of the plasma membrane and oligomerizes to form pores in the plasma membrane. When caspase-8 is inhibited, RipK1 binds to RipK3, which results in auto- and trans-phosphorylation of RipK3. This results in the recruitment and consequent phosphorylation of MLKL by RipK3. The phosphorylated MLKL then translocates from the cytosol to the plasma- and intracellular- membranes. The oligomerization of MLKL results in membrane pore formation, causing membrane rupture and eventually necroptosis. Ion influx as a result of pore formation induces activation of NLRP3 inflammasome. TNFR, TNF receptor 1; TNFα, tumor necrosis factor; TRADD, TNF-receptor-associated death domain; TRAF, TNF-receptor-associated factor; RipK, receptor-interacting serine/threonine kinase; cIAP, cellular inhibitor of apoptosis; TAK1, transforming growth factor-activated kinase 1; TAB, TAK1-binding protein; IKK, inhibitor of NFκB kinase; NEMO, NFκB essential modulator; FADD, Fas-associated protein with death domain; GSMD, gasdermin; MLKL, mixed lineage kinase domain like protein.



IAP depletion
(XIAP loss essential)
IAP Active
TNFα
TNFR
Membrane rupture
Ion Efflux
MLKL
MLKL
MLKL
P
Necroptosis
GSMD
RIPK1
RIPK3
IL-1β
NLRP3
Caspase-1
RIPK1
RIPK3
FADD
Casp8
Apoptosis
TRADD
TRAF5
RIPK1
TRAF2
cIAP1/2
K63: Poly-Ub
LUBAC
TAB2/3
TAK1
NEMO
IKKα/β
IκB
NFκB
Inflammatory
signaling
Proteosomal
Degradation
Ubiquitin
Activation
Phosphorylation
Inhibition

Figure 2. T cell response.

A) T cell response can be divided into 3 phases. First, the antigen specific T cells expand rapidly in response to antigen-presentation, peaking at day 7/8 post infection; and then differentiate into effector cells. Majority of effector T cells are removed during the contraction phase, leaving a small stable pool of memory T cells. **B)** T cells must receive very specific stimulation in order to become activated. T cells continually scan the MHC-peptide complexes presented by antigen presenting cells (APCs). Endogenous antigen from phagocytized or cytosolic pathogens are processed and presented on MHC-1 complex on the surface of APCs. **C)** Along with MHC-1 plus antigen complex, APCs provide co-stimulatory signals to promote (+) or inhibit (-) T cell activation. Activated T cells undergo rapid proliferation. Finally, cytokines released by T cells and APCs influence the activation and cycling of T cells. This figure was adapted from Nature Reviews Cancer publication titled "Immunostimulatory monoclonal antibodies for cancer therapy" by Melero *et al.* (130).



A
antigen specific CD8 T cells
Peak
Expansion
Contraction
Priming
memory
Pathogen
encounter
Day 7-8
Day 25
Day >90
B
Phagocytosis
APCs
Antigen
processing
IL-12,
IL-6
IFNγ,
TNFα
Cell cycling
CD8
T cells
IL-2
C
T cell –APC interaction
GITRL/AITRL
GITR/AITR
HVEM
BTLA
B7-H4
?
B7-DC/PD-L2
B7-H1/PD-L1
PD-1
?
B7-2 (CD86)
B7-1 (CD80)
CTLA-4
CD28
Signal 1
MHC-I/II
TCR
CD40
CD40L
CD70
CD27
LIGHT
HVEM
B7H3
?
OX40L
OX40
4-1BBL
4-1BB
APC
T cell
TNFR family
TNF family
B7 family
CD28 family
Postulated receptor
Pathogen
Endogenous antigens
Pattern recognition receptor

2. Materials and Methods

2.1 Mice strains and infections

All mouse strains were bred and maintained in a specific pathogen free facility at the Animal Care and Veterinary Services at Roger Guindon Hall (University of Ottawa, Ottawa, Ontario). All procedures were done in compliance with the Canadian Council on Animal Care guidelines. Wildtype (WT) C57BL/6J mice, OT-1 TCR transgenic mice and B6.SJL mice were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA). *Xiap*$^{-/-}$ mice were obtained from Dr. Robert Korneluk laboratory (Children's Hospital of Eastern Ontario, Canada). *Xiap*$^{-/-}$ OT-1 mice were generated by mating *Xiap*$^{-/-}$ mice (CD45.1^{+}) with OT-1 mice (CD45.2^{+}). WT OT-1 (CD45.1^{+} CD45.2^{+}) mice were generated by mating B6.SJL (CD45.1^{+}) mice with OT-1 (CD45.2^{+}) mice. OT-1 TCR expression on CD8 T was measured by flow cytometry using H2-K^{b}-OVA$_{257\text{-}264}$ dextramer, a MHC-1 multimer conjugated to PE (Immudex, Denmark). All mice used in my study were between the ages of 6-8 weeks.

2.2 Flow cytometry

For cell surface staining, anti-CD8α (53-6.7), anti-CD11b (M170), anti CD11c (HL3), anti-CD19 (1D3), anti-CD45.1 (A20), anti-CD45.2 (104), anti-Ly6C (HK1.4), anti-Ly6G (RB6-8C5), anti-CD4 (RM4-5), anti-CD3 (17A2), anti-NK1.1 (PK136), anti-MHC2 (M5/114.15.2), anti-CD62L (MEL-14), anti-CD127 (A7R34), anti-KLRG1 (2F1), anti-MHC1-H2k^{b} (AF6-88.5.5.3), and anti-CD49b (DX5) antibodies were purchased from eBioscience (San Diego, CA). Cell surface staining was performed in phosphate buffered saline (PBS) solution containing 1% BSA at a concentration of 10^{6} cells/tube. Cells were

treated with anti-mouse Fc block (anti-CD16/32) for 10 minutes at 4°C followed by 0.5μL of the surface staining antibodies for 30 minutes at 4°C. H2-K^b-$OVA_{257-264}$. Dextramer staining was performed, for 10 minutes, prior to Fc block and surface staining at room temperature in the dark. Intracellular staining was conducting following cell surface staining using anti-Ki67 (SolA15), anti-Cleaved caspase-3 Asp175 (5A1E), anti-FoxP3 (FJK-16s) and anti-RORγT (AFKJS-9). BioLegend's True-Nuclear Transcription Factor Buffer Set was used for intracellular staining. Live dead staining was conducted using Zombie dye from Biolegend (San Diego, CA).

2.3 Bacteria and infections

Salmonella enterica serovar Typhimurium (ST, SL1344), a recombinant of ST that expresses model antigen OVA (ST-OVA) and a recombinant of *Listeria monocytogenes* (LM) that expresses OVA (LM-OVA) were all used for *in vitro* and *in vivo* infection models. All bacterial strains were used from frozen stock. Generation of LM-OVA (131) and ST-OVA (132) has been previously reported.

For *in vitro* infections, BMDMs/BMDCs were plated at 10^5 cells per well in a round flat bottom 96-well plate in RPMI media (Rosswell Park Memorial Institute (RPMI) -1640 + 50μM β-mercaptoethanol) with 8% fetal bovine serum (FBS), also known as R8%, without antibiotics. Frozen bacterial stock were thawed, resuspended in R8% without antibiotics and added to the cells at various multiplicity of infection (MOIs). Plates were centrifuged at 2500 rpm for 6 minutes to synchronize bacterial uptake. Cells were infected with bacteria for 30 min, followed by a two-hour treatment with high gentamycin (50 μg/ml) to remove extracellular bacterium. Following a 2hr incubation, the media was

replaced with fresh R8% containing low gentamycin (10 μg/ml) and incubated for 24 hrs. Cell death was measured using neutral red uptake and Zombie assay.

For *in vivo* infection with ST, ST-OVA, or LM-OVA, frozen stocks were thawed and diluted in PBS. Mice were immunized with $1x10^3$ LM-OVA or ST-OVA suspended in 100 μl of PBS, via the lateral tail vein injection. For ST infection, mice were inoculated with 200 ST suspended in 100 μl of PBS, via the lateral tail vein injection.

2.4 ELISPOT

Enumeration of IFN-γ-secreting cells was done by Enzyme-linked immunospot assays (ELISPOT) on spleens harvested from mice challenged with OVA expressing pathogens. Multiscreen IP plates purchased from Millipore (Billerica, MA) were coated with 100 μl of a primary anti-IFNγ antibody (clone R46A2 in PBS) at 10 μg/ml. Spleens were harvested from naïve and infected mice at various time points following infection, and mechanically disrupted using frosted glass slides. Followed by treatment with ammonium-chloride-potassium (ACK) lysis buffer from Sigma (St. Louis, MO) for 1 minute. ACK buffer induces lysis of red blood cells. ACK lysis buffer was diluted by washing with R8% media. Cells were counted and resuspended in RPMI media with 10% FBS known as R10%. A total number of $5x10^5$ cells were plated in all wells. Cells from infected mice were plated at various concentrations of $5x10^5$, $1x10^5$ or $2x10^4$ cells per well; the remaining cells were supplemented with naïve feeder cells to maintain a total number of $5x10^5$ cells in each well. All cells were cultured in the presence of IL-2 (0.1ng/mL) to promote T cell growth. T cells were stimulated with peptide ($OVA_{257-264}$, 5μg/mL) or media for 30 hours. Wells were washed with PBS-Tween20 (0.05% Tween20 in PBS) buffer and incubated overnight, at 4 °C, with 75 μl of 2μg/ml biotinylated anti-IFNγ antibody (clone XMG1.2)

in PBS-Tween20 buffer. Following a few stringent washes, wells were washed and incubated with streptavidin-horse radish peroxidase (HRP) from Sigma (St. Louis, MO) for 1 hour. After HRP treatment, wells were washed and incubated with 3-amino-9-ethylcarbazole (AEC) substrate, from Sigma (St. Louis, MO), to quantify IFNγ producing spots in each well. Spots were counted using Immunospot Analyzer from Immunospot (Cleveland, OH).

2.5 Cell death

Cell death was measured using neutral red uptake assay. Cells were plated into 96 well plates at a concentration of 10^5 cells per well. Following various stimulations or infections, neutral red dye was added to each well at a concentration of 0.17g/mL diluted in R8% media for 5-10 minutes at 37°C. Live cells take up the dye and are visibly red, determined by viewing under the light microscope. Wells were then washed with PBS, to remove excess dye. Solubilization solution (50% ethanol, Et-OH; 50% water, H_2O and 1mL glacial acetic acid, CH3COOH) was added to each well. Absorbance was measured at 570-650nm using the FilterMax F5 microplate reader from Molecular Devices (Sunnyvale, CA). Percent cell death was calculated relative to absorbance values from control wells. These wells were assumed to have no death (0 %).

Death of infected cells was also measured using a Zombie dye. Zombie is an amine-reactive fluorescent dye that is permeant to cells with compromised membranes and non-permeant to live cells. Cells were washed with PBS to remove media and incubated with Zombie dye in PBS for 30 minutes at 37°C. Excess dye was washed using 1% BSA in PBS and cell death was measured using flow cytometry.

2.6 BMDCs/BMDMs generation

Bone marrow derived dendritic cells (BMDC) and bone marrow derived macrophages (BMDM) cell cultures were grown *in vitro* from bone marrow progenitors and various growth factors. Bone marrow progenitors isolated from the femur and tibia of representative mouse strains. Bone marrow progenitors were cultured at a concentration of 1 million cells/ml in 10 ml R8% medium in the presence of 5ng/ml growth factor GM-CSF from Empire Genomics (Buffalo, NY) in T25 flasks at 37°C to generated BMDCs. The media was replaced on day 2 and 4 to remove any floating cells. By day 7-8, GM-CSF DCs differentiate from adherent cells and become floating cells that can be collected from the supernatant. BMDCs generated *in vitro* using GM-CSF mimic monocyte DCs. BMDMs were generated by coating a petridish with 50 ng of M-CSF and plating 10 million bone marrow progenitors in 10 ml R8% medium. On day 7, petridishes were washes with PBS and the adherent cells, BMDMs, were detached using a cell lifter.

2.7 *In vitro* antigen presentation

The harvested BMDMs/BMDCs were plated at $5x10^4$ cells per well and infected with ST-OVA or LM-OVA for 1 hour as described in the Bacterial and Infections section. Following 1 hour infection with bacteria, APCs were washed with R8% containing gentamycin (50µg/ml) to remove any extracellular bacterium. APCs were co-cultured with $5x10^4$ OT-1 CD8 T cells per well, in gentamycin containing R8% medium (10mg/mL). Cell death and proliferation of the T cell was evaluated at various timepoints using flow cytometry. Exogenous recombinant murine IL-6 (rm IL-6), from Peprotech (Cranbury, NJ) was added at 50 ng/ml after 24 hr post-infection and supplemented after 72 hr post-infection.

Leukocytes were harvested from spleens of OT-1 mice. Spleens were mechanically disrupted and treated with ACK buffer, to remove red blood cells (RBCs). Leukocytes were then labelled with Carboxyfluresceine succinimidyl ester (CFSE), obtained from Sigma (St Louis, MO), to assess cell proliferation. $1x10^7$ splenocytes per ml were labeled with 0.125μM CFSE in PBS at 37°C for 8 min. Excess CFSE was quenched by adding an equal volume of equine serum and placed on ice for 5 minutes, followed by 2 washes with PBS. CD8 T cells isolated from CFSE-labelled splenocytes, using EasySep CD8 T cells negative selection kits from Stemcell (Vancouver, British Columbia).

2.8 Inhibitor and other reagents

Various inhibitors were used diluted from stocks in dimethyl sulfoxide (DMSO). Control cells were maintained in media containing comparable amounts of DMSO.

2.8.1 Caspase-1 inhibitor

Caspase-1 inhibitor Z-YVAD-FMK was obtained from Calbiochem (San Diego, CA). Prior to *in vitro* antigen presentation using ST-OVA infection, BMDCs were treated with 10 μM Caspase-1 inhibitor overnight.

2.8.2 RipK1 inhibitor

Following the 2 hour incubation in R8% containing high gentamycin (50 μg/ml), ST-infected cells were treated with Nec-1 in R8% containing low gentamycin (10 μg/ml) and incubated for 24 hours. RipK1 inhibitor, Necrostatin-1 (Nec-1), was obtained from Sigma (St Louis, MO).

2.8.3 TNFα stimulation

Cells ($1x10^5$ cells per well in 96-well plates) were treated with recombinant murine TNFα was obtained from R&D Systems (410-MT), for 24 hours.

2.9 Canonical NLRP3 activation

Host immune cells release ATP as an inflammatory signal, which in turn activates NLRP3 inflammasome in primed cells. BMDMs were primed with 100ng/mL of LPS from Sigma (St. Louis, MO). Cells were then treated with 2.5 μM ATP from Sigma (St. Louis, MO), at 0 or 4 hours following LPS treatment. Cell death and IL-1β expression was measured at 4.5 hours following LPS treatment.

2.10 Cytokine expression

Supernatants from *in vitro* experiments were collected and analyzed for the presence of cytokines. Cytokines IFN-γ, IL-1β, IL-6, TNF, IL-10 and IL-12 were analyzed using enzyme-linked immunosorbent assay (ELISA) kits from BD Bioscience (San Diego, CA). **TMB** (3,3',5,5'-tetramethylbenzidine) soluble substrates was chromogenic substrate. TMB forms a water-soluble blue reaction product, upon oxidation by HRP. Addition of stop solution (0.2 M sulfuric acid) the reaction product turns yellow. Absorbance was measured at 450nm using the FilterMax F5 microplate reader from Molecular Devices (Sunnyvale, CA).

Type I interferon (IFN-1) was measured using an L929 cell line (obtained from B. Beutler) with a luciferase reporter gene cloned under the regulation of an interferon-stimulated response element (ISRE) promoter. These ISRE-L929 cells (5×10^4 cells per well in 96-well plates) were incubated for 4 h with 40 μl cell culture supernatant. Cells were then lysed and luciferase activity was determined with a Luciferase Assay System according to the manufacturer's protocol (Promega).

2.11 Adoptive transfer

CD8 T cells, in OT-1 mice, express a transgenic TCR that is specific for H2-K^b-$OVA_{257-264}$ and thus provides an ideal model for tracking pathogens expressing the model antigen OVA. Three days prior to bacterial infection, OT-1 cells were injected (i.v.) into recipient mice. To acquire the cell to transfer, spleens from OT-1 mice were mechanically disrupted using frosted glass slides, followed by treatment with ACK buffer to remove RBCs. Following infection, T cell response was measure by assessing the number of adoptively transferred OT-1 CD8 T cells present in the recipient mouse. Splenocytes were harvest as described above and stained for CD45.1 and CD45.2 and assessed using flow cytometry to determine relative percentages of CD8 T cells

2.11.1 Cell-Intrinsic Model

10^4 of WT-OT1 (expressing CD45.2^+ CD45.1^-) and 10^4 of *Xiap*$^{-/-}$ OT-1 (expressing CD45.2^+ CD45.1^+) splenocytes were isolated, mixed, and injected into B6.SJL(expressing CD45.2^- CD45.1^+) mice.

2.11.2 Cell-Extrinsic Model

$5x10^4$ of WT-OT1 (expressing CD45.2^+ CD45.1^+) splenocytes were isolated and injected into *Xiap*$^{-/-}$ (expressing CD45.2^- CD45.1^+) and WT (expressing CD45.2^+ CD45.1^-) mice.

2.12 Statistical analysis

All experiments were performed in triplicates. All values were compared using two-way ANOVA, paired or unpaired student-t test depending on the number of variables involved and factors to be compared. The individual figure legends indicate the statistical test used for the analyses and the p values. Statistical analyses were done using the Prism

software (GraphPad, version 8). The differences were considered significant when the p value was <0.05.

