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cellular Inhibitor of Apoptosis Protein2 – A critical regulator of neuroinflammation

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A dissertation submitted in partial fulfilment of the requirement for the degree of Doctor of Philosophy at Virginia Commonwealth University

By

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LIST OF ABBREVIATIONS

APC	Antigen Presenting Cells
BIR	Baculoviral IAP repeats
cFLIP	Cellular FLICE-Like Inhibitory Protein
cIAP	cellular Inhibitor of Apoptotic Protein
CNS	Central Nervous System
DAMP	Danger associated Molecular Pattern
EAE	Experimental autoimmune encephalomyelitis
GFAP	Glial fibrillary acidic protein
IAP	Inhibitor of Apoptosis Protein
IL	Interleukin
IKK	IκB kinase

IκB	inhibitor of kappa-B kinase
IFNγ	Interferon-gamma
IRAK	Interleukin-1 Receptor-associated Kinase.
IRF	Interferon Regulatory Factor
MiDM	Microglia Derived Macrophages
MoDM	Monocyte Derived Macrophages
MLKL	Mixed lineage kinase domain-like
MS	Multiple Sclerosis
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NIK	NF-κB-inducing kinase
NLR	NOD-like receptors
PRR	Pattern recognition receptors

SMAC	second mitochondrial derived activator of caspase-s
RIPK	Receptor-interacting serine/threonine-protein kinase
TLR	Toll like Receptors
TNF-α	Tumor necrosis factor-alpha
TNFR	Tumor necrosis factor receptor
TRADD	Tumor necrosis factor receptor type 1-associated DEATH domain protein
TRAF	TNF receptor associated factors

ABSTRACT

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Inhibitors of apoptosis (IAPs) modulate cell death and play critical role in signal transduction that promotes inflammation. Recently, Smac mimetics, which are IAP antagonists, have attracted attention as novel cancer therapeutics. Cellular Inhibitor of Apoptosis 2 (cIAP2), a member of IAP family, positively affects both NF-κB and MAPK activation in response to many inflammatory stimuli. We observed that the lack of cIAP2 ablates LPS-induced neuroinflammation. Also, cIAP2$^{-/-}$ macrophages demonstrated diminished antigen presentation potential that could contribute to ablated immunity. In addition to these functions, we have previously reported that cIAP2 also regulates the activation of Interferon Regulatory Factor 1 (IRF1). Since IRF1$^{-/-}$ mice are resistant to experimental autoimmune encephalomyelitis (EAE), we hypothesized that cIAP2$^{-/-}$ mice

will be protected from the disease. Surprisingly, induction of EAE in cIAP2$^{-/-}$ mice resulted in exaggerated infiltration of immune cells increased expression of proinflammatory cytokines and demyelination within CNS. We found that the lack of cIAP2 induces caspase-8 expression in microglia derived macrophages, contributing to their activation and polarization towards M1 phenotype, and exacerbates the symptoms of EAE. These findings suggest that cIAP2 limits neuroinflammation in the CNS and thus the use of Smac mimetics as chemotherapeutics needs to be reevaluated.

CHAPTER 1

General Introduction

1.1 Neuroinflammation

1.1.1 Components of the CNS

The central nervous system (CNS) consists of the brain and spinal cord. The CNS integrates information received from the body and coordinates bodily activities and organ function. The spinal cord innervates the peripheral nervous system through spinal nerves. These nerves allow the transmission of efferent motor signals and afferent sensory signals.

Histologically, the CNS can be divided into two parts: white matter and gray matter. White matter consists of neuronal projections, called axons, while the gray matter is comprised of neuron cell bodies. Additionally, white matter and gray matter consists of non-neuronal cells called glial cells.

Neurons are electrically excitable cells that receive, process, and transmit information using electrical and chemical signals. Proper functioning of neurons is critical for the coordination of complex processes. Glial cells were discovered in 1856 and were thought to be non-functioning cells that only act as “glue” for the nervous system. However, investigations of glial cell function have highlighted the importance of these cells. In the CNS, glial cells are composed of four different cell types: astrocytes, oligodendrocytes, microglia, and progenitor cells.(Purves et al., 2001)

Approximately 20-40% of the cell population in the CNS is comprised of star-shaped glial cells called astrocytes. Astrocytes are critical for the CNS microenvironment and contribute to several significant functions, including preserving blood brain barrier integrity, regulating extracellular ion balance, forming and pruning synapses by secretion

of growth factor and cytokines. Astrocytes also play an important role in CNS repair and scarring following neuroinflammation (Liddelow and Barres, 2017, Sofroniew, 2014) Reactive astrocytes can be categorized into two subtypes, based on their inflammatory profile. A1 astrocytes are pro-inflammatory and cause tissue damage while A2 astrocytes are anti-inflammatory and demonstrate a resolving phenotype (Liddelow et al., 2017).

Oligodendrocytes are glial cells found in the CNS that support axons and provide electrical insulation in the form of myelin sheath. The myelin sheath enables fast action potential propagation and high fidelity transmission in the CNS. Oligodendrocytes also provide trophic support for neighboring neurons by secreting several proteins, including glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), and insulin-like growth factor-1 (IGF-1) (Wilkins et al., 2003). Similar to oligodendrocytes are Schwann cells of the peripheral nervous system. Schwann cells wrap around axons of peripheral nerves, including motor and sensory nerves, to form myelin sheaths. Like the myelin sheaths produced by oligodendrocytes, myelin sheaths of Schwann cells contribute to the conduction and propagation of impulses traveling along peripheral axons.

An additional group of glial cells are NG2-glial cells. These unique glial cells are precursor cells that predominately differentiate into oligodendrocytes. Interestingly, NG2-glial cells can also differentiate into neurons and astrocytes (Covacu et al., 2014).

The final group of glial cells found in the nervous system are microglia. Microglia are residential immune cells, like macrophages, found in the CNS. Unlike macrophages/monocytes, which originate in the bone marrow and infiltrate into the brain, microglia arise from early erythro-myeloid precursors in the embryonic yolk sac and migrate to the brain mesenchyme before the formation of the blood brain barrier(Ginhoux and Prinz, 2015; Gomez Perdiguero et al., 2015).

This contrasts with that macrophages/monocytes that originate in the bone marrow and can also infiltrate the brain at later stage. Microglia act as sentinel cells that constantly survey the CNS microenvironment (Nimmerjahn et al., 2005). In normal conditions, microglial cells demonstrate a *ramified* phenotype and are immunologically inactive. However, once "*activated*" via various triggers, microglia assume an *amoeboid* morphology and act as antigen-presenting, phagocytic, and cytotoxic cells. Phagocytic microglial cells play an important role in CNS development by contributing to synaptic pruning and neurogenesis (Wolf et al., 2017)(Graeber et al., 2011).

Like astrocytes, microglia can be categorized into two subgroups: M1 and M2 microglia. During neuroinflammation, microglia polarize into M1 pro-inflammatory cells. In this state, microglia secrete cytokines and chemokines to activate astrocytes and recruit immune cells. Conversely, anti-inflammatory M2 microglia by secreting factors that help to resolve inflammation (Orihuela et al., 2016).

1.1.2 Sterile neuroinflammation

Inflammation is a response of the innate immune system to retain the hemostasis of the body. Inflammation can be triggered by infection or injury. Although inflammation is important for tissue repair and the elimination of dangerous pathogens, unresolved and long-lasting inflammation be detrimental to the host. A major characteristic of unresolved inflammation in the CNS includes activation of microglia. Chronic activation of microglia is witnessed during neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and multiple sclerosis (MS). Further, chronic inflammation causes overall upregulation of pro-inflammatory cytokines and chemokines in the CNS.(Allensworth et al., 2013; McComb et al., 2016)

The CNS has historically been considered an immune privileged site due to the presence of the highly restrictive blood-brain barrier (BBB). However, it has been identified that neuroinflammation can occur through a broad range processes, including immune responses initiated in the periphery or within the CNS. During peripheral inflammation, proinflammatory mediators can permeate the BBB and permit the migration of leukocytes into the CNS. The entry of peripheral leukocytes damages the BBB and can create an inflammatory state similar to that seen in peripheral sites.

During sterile neuroinflammation, astrocytes and microglia can express specialized pattern-recognition receptors (PRRs) which can further trigger inflammatory pathways. These PRRs recognize microbial molecules, termed pathogen-associated molecular

patterns (PAMPs). PRRs also recognize host-derived endogenous molecules, or danger/damage-associated molecular patterns (DAMPs). DAMPs can be misfolded proteins, aggregated peptides, chromatin associated protein high-mobility group box 1(HGMB1), heat shock proteins (HSPs), ATP, uric acid (Tang et al., 2012) or mis localized nucleic acids found in diseased brain. DAMPs can signal PRRs expressed on astrocytes and microglia, allowing the activation of these cells. Activation of these cells depend on the injury site, severity of brain injury, surrounding environment, and signaling strength of the stimulus. Once activated, astrocytes and microglia either remove stimulants or secrete additional inflammatory mediators (Banjara and Ghosh, 2017).

Inflammation is controlled by a host of mediators, including cytokines, growth factors, complements, lipids and peptides. Recently the role of inhibitor of apoptosis (IAP) family has attained a lot of attention as a key regulator of signal transduction pathways that regulate cell proliferation, differentiation and cell death during inflammation.

1.2 Inhibitor of Apoptosis Proteins

The family of Inhibitor of Apoptosis (IAP) proteins were discovered in the genome of baculoviral and contain a domain of approximately 70 amino acids termed the baculoviral IAP repeat (BIR). Up to three tandem copies of the BIR domain occur in a wide range of eukaryotic species, including fission yeast, Caenorhabditis elegans, Drosophila melanogaster, and several mammalian species including mice, rats, chickens, pigs, and humans, which contain eight IAPs (Figure.1-1). Previously, the IAP family proteins were believed to be have functions related to apoptosis inhibition only. However, recent

studies have found that members of the IAP family play important roles in the regulation of inflammatory responses. The cellular Inhibitor of Apoptosis2 (cIAP2), also known as HIAP1 or BIRC3, is a member of the IAP family and has been shown to play a role in inflammation.

1.2.1 Structure of cIAP2

The activation of the nuclear factor-κB (NF-κB) family of transcription factors rapidly induces the upregulation of inflammatory and antiapoptotic genes, including cIAP2. cIAP2 has a type II BIR domain, which contains a hydrophobic cleft that binds to the N-terminal tetrapeptides of the IAP-binding motif. This motif is present in certain caspase-s and in IAP antagonists, such as second mitochondrial derived activator of caspase- (SMAC). Alternatively, type I BIR domains (BIR1) interact with proteins such as tumor necrosis factor receptor-associated factor (TRAF).cIAP2 also possess a RING domain that confers E3 ubiquitin ligase activity (Sharma et al., 2017). Additionally, cIAP2 can promote ubiquitin-dependent proteasomal degradation of caspase-s (Figure 1-2). cIAP2is a highly inducible gene during inflammation that, along with cIAP1, binds with TRAF2 and plays a vital role in TNF alpha (TNF-α) signaling (Alvarez et al., 2010; Mahoney et al., 2008). cIAP2 has been shown to inhibit cell death by directly repressing the proapoptotic activity of caspase-8, as well as RIPK3 dependent necroptosis. Despite these findings, the precise antiapoptotic mechanisms, as well as the pathophysiological role of cIAP2 in sterile neuroinflammation remains unknown.

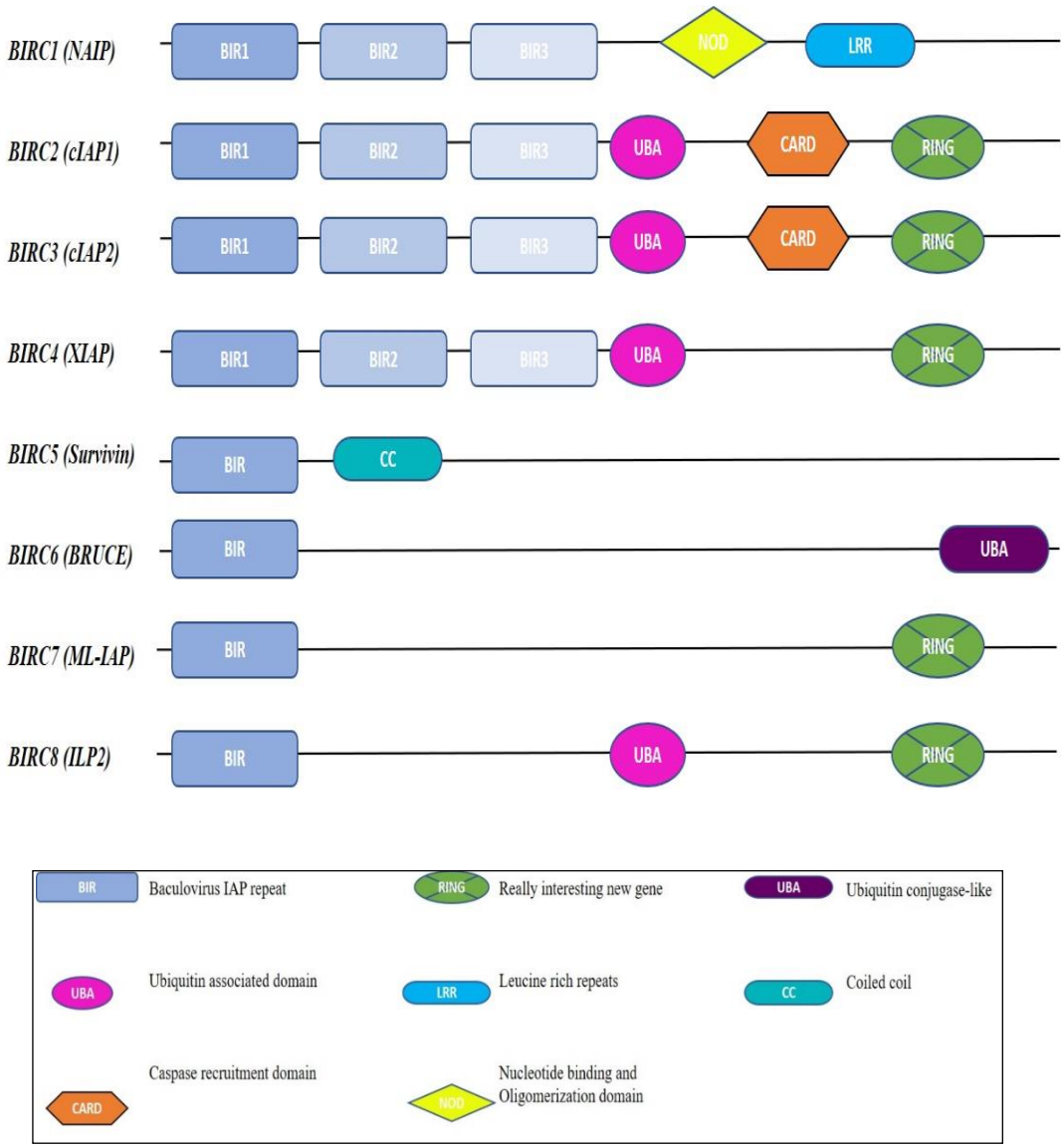


Figure 1- 1: IAP gene family known in human (modified from Modulation of immune signaling by inhibitors of apoptosis, *Trends in immunology*, Volume 33, Issue 11, November 2012)

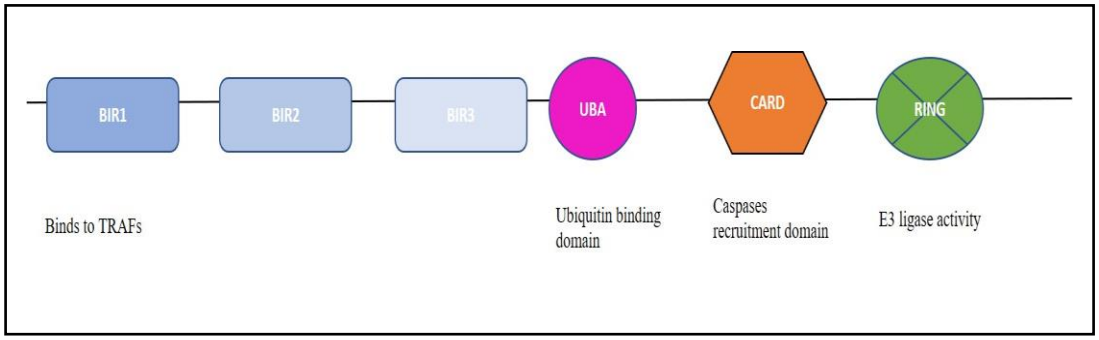


Figure 1- 2: Schematic diagram for cellular Inhibitor of apoptosis protein (cIAP2).

cIAP2 is characterized by the presence of the BIR domain, which is involved in protein-protein interactions such as TRAFs. Polyubiquitin chains of cIAP2 bind to the Ubiquitin binding domain

1.2.2 Regulation of innate and adaptive immunity by cIAP2

Innate and adaptive immunity are two arms of a sophisticated system that defends a living organism by detecting and invading, harmful microorganisms. Innate immunity, the first line of defense, provides a quick immune response, while the adaptive immune response requires extended time to initiate and involves antigen-specific clonal expansion of T and B lymphocytes.

Innate immunity is conducted by several myeloid-derived cells, including dendritic cells, macrophages and granulocytes, as well as non-hematopoietic cells, such as epithelial cells, fibroblasts and endothelial cells. Both groups of cells express pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), NOD-like receptors (NLRs) and retinoic acid-inducible gene (RIG)-I-like receptors, which can detect the presence of conserved microbial components (PAMPs), and endogenous intracellular molecules (DAMPs).

Recent studies have shown that ubiquitylation is critical for regulating various levels of the inflammatory response. The E3 ligase such as cIAP2, are found to be promoting ubiquitylation both downstream of various PRRs.

i. Role of cIAP2 in innate immunity

The first line of the innate immune response begins with the recognition of PRRs. This drives the production of proinflammatory cytokines, chemokines and interferons. Subsequently, this promotes the formation of inflammasomes.

NOD-like receptor signaling

NOD1 receptors recognize D-glutamyl meso-diaminopimelic acid found in Gram-negative bacteria, while NOD2 receptors identifies muramyl dipeptide present in both Gram positive and Gram-negative bacteria. Once activated, these receptors and Receptor-interacting serine/threonine-protein kinase2 (RIPK2) (Hasegawa et al., 2008; Yang et al., 2007). It has been shown that cIAP1/cIAP2 can conjugate K63-linked chains to RIPK2. Furthermore, TAK1/TAB2/TAB3 complexes are recruited to ubiquitin chains and activates p38, ERK, MAP kinases and NF-κB pathways. Studies have shown that macrophages derived from cIAP1/ cIAP2 deficient mice and human colonocytes deficient of cIAPs demonstrate reduced K63 polyubiquitin of RIPK2, leading to attenuated activation of MAPK kinases and NF-κB pathways. Similarly, $cIAP2^{-/-}$ mice injected with Diaminopimelic acid, or other synthetic NOD1 agonists, demonstrate blunted cytokine expression or diminished neutrophil recruitment in peritoneum (Bertrand et al., 2009).

Toll like receptor (TLR) signaling

TLRs have been subcategorized into two groups. The first group consists of TLRs (TLR1, -2, -4, -5, -6 and -11) that are present at the cell surface. These TLRs detect microbial membranes, such as proteins, lipoproteins, and lipids. The second group is composed of TLR3, 7, 8 and 9. These TLRs are located at the intracellular endosome/lysosome membranes and can detect nucleic acids from microbes and host cells (Kawai and Akira, 2010). Depending on the receptor, signaling downstream of TLRs occurs *via* recruitment of MyD88 or TRIF. TLR4, however, initiates both MyD88-and TRIF-dependent responses.(Kawasaki and Kawai, 2014)

Recently, an *in vitro* study identified roles of cIAPs in the regulation of MyD88-dependent MAPKs activation by degrading TRAF3 (Tseng et al., 2010). Upon TLR4 activation, cIAP1/2, TRAF3 and TRAF6, are recruited to the MyD88-signaling complex. Activation of TRAF6 in the complex leads to K63-linked polyubiquitylation of TRAF6 and cIAP1/2. The IKK complex recruited to these TRAF6 conjugated chains allows the activation of the canonical NF-κB pathway. Also, the TAB2/3-TAK1 complex, which is recruited to TRAF6 ubiquitin chains, translocate to the cytosol and activates the MAPK pathway. This process is inhibited by TRAF3. Upon activation of TLR4, cIAPs conjugate K48 polyubiquitin chains on TRAF3, leading to the proteasomal degradation of TRAF3. This allows TAK1 translocation and the activation of MAPKs.

Inflammasome activation

Upon sensing DAMPs, innate immune cells are primed by the activation of PRRs, such as NOD1/2, RIG-1, TNFR1 and IL-1R. This triggers the expression of inactive precursors of pro-IL-1β and pro-IL-18. The members of the NLP family, including NLRP1, NLRP3, NLP6, NLRC4 and cytosolic DNA sensor absent in melanoma (AIM 2), assemble into a caspase-1 dependent complex inflammasome, promoting the maturation of the proinflammatory cytokines IL-1β and IL-18 from their inactive precursors. (Dagenais et al., 2012; Guo et al., 2015)

Recently, contradictory roles of cIAPs in caspase-1 activation and pro-IL-1 maturation have been described. Labbe et al. has shown that cIAP1/2 can facilitate caspase- 1 activation by conjugating K63 linked polyubiquitin chains to caspase-1, *in vitro*. This allows the activation of NLRP3 and NLRP4 inflammasome (Labbé et al., 2011). However,

Vince et al. has demonstrated that deletion of XIAP, cIAP1 and cIAP2 results in NLRP3 activation. Additionally, they showed that the processing of pro-IL-1 triggered by IAPs was independent of caspase-1 but dependent on caspase-8 and RIPK3-dependent ROS generation (Vince et al., 2012). Further, *in vivo* studies have demonstrated that cIAP2-deficient mice have exacerbated colitis phenotype due to increased cell death and impaired activation of the inflammasome–interleukin-18 (IL-18) pathway, which is essential for tissue repair following injury (Dagenais et al., 2016)

ii. Role of cIAP2 in adaptive immunity

cIAP1 and cIAP2 negatively regulate the non-canonical NF-κB pathway in adaptive immune cells, including T and B cells. Diminution of cIAP1/2 in B cells leads to increased cell survival independent of BAFF-R. This also led to inability to form germinal centers in these mice, which are required for antibody-mediated immunity (Gardam et al., 2011). Similarly, in T cells, deficiency of cIAP1 and cIAP2 results in increased stabilization of NIK, followed by the processing of p100 to p52. This leads to activation of the non-canonical NF-κB pathway in resting lymphocytes, promoting their proliferation and activation in the absence of co-stimulation (Giardino Torchia et al., 2013a, 2013b). Together the research so far shows that IAPs play a significant role in regulating innate and adaptive immunity and can be targeted for therapies against pathogen-induced or sterile inflammation.

cIAPs can function as a regulator of signaling platform that activates intracellular signaling pathways, including the activation of nuclear factor κB (NF-κB) and mitogen-

activated protein kinase (MAPK). These pathways provide the expression of genes controlling innate and adaptive immunity, inflammation, cell proliferation, differentiation, migration, survival and death. TNF-α and IL-1β are significant proinflammatory cytokines involved during sterile inflammation. Several studies have showed that cIAP2 plays critical role upon activation of TNF-R1 and IL-1R receptors.

1.2.3 Role of cIAP2 in TNFR-1 dependent pathway

The role of cIAP2 is extensively studied in context of NF-κB pathway and MAPK kinase pathway downstream of the TNF-a signaling pathway.

i. Positive regulatory function of cIAP2 in TNF-R1 mediated NF-κB pathway

TNF-α is a pleiotropic cytokine that plays a role in inflammation and induces cell death. cIAP1 and cIAP2 can control the TNFα-mediated activation, survival and cell death by regulating both canonical and non-canonical arms of NF-kB pathways. Downstream of TNFR1 activation, RIPK1 when conjugated with K63 linkage ubiquitin provides a platform for proinflammatory signaling and cell survival through activation of NF-κB and MAPK pathways. Therefore, activation of RIPK1 and its post translational modification dictates the fate of the cell to either survive or die(Vandenabeele et al., 2010).

Activation of TNFR1 triggers assembly of the receptor associated signaling complex I, that includes TRADD, TRAF2, TRAF5, cIAP1and cIAP2. As shown in Figure 1-3, the E3 ligases with their RING domains, cIAP-1 and cIAP-2, facilitates the polyubiquitination of RIPK1 with K63 linkage that serves as a docking site for TAK1/TAB2/TAB3, and the

inhibitor of κB (I κB) kinases and (IKK) α/β/γ complexes. TAK1 phosphorylates IKKβ, that further phosphorylates IκBα, promoting its proteasomal degradation to allow NF-κB subunits(p65/p50) to enter the nucleus and activate transcription of target genes. These genes encode for the proinflammatory cytokines such as TNF, IL-1B and chemokines such as CCL5, CXCL10, and pro-survival FLICE inhibitory protein (c-FLIP) and cIAP-2.

Recruitment of TAK1/TAB2/3 complexes by K63 polyubiquitinated RIPK1 also triggers MAPK kinase pathway. This pathway functions to prevent apoptosis and promote cell survival, as well as to express proinflammatory cytokines

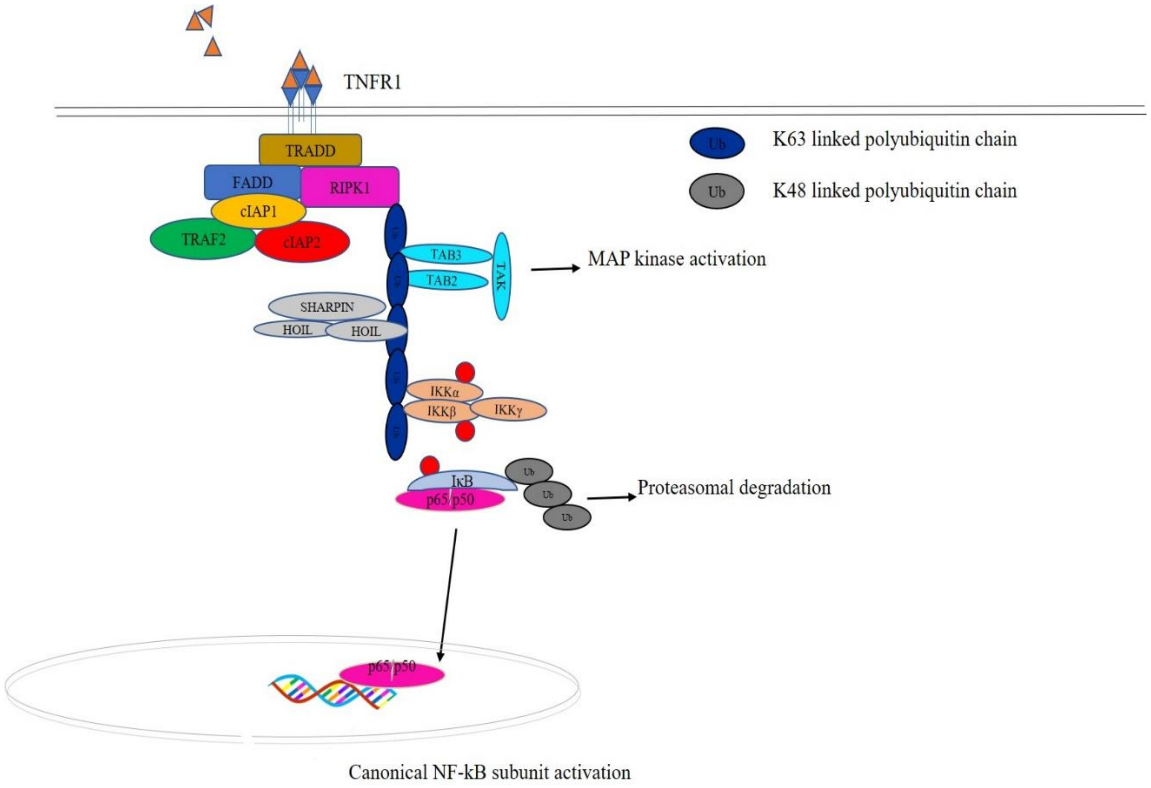


Figure 4-3: Role of cIAP2 in TNF-R1 signaling inducing NF-κB pathway

Binding of TNFα to TNF-R1 induces the formation of signaling complex I, consisting of TNF-R1, cIAP1/2, TRAF2 E3 ligase complex, TRADD, and RIPK1. cIAP1/2 and TRAF2 polyubiquitinates RIPK1 with K63 ubiquitin chain and recruit LUBAC to promotes linear ubiquitination. The attached polyubiquitin chains serve as docking sites for the TAB2/TAB3/TAK1 and IKK complexes. This leads to activation of TAK1 and IKK, in turn activates MAPK and NF-κB signaling pathway.

ii. Negative regulatory function of cIAP2 in TNFR1-mediated cell death

Dissociation of RIPK1 from TNFR1 can lead to several other destinies for the cell. This dissociation can either lead to formation of cell death promoting secondary cytosolic

complexes called ripoptosome (complex IIa) or Necroptosome (complex IIb)(Newton, 2015; Orozco et al., 2014)

RIPK1 becomes phosphorylated and interacts with Fas associated death domain (FADD), that recruits procaspase-8 to the complex. Ripoptosome contains TRADD, FADD, RIPK1, cellular FLICE-like inhibitory protein (c-FLIP), and procaspase-8. There are two forms of cFLIP- $cFLIP_L$ and cFLIPs. $cFLIP_L$ restricts processing of caspase- 8 from procaspase-8 while cFLIPs promoted cell death induced by RIPK3. On activation and processing of procaspase-8 into mature caspase-8, activates a cascade of caspase-s such as caspase-3 and caspase-7 and caspase-9 that lead to apoptosis.(Kondylis et al., 2017)

Conte *et al* reported that $cIAP2^{-/-}$ mice were protected from lipopolysaccharide (LPS)-induced sepsis with because of diminished inflammatory response. They concluded that $cIAP2^{-/-}$ macrophages undergo apoptosis that renders protection to the mice from sepsis. Also, Kavanagh *et al* showed that cIAP2 regulates caspase- 3 activation in BV2 microglial cells. Pharmacological inhibition of cIAP2, exhibited increased cell death when stimulated with LPS (Kavanagh et al., 2014).

To form a necroptosome, phosphorylated RIPK1 can interact with RIPK3 by binding via their shared RIPK hemolytic interaction motifs (RHIM) that in turn interact with mixed lineage kinase domain-like) MLKL and PGAM5 which activates DRP-1 to cause mitochondrial dysfunction and release of reactive oxygen species.(Remijsen et al., 2014; Zhou and Yuan, 2014)

McComb *et al* described that pharmacological inhibition of cIAPs resulted in death of bone marrow derived macrophages due to caspase- independent necroptosis. They also showed that cIAP1$^{-/-}$ or cIAP2$^{-/-}$ mice exhibited increased macrophage cell death than wildtype mice when infected with intracellular bacteria *Listeria monocytogenes* due to necroptosis that lead to increased bacterial burden in the mice(McComb et al., 2012).

Another group elucidated that when cIAP2$^{-/-}$ mice infected with influenza A virus had increased mortality. They reported that in absence of cIAP2, lung epithelial cells were dead due to necroptosis which caused exacerbated bronchiole epithelial degeneration. These symptoms were rescued in cIAP2$^{-/-}$ mice by pharmacological inhibition of RIPK1 or genetic deletion of r*ipk3* (Rodrigue-Gervais et al., 2014).

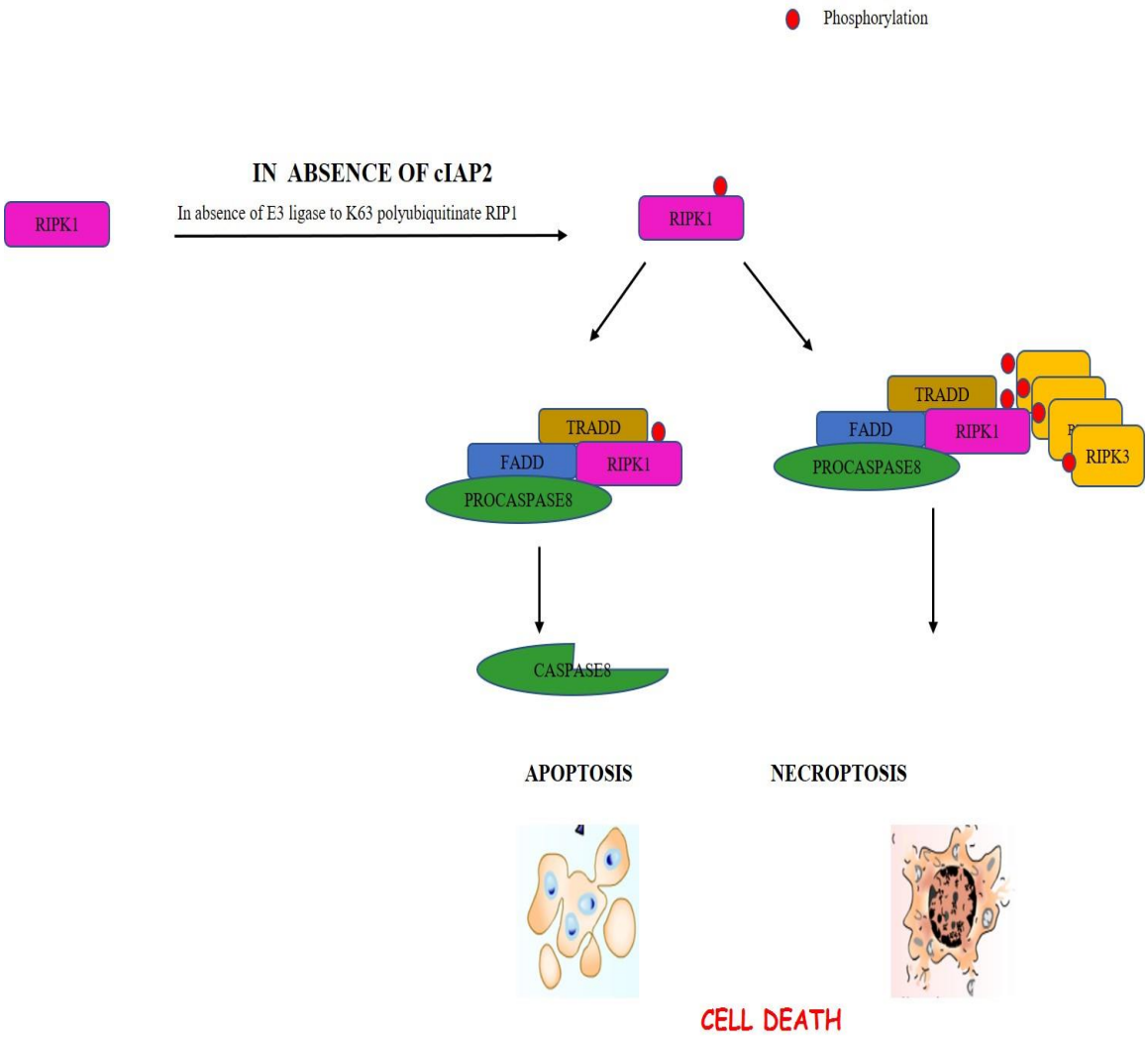


Figure 5-4: Absence of cIAP2 in TNF-R1 signaling leads to apoptosis or necroptosis mediated cell death

In absence of cIAPs, de-ubiquitinated RIPK1, promote formation of complex containing TRADD, FADD, c-FLIP and procaspase-8, which on activation causes caspase-8 dependent apoptosis. When caspase-8 activation is inhibited, auto- and cross-phosphorylation events between RIPK1, RIPK3 and MLKL lead to the formation of a RIPK3-MLKL-dependent necrosome complex, which induces cell death via necroptosis

iii. Regulatory function of cIAP2 in non-canonical NF-κB pathway

Other members of TNF receptor family members, such as TWEAK, BAFF, CD40 ligands that are responsible for non-canonical activation of NF-κB pathway, are negatively regulated by cIAP2.