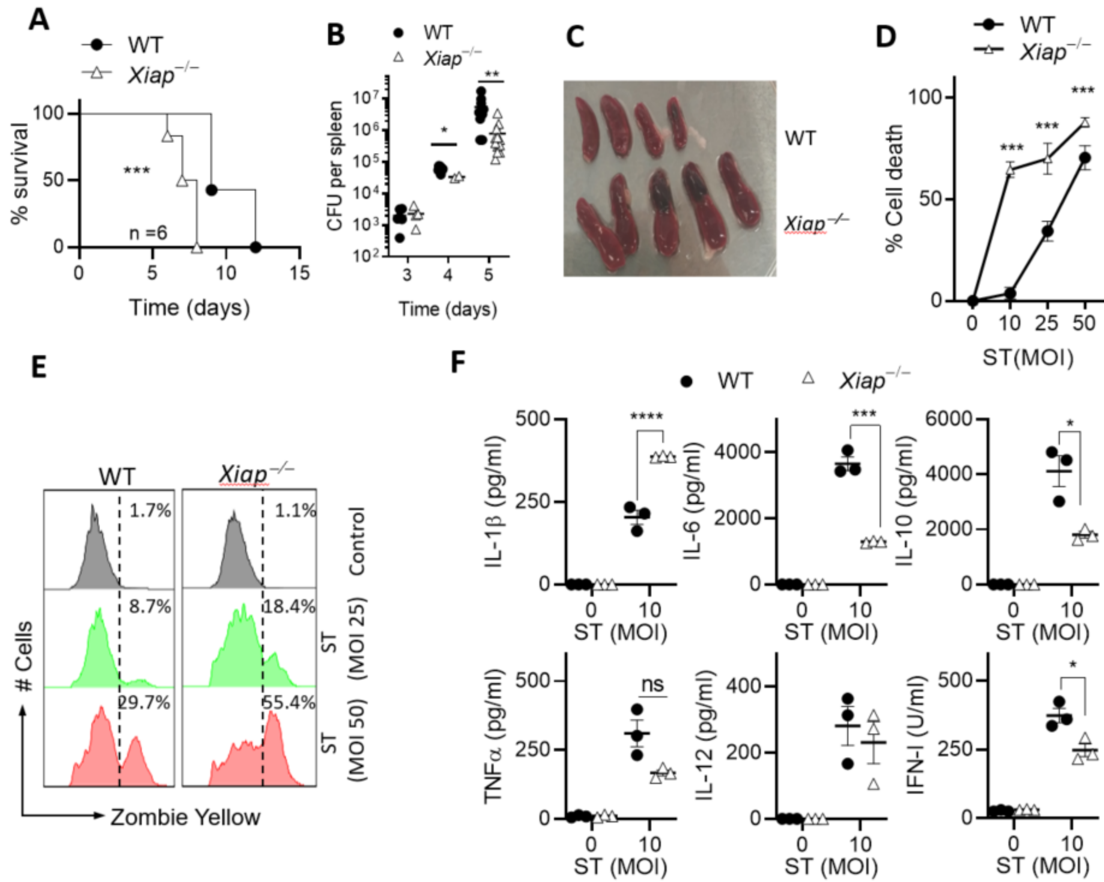
3. Results

3.1 Objective 1: To assess the impact of XIAP on mounting an antigen-specific CD8 T cell response.

3.1.1 XIAP regulates cell death and control of ST.

The first goal of my thesis was to investigate the role of XIAP during an antigen-specific CD8 T cell response. ST is a highly virulent intracellular bacterium that requires a complex interplay between innate and adaptive immune responses to limit the infection. Given that ST infects various host innate immune cells, such as macrophages and DCs, which, in turn, play an important role in priming the adaptive immune response, I began my investigation by evaluating the innate immune response to ST infection. To assess the impact of XIAP during an immune response to ST, I infected WT and *Xiap*$^{-/-}$ mice with ST ($2x10^2$ CFU, i.v.) and monitored the survival of mice. *Xiap*$^{-/-}$ mice were slightly more susceptible to infection with ST in comparison to WT mice **(Fig. 3 A)**. Surprisingly, the bacterial burden was slightly reduced in the spleens of *Xiap*$^{-/-}$ mice in contrast to WT mice **(Fig. 3 B**). This suggested that the increased susceptibility of *Xiap*$^{-/-}$ mice to ST infection was not a result of a failure to control the bacterial burden. The early fatality of *Xiap*$^{-/-}$ mice coincided with a pronounced splenic enlargement **(Fig. 3 C)**. The induction of inflammatory cell death (pyroptosis) in myeloid cells, such as macrophages, has been known to facilitate the control of ST (37, 76, 78). Additionally, the expression of IL-1 cytokine family and other cytokines by macrophages promotes the initial control of ST infection (65, 78, 133, 134). Since XIAP is a potent endogenous inhibitor of cell death and has been associated with regulating inflammatory signaling pathways, I evaluated the impact of XIAP on activation of cell death, and the expression of inflammatory cytokines

Figure 3. XIAP protects from inflammasome-induced cell death during ST infection. **(A-C)** WT and *Xiap*$^{-/-}$ mice were infected with ST (200 CFU, i.v.) **A)** Survival curve of the infected mice. **B)** CFU in spleen harvested from the infected mice, at days 3, 4 and 5 post-infection. **C)** Representative image of spleens from infected mice on day 5 post-infection. **(D, E)** BMDMs generated from WT and *Xiap*$^{-/-}$ mice were infected with ST as described in the methods section. **D)** Cell death evaluated at 24 hrs post-infection by neutral red uptake assay and **E)** zombie staining. **F)** Supernatants were collected at 6hr post-infection, and the expression of IL-1β, TNFα, IL-10, IL-6 and IL-12 was assessed by ELISA. IFN-I levels were assessed by luciferase bioassay. Data are shown as mean ±SEM and are representative of 2–3 separate experiments. Statistical analysis was performed by unpaired student *t*-test (**B-E**) and log-rank test (**A**) (**P*<0.05, ***P*<0.01, *****P*<0.0001)

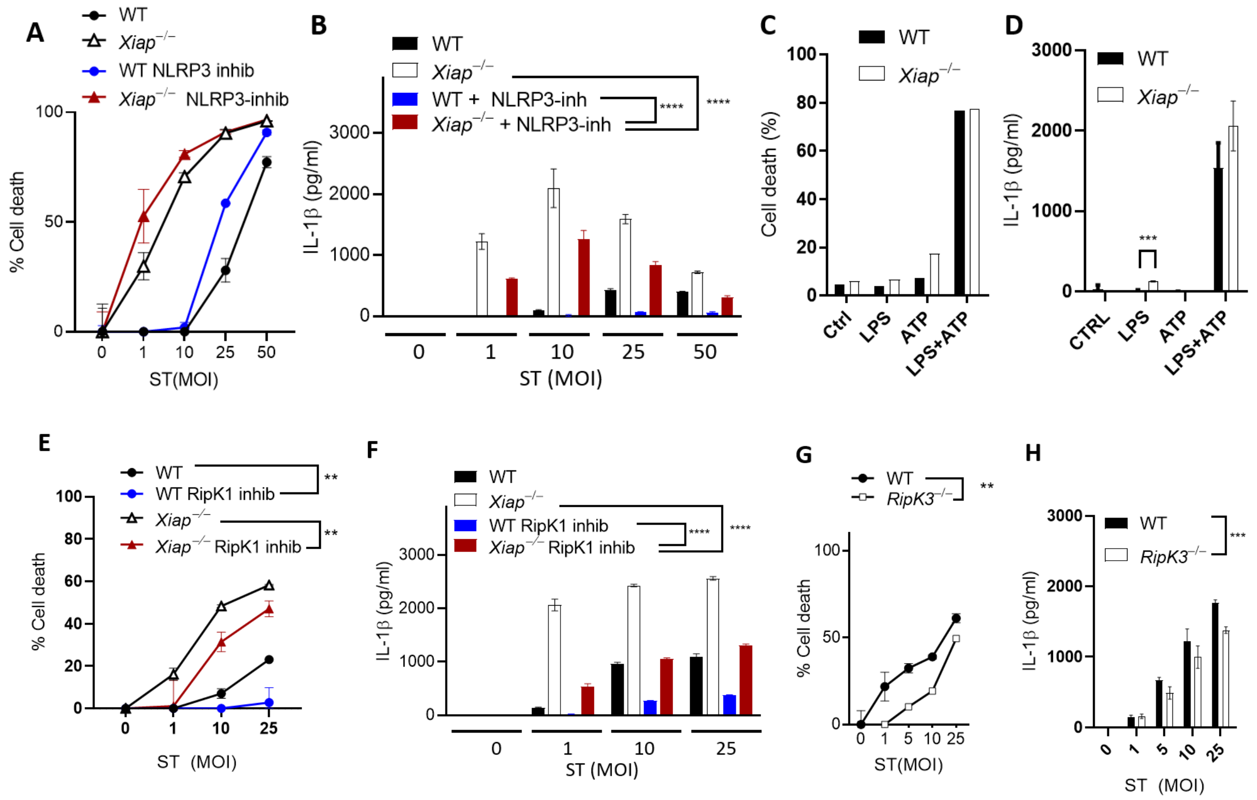

A
WT
Xiap−/−
% survival
***
n =6
Time (days)
B
CFU per spleen
Time (days)
C
WT
Xiap−/−
D
% Cell death
ST(MOI)
E
Control
ST (MOI 25)
ST (MOI 50)
Cells
Zombie Yellow
1.7%
1.1%
8.7%
18.4%
29.7%
55.4%
F
IL-1β (pg/ml)
IL-6 (pg/ml)
IL-10 (pg/ml)
TNFα (pg/ml)
IL-12 (pg/ml)
IFN-I (U/ml)
ST (MOI)
ns

by macrophages. Cell death of macrophages in response to infection with ST has been shown to be mainly mediated by inflammasome-induced pyroptosis (14, 78, 135, 136). Bone marrow-derived macrophages (BMDMs) generated from WT and *Xiap*$^{-/-}$ mice were infected with ST. Cell death was measured by staining cells with Zombie, which is an amine-reactive fluorescent dye that is incorporated by cells with compromised cell membranes, or by measuring the uptake of neutral red dye by viable cells. *Xiap*$^{-/-}$ macrophages underwent increased cell death following ST infection **(Fig. 3 D, E**). Commensurate with increased pyroptotic cell death, *Xiap*$^{-/-}$ macrophages secreted increased levels of IL-1β in comparison to WT macrophages (**Fig. 3 F**). Interestingly, the expression of IL-6 and IL-10 was reduced in *Xiap*$^{-/-}$ macrophages in comparison to WT macrophages **(Fig. 3 F**). Thus, XIAP plays and important role in modulating the innate immune response to ST, by supressing the inflammasome signaling and promoting the expression of IL-6 and IL-10.

Given that XIAP has been reported to inhibit the NLRP3 inflammasome activation downstream of RipK1 signaling (121, 137, 138), I used selective inhibitors against NLRP3 inflammasome (MCC950) and RipK1 (Necrotatin-1, Nec-1) activity in macrophages infected with ST. Inhibition of the NLRP3 did not have any impact on cell death, but IL-1β expression was substantially reduced in *Xiap*$^{-/-}$ macrophages **(Fig 4 A, B)**. Similarly, inhibition of RipK1 by Nec-1 resulted in the reduction of IL-1β expression by *Xiap*$^{-/-}$ macrophages. Additionally, inhibition of RipK1 also resulted in a slight decrease in cell death of *Xiap*$^{-/-}$ macrophages **(Fig 4 E, F)**. RipK1-dependent NLRP3 inflammasome activity is referred to as “alternative” inflammasome activation pathway (44, 139). RipK1-

Figure 4. Inhibiting NLRP3 and RipK1 inflammasome activity reduces the elevated IL1β secretion in *Xiap*$^{-/-}$ macrophages.

BMDMs were generated from WT and *Xiap*$^{-/-}$ mice and infected at various MOIs with ST as described in the methods section. **A, B)** Cells were treated with NLRP3 inhibitor (MCC950, 10μM). **A)** Cell death and **B)** IL-1β cytokine in the supernatant was measured at 24 hrs post-infection using neutral red uptake assay and ELISA, respectively. **C, D)** Cells were treated with LPS(100 ng/ml) for 4 hours and ATP(2.5μM) was added after 0hr **(D)** or 4 hours **(E). C)** Cell death measured using Zombie at 4 hours post LPS, and **D)** IL-1β levels were measured in the supernatant at 4.5 hours post-LPS-stimulation using ELISA. **E, F)** Cell were treated with Ripk1 inhibitor (Nec-1, 30μM) during incubation with 10μg/ml gentamycin. **E)** Cell death and **F)** IL-1β cytokine in the supernatant was measured at 24 hrs post-infection using neutral red uptake assay and ELISA, respectively. **G, H)** BMDMs generated from Ripk3-deficient mice were infected with ST. **G)** Cell death and **H)** IL-1β cytokine in the supernatant was measured at 24 hrs post-infection using neutral red uptake assay and ELISA, respectively. Data presented is a mean ± SEM. Data presented is a representative of 2-3 experiments. Statistical analysis was performed by student *t*-test (D) and 2-way ANOVA(B, E, F, G). (**$P<0.01$, ***$P<0.001$, ****$P<0.0001$)



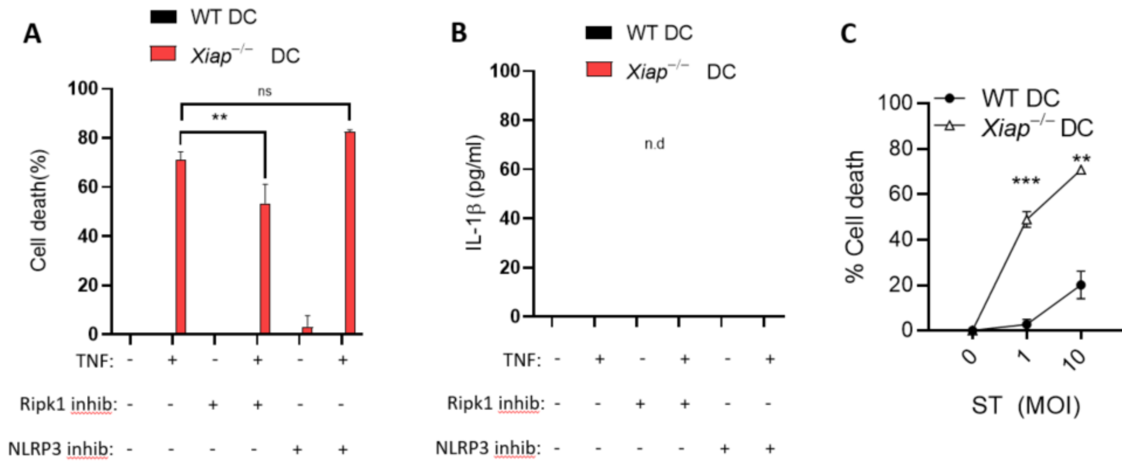
A
B
C
D
E
F
G
H

independent (canonical) NLRP3 inflammasome activation requires an activation signal provided by a variety of stimuli, including extracellular ATP, pore-forming toxins, RNA viruses, and particulate matter. To evaluate the role of canonical NLRP3 inflammasome activation, I primed macrophages with LPS treatment to induce PRR mediated transcriptional upregulation of pro-IL-1β and NLRP3. Extracellular ATP functions as an activation signal to induce the formation of NLRP3 inflammasome (140, 141). Co-stimulation of cells with priming and activating signaling (LPS+ATP) resulted in rapid cell death **(Fig 4 C)**. Premature cell death impeded the detection of secreted cytokines **(data not shown)**. Thus, to avoid early cell death activation, LPS and ATP treatments were provided sequentially. Activation of NLRP3 inflammasome resulted in similar cell death **(Fig 4 C)** and IL-1β expression **(Fig 4 D)** in WT and $Xiap^{-/-}$ macrophages. Finally, since RipK3 functions downstream of RipK1 signaling to promote NLRP3 inflammasome activation, I evaluated ST-induced cell death in $RipK3^{-/-}$ macrophages. I observed a slightly reduced cell death and IL-1β expression in $RipK3^{-/-}$ macrophages **(Fig 4 G, H)**. These results indicate that XIAP suppresses the alternate NLRP3 inflammasome activation that is induced via RipK1, RipK3 and NLRP3 signaling.

Interestingly, I noticed a small increase in IL-1β secretion, upon LPS stimulation alone, in the absence of XIAP **(Fig 4 D)**. Yabal and colleagues (47) have reported that XIAP protects from TNF-driven inflammasome formation, during autocrine TNFα signaling in LPS stimulated cells. However, I did not detect any inflammasome activity upon TNFα stimulation of macrophages **(data not shown)**. Yabal and colleagues (47) also reported that $Xiap^{-/-}$ DCs undergo pyroptotic cell death upon stimulation with TNFα, and secrete increased IL-1β. I evaluated the impact of XIAP in regulating TNFR signaling. My

Figure 5. Cell death in XIAP-deficient cells is mediated by RipK3 dependent fashion upon TNFα stimulation.

A, **B)** BMDCs were treated with recombinant murine TNFα (25 ng/ml), NLRP3 inhibitor (MCC950, 10μM), or RipK1 inhibitor (Nec-1, 10μM) and cell death evaluated by neutral red assay at 24h post-stimulation. **B)** IL-1β cytokine in the supernatant was also measured at 24 hrs post-stimulation by ELISA. **C)** BMDCs were generated from WT and *Xiap*$^{-/-}$ mice and infected at various MOIs with ST, as described in the methods section. Cell death was evaluated by neutral red uptake assay at 24hr post-infection. Data is presented as mean ± SEM. Data presented is a representative of 2-3 experiments. Statistical analysis was performed by student *t*-test. (***P*<0.01, ****P*<0.001)


A
WT DC
$Xiap^{-/-}$ DC
Cell death(%)
ns
**
TNF:
Ripk1 inhib:
NLRP3 inhib:
B
WT DC
$Xiap^{-/-}$ DC
IL-1β (pg/ml)
n.d
C
WT DC
$Xiap^{-/-}$ DC
% Cell death
***
**
ST (MOI)

results indicated an increased cell death of *Xiap*$^{-/-}$ BMDCs upon TNFα stimulation. Additionally, this cell death was partially dependent on RipK1 signaling **(Fig 5 A)**. However, I was not able to detect any IL-1β expression **(Fig 5 B)**. Similar to XIAP-deficient macrophages, cell death of *Xiap*$^{-/-}$ BMDCs was also higher than WT DCs, upon infection with ST **(Fig 5 C)**. These results indicated that NLRP3 and RipK1 promote enhanced cell death and IL-1β expression in *Xiap*$^{-/-}$ DCs and macrophages, as a response to ST infection.