In resting cells, E3 ligases such as cIAP1, cIAP2, TRAF2, TRAF3 adds K48-linked polyubiquitin chains on NIK and ultimately cause its degradation. Upon activation of the receptors, cIAP1/cIAP2/TRAF2 are recruited to the receptors that allows for addition of K48-linked ubiquitination to TRAF3 and its degradation. This permits stabilization of the NIK that further phosphorylates and activates IKKα. IKKα in turn phosphorylates p100 and causes its processing to form p52 that forms complex with RelB subunit. This complex can translocate to nuclei and induce target genes.(Giardino Torchia et al., 2013b; Razani et al., 2010; Sun, 2011)

In the absence of cIAPs, NIK cannot be degraded by TRAF3. Therefore, NIK is accumulated in the absence of ligands which leads to activation of the non-canonical NF-κB pathway. (Figure 1-5)

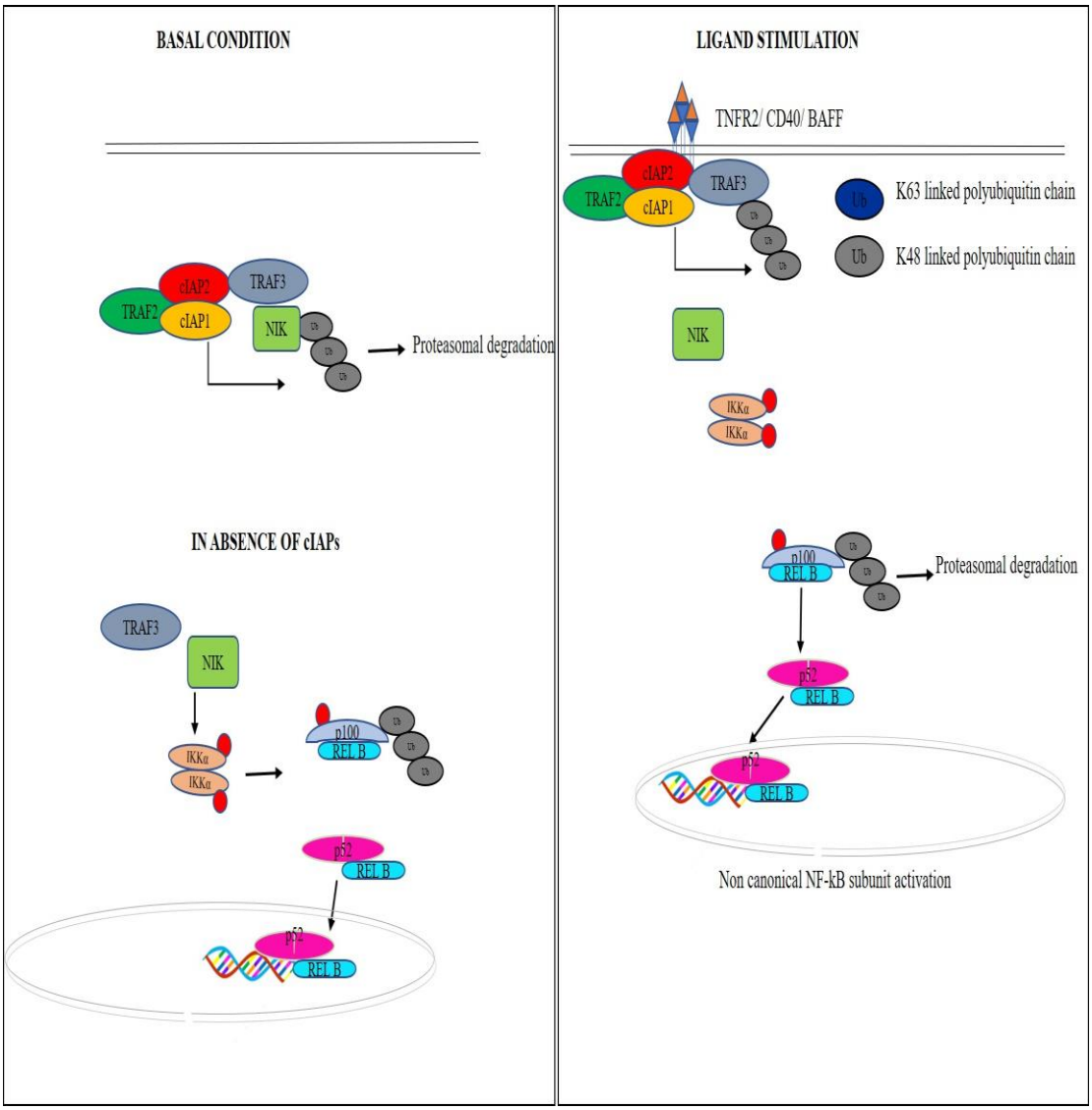


Figure 1-5: Role of cIAP2 in non-canonical NF-κB pathway.

(A) At basal condition, cIAP2 can cause degradation of NIK and thus inhibits activation of non-canonical NF-κB pathway. In absence of cIAPs, NIK accumulates and activates the non-canonical NF-κB pathway in absence of ligand.

(B) Activation of the TNFR2 receptors family can recruit E3 ligases such as cIAPs, TRAF3 at the receptor. This allows for the K48- linked polyubiquitination of TRAF3 and its degradation that consequently activate NIK induced non-canonical NF-κB pathway.

1.2.4 Role of cIAP2 in IL-1 signaling

Previously our group reported that interleukin1(IL-1) induces expression and activation of IRF-1, a transcription factor that upon activation stimulate expression of proinflammatory chemokine like CXCL10 and CCL5 which recruits adaptive immune cells to the site of inflammation .(Harikumar et al., 2014).

Upon stimulation with IL-1, the IL-1R receptors recruits MyD88 adapter, IRAK4, IRAK1, MEKK3, and TRAF6. Phosphorylation of IRAK1, and TRAF6-dependent K63-polyubiquitination chain that allows for the recruitment of the TAK/TAB1/TAK2 and IKKα/β/γ complexes and followed by activation of MAP kinases and NFκB pathway. These events induced the expression of IRF1, cIAP2 and a plethora of cytokines. The newly synthesized IRF1 is then K63polyubiquitinated by TRAF6-associated cIAP2. This K63linked polyubiquitination is regulated by intracellular S1P ("delayed" cIAP2/S1P regulated response). In turn, IRF1 translocate to the nucleus and activates expression of chemokines.

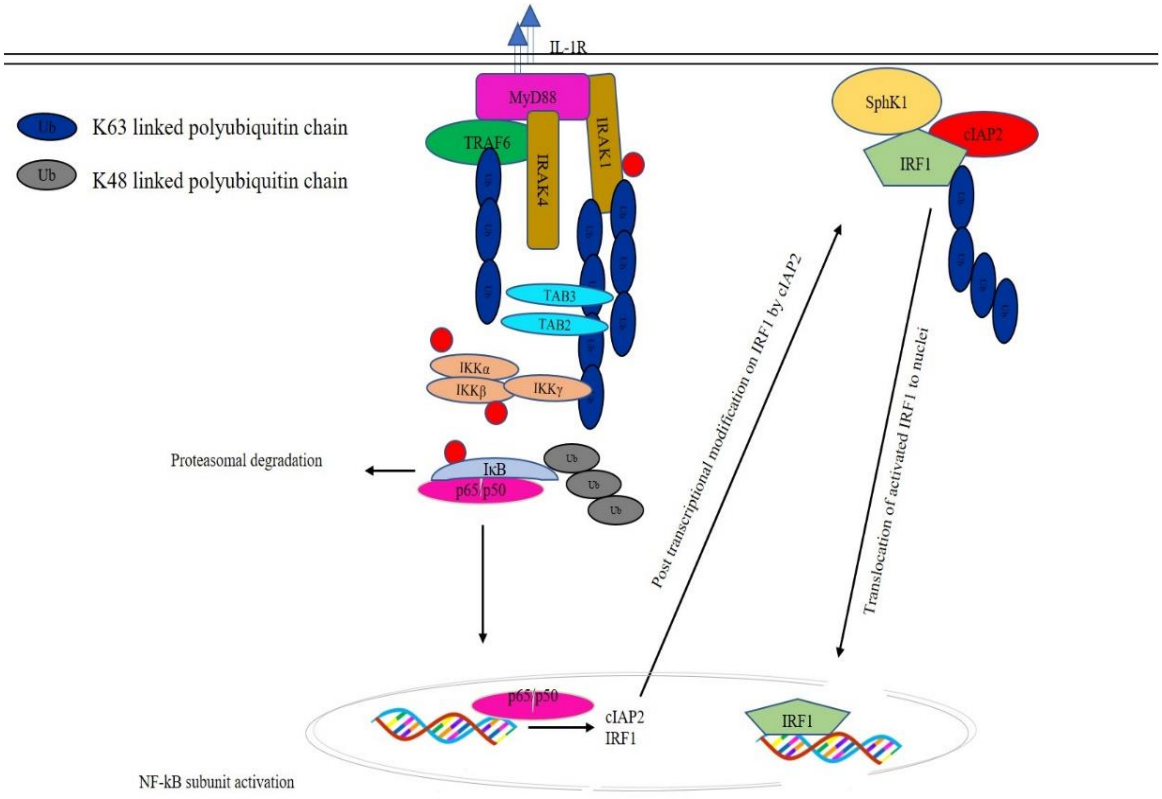


Figure 1-6: Role of cIAP2 in IL-1 signaling pathway

Upon stimulation with IL-1, the IL-1R, the expression of IRF-1 and cIAP2 is induced by NF-κB pathway. The newly synthesized IRF1 is then K63 polyubiquitinated by cIAP2 regulated by S1P. Activated IRF1 translocate to the nucleus and induce transcription of chemokines.

1.2.5 IAP antagonist SMAC and its implication in inflammation

During inflammation, the IAP family plays an important role in cell death evasion, which is a classic hallmark of cancer(Dubrez et al., 2013; LaCasse et al., 2008; Philchenkov and Miura, 2016). Currently, small-molecule antagonists of the IAPs, known as Smac mimetic compounds, are in development as cancer therapeutics. Smac mimetics are composed of the N-terminal four amino acid stretch found on the endogenous IAP antagonist Smac. Interaction of Smac mimetics with cIAPs enhances their E3 ligase activity, promoting their self-autoubiquitination and the proteasomal degradation (Fulda and Vucic, 2012; Varfolomeev et al., 2007). Smac mimetic–induced degradation of cIAPs cause accumulation of NIK, which activates non-canonical NF-kB signaling, and the upregulation of proinflammatory NF-kB target genes, including TNF-α. TNF-α can then stimulate the TNFR-1 receptor and, in the absence of cIAPs, can trigger caspase-8 mediated apoptosis or RIPK3 mediated necroptosis (Bertrand et al., 2008; Petersen et al., 2007).

A recent study has shown that the administration of Smac mimetics in mice converts M2 macrophages to an M1 phenotype, promoting the secretion of proinflammatory cytokines, including TNF-α. This led to necrosis of cancer cells in mice (Lecis et al., 2013).

Currently, five Smac mimetics are being used in phase I/II clinical trials as cancer therapeutics. In several trials, patients have demonstrated tolerance for Smac mimetics. However, in other studies toxicities, including cytokine release syndrome, has been observed (Infante et al., 2014).

Smac mimetic-stimulated upregulation of cytokines has been shown to act as an essential mediator for tumor cell death. Therefore, the side effects of Smac mimetics requires further examination before confirming its credibility as a treatment for cancer.

Although cIAP2 has been recognized as a regulator of cell death and immune signaling pathways, the role of cIAP2 in neuroinflammation remains elusive. Therefore, in this project we explored the role of cIAP2 in innate and adaptive immunity-mediated neuroinflammation.

CHAPTER 2

Materials and Methods

2.1 Mice

cIAP2$^{-/-}$ mice were provided by Dr. Korneluk, University of Ottawa and were housed in pathogen-free facilities according to guidelines of VCU Institutional Animal Care and Use Committee and the mouse protocols were approved by the institutional IACUC.

2.2 Experimental autoimmune encephalomyelitis model of Multiple sclerosis

EAE was induced in wildtype and cIAP2-deficient mice. Mice were immunized twice on day 0 and day 7, subcutaneously with 250 µg MOG (35–55) peptide (AnaSpec, Fremont, CA) emulsified in CFA containing 500 µg Mycobacterium tuberculosis H37Ra (Difco, Detroit, MI) on days 0 and 3, each mouse was injected intraperitoneally with 0.2 µg of purified *Bordetella pertussis* toxin (Enzo Life Sciences, Farmingdale, NY). Mice were weighed, and severity of the disease was scored daily for neurological signs using a five - point scale: 0, no symptoms; 1, limp tail; 2, limp tail with loss of righting; 3, paralysis of single hind limp; 4, paralysis of both hind limps; and 5, moribund state or death. Mice were sacrificed on the day 12 of second immunization. Brain tissue was collected for flow cytometry while spinal cord was collected for RNA analysis and immunostaining.

2.3 LPS induced neuroinflammation

Wildtype and cIAP2$^{-/-}$ mice were injected with PBS or lipopolysaccharide (5mg/kg) by intraperitoneal injection. After 6hours, the animals were sacrificed to harvest liver and brain tissues. These tissues were flash frozen, stored at -80C and used for mRNA analysis.

2.4 Reconstitution of bone marrow in the mice

Mice were irradiated for 6 min 15 sec at an intensity of 550 centi-gyre at an interval of 2 hours. Bone-marrow from naïve mice were harvested from the femur and tibia of the naïve wildtype and cIAP2$^{-/-}$ mice. 5 million bone marrow cells were injected in the irradiated mice by tail vein injection. EAE was induced in these mice after 8 weeks of reconstitution of bone marrow.

2.5 Genotyping and confirmation of reconstitution

To isolate DNA from the bone marrow, the cells from the femur were flushed out with PBS. To isolate DNA from the cells, the cells were pelleted by centrifugation and digested overnight in 100µl of PCR tail mix (Viagen Biotech Direct PCR Lysis Reagents) at 55^{0}C to isolate DNA. The concentration of the DNA from the digest was quantified using nanodrop. 100ng of DNA was amplified using forward and reverse primers for cIAP2 and lacZ forward and reverse primers and ran on DNA electrophoresis gel.

2.6 Blood brain barrier permeability assay

100µl of sodium fluorescein dye (100mg/ml) was administered via intraperitoneal injection. After 45min, blood was collected by cardiac puncture. Mice were perfused with PBS and CNS tissues were harvested, homogenized in PBS, clarified by centrifugation, precipitated in 1% trichloroacetic acid, and neutralized with borate buffer. Fluorescence was excited at 485nm and read at 528nm was determined using microplate reader (Parkin Elmer 2000). Fluorescein concentration was calculated from standard curve and tissue fluorescence values were normalized to plasma fluorescence value of the same mouse.

2.7 Immunoblotting tissue from spinal cord

Tissue lysates from spinal cord were prepared in 20 mM tris pH 7.4 containing 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.5% NP-40, 1 mM Sodium orthovanadate, 1 mM PMSF, 1:500 protease inhibitor cocktail (Sigma-Aldrich). Western blots were performed using 12% polyacrylamide gel. Blots were incubated with anti RIPK1(B D Biosciences), anti RIPK3(Santa Cruz Biotechnologies), or GAPDH (Cell signaling).

2.8 Isolation and flow cytometry analysis of CNS cells

Brains from the mice were homogenized using Wheaton Dounce glass tissue grinders. They were further centrifuged at 1500 rpm for 5 min at 4°C. Pelleted cells were resuspended in 10 ml of 30% Percoll (Amersham Bioscience) and centrifuged onto a 70% Percoll and centrifuged for 30min at 2600rpm. Cells that were collected at the 30–70%

interface were strained through 70 µm filter and stained with fluorescence-conjugated monoclonal antibodies against CD45(clone 30-F11), CD11b (clone M1/70), CD4 (clone GK 1.5), CD8 (clone 53-6.7), and Ly6C (clone HK1.4), F4/80 (clone BM8) and isotype controls were used to analyze cells (Biolegend). Fluorescence data were collected on Fortessa and analyzed using FACS-Diva software (BD Biosciences).