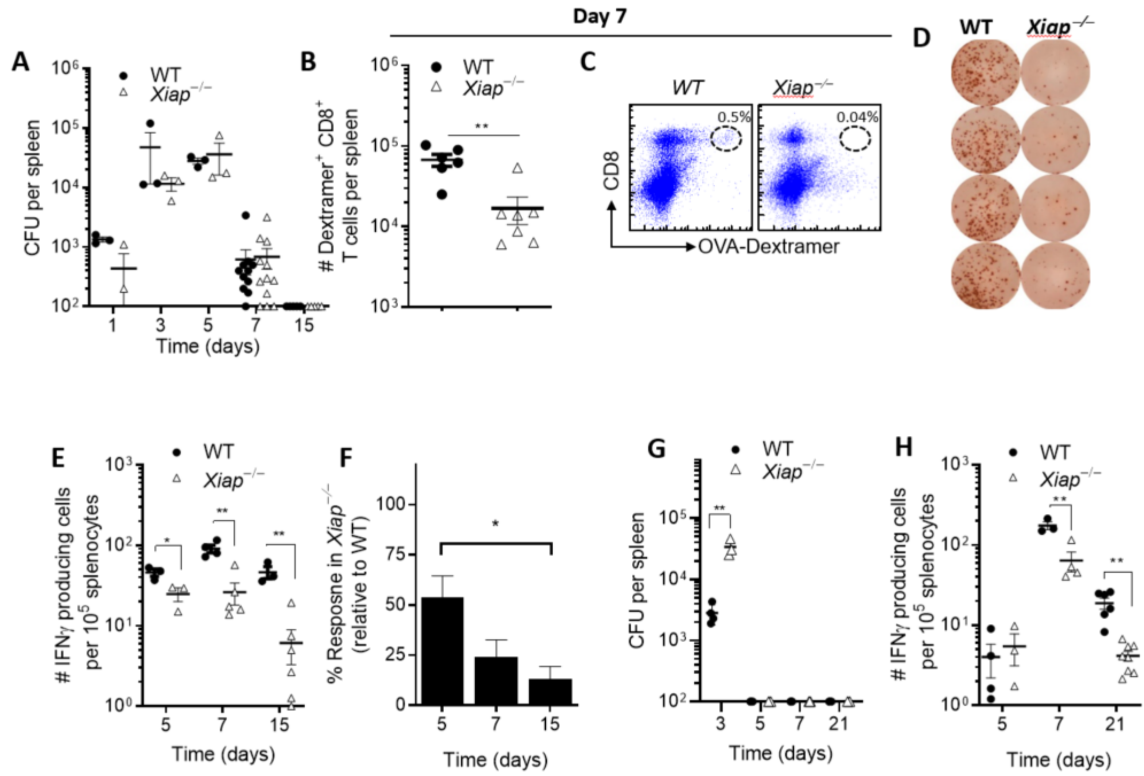
3.1.2 XIAP regulates the CD8 T cell response against intracellular bacteria.

Due to the early fatality of the mice during infection with ST, tracking the expansion and contraction of a CD8 T cell response was not possible. B6 mice are highly susceptible to infection with ST as a result of a mutation in the *Nramp1* gene, which is associated with inducing harsh anti-microbial environment in the phagosome, resulting in the elimination of phagosomal ST and control over infection spread (142). However, our lab has previously shown that early antigen presentation associated with expression and cytoplasmic transport of immunogenic proteins, such as Ovalbumin (OVA), by ST induced a potent CD8 T cell response resulting in control of infection in B6 mice. ST-OVA is a recombinant strain, which carries a plasmid for expression of a chimeric OVA protein with translocation domain from Yersinia outer membrane protein E (YopE). Translocation of the chimeric protein to the cytosol is mediated through the YopE-dependent recognition by ST T3SS. Cytosolic OVA is then presented on MHC-1 complexes to induce a potent CD8 T cell response (132, 143). Therefore, to assess the impact of XIAP on the development of an effective antigen-specific response, I infected WT and *Xiap*$^{-/-}$ mice with ST-OVA (10^3 CFU, i.v), and evaluated the CD8 T cell response against $OVA_{257-264}$ (SIINFEKL) peptide.

Xiap$^{-/-}$ mice were observed to have a comparable bacterial burden to WT mice, during both the early and later stages of infection **(Fig 6 A)**. On day 7 post-infection, I evaluated the OVA-specific CD8 T cell response by directly staining $OVA_{257-264}$ specific CD8 T cells using H2-K^b-$OVA_{257-264}$ dextramers. Interestingly, the number of $OVA_{257-264}$ -specific CD8 T cells observed at the peak of response (day 7) was significantly reduced in *Xiap*$^{-/-}$ mice **(Fig 6 B, C)**. This suggests that XIAP impacts the CD8 T cell response during the expansion phase of the response. OVA-specific CD8 T cell response was also evaluated by an ELISPOT assay. On day 7 post-infection, IFNγ-secreting CD8 T cells against $OVA_{257-264}$ (SIINFEKL) were reduced in *Xiap*$^{-/-}$ mice **(Fig 6 D, E)**. While the number of antigen-specific CD8 T cells observed was reduced in *Xiap*$^{-/-}$ mice at all the time intervals tested, *Xiap*$^{-/-}$ mice had an augmented decline in antigen-specific T cells during the contraction phase (**Fig 6 F)**. At day 5 post-infection *Xiap*$^{-/-}$ mice had a 50% reduction in OVA-specific CD8 T cells relative to WT mice, and this was reduced to ~12.5 % at day 15 (**Fig. 6 F**).

I tested the impact of XIAP on the development of CD8 T cell response against another infection model to determine whether the impact of XIAP is more generalized. I tested the impact of XIAP in during *Listeria monocytogenes* (LM) infection mouse model, using a recombinant LM strain that expresses OVA (LM-OVA). Unlike ST, LM is a non-phagosomal bacterium that actively facilitates its release from the phagosome and expresses OVA within the cytosol to generate a robust T cell response (144, 145). Interestingly, *Xiap*$^{-/-}$ mice had a higher bacterial burden at day 3 (peak of infection) in comparison to WT mice **(Fig 6 G)**. This is in contrast to ST infection model where the burden was slightly reduced in *Xiap*$^{-/-}$ mice. Consistent with ST-OVA infection model, *Xiap*$^{-/-}$ mice infected with LM-OVA (10^3 CFU, i.v.) were observed to have a lower

Figure 6. Poor CD8 T cell response to intracellular bacterial pathogens in *Xiap*$^{-/-}$ mice. (**A-F**) WT and *Xiap*$^{-/-}$ mice were infected with ST-OVA (10^3 CFU, i.v.) and the spleens were harvested from the infected mice at various timepoints post-infection to evaluate the antigen-specific CD8 T cell response to $OVA_{257\text{-}264}$ peptide. **A**) Bacterial burden in the harvest spleens. **B**) Numbers and **C**) percentage of $OVA_{257\text{-}264}$ (SIINFEKL)-specific CD8 T cells, on day 7 post-infection were evaluated in the spleens of infected mice by staining with anti-CD8 antibody and H2-K^b-$OVA_{257\text{-}264}$ Dextramer. (**D-F**) ELISPOT assay was performed on spleen cells stimulated with the $OVA_{257\text{-}264}$ peptide *in vitro*. **D**) Representative IFNγ positive spots in an ELISPOT assay plate. **E**) Number of $OVA_{257\text{-}264}$ specific cells secreting IFNγ in response to $OVA_{257\text{-}264}$. **F**) Relative number of antigen-specific cells in *Xiap*$^{-/-}$ mice in comparison to the average number in WT mice. (**G, H**) WT and *Xiap*$^{-/-}$ mice were infected with LM-OVA (10^3 CFU, i.v.). **G**) Bacterial burden in the harvest spleens of infected mice at various time intervals. **H**) Number of $OVA_{257\text{-}264}$ specific cells secreting IFNγ, in response to $OVA_{257\text{-}264}$ peptide, evaluated by ELISPOT assay. Data is presented as mean ±SEM and is a combination of 2-3 experiments. Statistical analysis was performed by unpaired student t-test (*P<0.05, **P<0.01, ****P<0.0001)


Day 7
A
CFU per spleen
Time (days)
WT
Xiap−/−
B
Dextramer+ CD8+ T cells per spleen
C
WT
Xiap−/−
0.5%
0.04%
CD8
OVA-Dextramer
D
E
IFNγ producing cells per 10^5 splenocytes
F
% Response in Xiap−/− (relative to WT)
G
H

number of antigen-specific T cells, compared to the WT mice. At day 5 post-infection, the CD8 T cell response was similar between WT and *Xiap*$^{-/-}$ mice **(Fig 6 H)**. However, at subsequent time intervals, the response was significantly reduced in *Xiap*$^{-/-}$mice.

I also evaluated whether there is a selective modulation of various immune cell subsets in naïve and infected mice. Spleen cells were stained with various antibodies to discriminate various cell subsets of the innate and acquired immune compartments. XIAP expression did not generate any selective modulation in the relative numbers of various cellular subsets of the immune system, in naïve or ST-OVA infected mice **(Fig 7)**. Similar to infection with ST-OVA, LM-OVA infection did not reveal any difference in the relative number of various immune cell subsets between WT and *Xiap*$^{-/-}$ mice **(data not shown)**. These results indicate that XIAP promotes CD8 T cell response independent of bacterial burden.

Figure 7. Comparison of immune compartment between WT and *Xiap*$^{-/-}$ mice.

Mice were infected with ST-OVA (10^3 CFU, i.v.). Spleens were harvested from **A)** naïve and **B)** day 5-infected mice, and spleen cells stained with various antibodies to identify various immune cell subsets. Plots presented are representative of 4-5 mice.

A Naive

WT

B cells 51% CD19 CD3 CD8 T cells 32.3% CD4 T cells 62.4% CD8 CD4

NK 20% CD49b NK1.1 Tregs 8.8% 1.4% TH17 FoxP3 RORγT

Xiap$^{-/-}$

B cells 47.6% CD19 CD3 CD8 T cells 32.3% CD4 T cells 62.4% CD8 CD4

NK 28.6% CD49b NK1.1 Tregs 10.6% 1.8% TH17 FoxP3 RORγT

WT

Neutrophils 1.7% 4.3% Monocytes Ly6G Ly6C

2.0% CD8 DC CD8 CD11b 5.1% Macrophages MHC2 CD11b 1.8% DCs CD11b CD11c

Xiap$^{-/-}$

Neutrophils 0.6% 3.8% Monocytes Ly6G Ly6C

1.2% CD8 DC CD8 CD11b 3.2% Macrophages MHC2 CD11b 1.7% DCs CD11b CD11c

B ST-OVA (day 5)

WT

B cells 59.7% CD19 CD3 CD8 T cells 45.4% CD4 T cells 48.6% CD8 CD4

NK 21.1% CD49b NK1.1 Tregs 11.8% 3.6% TH17 FoxP3 RORγT

Xiap$^{-/-}$

B cells 50.4% CD19 CD3 CD8 T cells 33.1% CD4 T cells 62.3% CD8 CD4

NK 12.0% CD49b NK1.1 Tregs 12.3% 2.6% TH17 FoxP3 RORγT

WT

Neutrophils 0.7% 6.9% Monocytes Ly6G Ly6C

2.5% CD8 DC CD8 CD11b 2.2% Macrophages MHC2 CD11b 6.4% DCs CD11b CD11c

Xiap$^{-/-}$

Neutrophils 1.2% 3.9% Monocytes Ly6G Ly6C

2.1% CD8 DC CD8 CD11b 1.8% Macrophages MHC2 CD11b 8.1% DCs CD11b CD11c

3.2 Objective 2: To assess the impact of T cell intrinsic XIAP during an immune response

3.2.1 XIAP regulates the contraction phase of CD8 T cell response in a cell intrinsic manner.

To evaluate the impact of cell intrinsic XIAP in modulating the CD8 T cell response, I crossbred *Xiap*$^{-/-}$ mice with OT-1 TCR transgenic mice to generate WT OT-1 (CD45.1^{-}) and *Xiap*$^{-/-}$ OT-1 (CD45.1^{+}) mice. CD8 T cells in OT-1 mice express a transgenic TCR that is specific for H2-K^{b}-OVA$_{257\text{-}264}$ and thus provides an ideal model for tracking the expansion and contraction of OVA-specific CD8 T cell response in response to a pathogen expressing OVA. I adoptively transferred an equal number of splenocytes (10^4 cells, i.v.) from both WT and *Xiap*$^{-/-}$ OT-1 mice into the same B6.SJL (WT) hosts (**Fig. 8 A)**. These adoptively transferred cells are undetectable in naïve mice (<0.1%) as the number of transferred cells is too low. Following infection with ST-OVA (10^3 CFU, i.v.), I monitored the expansion of both WT and *Xiap*$^{-/-}$ OT-1 CD8 T cells. At day 7 post-infection with ST-OVA, the percentages of WT OT-1 and *Xiap*$^{-/-}$ OT-1 cells in the WT host were similar. Both adoptively transferred cells had a relatively similar CD8 T expansion phase, suggesting that T cell intrinsic XIAP expression has no impact on T cell expansion. At day 15 post-infection, there was a decline in the numbers of OVA-specific CD8 T cells due to contraction of the response, and this decline was greater with *Xiap*$^{-/-}$ OT-1 cells (**Fig. 8 B lower panel**). These results indicate that the loss of XIAP leads to an increased culling of activated CD8 T cells. Thus, XIAP impacts the contraction of CD8 T cell response in a cell intrinsic manner (**Fig. 8 B, C**). While *Xiap*-deficient T cells stimulated *in vitro* are more prone to apoptosis, the role of XIAP in T cell differentiation and memory development has

not been well understood (146). On day 7 post-infection, I performed phenotypic characterization of activated OT-1 CD8 T cells (**Fig. 8 D**) based on the expression of various cell surface markers that discriminate the various subsets of CD8 T cells. Differential expression of KLRG1, L-selectin (CD62L) and α-chain of IL-7 receptor (CD127) on the cell surface were used to discriminate between effector and memory-precursor subsets. I observed slightly increased accumulation of effector subsets (SLECs and Teff) over memory subsets (Tcm, Tem and MPEC) within the *Xiap*$^{-/-}$ OT-1 CD8 population (**Fig. 8 E**). This suggested that XIAP expression in WT mice promotes the maintenance of memory phenotype cells in activated T cells.

I repeated the experiments described above in *Xiap*-deficient hosts. The overall expansion of transferred CD8 T cells in the *Xiap*-deficient recipients was much lower (**Fig. 9 C**) in comparison to when the cells were transferred to WT hosts (**Fig. 8 C**). Interestingly, *Xiap*$^{-/-}$ OT-1 cells underwent increased expansion relative to WT OT-1 cells upon transfer in *Xiap*$^{-/-}$ hosts at day 7 post-infection with ST-OVA (**Fig. 9 B, C**). At day 15 post infected, neither WT nor *Xiap*$^{-/-}$ OT-1 CD8 T cells were detectable in the *Xiap*$^{-/-}$ recipients, potentially owing to the poor expansion of OT-1 cells, as measured on day 7 post-infection. This impairment in the expansion of transferred OT-1 cells in *Xiap*-deficient hosts pointed towards a T cell extrinsic impact of XIAP in influencing T cell activation or expansion. The phenotypic analysis of CD8 T cell subsets of the transferred cells once again exhibited the inclination of *Xiap*$^{-/-}$ OT-1 to favor effector over memory subsets (**Fig. 9 C**). In both WT and *Xiap*$^{-/-}$ host, a greater proportion of the *Xiap*$^{-/-}$ OT-1 retained effector function, cell suggesting a potential role of XIAP in promoting memory formation and regulating effector activity in WT hosts.