2.9 Quantification of caspase- 8 activity in spinal cord tissue

Tissue extracts were prepared from lumbar of the spinal cord from mice and flash frozen in liquid nitrogen. Frozen tissue was powdered and resuspended in 450µl of frozen extraction buffer (20 mM HEPES, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 1 mM EDTA; pH 7.5) Samples were homogenized using sonicator and centrifuged, and the supernatant (100 µg protein/assay) was used for the assessment of caspase-8 activity using the reagents and methods supplied in Caspase-Glo® 8 Assay kit (Promega). Results were expressed as relative luminescent units per 100 µg protein.

2.10 Histological analysis

The mice were fixed with 4% paraformaldehyde solution cryopreserved with 30% sucrose for 48hours Then these tissues were paraffin embedded. 20µM thick sections were stained with hematoxylin and eosin. The images were taken by Zeiss Axio imager A1 microscope.

2.11 Luxol fast blue staining

Paraffin embedded spinal cord lumbar tissues (20µM) were deparaffinized in xylene and stained with Luxol fast blue overnight at 60C. The sections were differentiated in lithium carbonate and 70% ethanol till gray matter was clear and white matter was sharply defined. All these sections were also counterstained with hematoxylin. The images were taken by Zeiss Axio imager A1 microscope and processed using Zen 2012 Blue acquisition software (Zeiss Inc.) Quantitative analysis of the cells were determined using Image J software

2.12 Immunofluorescence

The mice were fixed with 4% paraformaldehyde solution cryopreserved with 30% sucrose for 48hours. The tissues were then embedded in Tissue-Tek OCT Compound (VWR, Radnor, PA, USA) and stained as sectioned with 30µm thickness. The primary antibodies used in this study include: anti GFAP (Cell signaling technology, Danvers, MA (1: 300)) and anti IBA ((Wako Chemicals USA Inc., Richmond, VA USA (1:1000), anti CC1 (Millipore, (1:300)).

Secondary antibodies were Alexa Fluor Dyes (ThermoFisher Scientific, Walham, MA, USA). Nuclei were counterstained with Hoechst. Sections were examined using LSM 700 confocal microscope. Sections were examined on a Zeiss LSM700 confocal microscope using a 40x oil-immersion lens. Maximum projection images from confocal z-stacks were acquired Care was taken to minimize pixel saturation while imaging each z-stack.

Captured images were processed using Zen 2012 Blue acquisition software (Zeiss Inc.) Quantitative analysis of the cells were determined using Image J software.

No fluorescence crossover was found between the channel and images were collected separately using appropriate laser excitation.

2.13 TUNEL assay

As mentioned above, lumbar tissue was in embedded in OCT with section thickness of 30µm. They were processed for terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL) as per as manufacture's protocol (Roche) and co-stained for either IBA1or CC1 antibodies (details as mentioned above). Images were either captured on Zeiss Axio imager A1 microscope or LSM 700 confocal microscope.

2.14 Isolation and culturing of primary cells from mice brain

Mixed glial culture was harvested from P0-P3 pups. After removal of meninges, whole brain was minced and trypsinized for 30 min at 37 °C followed by sieving them through 100µM nylon cell strainer to obtain single-cell suspensions. Cells were then washed and were cultured in poly-D-lysine-coated flasks in DMEM medium with 10% FBS. The culture was intermediately fed after every 2 days.

2.15 Preparation of macrophages from bone marrow of mice

Bone marrow cells were isolated from the femur of the mice and cultured in 10% FBS containing RPMI 1640 media. For differentiation of macrophages, the media was supplemented with 30 ng/ml of M-CSF (Biolegend) for 5 days. The adherent cells were used for further experiment.

2.16 Antigen presentation assay

Bone marrow derived macrophages were obtained using the protocol mentioned above. were stimulated with IFN-g (50ng/ml) for 24 hours followed by treatment with OVA antigen conjugated with fluorochrome AF-647 Ova (50ng/ml). After next 24 hours, the cells were harvested and quantified for APC positive cells and MHC II expression on the cells (Biolegend) by flow cytometry.

2.17 Lactate dehydrogenase assay

The mixed glial cells or microglial cells isolated *in vitro* (as mentioned above) were treated with LPS (100 ng/ml), TNF-α (30 ng/ml) and IL-1β (20n g/ml) for 24 hours. The lactate released by the dead cells were quantified using LDH assay kit (Dojindo Molecular Technologies, Inc) as per manufacturer's protocol.

2.18 Quantitative PCR

The lumbar tissue from spinal cord, liver or brain were flash freezed in liquid nitrogen. Tissues were grinded in cold mortar and pestle and RNA was harvested using Trizol (Invitrogen, Grand Island, NY). 1 µg RNA was reverse-transcribed using the High Capacity cDNA Archive kit (Applied Biosystems). The cDNAs were diluted 10-fold (for the target genes) and 100-fold for GAPDH (housekeeping gene) and amplified using (CFX CONNECT ^{TM} Real time system, Biorad). All gene expression levels were normalized to GAPDH and presented as a fold induction as compared to non-treated controls or PBS injected controls (as indicated in the text).

For cell culture, mixed glial cells were treated at different time with TNF-α (30 ng/ml) or IL-1β (20 ng/ml) for 24hours and RNA was isolated by Trizol.

2.19 Statistical analysis

Statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software, Inc. La Jolla, CA). Quantitative data are expressed as mean ± SEM or mean ± S.D. (as specified). Sample size is indicated for each figure. Differences across groups with multiple comparisons were analyzed with Two-way ANOVA with Tukey's Multiple Comparison. With the goal of achieving stringent and accurate measures of difference between different groups, the significance level of $p<0.05$ was considered statistically significant.

CHAPTER 3

cIAP2 propagates neuroinflammation induced by lipopolysaccharide

3.1 Introduction

Entry of pathogens induces an inflammatory response in the host that helps invading of pathogens and cleaning of debris. However, the entry of bacteria or exposure to lipopolysaccharide (LPS), a bacterial endotoxin, can also induce hyperinflammation. LPS binds to the Toll like receptors (TLR4) which initiates proinflammatory cascades, allowing for the recruitment of signaling proteins that ultimately activate NF-κB and MAP kinase pathways and induce expression of proinflammatory cytokines and chemokines that recruit immune cells. A single dose of LPS can trigger activation of macrophages to induce generation of inflammatory cytokines such as TNF-α and IL-1β, resulting in systemic cytokine storm (Płóciennikowska et al., 2015). This inflammation can lead to sepsis, hypotension, tachycardia, systemic edema and finally multiple organ failure.

cIAP2 is a key survival factor induced by the activation of the NF-κB pathway. It has been shown that cIAP2$^{-/-}$ mice displayed a robust resistance to the systemic administration of LPS and an attenuated inflammatory response. They also demonstrated that macrophages deficient of cIAP2 are highly susceptible to apoptosis in LPS mediated proinflammatory environment (Conte et al., 2006).

Also, another study reported that cIAP2 deficient microglia upon treatment with LPS, undergo caspase-3-mediated apoptosis. However, CNS is usually protected by the blood brain barrier, and systemically administered LPS cannot enter the brain. Qin et al showed that systemic administration of LPS peripherally elevates the level of proinflammatory

cytokine such as TNF-α. Consequently, TNF-α can cross the blood brain barrier to activate microglia at several regions of brain as substantia nigra, hippocampus and cortex(Iravani et al., 2014; Järlestedt et al., 2013). This activation of microglia promotes the secretion of proinflammatory cytokines including TNF-α, IL-1β or IL-6 and initiate a second wave of inflammation in the central nervous system, which can persist for months leading to chronic neuroinflammation and progressive neuro-degradation. (Banks et al., 2015; Qin et al., 2007)

We investigated weather cIAP2 affects neuroinflammation induced by activation of innate immune response with systemic LPS.

3.2 Results

3.2.1 cIAP2 is critical for systemic cytokine storm induced by LPS

Binding of LPS to TLR-4 receptors on the macrophages activate NF-κB or MAP kinase pathways and generates a cytokine storm that ultimately results in endotoxin shock, and sepsis. To investigate if cIAP2 affects innate immune response, wildtype and cIAP2 deficient mice were systematically administered with LPS. In response to LPS, Kupffer cells, macrophage-like cells in the liver secrete proinflammatory cytokines. Concurrently, cytokine stimulated hepatocytes secrete acute phase proteins. Increased expression of these acute phase proteins can trigger sepsis and are used as a marker of inflammation. We observed that the expression of acute phase proteins such as Serum Amyloid A, Serum Amyloid P and Alpha -1- glycoprotein were comparable in the wild type and cIAP2$^{-/-}$ mice livers (Figure 3.1-B), indicating ongoing acute phase response.

During inflammation, Kupffer cells in the liver are one of the major source of circulating proinflammatory cytokines (Kumins et al., 1996). To understand if cIAP2 modulates expression of cytokines systemically, we investigated the expression of the proinflammatory cytokines in the liver. We found that the expression of TNF-α was significantly reduced in the cIAP2$^{-/-}$ mice in comparison to the wildtype littermate. However, the expression of cytokines such as IL-1β and IL-6 was comparable. (Figure 3.1-C)

Previously Conte et al showed that cIAP2 deficient peritoneal macrophages die in response to LPS and thus the cIAP2$^{-/-}$ mice are protected from LPS endo-toxin shock.

However, we observed a similar elevated level of CD14 expression (a macrophage specific receptor) in the liver of upon LPS treatment the wildtype and cIAP2$^{-/-}$ mice (Figure 3.1 D), suggesting that although cIAP2 deficient Kupffer cells survived, the expression of some of the proinflammatory cytokines was attenuated systemically.

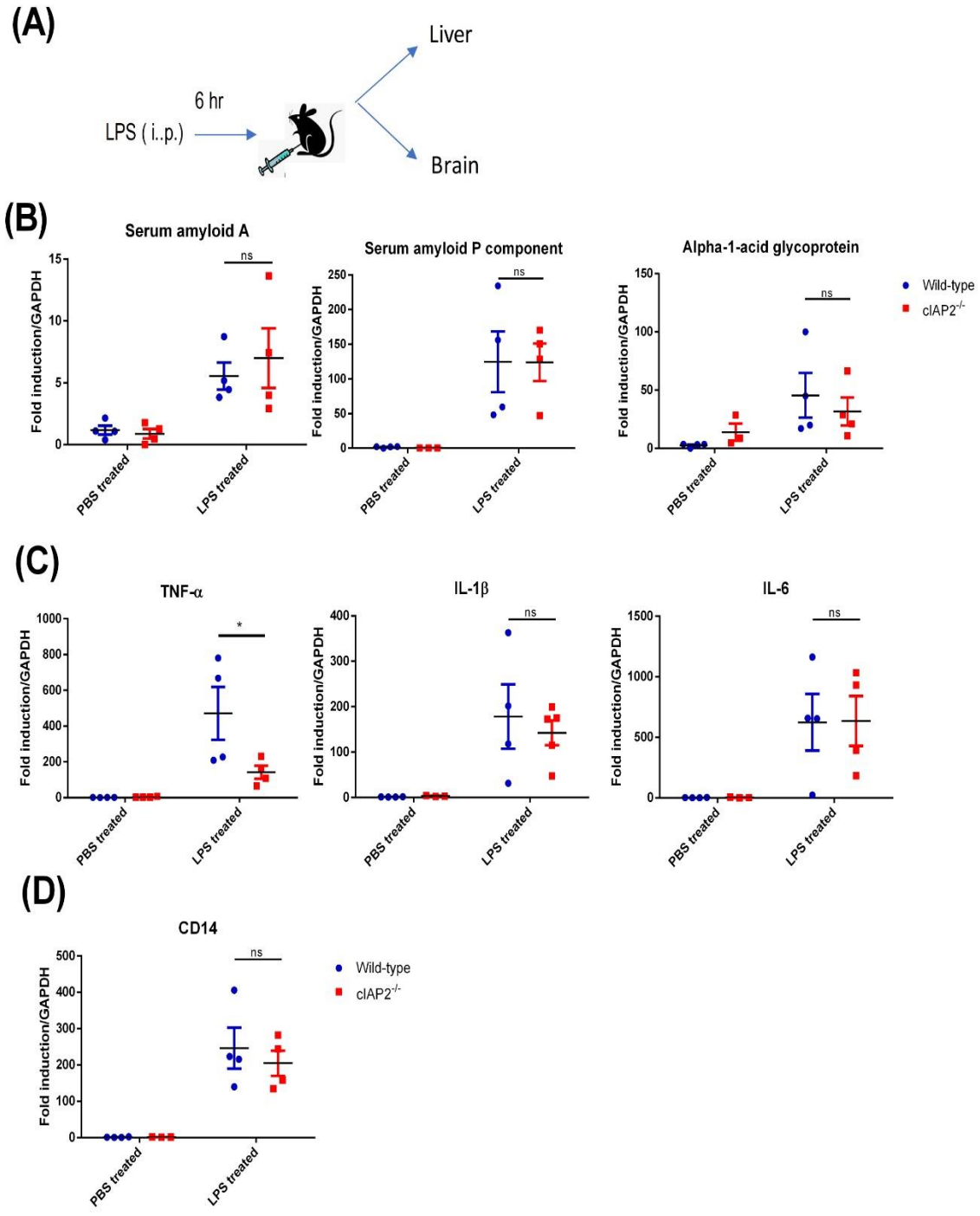

(A)
Liver
6 hr
LPS (i..p.)
Brain
(B)
Serum amyloid A
Serum amyloid P component
Alpha-1-acid glycoprotein
Fold induction/GAPDH
ns
PBS treated
LPS treated
Wild-type
cIAP2-/-
(C)
TNF-α
IL-1β
IL-6
*
(D)
CD14

Figure 3- 1: cIAP2 regulates expression of proinflammatory cytokines in the liver upon systemic administration of LPS

(A) Schematic representation of systemic LPS treatment. Wildtype and cIAP2$^{-/-}$ mice were systemically administered with PBS or 5 mg/kg of LPS. Liver and brain were harvested from the mice after 6 hours. The relative gene expression of (B) the acute phase proteins including Serum Amyloid A, Serum Amyloid P and Alpha-1-glycoprotein analyzed in the liver, (C) proinflammatory genes (TNF-α, IL-1β and IL-6) and (D) CD14 was quantified in the livers. 4 mice per group, *$p<0.05$, two-way ANOVA. Error bar = SEM

3.2.2 cIAP2$^{-/-}$ mice display an attenuated neuroinflammation in response to systemic LPS

TNF-α secreted by LPS stimulated by macrophages can cross the blood brain barrier to activate glial cells. Since we observed dampened expression of TNF-α systemically, we asked if lack of cIAP2, can also affect neuroinflammation induced by systemic exposure to LPS. To answer this question, we harvested the brains from the wildtype and cIAP2$^{-/-}$ mice following systemic LPS administration. We observed that the expression of cIAP2 increased approximately to 20 times in the CNS of the wildtype mice compared to that of PBS treated mice (Figure 3.2-A), suggests that cIAP2 may be important during neuroinflammation in the CNS.

Interestingly, we also observed that the expression of TNF-α, IL-1β and IL-6 is remarkably reduced in the brains of cIAP2$^{-/-}$ mice in comparison to the wildtype littermate (figure 3.2-B). Also, by probing for the expression of CD14 and LCN2, markers for activated microglia and astrocytes, we found a decreasing trend of glial activation in cIAP2$^{-/-}$ mice (Figure 3.2 C). We conclude that cIAP2$^{-/-}$ mice had lesser extent of glial activation that led to reduced expression of cytokines during an acute neuroinflammation induced by systemic LPS.