Figure 8. ***Xiap*$^{-/-}$ CD8 T cells undergo increased contraction in infected mice.**

A) Schematic representation of the adoptive transfer protocol. Splenocytes from both WT OT1 (CD45.1^{-}CD45.2^{+}) and *Xiap*$^{-/-}$ OT1 (CD45.1^{+}CD45.2^{+}) were mixed 1:1 and injected (10^{4} cells each, i.v.) into B6.SJL mice (CD45.1^{+} CD45.2^{-}). After two days, the recipient B6.SJL (CD45.1^{+}) mice were infected with ST-OVA (10^{3} CFU, i.v.). At day 7 and 15 post-infection, the spleens of the recipient mice were harvested, and the donor OT-1 cell numbers evaluated by flow cytometry using antibodies against CD8, CD45.1 and CD45.2. **B)** Representative dot plots, and **C)** percent population of the WT and *Xiap*$^{-/-}$ OT-1 CD8 T cells in the same recipient mouse. **D)** Representative dot plots and **E)** percent distribution of various CD8 T cell subsets in the same host, based on flow cytometric analysis following staining with CD8, CD3, KLRG1, CD62L, CD127 and live-dead staining with Zombie. Short-lived effector cells (SLEC, CD127^{low}KLRG1^{high}), Memory precursor effector cells (MPEC, CD127^{high}KLRG1^{low}), Effector T cells (Teff, CD127^{low}CD62L^{low}), Effector-memory T cells (Tem, CD127^{high}CD62L^{low}), and Central-memory T cells (Tcm, CD127^{high}CD62L^{high}). Data presented is a combination of 2 experiments. Statistical analysis was performed by paired student *t*-test (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

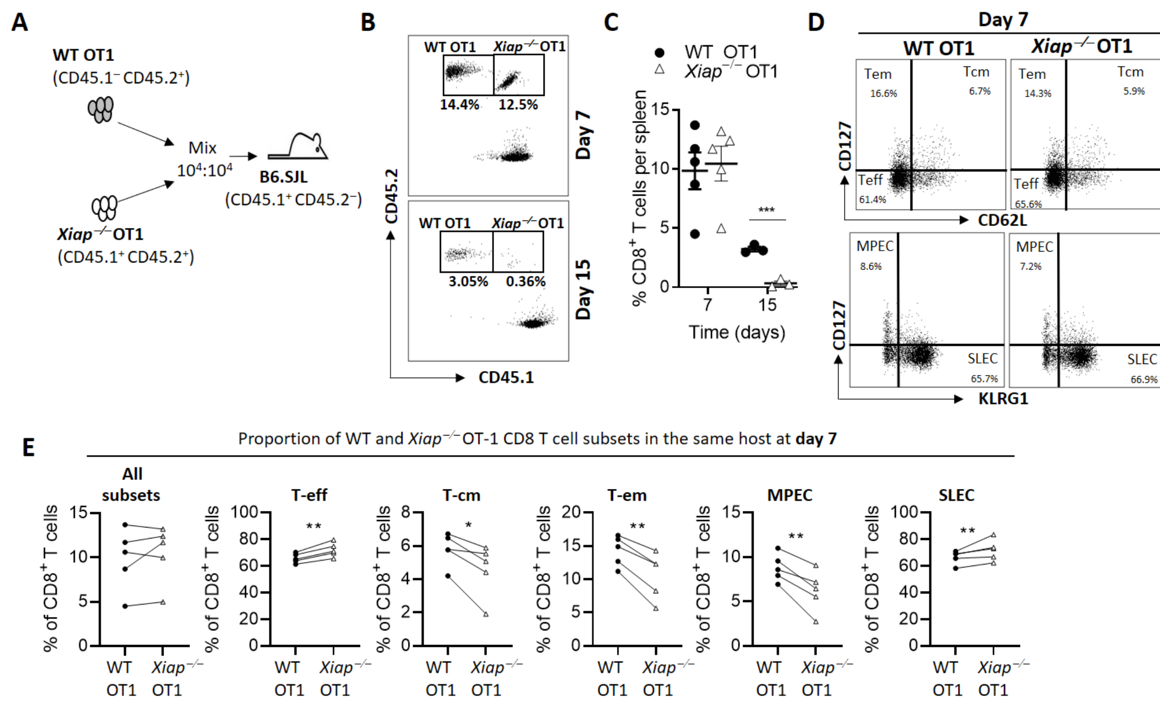
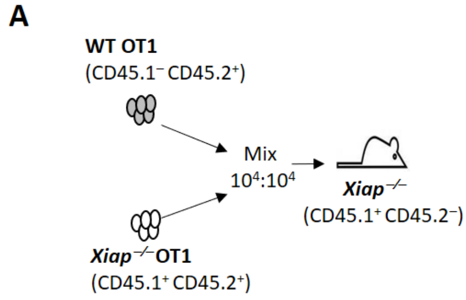
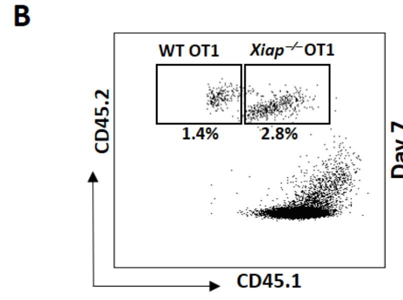
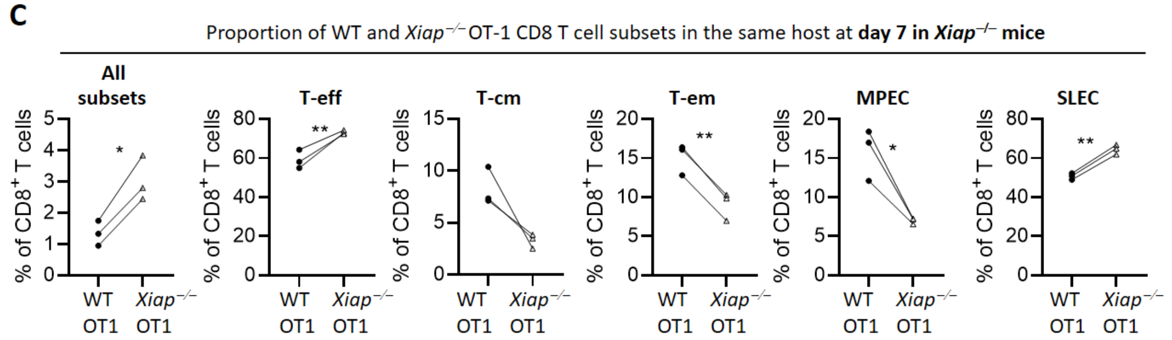

A
WT OT1
(CD45.1⁻ CD45.2⁺)
Mix
10⁴:10⁴
B6.SJL
(CD45.1⁺ CD45.2⁻)
Xiap⁻/⁻OT1
(CD45.1⁺ CD45.2⁺)
B
WT OT1
Xiap⁻/⁻OT1
14.4%
12.5%
Day 7
3.05%
0.36%
Day 15
CD45.2
CD45.1
C
WT OT1
Xiap⁻/⁻ OT1
% CD8⁺ T cells per spleen
Time (days)
***
D
Day 7
WT OT1
Xiap⁻/⁻OT1
Tem
16.6%
Tcm
6.7%
Teff
61.4%
Tem
14.3%
Tcm
5.9%
Teff
65.6%
CD127
CD62L
MPEC
8.6%
SLEC
65.7%
MPEC
7.2%
SLEC
66.9%
KLRG1
E
Proportion of WT and Xiap⁻/⁻ OT-1 CD8 T cell subsets in the same host at day 7
All subsets
T-eff
T-cm
T-em
MPEC
SLEC
% of CD8⁺ T cells
WT OT1
Xiap⁻/⁻ OT1

Figure 9. Poor expansion of CD8 T cell response in *Xiap*$^{-/-}$ mice is not dependent on the expression of XIAP in CD8 T cells.

A) Schematic representation of the adoptive transfer protocol. Splenocytes from both WT OT1 ($CD45.1^{-}CD45.2^{+}$) and *Xiap*$^{-/-}$ OT1 ($CD45.1^{+}CD45.2^{+}$) were mixed 1:1 and injected (10^{4} cells each, i.v.) into *Xiap*$^{-/-}$ mice ($CD45.1^{+}$ $CD45.2^{-}$). After three days, the recipient mice were infected with ST-OVA (10^{3} CFU, i.v.). **B)** At day 7 post-infection, the spleens from the recipient mice were harvested and the proportion of donor OT1 cells was evaluated by flow cytometry following staining with antibodies against CD8, CD45.1 and CD45.2. **C)** Cells were also stained with various antibodies to identify various subsets of activated CD8 T cells described in Figure 8. Percent distribution of the CD8 T cell subsets among the transferred OT1 cells is shown. Data presented is representative of 2 experiments. Statistical analysis was performed by paired student *t*-test (*P<0.05, **P<0.01)


A
WT OT1
(CD45.1⁻ CD45.2⁺)
Mix
10⁴:10⁴
Xiap⁻/⁻
(CD45.1⁺ CD45.2⁻)
Xiap⁻/⁻ OT1
(CD45.1⁺ CD45.2⁺)


B
WT OT1
Xiap⁻/⁻ OT1
1.4%
2.8%
CD45.2
CD45.1
Day 7


C
Proportion of WT and Xiap⁻/⁻ OT-1 CD8 T cell subsets in the same host at day 7 in Xiap⁻/⁻ mice
All subsets
T-eff
T-cm
T-em
MPEC
SLEC
% of CD8⁺ T cells
WT OT1
Xiap⁻/⁻ OT1

3.2.2 XIAP deficient mice exhibit weaker memory response

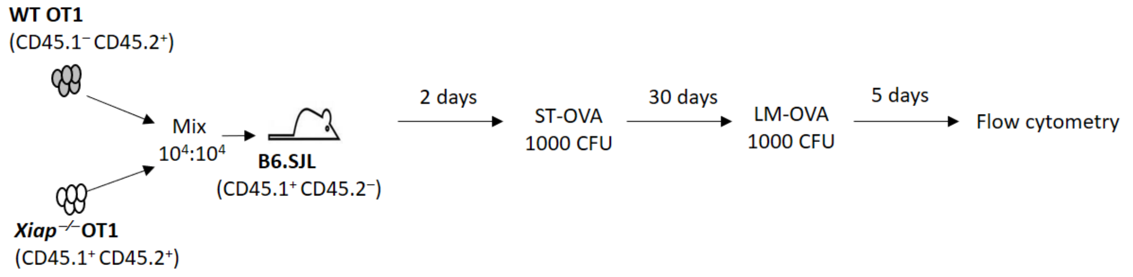
Xiap$^{-/-}$ OT-1 CD8 T cells exhibited a potential impairment in the distribution of memory forming CD8 T cell subsets, MPECs **(Fig 8, 9)**. Therefore, I tested the response of WT and *Xiap*$^{-/-}$ OT-1 during a secondary challenge with OVA. WT OT-1 and *Xiap*$^{-/-}$ OT-1 splenocytes were transferred into a B6.SJL host and infected with ST-OVA as indicated in **Fig. 8 A**. At day 30 days post-infection following the initial challenge, the WT recipients mice were infected challenged with LM-OVA. At day 5, following secondary challenge with LM-OVA, the WT OT-1 cells had undergone substantial expansion, whereas *Xiap*$^{-/-}$ OT-1 cells were undetectable. I observed a nominal expansion of *Xiap*$^{-/-}$ OT-1 cells compared to WT OT-1 cell (**Fig. 10 B, C**). These results suggest that XIAP plays a key role in promoting memory formation and suggests a potential defect in memory response in the absence of XIAP.

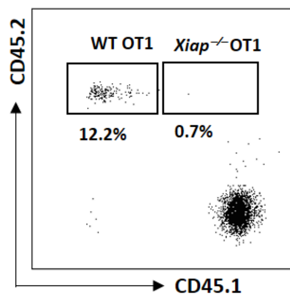
3.2.3 *Xiap*$^{-/-}$ OT1 cells undergo increased activation following antigen-presentation *in vitro*.

Since DCs are the critical antigen-presenting cells that induce the development of an adaptive immune response, I tested the impact of XIAP on T cell expansion in an *in vitro* antigen presentation model using bone marrow-derived dendritic cells (BMDCs). I infected WT BMDCs with ST-OVA *in vitro* and cultured them along with CFSE labeled CD8 T cells, isolated from WT OT-1 or *Xiap*$^{-/-}$ OT-1 mice. *Xiap*$^{-/-}$ OT-1 CD8 T cells exhibited greater proliferation indicated by a slightly larger population of viable proliferated cells (CFSE^{low}Zombie^{low}) **(Fig. 11 A)** compared with WT OT-1 CD8 T cells. Interestingly, the proportion of proliferating, dying cells, CFSE^{low}Zombie^{high}, was not modulated by XIAP during this initial time period **(Fig. 11 A, B)**. Intracellular staining for

Figure 10. XIAP$^{-/-}$ OT-1s failed to induce memory response upon secondary challenge.

A) Schematic representation of the adoptive transfer protocol. Splenocytes from both WT OT1 ($CD45.1^{-}CD45.2^{+}$) and *Xiap*$^{-/-}$ OT1 ($CD45.1^{+}CD45.2^{+}$) were mixed 1:1 and injected (10^{4} cells each, i.v.) into WT mice ($CD45.1^{+}$ $CD45.2^{-}$). After three days, the recipient mice were infected with ST-OVA (10^{3} CFU, i.v.). 30 days following the initial challenge, the mice were rechallenged with LM-OVA. At day 5 post-infection, the spleens from the recipient mice were harvested and the proportion of donor OT1 cells was evaluated by flow cytometry following staining with antibodies against CD8, CD45.1 and CD45.2. **B)** Representative dot plot and **C) p**ercent population of the WT and *Xiap*$^{-/-}$ OT1 CD8 T cells in the recipient mice. Data presented is representative of 2 experiments. Statistical analysis was performed by paired student *t*-test (*$P<0.05$)


A
WT OT1
(CD45.1⁻ CD45.2⁺)
Mix
10⁴:10⁴
B6.SJL
(CD45.1⁺ CD45.2⁻)
Xiap⁻/⁻ OT1
(CD45.1⁺ CD45.2⁺)
2 days
ST-OVA
1000 CFU
30 days
LM-OVA
1000 CFU
5 days
Flow cytometry


B
CD45.2
WT OT1
Xiap⁻/⁻ OT1
12.2%
0.7%
CD45.1

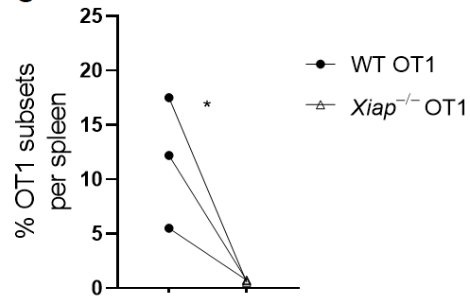
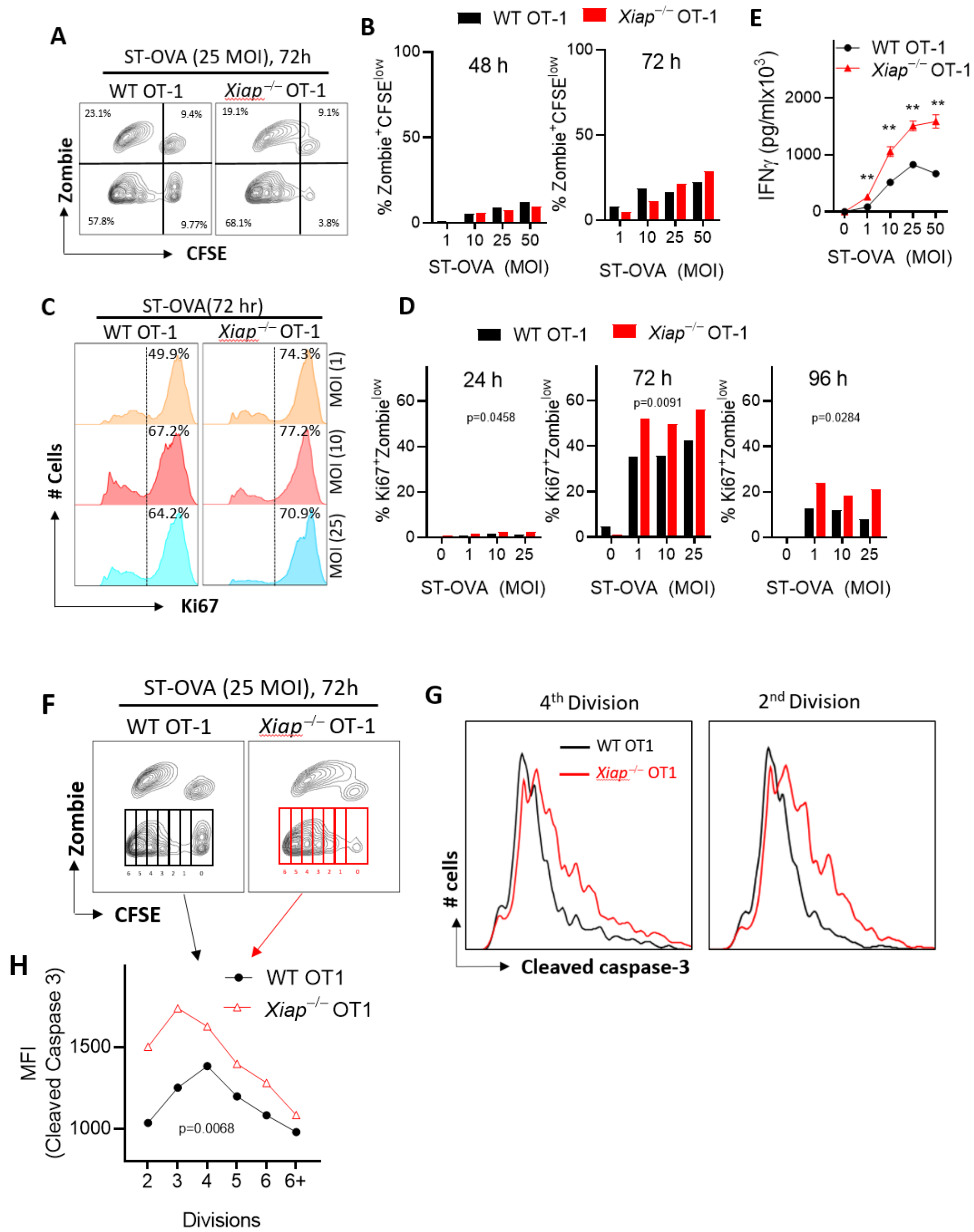

C
% OT1 subsets per spleen
25
20
15
10
5
0
*
WT OT1
Xiap⁻/⁻ OT1

Figure 11. ***Xiap*$^{-/-}$ CD8 T cells undergo increased proliferation following ST-OVA antigen presentation *in vitro*.**