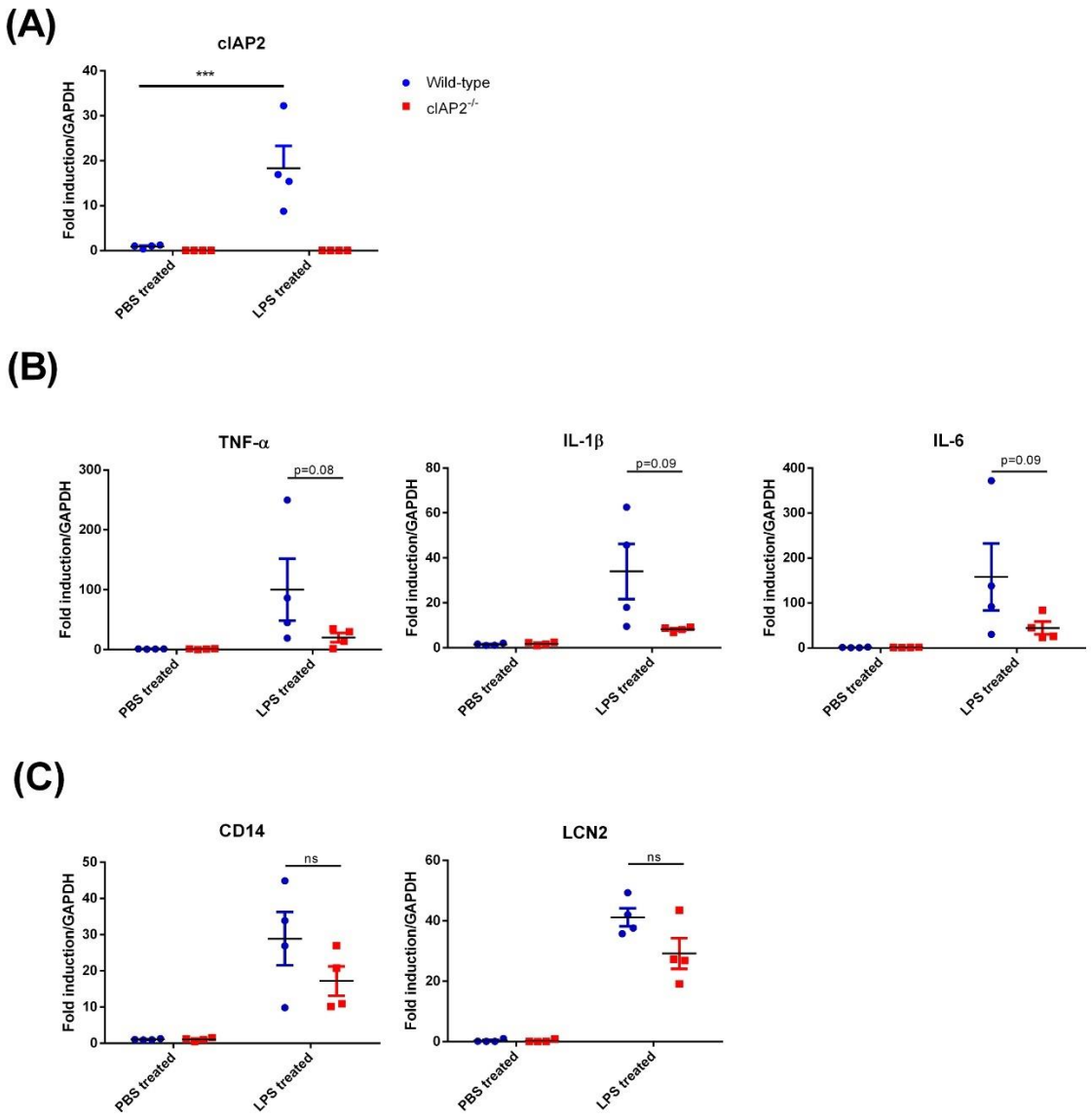

(A)
cIAP2
***
Fold induction/GAPDH
Wild-type
cIAP2-/-
PBS treated
LPS treated
(B)
TNF-α
p=0.08
IL-1β
p=0.09
IL-6
p=0.09
(C)
CD14
ns
LCN2
ns

Figure 3- 2: Loss of cIAP2 reduces neuroinflammation in LPS treated mice.

Gene expression of (A) cIAP2, (B) proinflammatory cytokines (C)Markers of reactive microglia and astrocyte were quantified by qPCR in the brains of wildtype and cIAP2$^{-/-}$ mice after systemic LPS administration. 4 mice per group, *$p<0.05$, two-way ANOVA. Error bar = SEM

3.3 Chapter summary

cIAP2 is a ubiquitously expressed protein and its expression dramatically increases within hours of inflammation. Our data show that the hepatocytes of wildtype and cIAP2$^{-/-}$ mice express similar level of acute phase proteins after systemic LPS administration. However, the expression of the proinflammatory cytokines produced by the Kupffer cells in the liver, such as TNF-α, were significantly diminished in cIAP2$^{-/-}$ mice. These data corelate with previous studies, that reported that attenuated systemic cytokine production in cIAP2$^{-/-}$ mice due to apoptotic death of peritoneal macrophages induced by LPS(Conte et al., 2006).

It has been reported that peripherally circulating TNF-α can enter the CNS and activate microglia within a short duration of time post systemic LPS treatment.(Qin et al., 2007) Our results show that, although limited amount of peripheral TNF-α was generated in cIAP2$^{-/-}$ mice, it was sufficient enough to activate microglia and astrocytes. However, these glia cells failed to produce proinflammatory cytokines such as TNF-α, IL-1β and IL-6. Our findings revealed that cIAP2 was critical in regulating neuro-inflammation induced by LPS.

It has been well documented that cIAP2 is an E3 ligase playing a crucial role in TNF-α signaling. Once TNFR1 is activated, RIPK1 kinase, is recruited to the receptor. cIAP1/cIAP2/TRAF2, an E3 ligase complex, can together efficiently trigger K63 linked polyubiquitination of RIPK1. This provides a platform for recruitment of IKK complex,

which further degrades IκB, releasing NF-κB subunits. This allows the initiation of pro-survival NF-κB signaling to produce pro inflammatory cytokines and chemokines, to recruit immune cells at the site of inflammation. It has been demonstrated by many studies that lack of cIAPs (together or individually) can attribute to inhibition of NF-κB pathway thus ablating inflammation.

However, some studies also demonstrate that absence of cIAP2 leads to RIPK1 phosphorylation and activate caspase-8 mediated apoptotic cell death. (Wang et al., 1998)

Therefore, there are two likely possibilities by which cIAP2 can regulate neuroinflammation (1) by activation of NF-kB signaling or (2) by modulating macrophage survival. Thus, further studies are required to understand the mechanism involved in cIAP2 mediated protection from the cytokine storm induced by systemic LPS treatment.

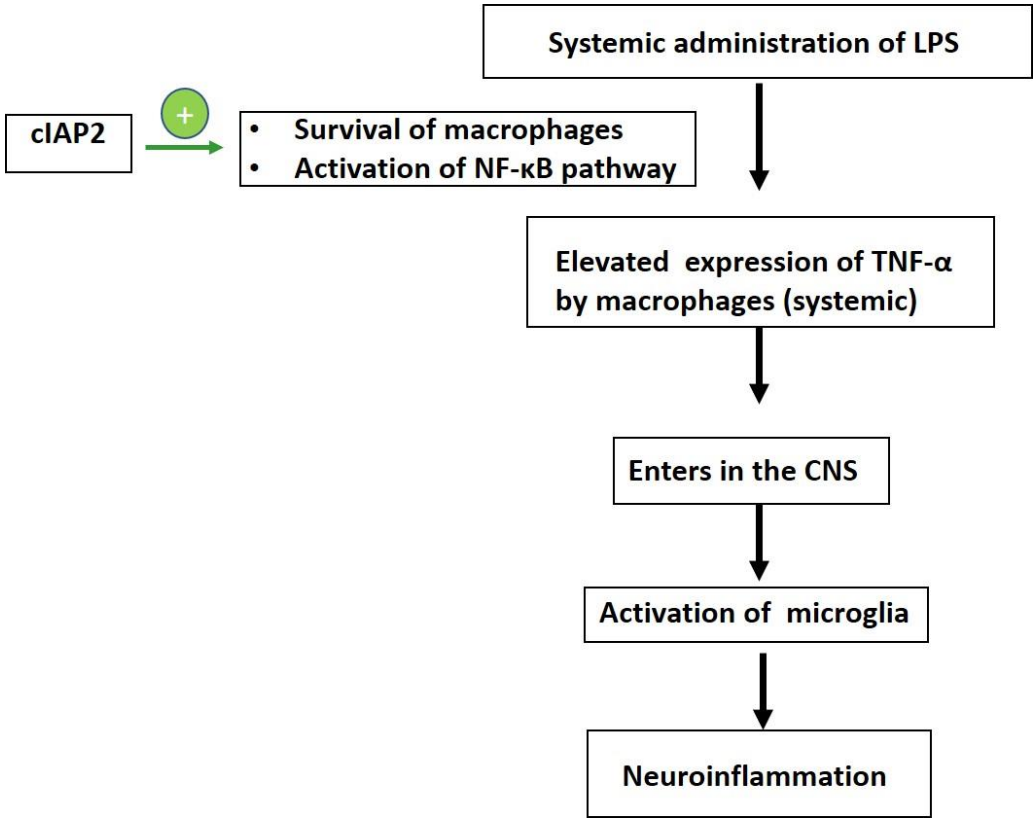


Figure 3- 3 Role of cIAP2 in neuroinflammation mediated by innate immune response during systemic administration of LPS

cIAP2 in the macrophages (Kupffer cells) regulates the expression of TNF- α upon exposure to LPS. These cytokines can cross the blood brain barrier and consequently activate microglia to generate proinflammatory cytokines.

CHAPTER 4

cIAP2 in CNS impedes neuroinflammation induced by adaptive immunity

4.1 Introduction

Sterile inflammation is essential for a proper development of brain and tissue repair. But once uncontrolled, it leads to detrimental pathogenesis associate with several diseases. For the longest time, it was believed that the CNS is an immunologically privileged site due to protection rendered by the blood brain barrier. However, CNS can be attacked by various viruses, bacteria, fungi or protozoa that cause inflammation. Neuroinflammation is also evident in various CNS disorders, including Alzheimer's, Parkinson's and Huntington's disease, traumatic brain injury, amyotrophic lateral sclerosis, and multiple sclerosis (MS) which are associated with non-pathogenic sterile neuroinflammation.

MS is an autoimmune disease characterized by multiple lesions in the white matter of the brain and spinal cord (Noseworthy et al., 2000; Pender, 2000). MS is an autoimmune disease mediated by CD4+ T cells, including Th1, Th17, Th9 and γδ T cells, which attack the myelin sheath, oligodendrocytes and neurons (Elyaman and Khoury, 2017; Komiyama et al., 2006; Zhang et al., 2017). The factors that cause the onset of MS remain elusive, however it has been proposed that viral infection, smoking, and vitamin D deficiency are major risk factors (Koch et al., 2013; O'Gorman et al., 2012). Further, the mechanisms underlying the pathophysiology of MS are unknown.

Early MS lesions are caused by trafficking of lymphocytes or macrophages that are activated peripherally and cross the blood brain barrier. These recruited cells release various proinflammatory cytokines, which activate residential cells and polarize them

towards M1 proinflammatory microglia and astrocytes. The cytokines associated with MS includes IL-1β, TNF-α, IL-6, IFN-γ, CCL2 (Frei et al., 1991; Malmeström et al., 2006) and chemokines CCL5, CXCL10, CXCL12 that recruit $CD4^+$ T cells, monocytes and neutrophils (Franciotta et al., 2006). This inflammatory environment along with metalloproteases secreted by inflamed cells damage the blood brain barrier and attract more leukocytes to the CNS. These cascades of inflammatory programs accentuate myelin degradation, oligodendrocyte death and axonal loss, leading to neurological dysfunction. Simultaneously, immunoregulatory cells, such as Treg cells or M2 anti-inflammatory microglia, provide an anti-inflammatory milieu for tissue repair.

There are several animal models of MS. EAE can be induced in non-human primates and rodents using various methods. EAE can be induced by viral infections that demyelinate the CNS or by consumption of toxins, such as cuprizone, that selectively kill oligodendrocytes required for remyelinating axons. The most common model used to study demyelination and inflammation during MS is Experimental Autoimmune Encephalomyelitis (EAE). EAE is induced by the immunization of mice with antigens like myelin oligodendrocyte glycoprotein (MOG) with M*ycobacterium tuberculosis* extract and pertussis toxin. This model causes damage to the blood brain barrier and allows $CD4^+$ T cells in the CNS to proliferate (Constantinescu et al., 2011).

Not much has been reported regarding the role of cIAP2 in neuroinflammation. It has been reported that cIAP2 can modulate Caspase-3 in BV2 microglial cells, dictating microglia cell survival (Kavanagh et al., 2014b). Further, Kavanagh et al. showed that

cIAP2 has the potential to inhibit the cleavage of pro-Caspase-3 to p17/p12 Caspase- 3 complexes, directing cell death. The presence of cIAP2 can also promote orchestrated inflammation by activating PKC δ.

The studies by Torchia et al. (Gardam et al., 2011; Giardino Torchia et al., 2013b) suggest that cIAP2 controls non canonical NF-κB pathways in T and B adaptive immune cells to regulate its activity and proliferation, and modulate the architecture of secondary lymphoid tissues. However, it is unclear if deficiency in cIAP2 plays a role in autoimmunity mediated neuroinflammation, such as MS-related inflammation.

Our lab has recently reported that cIAP2 is an E3 ligase that promotes the assembly of K63 linked polyubiquitin chains on Interferon Regulatory Factor (IRF-1), a transcription factor required for expression of chemokines, such as CCL5 and CXCL10 (Harikumar et al., 2014). Additionally, others have shown that IRF1$^{-/-}$ mice are resistant to EAE (Ren et al., 2011). Thus, we speculated that absence of cIAP2 will prevent activation of IRF1, production of chemokines and recruitment of T cells, and consequently protect the cIAP2$^{-/-}$ mice from neuroinflammation.

4.2 Results

4.2.1 Exacerbated inflammatory responses of cIAP2$^{-/-}$ mice during EAE.

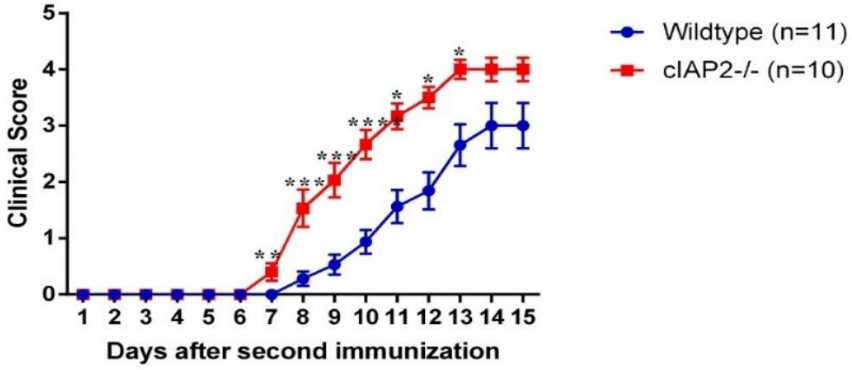
To assess the role of cIAP2 during the adaptive immune induced disease, we induced EAE in wildtype and cIAP2$^{-/-}$ mice. To our surprise, unlike the attenuated inflammatory response induced by the innate immune activation, cIAP2 deficient mice displayed a higher clinical score of EAE than that of wildtype mice (Figure 4-1 A). cIAP2$^{-/-}$ mice not only exhibited an earlier onset of disease but attained the peak symptoms of EAE (score 4) significantly faster than that of wildtype mice (Figure 4-1B, C). cIAP2$^{-/-}$ mice reached score 4 by day 12 while wildtype mice reached its maximum score of 4 by day 15. Further, we found that about 40% of cIAP2$^{-/-}$ mice died due to worsening of the symptoms while wildtype mice survived for a prolonged period of time (Figure 4-1 D).

Clinically, patients with MS present elevated levels of cytokines and chemokines. For instance, IFN-γ and TNF-α are cytokines elevated in the cerebrospinal fluid of MS patients. This can promote oligodendrocyte cell death and axonal damage(Cudrici et al., 2006; Ozawa et al., 1994; Wolswijk, 2000). IFN-γ secreted by NK cells and is required for CD4^{+} T cell differentiation. Cytokines, such as TNFα, IL-1β and IL-6 are secreted by monocytes, macrophages, endothelial cells and lymphocytes during the onset of the disease. These cytokines can also be released by microglia and astrocytes in the CNS and can sustain inflammation in MS patients, as well as in EAE animals(FILION et al., 2003; Sørensen and Sellebjerg, 2001).

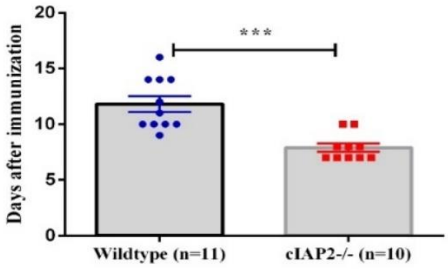
Higher levels of chemokines allow for the migration of immune cells toward the site of inflammation. Chemokines such as CCL5 and CXCL10 correlate with the accumulation of leukocytes in the CNS(Di Prisco et al., 2013; Mills Ko et al., 2014)

We analyzed the level of cytokines and chemokines in the spinal cords of wildtype and cIAP2$^{-/-}$ mice after inducing EAE. In agreement with the higher clinical scores demonstrated by cIAP2$^{-/-}$ mice, these mice expressed higher levels of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IFN-γ, when compared to wildtype mice (Figure 4.2). Chemokines such as CCL5 and CXCL10 were also significantly higher in cIAP2 deficient mice than in wildtype littermates (Figure 4-3).

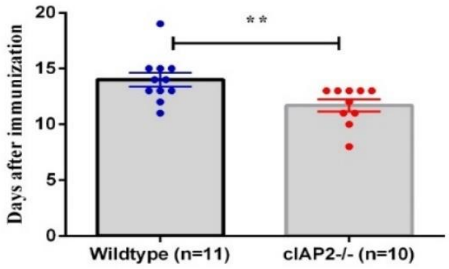
(A)


Clinical Score
Days after second immunization
Wildtype (n=11)
cIAP2-/- (n=10)

(B)


Days after immunization
Wildtype (n=11)
cIAP2-/- (n=10)

(C)


Days after immunization
Wildtype (n=11)
cIAP2-/- (n=10)

(D)

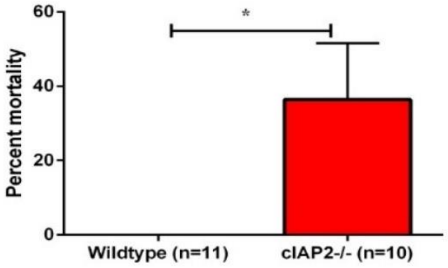

Percent mortality
Wildtype (n=11)
cIAP2-/- (n=10)

Figure 4- 1 cIAP2$^{-/-}$ mice exhibit exacerbated symptoms of EAE in comparison to wildtype mice.