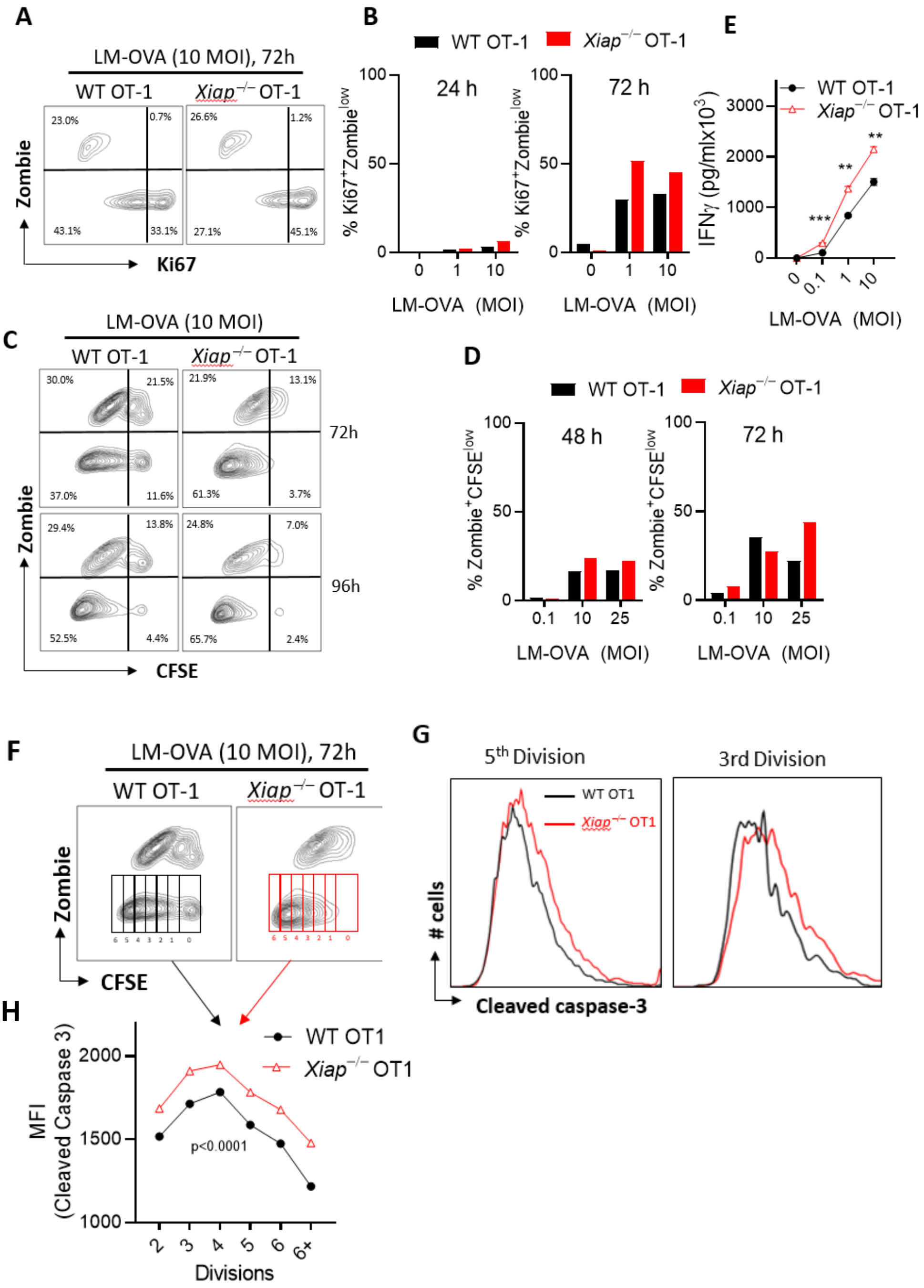
BMDCs were generated from WT mice and infected with ST-OVA as described in the methods section. CD8 T cells were purified from WT OT1 and *Xiap*$^{-/-}$ OT1 spleens and labeled with CFSE and incubated with the infected BMDCs. Cells were stained with Zombie, and antibodies against Ki67, cleaved caspase-3 and CD8 and evaluated for proliferation and viability via flow cytometry. **A**) Representative contour plot showing proliferation and cell death, and **B**) cell death (Zombie^{+}) of CD8 T cells that have undergone proliferation (CFSE^{low}). **C, D**) Percent Ki67^{+} of CD8 T cells. **E**) Supernatants were collected at 24hr post-infection and IFNγ secretion evaluated by ELISA. **F-H)** Cleaved caspase 3 expression in Zombie^{low} cells following various cell divisions(based on CFSE dilution. **G)** Representative histogram for cleaved caspase-3 in WT and *Xiap*$^{-/-}$ OT1 cell at 4th and 2nd division, and **H)** MFI for cleaved caspase-3 at various divisions. Data presented is a representative of 2 experiments. Statistical analysis was performed by unpaired student *t*-test (E) and 2-way ANOVA(D, F). (***P*<0.01).



A
ST-OVA (25 MOI), 72h
WT OT-1
Xiap−/− OT-1
Zombie
CFSE
B
WT OT-1
Xiap−/− OT-1
48 h
72 h
% Zombie+CFSElow
ST-OVA (MOI)
E
IFNγ (pg/mlx10³)
ST-OVA (MOI)
C
ST-OVA(72 hr)
Cells
Ki67
MOI (1)
MOI (10)
MOI (25)
D
24 h
72 h
96 h
p=0.0458
p=0.0091
p=0.0284
% Ki67+Zombielow
F
G
4th Division
2nd Division
WT OT1
Xiap−/− OT1
cells
Cleaved caspase-3
H
MFI (Cleaved Caspase 3)
p=0.0068
Divisions

Figure 12. ***Xiap*$^{-/-}$ CD8 T cells undergo increased proliferation upon LM-OVA antigen-presentation *in vitro.***

BMDCs were generated from WT mice and infected with LM-OVA as described in the methods section. CD8 T cells were purified from WT OT1 and *Xiap*$^{-/-}$ OT1 spleens and labeled with CFSE and incubated with the infected BMDCs. Cells were stained with Zombie and antibodies against Ki67, cleaved caspase-3 and CD8 and evaluated for proliferation and viability via flow cytometry. **A**) Representative **c**ontour plots at 72h is shown. **B**) Percent of Ki67^{+} cells among viable cells (Zombie^{-}) is shown. **C**) Representative contour plot of Zombie versus CFSE is shown. **D**) Percent of cycled cells (CFSE^{low}) that have died (Zombie^{+}). **E**) Supernatant was collected at 24hr post-infection and analyzed for IFNγ secretion by ELISA. **F- H)** Cleaved caspase 3 expression in Zombie^{low} cells following various cell divisions(based on CFSE dilution. **G)** Representative histogram for cleaved caspase-3 in WT and *Xiap*$^{-/-}$ OT1 cell at 5th and 3rd division, and **H)** MFI for cleaved caspase-3 at various divisions. Data presented is a representative of 2 experiments. Statistical analysis was performed by unpaired student *t*-test (E) (***P*<0.01, ****P*<0.001).


A
LM-OVA (10 MOI), 72h
WT OT-1
Xiap−/− OT-1
Zombie
Ki67
B
WT OT-1
Xiap−/− OT-1
24 h
72 h
% Ki67+Zombielow
LM-OVA (MOI)
E
IFNγ (pg/mlx10³)
LM-OVA (MOI)
C
LM-OVA (10 MOI)
72h
96h
CFSE
D
48 h
% Zombie+CFSElow
F
H
WT OT1
Xiap−/− OT1
MFI (Cleaved Caspase 3)
p<0.0001
Divisions
G
5th Division
3rd Division
cells
Cleaved caspase-3

Ki67, a nuclear protein that is associated with cellular cycling, also indicated that higher portions of *Xiap*$^{-/-}$ OT-1 CD8 T cells were undergoing proliferation (**Fig. 11 C, D**). *Xiap*$^{-/-}$ OT-1 cells produced more IFNγ than WT OT-1, following culture with ST-OVA infected DCs; further indicating increased activation of *Xiap*$^{-/-}$ OT-1 CD8 T cells (**Fig. 11 E**). Finally, levels of intracellular active (cleaved) caspase-3 were higher in *Xiap*$^{-/-}$ OT-1 cells (**Fig. 11 F-H**).

I also performed the *in vitro* antigen presentation with DCs infected with LM-OVA. A larger proportion of *Xiap*$^{-/-}$ OT-1 was CFSE^{low}Zombie^{low} and Ki67^{+} (**Fig. 12 A, B**). Similar to antigen presentation by ST-OVA infected DCs, *Xiap*$^{-/-}$ OT-1 cells stimulated with LM-OVA infected BMDCs also underwent increased proliferation and expansion, as indicated with a larger proportion of CFSE^{low} cells (**Fig. 12 C, D**). The difference in proliferation between WT and *Xiap*$^{-/-}$ OT-1 cells was more profound at lower MOIs. Here again, *Xiap*$^{-/-}$ OT-1 cells produced more IFNγ than WT OT-1 following antigen-presentation by LM-OVA-infected DCs (**Fig. 12 E**). Additionally, *Xiap*$^{-/-}$ OT-1 cells were observed to have increased cleaved caspase-3 expression (**Fig. 11 F-H**). These results indicate that *Xiap*-deficient T cells undergo increased proliferation and activation, relative to WT cells.

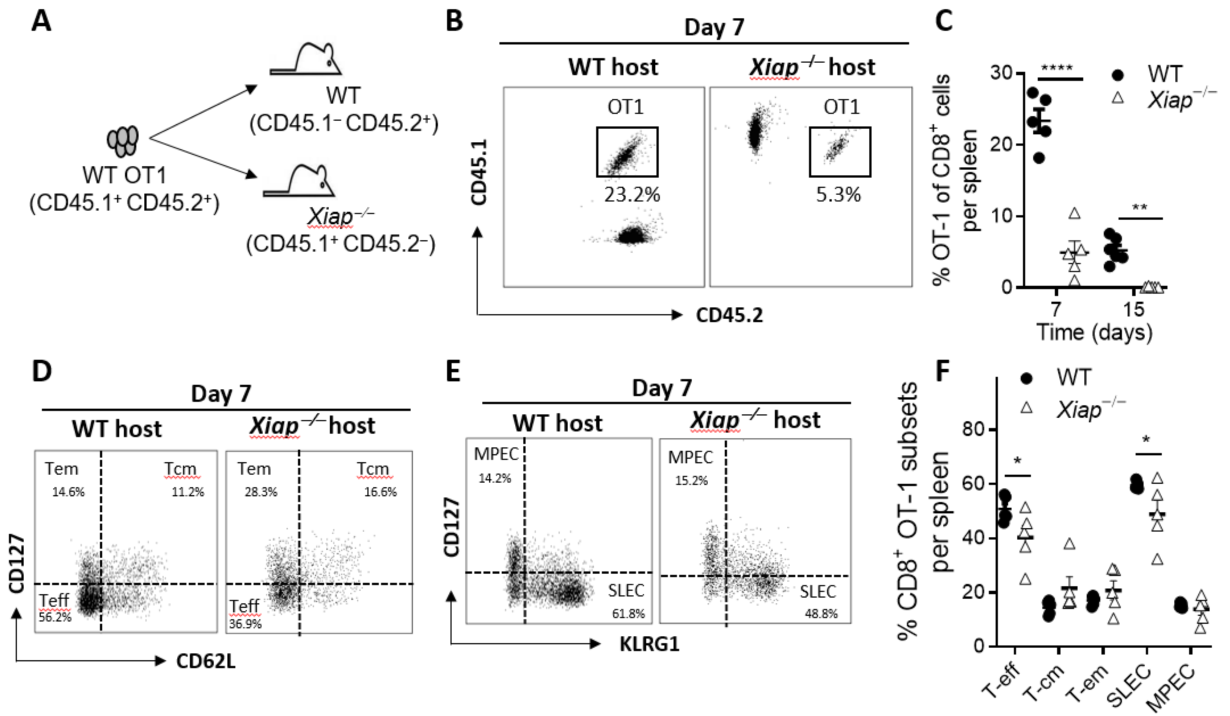
3.3 Objective 3: To assess the impact of XIAP-deficient APCs on CD8 T cell response.

3.3.1 XIAP regulates the expansion of CD8 T cell response in a cell extrinsic manner.

The relatively poor expansion of both WT and *Xiap*$^{-/-}$ OT-1 in XIAP-deficient recipients **(Fig 9)** compared to a WT recipients **(Fig 8)** suggests that there may be a cell extrinsic impact of XIAP in controlling the expansion of activated CD8 T cells. Therefore, I examined the role of T cell extrinsic XIAP on generating an effective T cell response. To assess the impact of T cell extrinsic XIAP, I adoptively transferred WT OT-1 (CD45.1^{+}CD45.2^{+}) splenocytes (5x10^{4} cells, i.v.) into WT and *Xiap*$^{-/-}$ mice and measured the expansion of activated WT OT-1 cells at day 7 and 15 post-infection with ST-OVA (**Fig. 13 A**)**.** On both day 7 and day 15 post-infection, the proportion of WT OT-1 cells was smaller in *Xiap*$^{-/-}$ mice compared to that in WT mice **(Fig. 13 B, C)**. Expansion of WT OT-1, at the peak of T cell response at day 7 post-infection was weaker in *Xiap*$^{-/-}$ mice, relative to their expansion in WT mice. Transitioning from day 7 to day 15 post-infection, the magnitude of the decline in the number of WT OT-1 T cells was similar in both WT and *Xiap*$^{-/-}$ mice. This indicated that the contraction of WT OT-1 was relatively similar in both WT and *Xiap*$^{-/-}$ recipients. Interestingly, the phenotypical analysis of CD8 OT-1 cells on day-7 post-infection, only revealed a reduction in the OVA-specific effector-phenotype CD8 T cells **(Fig 13 D-F)**. The ratio of memory-precursor subsets of the expanded OT-1 CD8 T cell population was similar in both WT and *Xiap*$^{-/-}$ mice. These results indicated that along with a cell intrinsic impact of XIAP in CD8 T cells, XIAP also exerts a T cell extrinsic effect on CD8 T cell response. T cell extrinsic XIAP plays a key role in the expansion of CD8 T cell response.

Figure 13. WT CD8 T cells undergo poor expansion in infected *Xiap*$^{-/-}$ mice.

A) Schematic representation of the experiment. Splenocytes from WT OT1 mice ($CD45.1^{+}CD45.2^{+}$) were injected (5×10^{4} cells, i.v.) into WT mice ($CD45.1^{-}CD45.2^{+}$) or *Xiap*$^{-/-}$ mice ($CD45.1^{+}CD45.2^{-}$). Three days later, the recipient mice were infected with ST-OVA (10^{3} CFU, i.v.). At day 7 and 15 post-infection, the spleens of the recipient mice were harvested, and the donor OT1 cells were tracked by flow cytometry using antibodies against CD8, CD45.1 and CD45.2. (**B**) Representative dot plots and (**C**) percent population of the WT OT1 CD8 T cells in the recipient mice. (**D, E**) Representative dot plots and (**F**) percent distribution of CD8 T cell subsets within the transferred OT1 population after staining with various antibodies described in the methods. Data presented is a combination of 2 experiments. Statistical analysis was performed by unpaired student *t*-test (*$P<0.05$, **$P<0.01$, ****$P<0.0001$).



A
WT
(CD45.1⁻ CD45.2⁺)
WT OT1
(CD45.1⁺ CD45.2⁺)
Xiap⁻/⁻
(CD45.1⁺ CD45.2⁻)
B
Day 7
WT host
Xiap⁻/⁻ host
OT1
23.2%
5.3%
CD45.1
CD45.2
C
% OT-1 of CD8⁺ cells per spleen
Time (days)
WT
Xiap⁻/⁻
D
Day 7
WT host
Xiap⁻/⁻ host
Tem
Tcm
Teff
14.6%
11.2%
56.2%
28.3%
16.6%
36.9%
CD127
CD62L
E
Day 7
WT host
Xiap⁻/⁻ host
MPEC
SLEC
14.2%
61.8%
15.2%
48.8%
CD127
KLRG1
F
% CD8⁺ OT-1 subsets per spleen
WT
Xiap⁻/⁻
T-eff
T-cm
T-em
SLEC
MPEC

3.3.2 Cell extrinsic XIAP promotes the antigen-presentation to CD8 T cells *in vitro*.

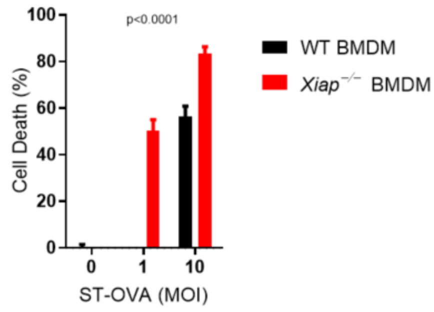
My *in vivo* adoptive transfer models indicated a potential impairment in the expansion of WT CD8 T cells in *Xiap*$^{-/-}$ mice. Since antigen-presentation governs the T cell activation, proliferation and survival, I assessed the impact of *Xiap*$^{-/-}$ APCs on the activation of CD8 T cells *in vitro*. I co-cultured CFSE labeled CD8 T cells isolated from WT OT-1 mice with ST-OVA infected APCs. Macrophages play an essential role in limiting pathogen spread and can also present antigen to CD8 T cells. Similar to infection with ST, *Xiap*$^{-/-}$ BMDMs also exhibited increased cell death upon infection with ST-OVA **(Fig 14 A)**. Interestingly, WT OT-1 cells underwent relatively reduced proliferation upon co-culture with ST-OVA infected *Xiap*$^{-/-}$ BMDMs. Only a small proportion of CD8 T cells had undergone 5 or more cell divisions upon stimulation with ST-OVA infected *Xiap*$^{-/-}$ BMDMs **(Fig 14 B)**. The expression of IFNγ expression did not appear to be potently modulated by the expression of XIAP in macrophages **(Fig 14 C)**.

I also tested the impact of XIAP in antigen-presentation by DCs. Upon ST-OVA infection, *Xiap*-deficient BMDCs induces reduced proliferation of WT OT-1 CD8 T cells. A significant proportion of the CD8 T cells did not undergo expansion (CFSE^{high} Zombie^{low}) upon activation by *Xiap*$^{-/-}$ BMDCs **(Fig 15 A)**. Additionally, intracellular staining for Ki67 also revealed that relatively fewer WT OT-1 cells were actively proliferating upon co-culture with *Xiap*-deficient DCs, in comparison to WT DCs **(Fig 15 C, D).** These results indicated that the expansion of WT CD8 T cells is compromised upon co-culture with infected *Xiap*$^{-/-}$ DCs. I also observed increased cell death in proliferating CD8 T cells (CFSE^{low} Zombie^{high}) that were incubated with *Xiap*$^{-/-}$ BMDCs infected with ST-OVA **(Fig 15 A, B)**. *In vitro* antigen presentation experiments were repeated with the

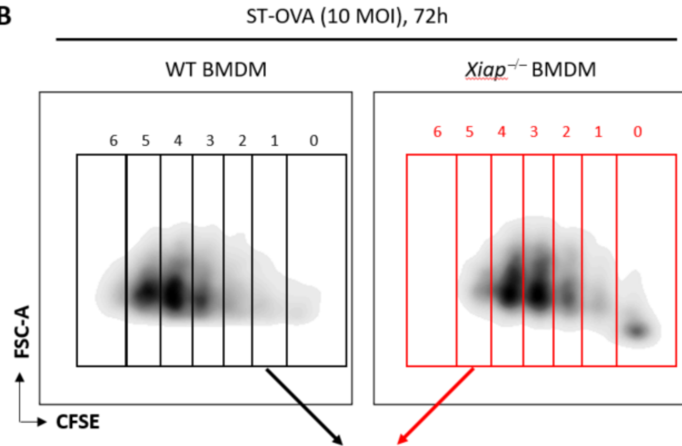
Figure 14. Poor *in vitro* expansion of WT OT-1 CD8 cells co-cultured with *Xiap*$^{-/-}$ macrophages.