EAE induced wildtype and cIAP2$^{-/-}$ mice were scored daily for 15 days after second immunization of MOG. (A) mean clinical score, (B) days required for the onset of the disease, (C) days required to reach peak symptoms, and (D) percentage of mortality are illustrated. Error bars = SEM; Student's t-test; *p<0.05; **p<0.01; ***p<0.001.

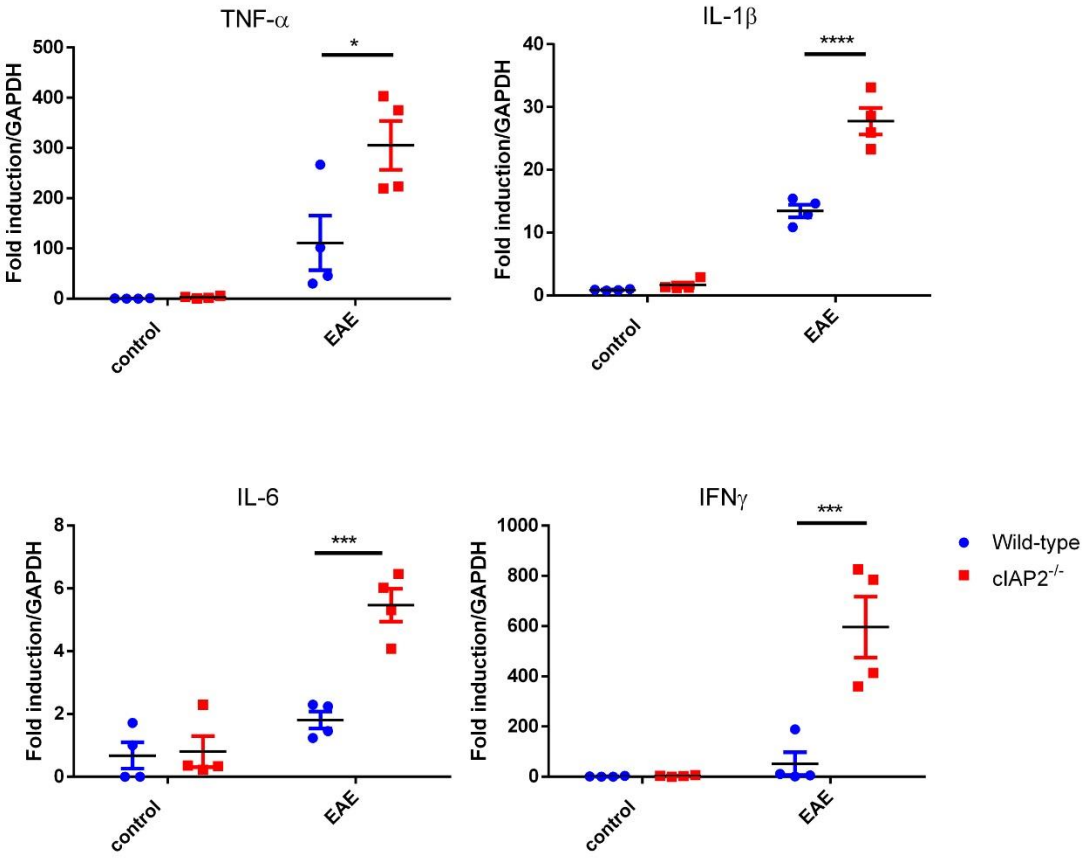

TNF-α
IL-1β
IL-6
IFNγ
Fold induction/GAPDH
control
EAE
*
****
***
***
Wild-type
cIAP2-/-

Figure 4- 2 Elevated levels of cytokines in the CNS of cIAP2$^{-/-}$ mice.

The expression of proinflammatory cytokines were analyzed in the lumbar region of the spinal cord of control mice (without MOG treatment) and EAE induced mice using real-time PCR 12 days after second immunization Each dot represents a mouse. *p<0.05, **p<0.01, ***p<0.001, ****p< 0.0001 comparing EAE cIAP2$^{-/-}$ vs wildtype mice; two-way ANOVA; error bars = SEM.

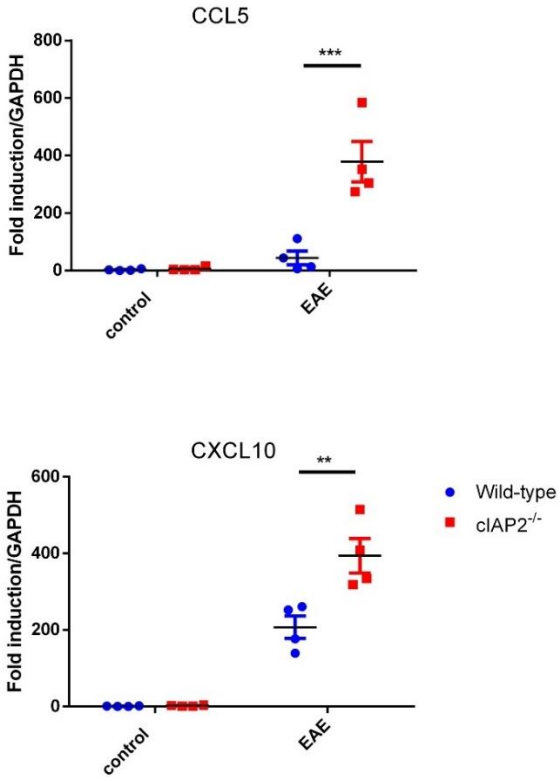

CCL5
Fold induction/GAPDH
800
600
400
200
0
***
control
EAE
CXCL10
Fold induction/GAPDH
600
400
200
0
**
control
EAE
Wild-type
cIAP2-/-

Figure 4- 3 Increased expression of chemokines in the CNS of cIAP2$^{-/-}$ mice.

The expression of CCL5 and CXL10 were analyzed in the lumbar region of the spinal cord of control mice (without MOG treatment) and EAE induced mice using real-time PCR 12 days after second immunization. **p<0.01, ***p<0.001, comparing EAE cIAP2$^{-/-}$ vs wildtype mice; two-way ANOVA; Error bars = SEM.

4.2.2 Absence of cIAP2 promotes the presence of inflammatory cells in the CNS

During EAE immune cells are recruited in the CNS. through the lumbar region of the spinal cord. We analyzed the histopathology of the lumbar region of the spinal cords of naïve and EAE induced mice. Our examination of the spinal cords from naïve wildtype and cIAP2$^{-/-}$ mice revealed no traces of infiltrated immune cells into the white matter of the lumbar. The spinal cord of the EAE induced wildtype mice showed moderate but significant infiltration of immune cells. In contrast, spinal cords from EAE induced cIAP2$^{-/-}$ mice demonstrated robust infiltration of immune cells across the tissue at multiple regions (Figure 4-4).

The hallmark symptom of MS is demyelination, where the myelin sheath or the oligodendrocyte cell bodies are destroyed by the inflammatory process. Hence, we investigated myelin sheath presentation in the lumbar tissue using Luxol fast blue dye. We observed that in comparison to EAE induced wildtype mice, cIAP2$^{-/-}$ mice exhibited extensive demyelination at the edge of the white matter. Importantly, wildtype and cIAP2$^{-/-}$ naïve mice demonstrated normal myelin sheath, suggesting that these histopathological features were not due to preexisting differences in the spinal cords (Figure 4-5).

Recent studies have demonstrated that T cells cause many of the pathologies of the autoimmune inflammatory diseases. The main effector T cells responsible for autoimmune inflammation associated with EAE are CD4^{+} T cells such as Th1, Th17 and γδ T cells.(Murphy et al., 2010; Zhang et al., 2017) These T cells are recruited to the CNS and play a pathogenic role in the development of autoimmune and chronic inflammatory

conditions. Therefore, we compared the recruitment of $CD4^+$ T cells to the brain of wildtype and $cIAP2^{-/-}$ mice after EAE induction. We found that in correlation with demyelination, cIAP2 deficient mice had significantly more $CD4^+$ T cell recruitment to the CNS than wildtype mice (Figure 4.6).

Along with immune cell infiltration, astrocytes are rapidly activated during EAE(Brambilla et al., 2014; Kothavale et al., 1995). This astrogliosis can be evidenced by increased expression of glial fibrillary acidic protein (GFAP). We investigated astrogliosis in the lumbar region of wildtype and $cIAP2^{-/-}$ mouse spinal cords by probing for the immunoreactivity of GFAP. In wildtype mice, EAE induced astrogliosis was identified by GFAP- staining. Significantly, the immunoreactivity of GFAP was significantly increased in the spinal cords of $cIAP2^{-/-}$ mice (Figure 4.7), indicating exacerbated activation of astrocytes.

It has been demonstrated that the myeloid cells in the spinal cord, namely microglia (CNS resident macrophages) and blood-derived macrophages, are critical for the pathogenesis of MS and EAE(Block et al., 2007). The activation of these cells contributes to the formation of lesions, myelin damage, phagocytosis and oligodendrocyte dysfunction (Hendricks,2005). Next, we used immunofluorescence to investigate the density and morphology of myeloid cells. The number and morphology of resting myeloid cells in the spinal cord of both naïve wildtype and cIAP2-/- mice were comparable. Interestingly, we found that EAE- induced wildtype mice had activated myeloid cells that lost their dendritic processes and transformed to phagocytic macrophages. Significantly, the number of these activated cells were significantly higher in cIAP2 deficient spinal cord. (Figure 4-8)

To verify if the massive infiltration of immune cells in the cIAP2$^{-/-}$ mice were due to an increase in blood brain barrier permeability, we quantified the peripherally injected sodium fluorescence accumulation in the CNS of the naïve and EAE mice. We found that there was no difference in the blood brain permeability between wildtype and cIAP2$^{-/-}$ mice either before or after EAE induction (Figure 4.9). These findings reveal that in correlation with the disease score, lack of cIAP2 resulted in increased infiltration of immune cells, astrogliosis and myeloid cell activation that correlated with the extensive demyelination.

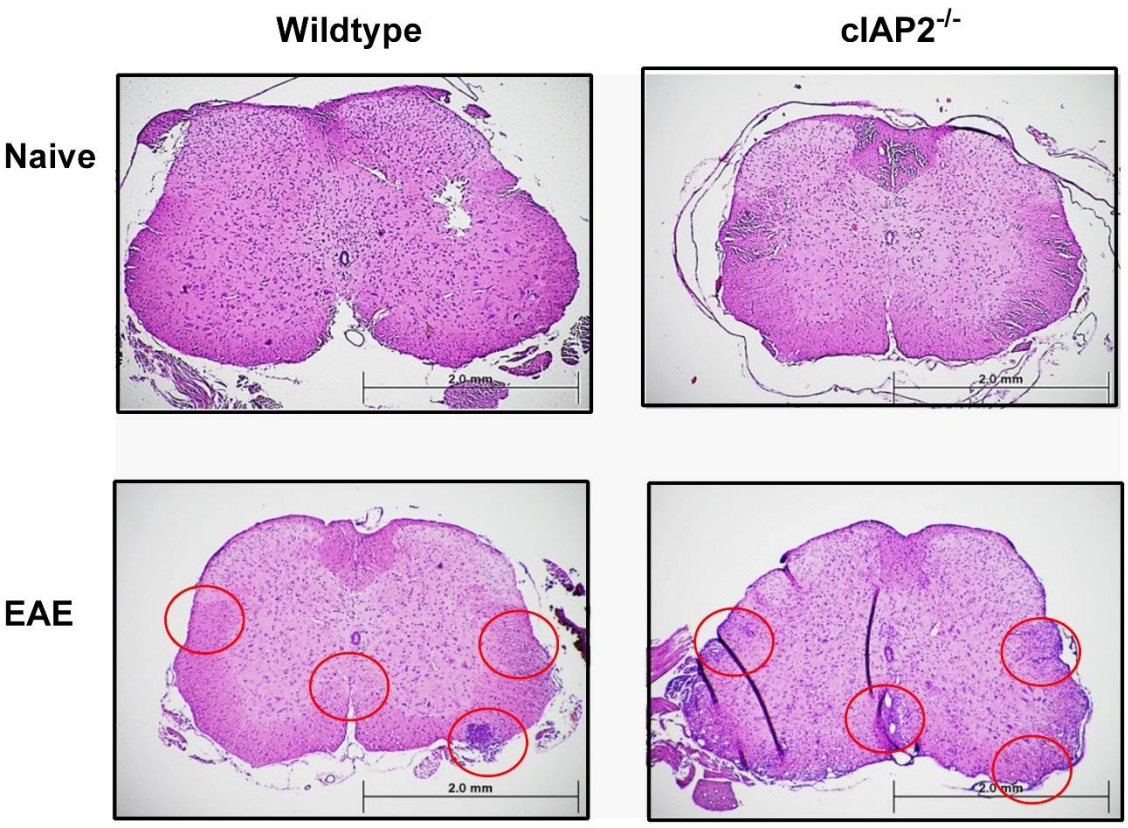

Wildtype
cIAP2-/-
Naive
EAE
2.0 mm
2.0 mm
2.0 mm
2.0 mm

Figure 4- 4 Histological analysis of lumbar regions of wildtype and cIAP2$^{-/-}$ mouse spinal cords.

Hematoxylin and eosin staining of lumbar tissue harvested from naïve and EAE induced wildtype and cIAP2$^{-/-}$ mice. Scale bar = 2.0 mm.

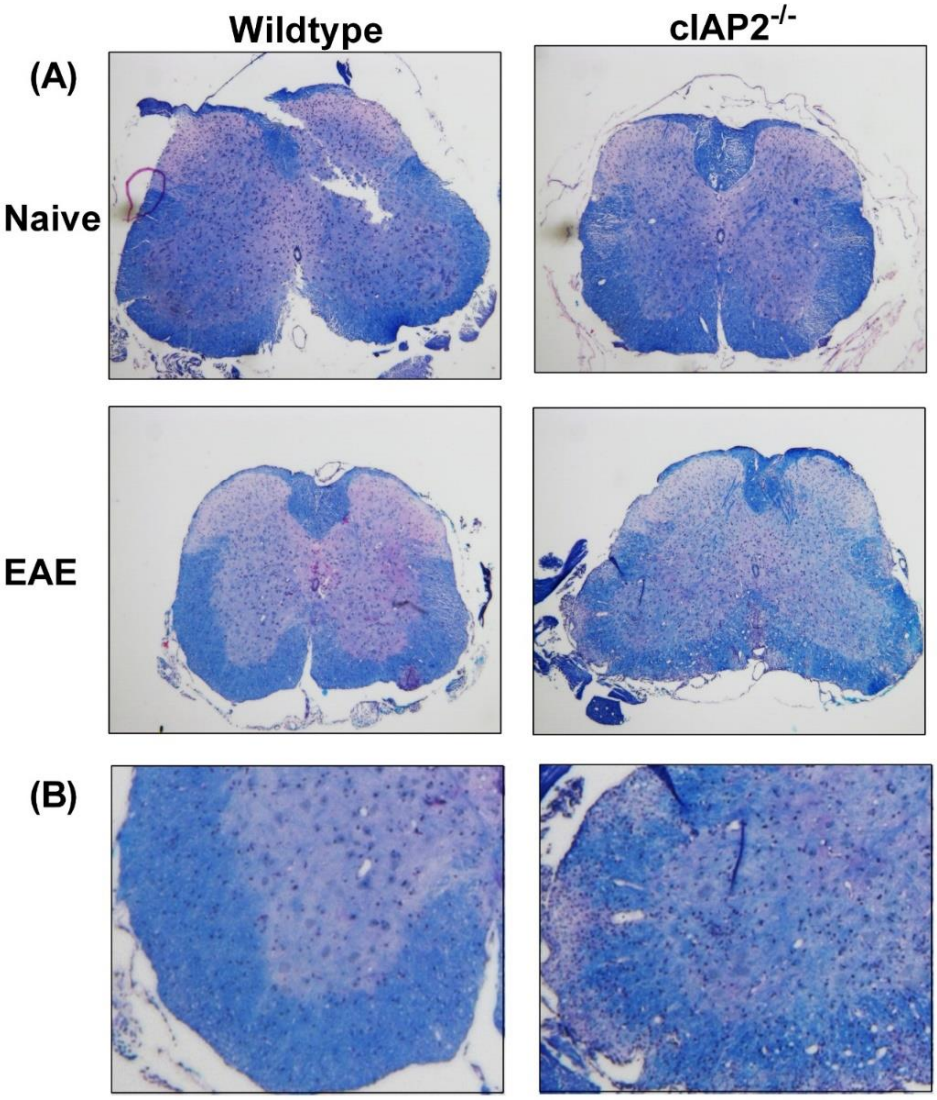

Wildtype
clAP2-/-
(A)
Naive
EAE
(B)

Figure 4- 5 Absence of cIAP2 increases demyelination in the lumbar region of mouse spinal cords.

Luxol fast blue staining for myelin at the lumbar region of the spinal cord of (A) naive and EAE induced wildtype and cIAP2$^{-/-}$ mice. (B) Magnified images of the white matter of the spinal cord of the EAE induced wildtype and cIAP2$^{-/-}$mice.

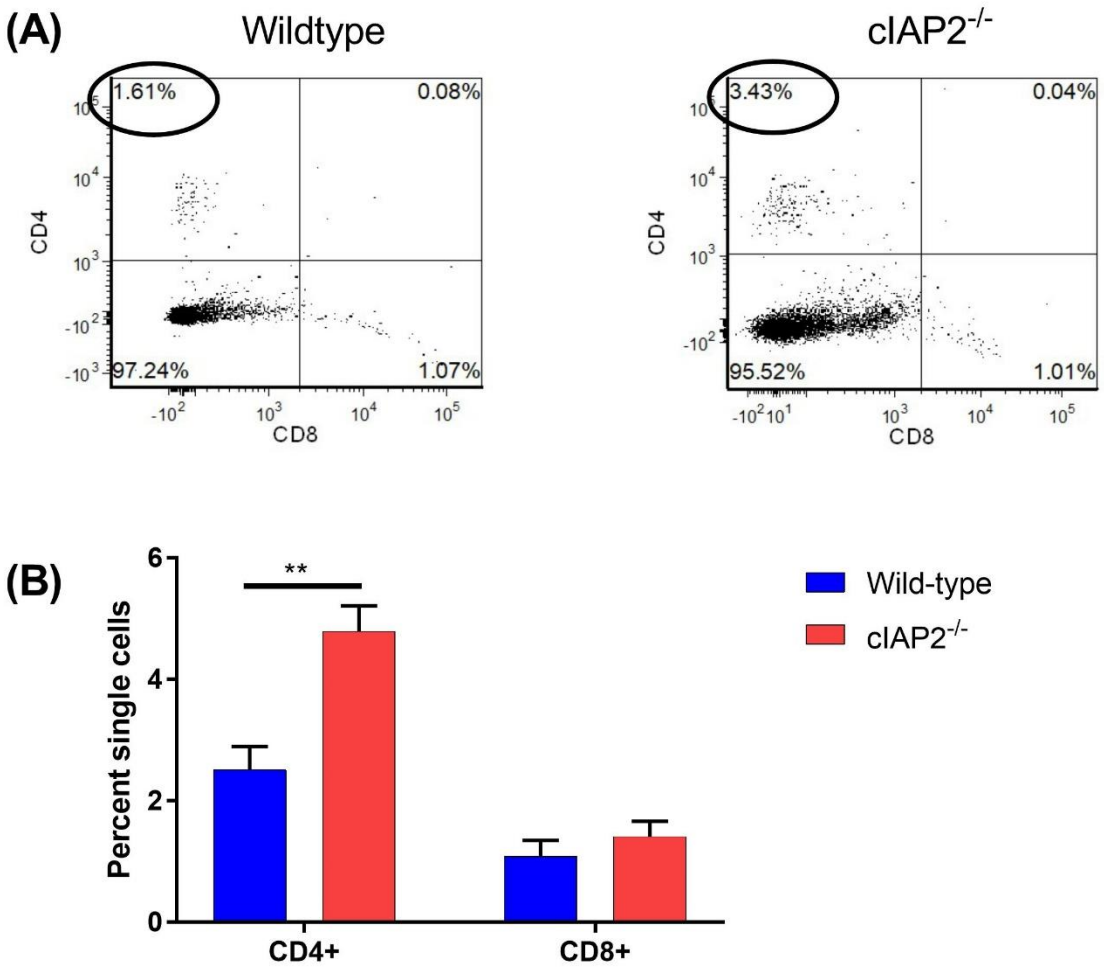

(A)
Wildtype
cIAP2-/-
1.61%
0.08%
97.24%
1.07%
3.43%
0.04%
95.52%
1.01%
CD4
CD8
(B)
Percent single cells
**
Wild-type
cIAP2-/-
CD4+
CD8+

Figure 4- 6: Increased recruitment of immune cells to the brain of cIAP2$^{-/-}$ mice.

Quantifications of recruited immune cells isolated from the brains of wildtype and cIAP2$^{-/-}$mice after 12 days of second immunization by flow cytometry (A) Representative histograms of $CD4^{+}$ and $CD8^{+}$ T cells in wildtype and cIAP2$^{-/-}$ mice. (B) Graphical representation of recruited T cells in the brain, 4 mice per group **$p<0.01$ EAE cIAP2$^{-/-}$ vs wildtype mice, two-way ANOVA; Error bar = SEM.

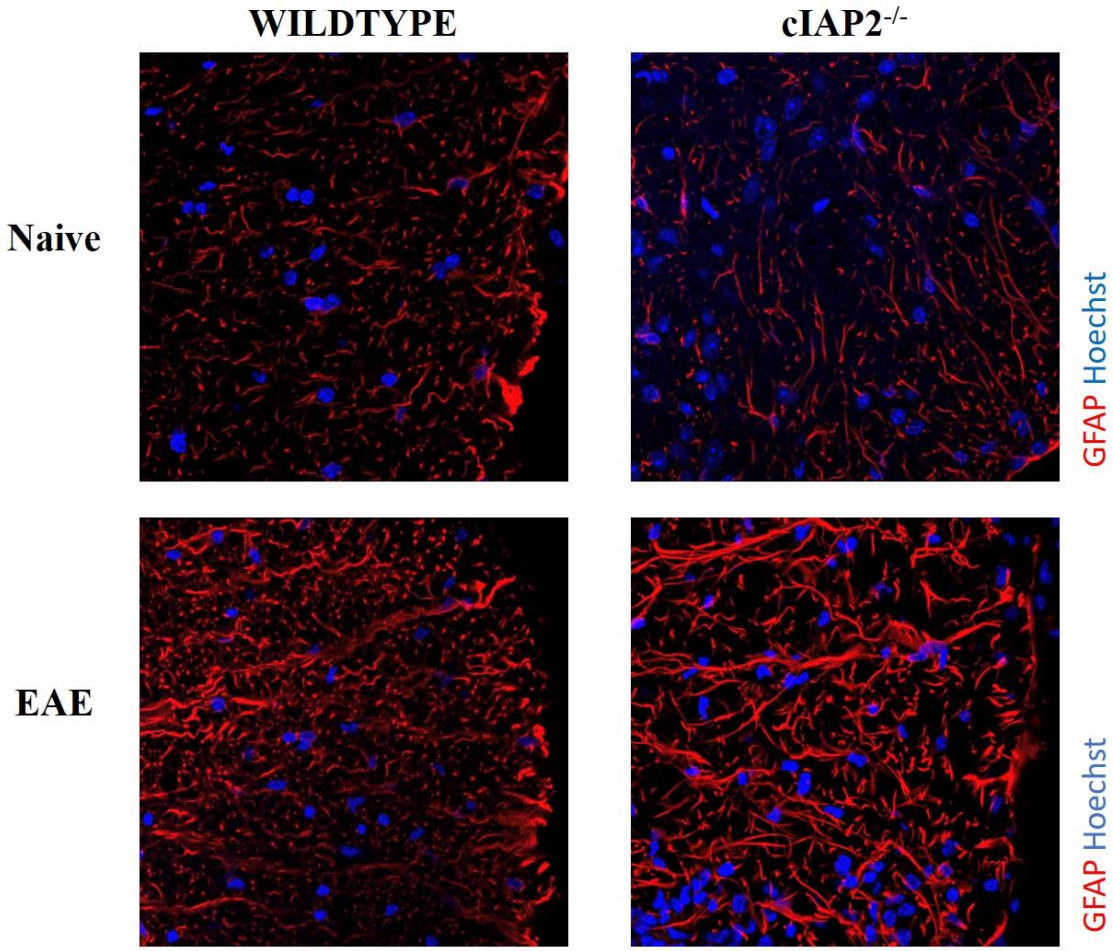
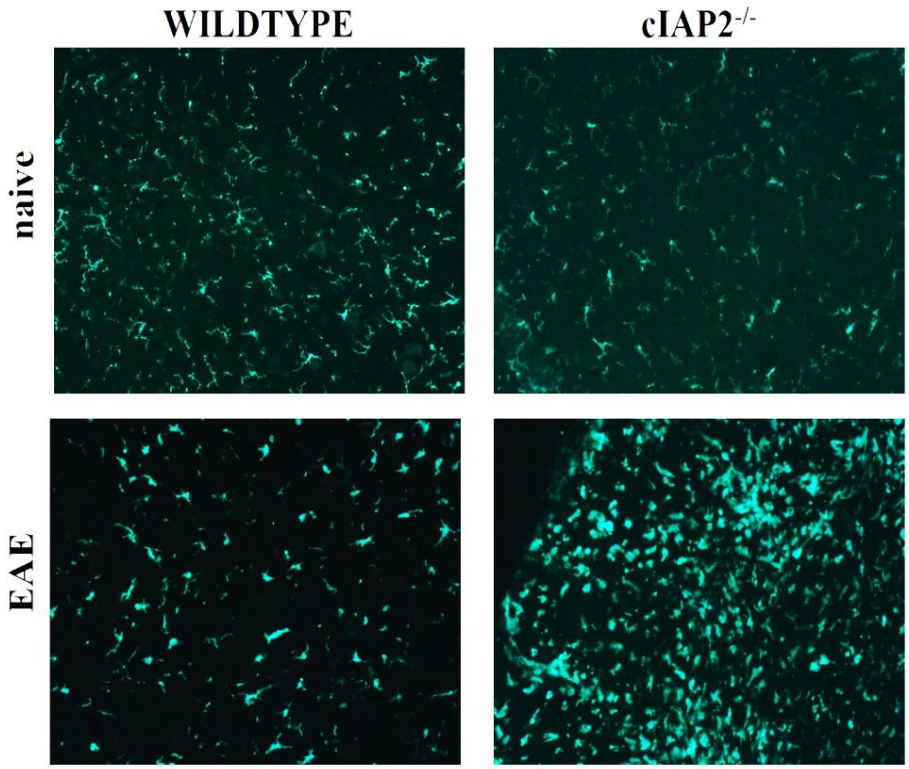

WILDTYPE
cIAP2-/-
Naive
EAE
GFAP Hoechst
GFAP Hoechst

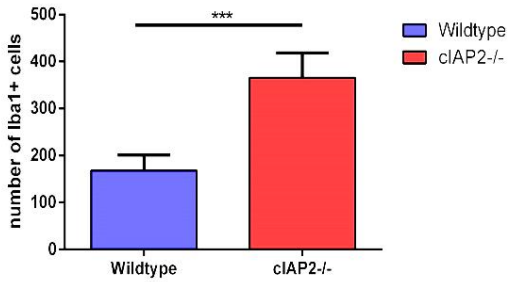
Figure 4- 7 Increased astrogliosis in the absence of cIAP2 after EAE induction.

Immunofluorescence staining of naïve and EAE-induced wildtype and cIAP2$^{-/-}$ mice with GFAP, shown in red, nuclei was stained by Hoechst.

(A)


WILDTYPE
cIAP2-/-
naive
EAE

(B)


***
number of Iba1+ cells
500
400
300
200
100
0
Wildtype
cIAP2-/-
Wildtype
cIAP2-/-

8

Figure 4- 8 Increased activation of myeloid cells in the absence of cIAP2 after induction of EAE.

(A) Immunofluorescence staining of naïve and EAE induced wildtype and cIAP2$^{-/-}$ mice with Iba1, marker of myeloid cells. (B) Graphical representation of total number of myeloid cells from five different regions of lumbar. **p<0.01 vs EAE Wild type mice, Student T. Test ; Error bars = SEM.

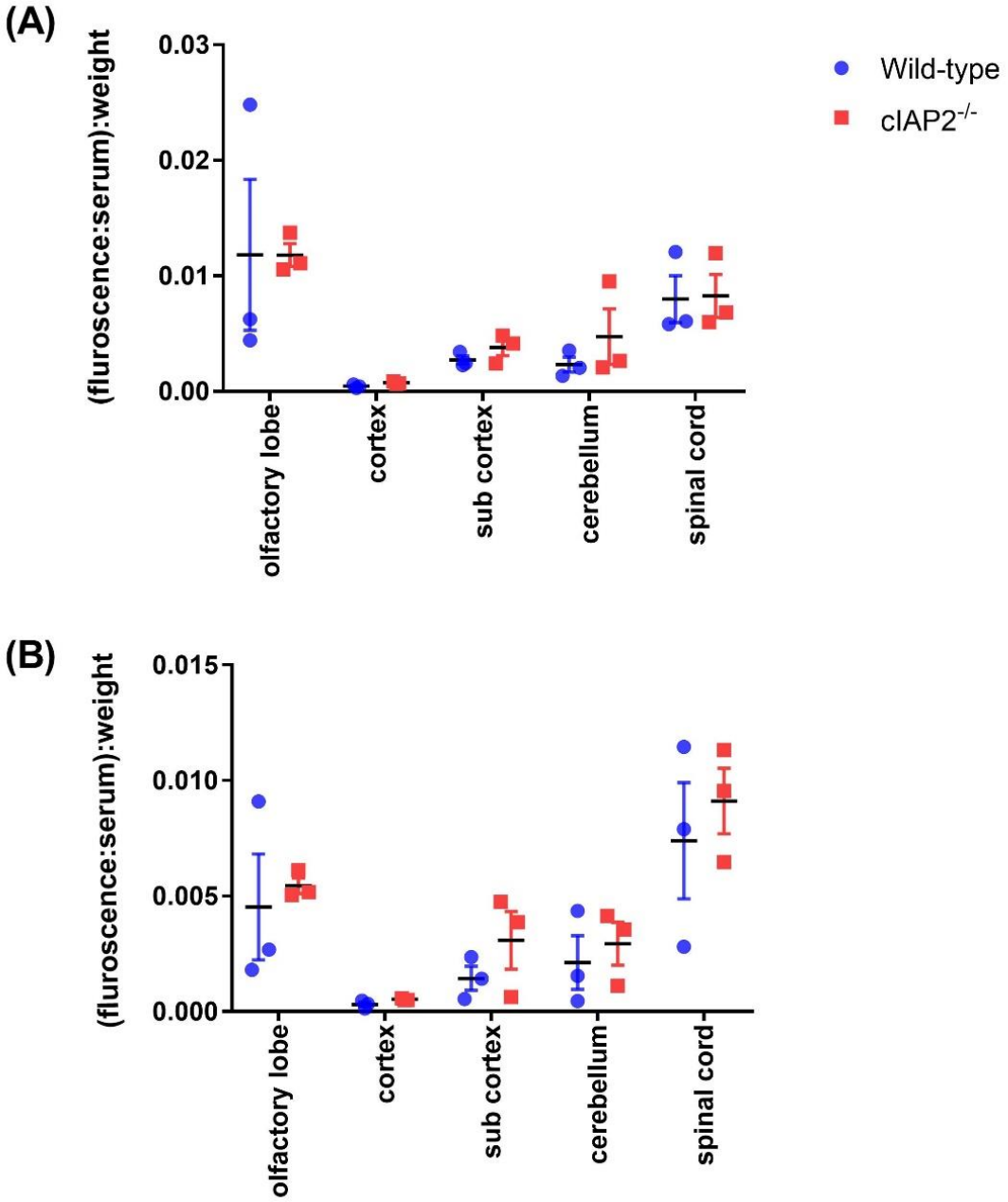

(A)
(fluroscence:serum):weight
0.03
0.02
0.01
0.00
olfactory lobe
cortex
sub cortex
cerebellum
spinal cord
Wild-type
cIAP2-/-
(B)
(fluroscence:serum):weight
0.015
0.010
0.005
0.000
olfactory lobe
cortex
sub cortex
cerebellum
spinal cord

Figure 4- 9: cIAP2 does not regulate blood brain barrier permeability.

BBB permeability was assessed (A) in naïve mice and (B) 12 days after induction of EAE, permeability was measured by the accumulation of sodium fluorescein dye in CNS tissues, 1 hour after intraperitoneal administration of the dye. Each dot represents one mice, * $p>0.05$, Student's T test. Error bar=SEM

4.2.3 cIAP2 restricts the proliferation of the microglia during inflammation in the CNS

Microglia are the immune cells of the CNS. Unlike monocytes, which differentiate from bone marrow hematopoietic stem cells, microglia are derived from yolk sac progenitors that infiltrate CNS during embryogenesis and self-renew independently(Ginhoux and Prinz, 2015). In MS and EAE, myeloid predominate demyelinated areas and their numbers correlate with the severity of symptoms in the disease. These myeloid cells are either microglia derived macrophages (MiDM) or infiltrating monocyte derived macrophages (MoDM).(Lewis et al., 2014)

Since we observed a substantial increase of (Iba1+) myeloid cells in cIAP2$^{-/-}$ mice with extensive demyelination, we asked if the macrophages that populate demyelinated lesions are MiDM or MoDM. These two types of cells have similar morphology and surface phenotypes and it is difficult to distinguish between these populations. Therefore, we addressed this questions with two different approaches. MoDM and MiDM express different gene profiles at different stages of EAE(Lewis et al., 2014) During EAE induced inflammation in the spinal cord, we detected that the mice lacking cIAP2 express higher MiDM specific markers (*cxc3r1*, *tgfbr1*, *csfr1* and *p2yr1*) than the wildtype mice. While the MoDM specific marker, *ccr2*, was not significantly different in the wildtype and cIAP2$^{-/-}$ mice. The levels of expression of ccr2 greatly varied in cIAP2$^{-/-}$ mice and did not corelate with the clinical scores (Figure 4-10). To confirm if absence of cIAP2 induced an increase in the population of MiDM in the brain similar to that in spinal cord, we quantified the MiDM (CD45^{low}/CD11b^{+}) and MoDM (CD45 ^{high}/CD11b^{+}) cells using flow cytometry. In

agreement with qPCR data, the number of MiDMs were nearly three times higher in cIAP2$^{-/-}$ mice than in the wildtype mice after induction of EAE. Interestingly the number of MoDM recruited to the brain of cIAP2$^{-/-}$ mice was also higher but was not statistically significant (Figure 4-11). The activation of the MiDM was induced by the development of the disease, since their numbers were comparable in the brain of naïve wildtype and cIAP2-/- mice (Figure 4-12). These novel findings suggest that cIAP2 limits proliferation and activation of microglia during EAE induced neuroinflammation.