BMDMs generated from WT and *Xiap*$^{-/-}$ mice and infected with ST-OVA as described in the methods section. Infected DCs were incubated with CFSE labeled purified WT OT1 cells. At various time intervals, cells were harvested, stained with and CD8 and evaluated for proliferation and viability via flow cytometry. **A)** Cell death in ST-OVA infected cells measured at 24hr post-infection using neutral red uptake assay. **B)** Representative contour plot showing level of proliferation in (indicated by diluted CFSE) CD8 T cells. **C)** Supernatant was collected at 24hr post-infection and analyzed for IFNγ secretion by ELISA. Data presented is ±SEM and is a representative of 2 experiments. Statistical analysis was performed by 2-way ANOVA (A) and student *t*-test(C) (*P<0.05)

A



B



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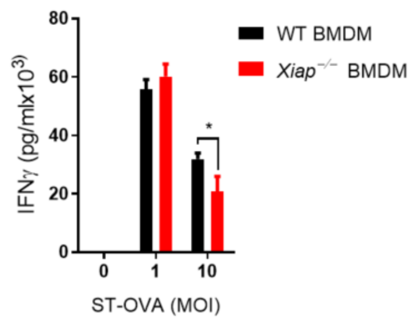
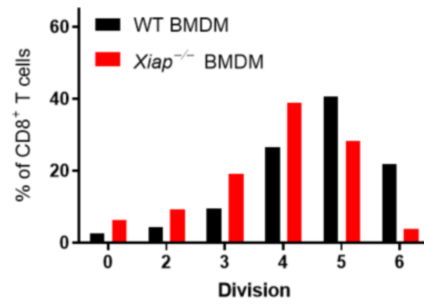




LM-OVA infection model. Here again, I observed increased cell death in activated CD8 T cells (CFSE^{low} Zombie^{high}) co-cultured with infected *Xiap*-deficient DCs **(Fig 15 E, F)**. Interestingly, the increase in cell death of proliferated cells was prominent at later timepoints (96 hrs) of co-culture of DCs and CD8 T cells **(Fig 15 B, F)**. These results suggest that XIAP expression in the APCs promotes the expansion and survival of proliferating CD8 cells.

Finally, the reduced T cell expansion observed upon co-culture with *Xiap*$^{-/-}$ APCs was not related to the expression of MHC class I, which is required for antigen presentation to OT-1 cells, as both WT and *Xiap*$^{-/-}$ DCs and macrophages expressed similar levels of MHC class I **(Fig 16)**.

3.3.3 Inhibition of caspase-1 potentiates T cell activation in *Xiap*-deficient DCs

Next, I evaluated the mechanism behind the poor antigen-presentation to CD8 T cells by *Xiap*$^{-/-}$ DCs. *Xiap*$^{-/-}$ DCs underwent increased cell death following ST-OVA infection in comparison to WT DCs **(Fig 17 A)**. Since cell death in the ST infection model is mainly mediated by inflammasome signaling; the processing and secretion of IL-1β was also potently enhanced in *Xiap*$^{-/-}$ DCs relative to WT DCs (**Fig. 17 A**).

Cell death of macrophages and DCs following infection with ST is mainly induced through inflammasome signaling (79, 147, 148). Enhanced inflammasome activity in *Xiap*-deficient DCs has been reported to induce the activation of caspase-1, which in turn drives the cell death and IL-1β secretion (47, 138). Enhanced inflammatory cell death of *Xiap*-deficient APCs, could limit the time required for APCs to stimulate CD8 T cells, which can result in a weaker activation and expansion of CD8 T cells. Our lab had previously reported that inhibition of caspase-1 driven cell death in APCs leads to increased proliferation

Figure 15. ***Xiap***$^{-/-}$ **DCs induce poor antigen presentation to WT CD8 T cells *in vitro*.**

BMDCs generated from WT and *Xiap*$^{-/-}$ mice and infected with ST-OVA (A-D) or LM-OVA (E, F) as described in the methods section. Infected DCs were incubated with CFSE labeled purified WT OT1 cells. At various time intervals, cells were harvested, stained with Zombie, and antibodies against Ki67 and CD8 and evaluated for proliferation and viability via flow cytometry. **A, E)** Representative contour plot and **B, F)** cell death of proliferated (indicated by diluted CFSE) CD8 T cells. **C)** Representative contour plot and **D)** percent Ki67^{+} of CD8 T cell. Data presented is a representative of 2 experiments. Statistical analysis was performed by 2-way ANOVA (B, D).

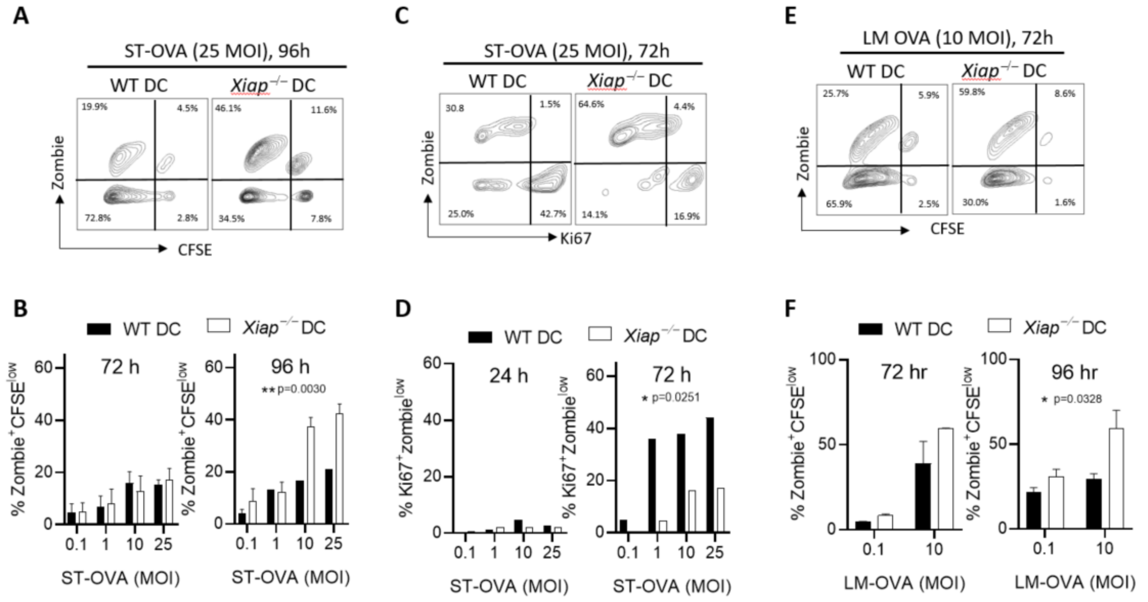
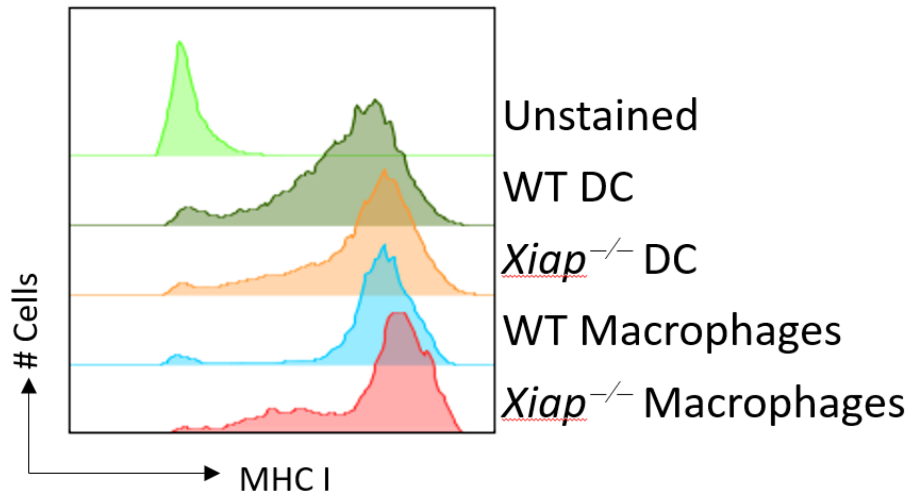

A
ST-OVA (25 MOI), 96h
WT DC
Xiap−/− DC
Zombie
CFSE
C
ST-OVA (25 MOI), 72h
Ki67
E
LM OVA (10 MOI), 72h
B
72 h
96 h
** p=0.0030
% Zombie+CFSElow
ST-OVA (MOI)
D
24 h
72 h
* p=0.0251
% Ki67+zombielow
F
72 hr
96 hr
* p=0.0328
LM-OVA (MOI)

Figure 16. *Xiap*$^{-/-}$ and WT mice have similar MHC class I expression.

Expression of H2k^{b} on BMDMs and BMDCs generated from WT and *Xiap*$^{-/-}$ mice was evaluated by flow cytometry following staining with anti-H2K^{b} antibody. Data presented is representative of 2 experiments.


Unstained
WT DC
Xiap−/− DC
WT Macrophages
Xiap−/− Macrophages
Cells
MHC I

in T cells (143). Therefore, I used a caspase-1 inhibitor, Z-yVAD-FMK, to determine whether inhibition of caspase-1 activity in *Xiap*$^{-/-}$ DCs influences the cycling of WT OT-1 cells during an *in vitro* antigen-presentation model. I observed an increase in the percentage of viable and cycling (CFSE^{low}Zombie^{low}) WT OT-1 cells when caspase-1 was inhibited in *Xiap*$^{-/-}$ DCs **(Fig 17 B)**. Additionally, the proportion of Ki67^{+}Zombie^{low} cells was also increased **(Fig 17 C)**. These results indicated that XIAP limits premature cell death of APCs to facilitate the activation and expansion of CD8 T cell expansion.

3.3.4 Addition of exogenous IL-6 rescues the poor antigen-presentation by *Xiap*-deficient DCs

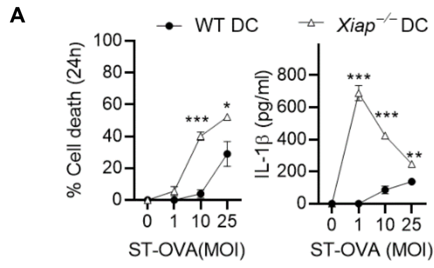
Next, I evaluated how the enhanced cell death of *Xiap*-deficient DCs result in poor activation of CD8^{+} T cells *in vitro*. Upon infection, DCs secrete cytokines that promote the survival of activated T cells (96). I measured the expression of various cytokines following infection of WT and *Xiap*$^{-/-}$ BMDCs with ST-OVA. The expression of IL-6 and IL-10 was reduced in *Xiap*$^{-/-}$ BMDCs, in comparison to WT BMDCs **(Fig 17 D)**. Additionally, this reduction in cytokine expression was augmented at higher MOIs, where more cell death was observed. Since IL-6 has been reported to promote T cell expansion and survival (149), I considered the possibility of increased cell death of *Xiap*-deficient DCs could limit the availability of the necessary amount of IL-6 required for the proliferation/survival of activated CD8 T cells(149, 150). Therefore, I added exogenous recombinant murine IL-6 to ST-OVA infected WT and *Xiap*$^{-/-}$ BMDCs co-cultured with WT OT-1 cells. The addition of exogenous IL-6 significantly increased the proliferation and survival of WT OT-1 cells cultured with infected *Xiap*$^{-/-}$ DCs **(Fig 17 E, F)**. Thus, poor cytokine signaling along with

the premature death of APCs, could explain the lack of T cell expansion in *Xiap*-deficient APCs.

Figure 17. Inhibition of inflammasome signaling and addition of exogenous IL-6 rescues the impaired priming of WT CD8 T cells by infected *Xiap*$^{-/-}$ DCs.

DCs were generated from WT and *Xiap*$^{-/-}$ mice and infected with ST-OVA, at various MOIs, as described in the methods section. (**A, D**) Cell death of DCs was measured at 24hr post-infection by neutral red assay (**A**). Supernatants were collected at 6hr and cytokines were measured by ELISA. (**B, C**) DCs were treated with 10μM of Caspase-1 inhibitor (Z-yVAD-FMK, 10uM) and then infected with ST OVA. Purified CD8 T cells from WT OT1 spleen were labeled with CFSE and incubated with the infected DCs. Cell cultures were harvested and CD8 T cells stained with Zombie and antibodies against Ki67 and CD8 followed by flow cytometric evaluation of proliferation and viability at 24 and 72hr. **B, C**) Representative contour plot of the CD8 T cell population is shown. (**E, F**) DCs were infected and cultured with purified WT OT1 CD8 T cells as described above, and exogenous rmIL-6 (50 ng/ml) supplemented at 24 and 72hr. Cell proliferation was measured by staining with Zombie, and antibodies against Ki67 and CD8 at indicated at 96hr. Data presented is representative of 2-3 experiments. Statistical analysis was performed by 2-way ANOVA (**F**) and unpaired student *t*-test (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

A



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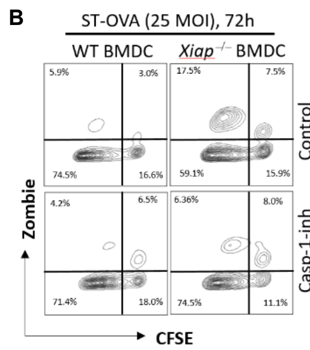

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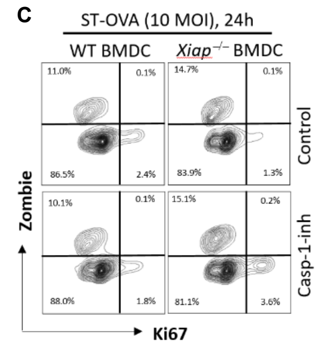
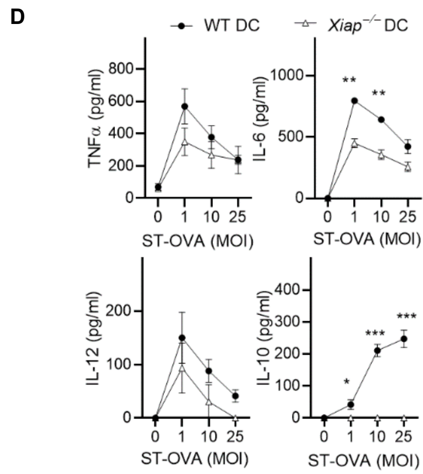
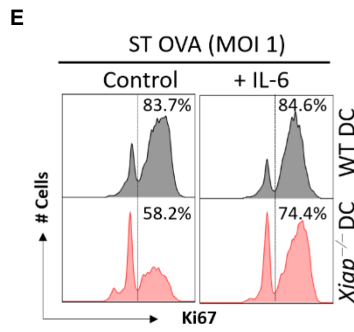


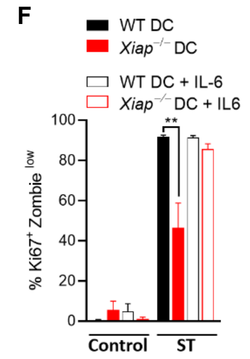
D



E



F



4. Discussion

4.1 Prelude

An effective CD8 T cell response is critical for the clearance of intracellular pathogens, as well as establishing a state of protection against future infections. The depletion of CD8 T cell population in mice increases susceptibility to viral infections, such as HIV (151) and LCMV (152), and intracellular bacterial infections, such as ST (153), LM (145) and *Mycobacterium tuberculosis* (144, 154). The CD8 T cell response peaks around day 7 of infection, and is immediately followed by a rapid contraction phase wherein ~90% of the activated CD8 T cells are culled to maintain homeostasis. Apoptotic cell death would seem to be an obvious mechanism for eliminating activated antigen-specific CD8 T cells during the contraction phase. The support for apoptosis as the mechanism of cell death of activated T cells came from reports wherein diminished contraction and increased accumulation of activated CD8 T cells was observed in mice with impaired Bim (155) or caspase-8 (117), respectively. XIAP is a bona fide endogenous inhibitor of apoptotic signals, which functions through the direct inhibition of executioner caspases (119). As predicted, the deletion of XIAP in T cells resulted in an augmented contraction phase **(Fig 6 F, 8)**. Surprisingly, in addition to the inhibition of apoptosis in activated T cells, the T cell-intrinsic XIAP was observed to be critical for differentiation and formation of memory cells **(Fig 8)** and memory response **(Fig 10)**. Cytokines and co-stimulatory signals expressed by APCs provided the key signaling mechanisms that promote the differentiation of CD8 T cells (96). I observed that XIAP expression by APC is necessary for their survival and secretion of cytokines, which subsequently supports the expansion and survival of activated CD8 T cells **(Fig 17)**. Taken together, my results indicated that T cell-

extrinsic expression of XIAP promotes survival of primed T cells during the expansion phase, and T cell intrinsic expression of XIAP is necessary for survival during the contraction phase of T cell immune response.

4.2 IAPs and inflammasome signaling

IAPs are best characterized for their ability to prevent apoptotic cell death by inhibiting the enzymatic activity of apoptotic caspases. XIAP is the only mammalian IAP with the ability to directly inhibit the enzymatic activity of two effector caspases, caspase-3 and -7, and an initiator caspase, caspase-9 (119, 120, 156). *Xiap*-deficient macrophages stimulated with LPS, TNF-α, IFN-γ, or excess NO were reported to display increased caspase-3 activity and, consequently, a much lower survival (126). My results indicate that *Xiap*$^{-/-}$ macrophages **(Fig 3 D-E)** and DCs **(Fig 5 C)** are more prone to cell death and express increased IL-1β, following infection with ST. Intracellular bacteria such as ST induce caspase-1/11 activity in infected cells, which results in cell death through an alternative pathway called pyroptosis. In addition, activated caspase-1 cleaves the pro-forms of IL-1β and IL-18 into their active forms. Furthermore, cleavage of gasdermin by caspase-1 or caspase-11 leads to inflammatory cell death (77, 78, 133, 135, 136, 157). This suggests that XIAP functions to limit pyroptotic cell death in innate immune cells.

During infection with ST, both NLRP3 and NLRC4 inflammasomes have been individually identified to induce caspase-1 activation, which then promotes pyroptosis and release of IL-1β and IL-18 (36, 135, 158). T3SS-2 expression by phagosomal ST enables the injection of ST effector proteins into the host cytosol. The T3SS-2 proteins and secreted effectors, such as flagellin, induce the activation of NLRC4 inflammasome (159). Whereas, NLRP3 inflammasome is activated by a large repertoire of PAMPs and DAMPs (36).

Interestingly, NLRP3 inhibition did not influence cell death during infection with ST. However, inhibition of NLRP3 inflammasome activity resulted in reduced IL-1β expression **(Fig 4)**. The inhibition of either inflammasome activity has been reported to only result in partial or nominal protection from ST-mediated cell death. Simultaneous inhibition of both NLRP3 and NLRC4 inflammasomes has been shown to result in the potent inhibition of cell death, similar to caspase-1 deletion (79, 133, 159, 160). Similarly, recent results in our lab appear to indicate that while the processing of IL-1β is mainly mediated by the NLRP3 inflammasome, cell death is synergistically dependent on both NLRP3 and NLRC4 (unpublished data). Use of immunofluorescence microscopy has revealed the formation of singular cytoplasmic ASC (apoptosis-associated speck-like protein containing C-terminal caspase recruitment domain [CARD]) speck, containing both NLRC4 and NLRP3, during infection with ST. ASC is an adaptor protein that interacts with various NLRs, such as NLRP3, NLRC4 and AIM2, to form caspase-1 activating platforms (79, 148, 160). Furthermore, NLCR4 has been shown to recruit NLRP3 to its inflammasome complex during ST infection to induce caspase-1 activation (15). This suggests NLRP3 function synergistically with NLRC4, as part of ASC speck, for inducing caspase-1 mediated cell death during infection with ST.

In addition to inflammasome mediated cell death through caspase-1 activity, a recent study described a non-canonical form of caspase-1 activation induced through the recognition of cytosolic LPS by caspase-11, which promotes cell death and processing of IL-1β (38, 39, 76, 161). LPS mediated caspase-11 activity has also been reported to be critical for inducing cell death, in the absence of caspase-1, during infection with ST-lacking flagellin expression (162). However, no reports have associated XIAP with either NLRC4 or caspase-11 activity.

My results indicated that the release of IL-1β from *Xiap*$^{-/-}$ macrophages was driven in an NLRP3-dependent fashion **(Fig 4 A, B)**. Canonical NLRP3 inflammasome activation requires two independent signaling steps: transcription (priming) and oligomerization (activation). In the priming step (Signal 1), inflammatory triggers mediated by TLRs or TNFR signaling, such as TLR4 agonist LPS, induces the NFκB mediated expression of NLRP3, pro-IL-1β and pro-caspase-1. In the activation step (Signal 2), various stimuli associated with infections including an increase in extracellular adenosine triphosphate (ATP), pH alterations, β-amyloid fibers and degradation of extracellular matrix components, increase in potassium efflux and generation of oxygen species (ROS), can promote NLRP3 inflammasome oligomerization and activation which leads to caspase-1 activation as well as IL-1β and IL-18 release (36). The sequential use of LPS and ATP treatment has been reported to induce NLRP3 inflammasome (140). Indeed, my results also show that ATP treatment of LPS primed cells results in the release of IL1β. However, XIAP expression had no impact on canonical activation of NLRP3 inflammasome, as LPS and ATP treatments resulted in similar expression of IL-1β and cell death between WT and *Xiap*-deficient macrophages **(Fig 4 C, D)**.

Interestingly, Gaidt et al. have reported an alternative form of NLRP3 inflammasome activation, which relies on the RipK3 signaling pathway (139). The activation of RipK3 signaling is sufficient for the IL-1β processing and secretion. RipK3 functions as a complex with RipK1 and caspase-8, to activate NLRP3 inflammasome and subsequent caspase-1 activation **(Fig 1)**(44). RipK1 is a key protein that drives both cell death and survival signaling. Ubiquitination and deubiquitination events on RipK1 are crucial for determining cell fate. RipK1 signaling can be triggered by the ligation of death

receptors, including TNFR1, TNFR2 and Fas (CD95), as well as members of the PRR families. The association of receptors to their cognate ligands results in the formation of a large receptor complex, which includes cIAPs. Ubiquitination of RipK1 by cIAPs is crucial for initiating cell survival response, via NFκB and MAPK signaling (163–165). Conjugation of ubiquitin chains, by cIAPs, at various lysine residues represses RipK1 kinase activation. Additionally, cIAPs limit the accumulation of cytotoxic levels of active RipK1 through K48-linked poly-ubiquitination, which destabilizes active RipK1 and result in proteasomal degradation (164). In the absence cIAP1/2 activity, RipK1 forms a distinct protein complex with RipK3, via RHIM–RHIM interactions, resulting in diminished protective effect of NFκB signaling and the initiation of cell death programs (164–166). While not part of the TNFR or TLR4 receptor complex, XIAP also regulates RipK1 ubiquitination to prevent RipK3 signaling. The absence of XIAP during TNFR signaling induces aberrant RipK1 ubiquitination, which results in increased RipK1/RipK3 activity (47, 138).

Additionally, RipK1/RipK3 signaling, in the absence of caspase-8, has been shown to mediate cell death through MLKL-dependent pore formation to promote NLRP3 inflammasome activation. Translocation of MLKL oligomer to the cellular membrane results in a decrease in intracellular potassium levels, which results in NLRP3 activation (45, 46). MLKL driven inflammasome activation has been observed in IAP-deficient macrophages, following TNFR2 stimulation (167).

Interestingly, inhibition of cIAPs does not induce IL-1β expression or cell death, during stimulation with LPS or TNFα. However, the loss of *Xiap* expression has been reported to promote RipK1/RipK3-mediated inflammasome activation in BMDCs, resulting in the promotion of IL-1β processing during stimulation with LPS or TNFα (47). I also

observed an increased RipK1-mediated cell death upon TNFα stimulation of *Xiap*-deficient BMDCs **(Fig 6).** Studies in neutrophils (168), macrophages (167) and DCs (47, 48) have identified XIAP as a central suppressor of RipK1/RipK3-driven induction of NLRP3 inflammasome, IL-1β, and cell death responses.

Many pathogenic infections such as *Yersinia* pestis, vesicular stomatitis virus (VSV), Sendai virus, and influenza virus can trigger RipK1/RipK3 complex-dependent inflammasome activation (169, 170). ST also appears to induce inflammasome activation through the RipK1/RipK3 complex. The silencing of RipK1 or RipK3 resulted in a reduction of the inflammasome activity **(Fig4 E-H).** Our lab has shown that RipK1/RipK3 signaling plays a complementary role to caspase-1/caspase-11 in mediate host protection from ST infection, by inducing cell death and IL-1β expression (171). My data indicated that the enhanced RipK1 driven inflammasome signaling in *Xiap*$^{-/-}$ cells might be responsible for the increased cell death and IL-1β expression during infection with ST **(Fig 4 E, F).** Thus, my results with ST infection model revealed that XIAP functions to limit RipK1/RipK3 signaling to prevent the alternative form of NLRP3 inflammasome activation.

4.3 Impact of IAPs on NFκB and MAPK signaling

In addition to blocking apoptosis and other immunogenic forms of cell death, IAPs have also been identified as potent regulators of innate immune responses. IAPs can regulate the activation of NFκB and MAPK signaling pathways in response to the stimulation of members of TNFR superfamily and PRRs. NFκB and MAPK signaling pathways promote the expression of genes for cell survival and inflammation. Along with the expression of inflammatory cytokines, such as TNFα, pro-IL-1β and IL-6, activation of

NFκB signaling pathway induces the expression of genes that prevent programmed cell death. It induces expression of several Bcl-2 family members, most notably Bcl-XL and A1/Bfl-1, to prevent apoptosis by limiting permeability and depolarization of the mitochondrial membrane, and subsequent cytosolic release of cytochrome-C. NFκB also promotes the expression of both cIAPs and XIAP, which prevent caspase activity and further augment the activation of NFκB signaling pathway (172–174).

IAPs promote PRR/TNFR induced activation of NFκB and MAPK signaling by regulating the ubiquitination of key signaling intermediates. IAPs such as XIAP, cIAP1 and cIAP2 carry a C-terminal RING domain that functions an E3-ubiquitin ligase, which catalyzes the conjugation of K6, K11, K27, K48, and K63-linked ubiquitin chains on a lysine residues on the substrate proteins (173). Ubiquitination is a post-translational modification, which was initially described to regulate proteasomal degradation of defective and misfolded proteins. However, it is a complex system capable of regulating the protein turnover and facilitating signal transduction within the cells. For example, conjugation of homotypic K48-linked ubiquitin chains results in the proteasomal degradation of the substrate protein. Whereas, the homotypic K63-, or the linear homotypic M1-linked ubiquitin chains signals for the assembly of intracellular multiprotein signaling platforms (173). cIAPs and XIAP contain three tandem repeats of BIR domains, that form a deep hydrophobic groove to specifically anchor a 4 amino-acid linear motif called IBM (IAP binding motif). The BIRs serve as ubiquitination substrate-binding components through association with IBMs present on the substrate proteins. Alternatively, substrates can be recruited through adaptors or intermediates, such as TRAF2 and TRAF3 (175, 176). A dimerization of the IAPs is required for ubiquitin transfer onto the substrate. The dimerization interface is located near the C-terminal RING domain. UBA and CARD

domain regulates the activating dimerization of cIAPs. Association of BIR with an IBM motif carrying substrate results in a conformational modification that exposes the RING and CARD domain, resulting in dimerization and subsequent E3-ligase activation. cIAPs require BIR3 or substrate (TRAF2) association to prime the activation of the RING domain. By contrast, XIAP is able to form a stable dimer whilst lacking a CARD domain; as such, XIAP does not require an activation step (177).

IAPs have been well understood in mediating the ubiquitination of RipKs for promoting NFκB signal transduction (164). cIAPs and XIAP are able to catalyze the conjugation of ubiquitin chains to RIPK1, 2, 3, and 4 (178). RipK1 is a death domain-containing protein that can bind TNFR superfamily and adaptor proteins via homotypic death domain interaction. TNFR complex **(Fig 1)** has been extensively studied. Briefly, TNFR engagement triggers the binding of RipK1 and adaptor TRADD to the cytoplasmic domain of TNFR. TRADD allows the recruitment of TRAF2 and cIAP1/2, which mediate the conjugation of ubiquitin chains on RipK1 to regulate its activity (163, 164). cIAP1 has the ability to control the scaffolding function of RipK1 through K11- and K63-linked ubiquitination at K377, of RipK1. Both cIAP1/2 and XIAP has been shown to promote K11- and K63 -linked ubiquitination of RipK1 in TNF-α-treated cells. K11 and K63-linked polyubiquitin chains conjugated on RipK1 are recognized by LUBAC, which in turn adds a linear (M1-linked) ubiquitin chain. Degradation or deletion of cIAPs, has been shown to inhibit the recruitment of LUBAC and activation of NFκB signaling (163, 179). Altogether, the K11, K63 and M1-linked polyubiquitin chains form a molecular scaffold that allows the recruitment of IKK and TAB1/TAB2/TAK1 complexes, which result in the activation of NFκB and MAPK signaling (180).

In addition to promoting TNFR-mediated NFκB signaling, IAPs also promote PRRs signaling. NOD receptors sense intracellular PAMPs, bacterial peptidoglycans, to initiate the innate immune response (61). RipK2 is recruited to NOD1 and NOD2 receptor, through homotypic CARD interaction, leading to the formation of a signaling complex. Similar to RipK1, polyubiquitination of RipK2 is critical for recruiting IKK and TAB1/TAB2/TAK1 complexes that subsequently drive NFκB and MAPKs signaling pathways. Both cIAPs and XIAP are able to induce the ubiquitination of RipK2. However, XIAP has been shown to be the primary determinant of potentiating NOD signal transduction. *Xiap*-deficient macrophages exhibited impaired NOD2-dependent activation of NFκB and MAPK(p38) and the subsequent production of proinflammatory cytokines and antimicrobial molecules (124). BIR2 domain on XIAP directly interacts with RipK2 to facilitate the conjugation of K63-linked ubiquitin chains on K209, K410, and K538 residues located in the kinase domain. XIAP mediated RipK2 ubiquitination promotes the recruitment of LUBAC for the conjugation of linear ubiquitin chains and efficient NFκB and MAPK activation (181). Patients with dysfunctional XIAP expression, display defective NOD2-mediated NFκB activation (182).

In addition to ubiquitinating RipKs, IAPs are also responsible for TAK1 ubiquitination (122, 183). TAK1 of the TAB1/TAB2/TAK1 complex functions in transducing MAPK signaling and participates in NFκB activation by activating IKK complex. XIAP directly binds TAK1 through its BIR1 domain to catalyze the conjugation of K63-linked polyubiquitin chains to TAK1. A direct K63-linked polyubiquitination is required for TAB1 binding to TAK1 (122, 183). It is also critical for TAK1 activation, which results in the phosphorylation of IKKβ and the activation of IKK complex. This leads to the phosphorylation, K48-linked ubiquitination and proteasomal degradation of the

inhibitor of κB (IκB). Loss of IκB results in the release and nuclear translocation of NFκB subunits. Additionally, TAK1 can also activate MAPKs, including JNK, p38 and ERK (184).

Modulation of NFκB signaling in cells by XIAP is bound to influence the cell death and the expression of inflammatory cytokines by myeloid cells. It has been previously reported that the inhibition of NFκB signaling pathway, following stimulation with TNFα, resulted in increased cell death (185). Similarly, direct inhibition of TAK1, which is responsible for mediating NFκB and MAPK signaling, has been shown to induce caspase-8 directed pyroptosis, and release of IL-1β and IL-18 (186). This is consistent with the cytokine expression profile that I observed in *Xiap*$^{-/-}$ DCs and macrophages following ST infection, in which I observed a sharp reduction in IL-10 and IL-6 expression along with increased IL-1β release **(Fig 3, 17)**. A similar increase in IL-1β expression has been reported with murine γ-herpesvirus 68 (MHV-68) infection (47). Infection of *Xiap*$^{-/-}$ cells with *C. albicans* (187), LM (123) and *C. pneumoniae* (126) have been shown to result in reduced NFκB signaling in comparison to WT cells. These studies also attributed the lack of pathogen control in mice to the reduced NFκB and MAPK (p38) signaling in *Xiap*-deficient myeloid cells.

IAPs have traditionally been evaluated in tumor cell lines, as cancer cells often express high levels of IAPs to evade cell death mechanisms. However, cancerous tissues typically do not efficiently express inflammatory cytokines (188–190). It has been previously reported that LPS+IFNγ primed macrophages had reduced expression of IL-6, IL-10 and TNFα upon LM infection (123). A similar loss in the production of proinflammatory cytokines was observed in the serum of *Xiap*$^{-/-}$ mice infected with

Candida *albicans* (187). Concurrent with the idea of poor-inflammatory signaling in the absence of XIAP, both studies reported an increased pathogen burden in *Xiap*$^{-/-}$ mice following a pathogen challenge. While my results also reveal a reduction in the expression of IL-6 and IL-10 in *Xiap*-deficient macrophages, I observed better control of ST infection in *Xiap*$^{-/-}$ mice **(Fig 3A, B)**. The control over ST infection could be attributed to the enhanced inflammasome activity I observed in *Xiap*-deficient innate immune cells, as the inflammasome activation has been attributed to limit the spread of ST infection (40). Despite the early control during ST infection, *Xiap*$^{-/-}$ mice succumb to infection earlier than WT mice. This suggests that while the enhanced inflammasome activation may facilitate early bacterial clearance, the early fatality of *Xiap*-deficient mice might be attributed to the excessive inflammation, irrespective of bacterial burden.

4.4 Cell extrinsic impact of XIAP

APCs are highly phagocytic sentinel immune cells that are critical for ensuring optimal development of an adaptive immune response. Premature cell death of infected APCs can limit the duration of APC-T cell interactions. DCs are the most efficient APCs for stimulating antigen-specific T cells. Hence, the early ablation of DCs by pathogen induced virulence factors can limit the antigen-specific CD8 T cell response in mice (191). Conversely, increased survival for DCs, by inhibition of apoptosis, may lead to over-accumulation of active/mature DCs and enhance antigen-specific T cell activation (192). It has previously reported that the rapid death of APCs by activated CD8 T cells restricts the full activation of CD8 T cells *in vitro* (193). While prolonging the survival of DCs increased T cell activation; increasing the survival of infected APCs resulted in CD8 T cell differentiation towards a dysfunctional phenotype (143). Similar to *in vitro* infection with

ST, my results also indicated increased cell death and IL-1β expression by *Xiap*-deficient APCs, following ST-OVA infection **(Fig 5 C, 17)**.

Our lab has previously reported that limiting inflammasome activity to prevent the cell death of APCs infected with ST-OVA results in prolonged antigen presentation and enhanced T cell expansion (63). Using ST (**Fig 3-5)** and ST-OVA **(Fig 17 A)** infection model, I showed that inflammasome signaling is exacerbated in the absence of XIAP**,** which could potentially limit APC-T cell interaction. Inflammasome mediated caspase-1 signaling is the primary mechanism that induces cell death of myeloid cells during infection with ST (78). As such, by inhibiting caspase-1 in *Xiap*$^{-/-}$ DC, I was able to rescue T cell proliferation **(Fig 17)**. This suggests that the reduced threshold for *Xiap*-deficient APCs to undergo cell death could explain the lack of expansion of WT OT1 activated in *Xiap*-deficient milieu **(Fig 13-15)**. Similarly, the attenuation of cell death upon infection with LM-OVA has been shown to result in increased antigen-specific T-cell activation (194).

Considering the defect in proinflammatory NFκB signaling in conjunction with the greater tendency of cell death in *Xiap*-deficient APCs; it is, therefore, conceivable that the increased cell death that I observed in *Xiap*-deficient DCs and macrophages limits their ability to express inflammatory cytokines for prolonged periods. Activated APCs need to express cytokines that provide the third signal that ensures increased cycling of stimulated T cells. Type I interferon and/or IL-12 has been shown to provide the third signal for promoting the cycling of stimulated cells (195). Additionally, IL-6 has been shown to promote the survival of proliferating T cells, and exogenous supplementation of IL-6 resulted in increased numbers of activated T cells. Therefore, it is plausible that poor expression of IL-6 by the APCs would subsequently compromise the priming/survival of

stimulated CD8 T cells (149, 150, 196). I was able to rescue the defective proliferation/survival of CD8 T cells upon stimulation by *Xiap*-deficient DCs, by the addition of exogenous IL-6 **(Fig-17)**. This suggests that XIAP potentiates cytokine production and prolongs cell death of DCs to promote the activation of CD8 T cells.

4.5 XIAP limits contraction of T cell immune response

Activated T cells undergo cell death through a variety of pathways. Passive cell death or cell death by neglect occurs through the deprivation of growth factors and critical cytokines required for cell survival (IL-2, IL-4, IL-7 and IL-15). Loss of some cytokines and growth factors coincides with pathogen clearance and the reduction in innate immune response. Effector T cells rely on the presence of cytokines for their survival (197). Cell death as a result of cytokine unavailability engages the intrinsic apoptosis pathway by inducing the activation of the pro-apoptotic BCL-2 family members, Bim and Puma (198, 199). Bim is essential for promoting apoptosis induced by deprivation of IL-2 (200, 201), IL-7 (202) or IL-15 (194). Signaling by the cell survival cytokines promotes the expression of the antiapoptotic proteins of the Bcl-2 family, such as Mcl-1, to antagonize Bim-dependent apoptosis (203).

Active cell death can occur upon repeated stimulation of T cells leading to activation-induced cell death (AICD), which is thought to be mediated by persistent engagement of the TNF-death receptor family. Ligation of CD95(Fas)-FasL is responsible for AICD of CD4 T cells and some CD8 T cells (204). *In vitro* engagement of Fas with anti-CD95 antibody or TCR-crosslinking with anti-CD3 antibody has been shown to induce increased cell death of XIAP deficient CD8 T cells (128, 205). Additionally, cell death signals transmitted upon TNF binding to TNFR-1/TNFR-2 has been implicated in inducing

cell death (206, 207). These pathways induce the extrinsic apoptotic pathways that result in caspase-8 activation, which in turn leads to caspase-3/7 activation. A defective *Fas* or *FasL* gene has been shown to resist AICD *in vitro* (208). However, our lab has previously shown that the contraction of CD8 T cell response during infection is not affected by Fas-FasL interactions (209). Similarly, a normal contraction has been observed in caspase-8 deficient mice (117).

While extrinsic apoptotic signaling is dispensable for contraction, Bid/Bim dependent intrinsic apoptotic signaling plays an important role in regulating the contraction of CD8 T cell response, after an acute viral infection with HSV (204, 210). Bid can be cleaved and activated by caspase-8 to trigger cell death in response to activation of cell surface death receptors, such as Fas, TNFR and TRAIL (211). Both Bim and Bid play a synergistic role in mediated activation of intrinsic apoptotic signaling in T cells. Bid preferentially activates Bak while Bim preferentially activates Ba (212). Both Bax and Bak, when activated, oligomerize and directly cause mitochondrial outer membrane permeabilization (MOMP), resulting in the release of cytochrome-C along with other pro-apoptotic proteins, such as SMAC/DIABLO and OMI/HTRA2. Released cytochrome-C, results in the formation of apoptosome that triggers caspase-9 cascade. Effector caspase-9 then activates caspase-3 and -7, which ultimately results in cell death of activated T cells, that precipitates the contraction of the T cell response (213). Activation of executioner caspase-3 and -7 is further potentiated by the release of SMAC and OMI, which inhibit IAPs (214).

JNK signaling can also promote mitochondrial/intrinsic apoptosis by promoting Bim/Bid activity. Upon activation by the upstream MAP2Ks, such as TAK1 and ASK1, the phosphorylated JNK translocates to the nucleus, where it induces phosphorylation mediated

activation of c-Jun resulting in the formation of AP-1. AP-1 is involved in the transcription of a wide variety of proteins, including several pro-apoptotic proteins (208). JNK promotes intrinsic apoptosis further by inducing the expression of BCL-2 family of pro-apoptotic proteins, such as Bax and Bim (215, 216). JNK also targets the phosphorylation and activation of the pro-apoptotic BCL-2 proteins, Bim and Bmf, to release them from their scaffolding proteins. Bim and Bmf, thus released, induce mitochondrial outer membrane permeabilization to initiate apoptosis(208). JNK-mediated phosphorylation leads to the cleavage and activation of Bid. The 21 kDa cleaved fragment of Bid translocates to mitochondria and selectively promotes the release of SMAC/DIABLO, OMI and cytochrome-C (208).

My results indicated that the expression of *Xiap* in CD8 T cells is critical for regulating the contraction phase of the response **(Fig 8)**. The loss of 90-95% of activated CD8 T cells occurs despite the presence of XIAP in WT OT1 cells, suggesting that XIAP is responsible for limiting rapid contraction.

By activating both WT and *Xiap*-deficient OT1 cells in the same environmental milieu, I controlled the impact of external stimuli in influencing T contraction. Internal stressors, such as ROS and DNA damage, and external stimuli, through death receptor or PRRs can trigger JNK activation (14). XIAP has been shown to limit the JNK signaling pathway in innate immune cells and Jurkat T cells. XIAP can inhibit JNK signaling by promoting K48-linked ubiquitination and proteasomal degradation of TAK1 (183, 217). Therefore, it is conceivable that increased JNK activation in *Xiap*$^{-/-}$ CD8 T cells might result in increased activation of Bim, which would lead to a more prominent contraction of the response (218).

Additionally, XIAP further limits intrinsic apoptosis by inhibiting initiator caspase-9 as well as executioner caspase-3/7 (120). XIAP binds to active caspases-9 and -3 with BIR3 and BIR2 domains, respectively. Additionally, by also associating with the Apaf-1 apoptosome, XIAP appears to limit the release of active caspase-3 form the apoptosome complex (219). Since the intrinsic pathway of apoptosis, mediated through activation of caspase-9, is mainly involved in the contraction of CD8 T cell response, it is likely that XIAP might play a significant role in the contraction of the CD8 T cell response by inhibiting caspase-9 activity.

4.6 XIAP limits CD8 T cell proliferation

Ex vivo activation of naïve *Xiap*-deficient T cells with anti-CD3/CD28 antibodies has been reported by multiple studies to induce cell death (128). This augmentation of cell death was attributed to the increased executioner caspase activity (caspase-3 in particular) and reduced survival signaling (NFκB) in *Xiap*-deficient T cells (187). However, these results are in contrast to my observations with infected APCs presenting an antigenic peptide, which revealed that XIAP is dispensable for T cell survival during activation **(Fig 15)**. Rather, I observed an increased proliferation of Xiap-deficient CD8 T cells in response to antigen-presented by an infected APC. Anti-CD3/CD28 mediated activation of T cells is an artificial model of T cell stimulation, which does not mimic the physiological mode of antigen presentation that involves cytokines and co-stimulatory molecules expressed by APCs. Furthermore, stimulation by the plate-bound anti-CD3 antibody results in potent, chronic and massive cross-linking of the TCR complex on all cells. However, antigen presentation by DCs to naive T cells includes a variety of co-stimulatory and inhibitory signals, which may increase or decrease the activation threshold. The presence of additional

survival cytokines expressed by APCs could make XIAP dispensable for inhibiting cell death pathways. Additionally, the results of my *in vitro* T cell stimulation model are consistent with my in vivo adoptive transfer model in WT mice, which also revealed that the deletion of *Xiap* from CD8 T cells does not augment their expansion **(Fig 8)**. Likewise, XIAP has been shown to have no impact on CD8 T cell expansion evaluated on day 7/8 of infection with *Lymphocytic Choriomeningitis Virus* (LCMV). Interestingly, additional treatment of *Xiap*$^{-/-}$ mice to inhibit cIAPs resulted in reduced numbers of antigen-specific CD8 T cells (220). T cell stimulation with anti-CD3 antibodies or APCs crucially induces activation of caspase-8 and caspase-3. XIAP and cIAPs have been reported to restrict caspase-3 from localization into the nucleus (221, 222). Therefore, cIAPs may play a compensatory role in the absence of XIAP to prevent aberrant cell death during expansion. Using just anti-CD3/CD28 antibodies to stimulate T cell activation has major limitations in examining the role of XIAP in regulating T cell response. One such artifact of anti-CD3/CD28 activation observed in my study arises from the ability of cytokine expression by *Xiap*-deficient T cell. Anti-CD3/CD28 stimulated *Xiap*-deficient T cells have exhibited a relatively reduced expression of IFNγ (187). However, my results using APCs to stimulate T cell activation reveal that the absence of XIAP results in increased IFNγ expression by T cells (Fig 11E, 12E). A similar increase in IFNγ production by T cells was observed in patients with XIAP deficiency(223).

Finally, I also observed that *Xiap*$^{-/-}$ CD8 T cells displayed increased proliferation during the expansion phase of the response **(Fig 9, 11, 12)**. APC stimulation, via Fas engagement and MHC-TCR interaction, induces caspase-8 and subsequent caspase-3 activation. Our lab and others have reported that caspase-3 activity in T cells is required

during the initial stages for promoting the proliferation of primed T cells (37, 38) and in the proliferation of sebaceous gland cells (224). Executioner caspases have been shown to induce cleavage-dependent inhibition of a potential inhibitor of cell cycling protein(224). It is, therefore, conceivable that increased caspase-3 activation that I observed in *Xiap*$^{-/-}$ CD8 T cells results in increased cell cycling during the initial stages of activation **(Fig 11, 12)**. Interestingly, this expansion was also evident in my adoptive transfer model using *Xiap*$^{-/-}$ hosts, which have a relatively weaker antigen presentation **(Fig 9)**.

4.7 XIAP potentiates formation of CD8 T cell memory response

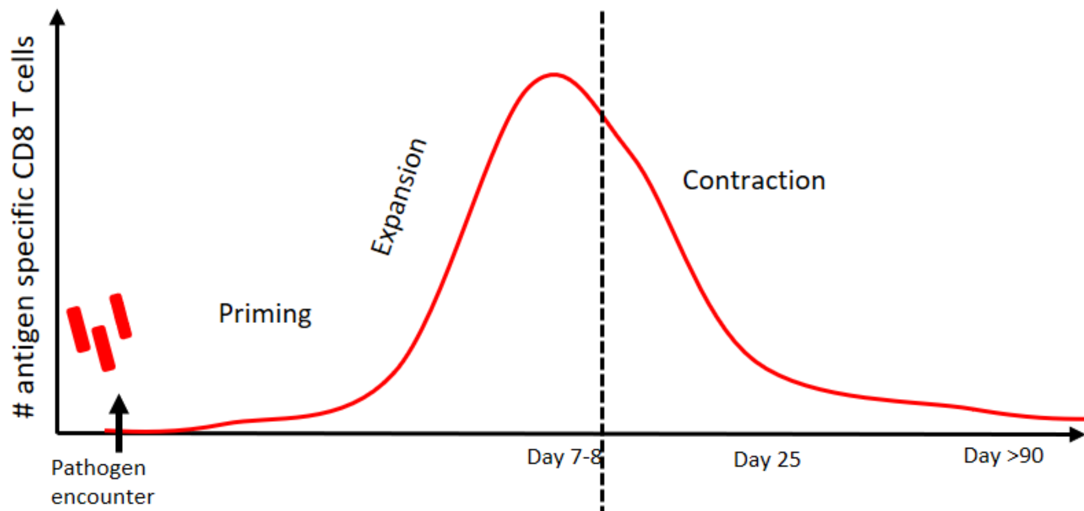
My results indicated that XIAP is necessary for cell signaling that promotes the differentiation of CD8 T cells towards memory cells **(Fig 8, 9)**. TCF-1 signaling through the Wnt pathway has been shown to promote T cells differentiation towards memory. TCF-1 and Wnt signaling are highly active and expressed in undifferentiated CD8 T and memory CD8 T cells, and inhibited during naïve T cell differentiation into effector cells. It is possible that XIAP might impact CD8 T cell differentiation by regulating Wnt signaling. XIAP was shown to promote Wnt signaling in mammalian cultured cells and *Xenopus* embryos by monoubiquitinating the transcriptional repressor TLE and reduce its affinity for binding TCF-1 (225, 226). T cells activated in the absence of XIAP might exhibit reduced Wnt signaling, resulting in reduced differentiation of activated T cells into memory subsets. Additionally, my results revealed a weaker secondary immune response in the absence of XIAP **(Fig 10)**. The lack of a memory recall response of *Xiap*-deficient cells could result in a defective differentiation of activated cells into memory precursors. This is in line with my observation of fewer CD8 T memory precursor subsets, in the absence of XIAP.

4.8 Conclusion and implications

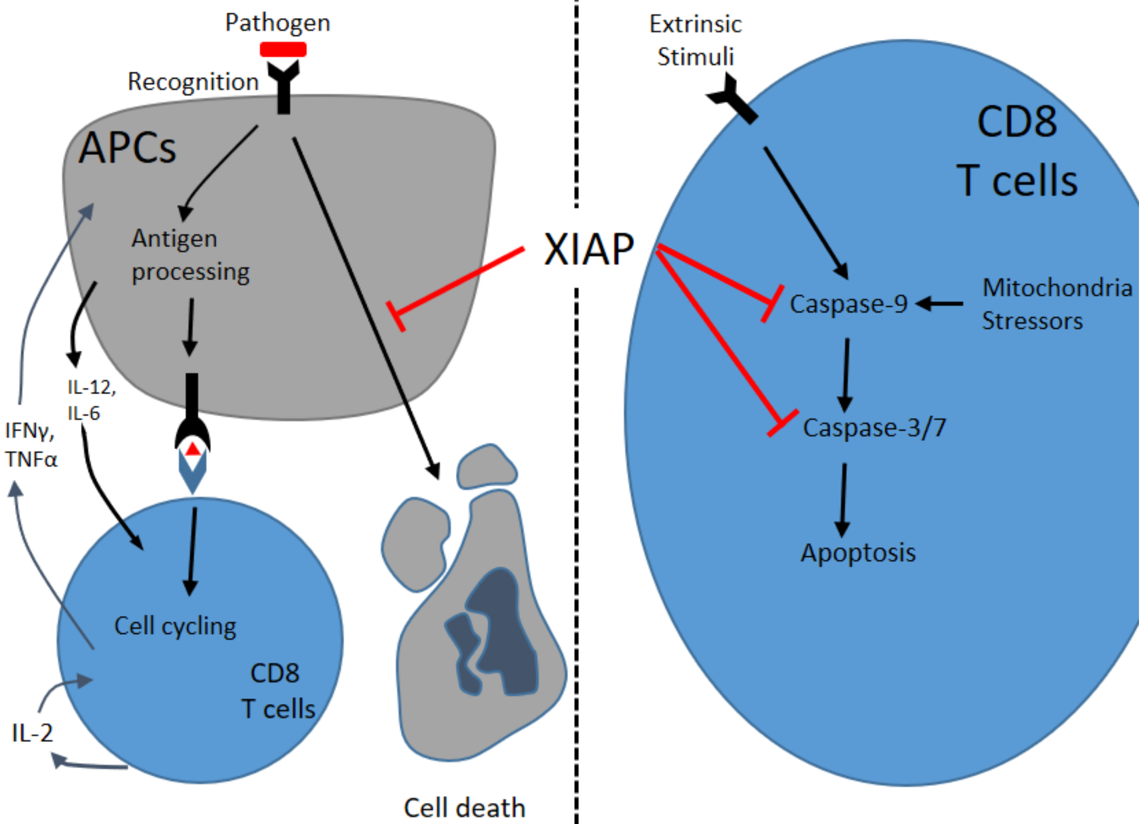
Overall, my results indicate that XIAP plays a critical role in generating an effective CD8 T cell immune response to infection. My study shows that XIAP protected activated CD8 T cells from overt cell death during the expansion and contraction phases of the response in a cell extrinsic- and cell intrinsic- manner, respectively. The combination of both increased contraction in *Xiap*$^{-/-}$ CD8 T cells and poor priming and activation by *Xiap*$^{-/-}$ APCs, due to their increased cell death, resulted in a weak T cell response to intracellular pathogens. My results could have major implications on the usage of IAP-antagonist as cancer therapeutics. High levels of IAPs expression has been observed in a number of tumors, and IAP antagonists sensitize tumor cells to death, through the inhibition of IAPs, including XIAP. My results show that the absence of XIAP results in defective immune responses to intracellular bacterium. Thus, IAP antagonists could also have powerful immunomodulatory capacities, which could render a patient susceptible to infections.

Figure 18 Thesis Overview

CD8 T cell response peaks around day 7/8 of infection, which is immediately followed by a rapid phase of contraction wherein ~90% of the activated CD8 T cells are eliminated. My study shows that XIAP acts in a CD8 T cell extrinsic and intrinsic manner to regulate the expansion and contraction phases of the CD8 T cell response, respectively. During the expansion phase, XIAP promotes the development of CD8 T cell response in a cell extrinsic manner. XIAP prevents the cell death of APCs to promote APC-T cell interaction and cytokine release, which facilitates the proliferation and survival of activated T cells. In the contraction phase of the immune response, XIAP functions in a cell intrinsic fashion to inhibit the pro-apoptotic signals in the activated CD8 T cells to prolong the immune response.



antigen specific CD8 T cells
Priming
Expansion
Contraction
Pathogen encounter
Day 7-8
Day 25
Day >90



Pathogen
Recognition
APCs
Antigen processing
IL-12, IL-6
IFNγ, TNFα
Cell cycling
CD8 T cells
IL-2
Cell death
XIAP
Extrinsic Stimuli
CD8 T cells
Caspase-9
Mitochondria Stressors
Caspase-3/7
Apoptosis

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