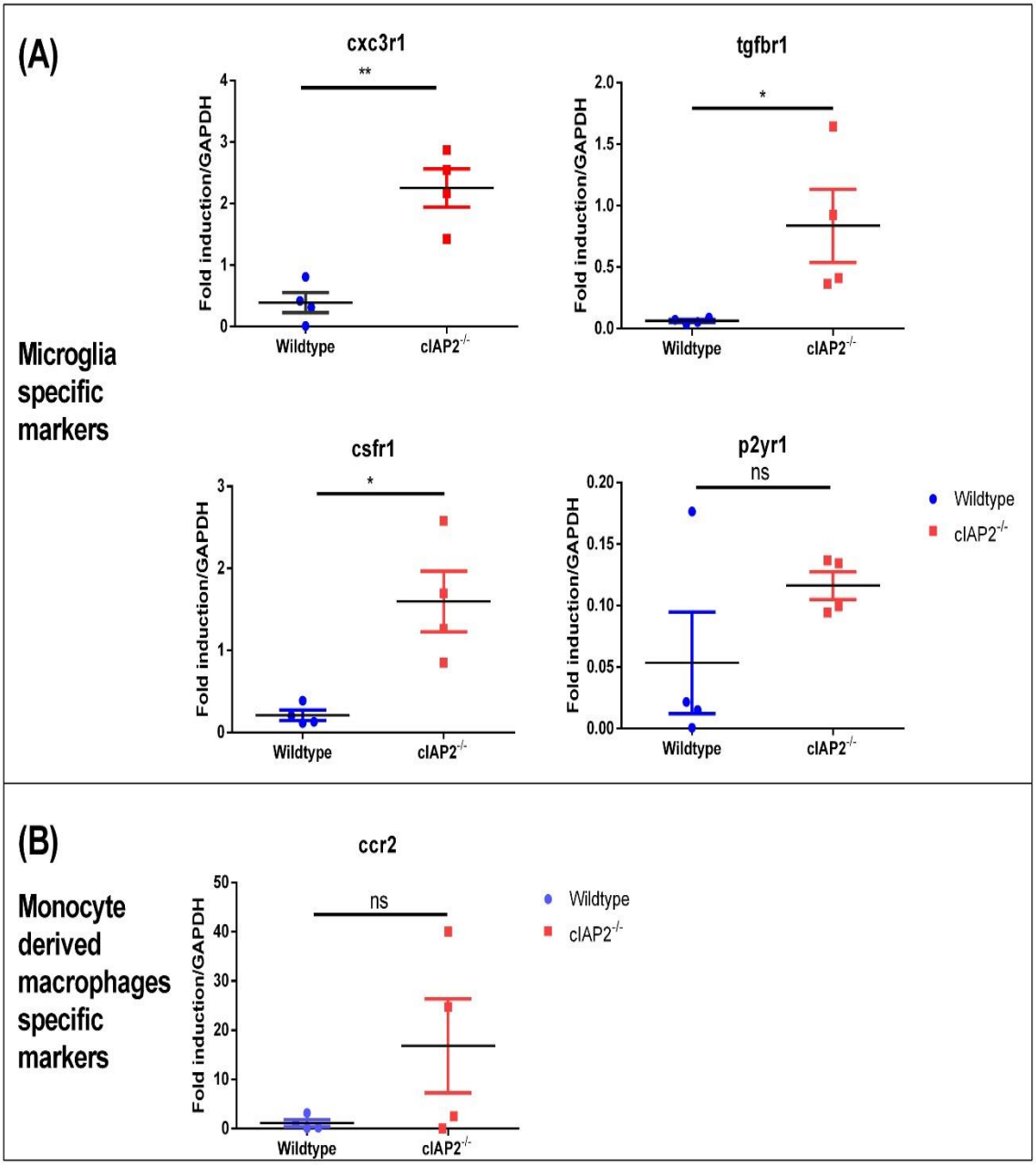
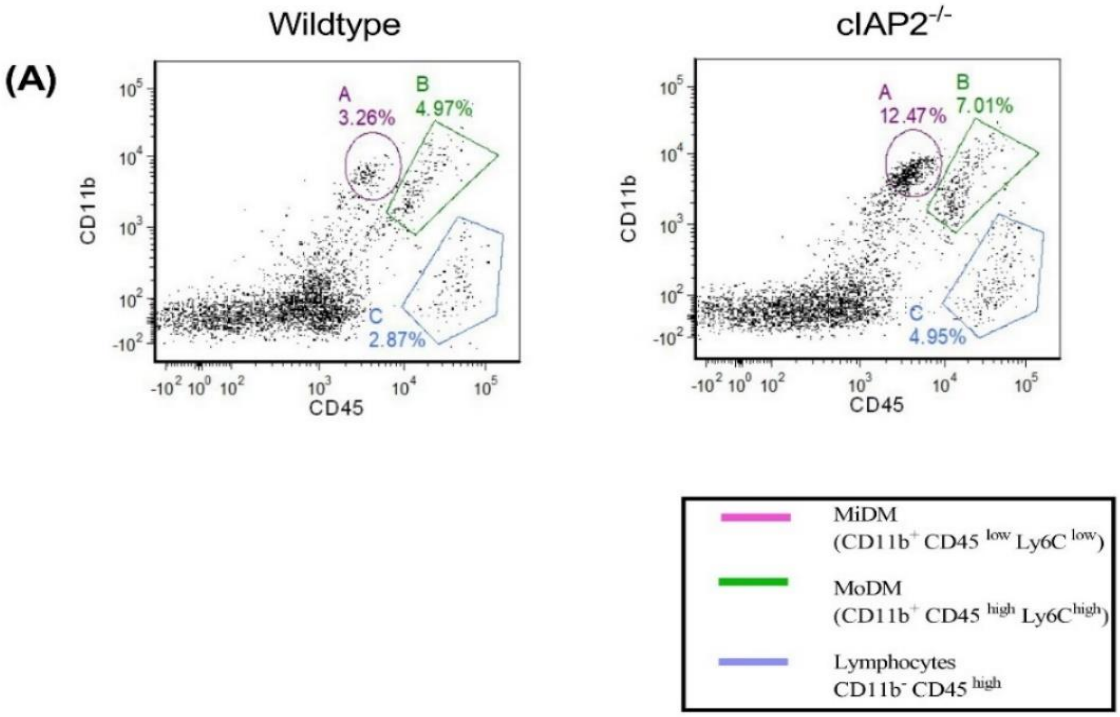

(A)
Microglia specific markers
cxc3r1
**
Fold induction/GAPDH
Wildtype
cIAP2-/-
tgfbr1
*
csfr1
*
p2yr1
ns
Wildtype
cIAP2-/-
(B)
Monocyte derived macrophages specific markers
ccr2
ns

Figure 4- 10: Lack of cIAP2 increases expression of MiDM in the CNS.

Analysis of cxc3r1, csfr1, p2yr1 (MiDM specific markers) and ccr2 (MoDM specific marker) expression in the lumbar region of EAE induced wildtype and cIAP2$^{-/-}$ mice using real time PCR. *$p<0.05$, Two-way ANOVA, Error bar= SEM


(A)
Wildtype
cIAP2-/-
A
3.26%
B
4.97%
C
2.87%
A
12.47%
B
7.01%
C
4.95%
CD11b
CD45
MiDM
(CD11b+ CD45 low Ly6C low)
MoDM
(CD11b+ CD45 high Ly6C high)
Lymphocytes
CD11b- CD45 high

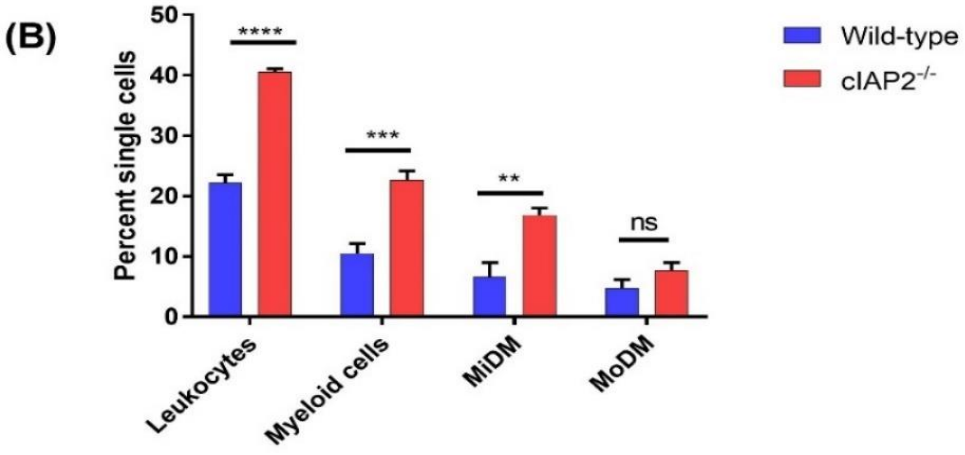

(B)
Percent single cells
****
***
**
ns
Leukocytes
Myeloid cells
MiDM
MoDM
Wild-type
cIAP2-/-

Figure 4- 11: cIAP2 inhibits response of microglia during EAE

Flow cytometry analyses of myeloid cells in the brain of EAE induced wildtype and cIAP2$^{-/-}$ mice. (A) Representative histogram of immune cells in the brain of wildtype and cIAP2$^{-/-}$ mice. (B) Graphical representation of the number of leukocytes, myeloid cells, Microglia- derived macrophages(MiDM) and Monocyte derived macrophages(MoDM) in the wildtype and cIAP2 $^{-/-}$ mice. 4 mice per group. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs EAE wildtype Student t test. Error bar = SEM.

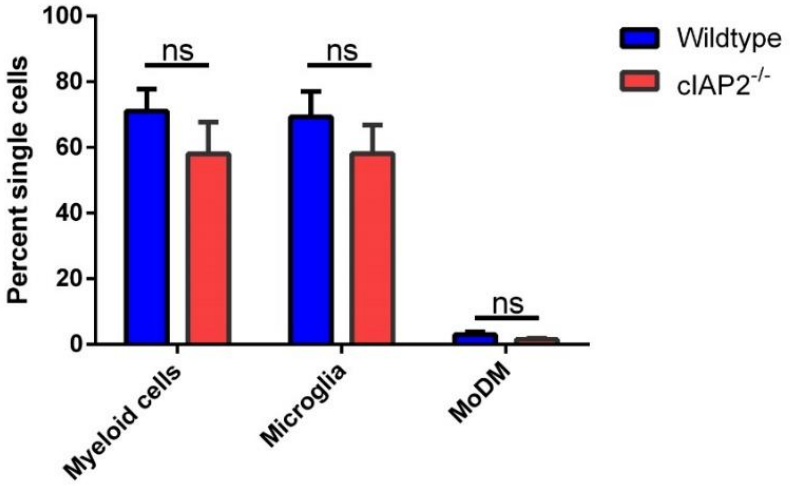

Percent single cells
100
80
60
40
20
0
ns
ns
ns
Myeloid cells
Microglia
MoDM
Wildtype
cIAP2-/-

Figure 4- 12: No difference in the number of microglia in naïve wildtype and cIAP2$^{-/-}$ mice.

Graphical representation of the percentage of leukocytes, myeloid cells, microglia, and MoDM from the brain of wildtype and cIAP2$^{-/-}$ mice analyzed by flow cytometry. 4 mice per group. *$p<0.05$, Student T. test; Error bar = SEM.

4.2.4 Exacerbated symptoms of EAE in cIAP2$^{-/-}$ mice is not dependent on bone marrow derived cells

To determine which cell type is responsible for exacerbated symptoms of EAE cIAP2$^{-/-}$ mice, we generated bone marrow chimeras in which only the hematopoietic (WT→cIAP2$^{-/-}$) or non-hematopoietic compartment (cIAP2$^{-/-}$→WT) expressed cIAP2. The reconstitution of the bone marrow was confirmed by genotyping the DNA isolated from the bone marrow of these mice at the end of the experiment. The wildtype bone marrow failed to protect cIAP2$^{-/-}$ mice not only from early onset of EAE, but also from developing rapid symptoms. This suggests that cIAP2 is primarily required for non-hematopoietic cells to protect mice from neuroinflammation during EAE (Figure 4.14 A). To our surprise, we found that cIAP2 deficient bone marrow delayed the onset of the disease in the wildtype mice. However, the subsequently the disease worsened and was comparable to that in wildtype mice (Figure4-14 B).

First, we asked if delayed onset of the disease of wildtype mice receiving cIAP2$^{-/-}$ bone marrow was caused due to dysfunction of cIAP2$^{-/-}$ cells. EAE is a Th cell driven autoimmune disease, however, for proliferation and activation of T cells, it requires the MOG antigen presentation by cells, such as macrophages(Almolda et al., 2011). To investigate if cIAP2 affects for antigen presentation, we treated the wildtype and cIAP2 deficient bone marrow derived macrophages (BMDM) with fluorescent conjugated AF-647 OVA antigen. We found that the uptake of the antigen in BMDM lacking cIAP2 was considerably reduced when compared to that of wildtype BMDM. Also, the MHC II expression on macrophages, which is required for activation of T cells, was significantly

decreased in the absence of cIAP2(Figure 4-16). This demonstrate that cIAP2 is required for optimal antigen presentation. And could explain delayed symptoms of EAE in wildtype mice receiving bone marrow devoid of cIAP2.

Second some studies show that cIAP2 is required for the survival of macrophages after exposure to LPS, therefore, we examined if the lack of cIAP2 lead to the death of macrophages induced by TNF-α, which is abundantly expressed during systemic inflammation induced by MOG. We found that lack of cIAP2 did not influence the survival of the BMDM were stimulated with TNF-α (Figure 4-15). Thus, we conclude that cIAP2 play opposite functions during inflammation in macrophages and the brain cells. Not only cIAP2 is required for optimal antigen presentation and drives Th cell dependent autoimmune diseases, but it also protects glial cells by limiting EAE driven neuroinflamation in the CNS.

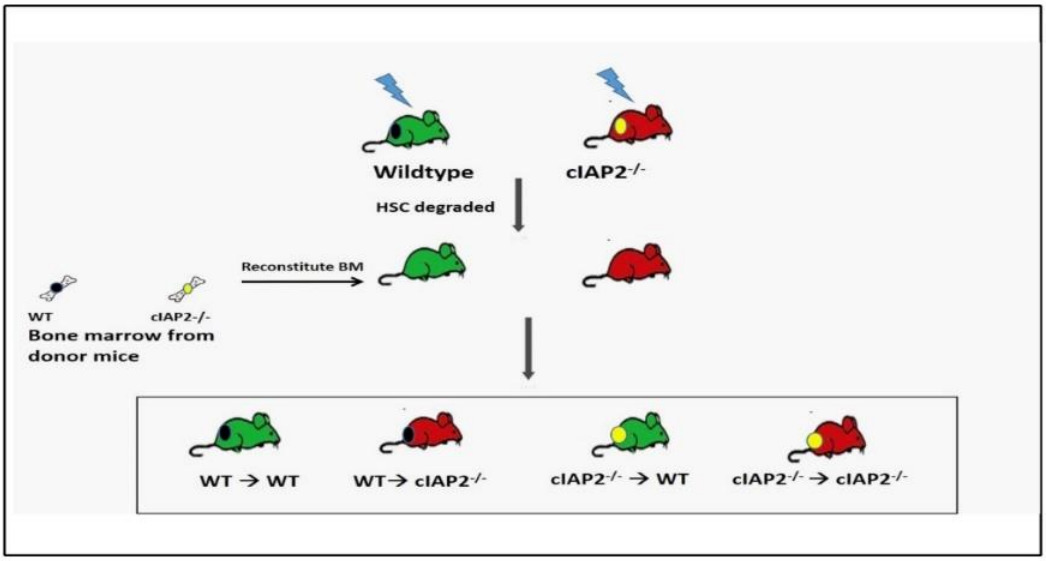
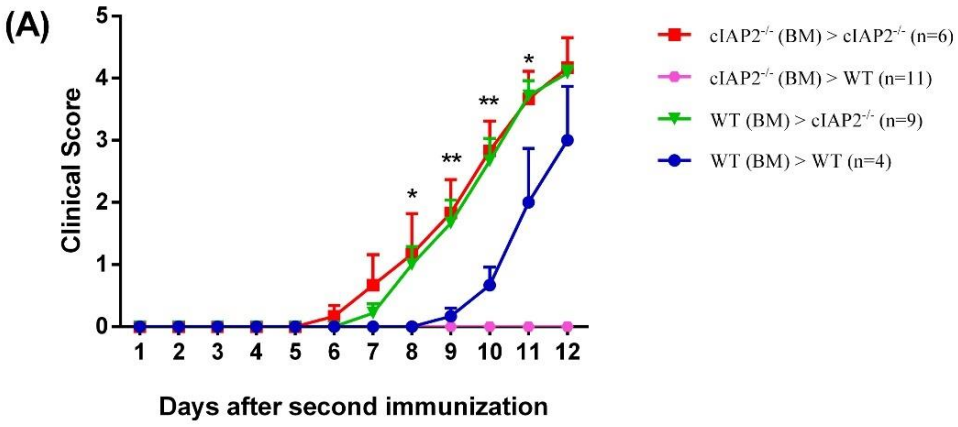

Wildtype
cIAP2-/-
HSC degraded
Reconstitute BM
WT
cIAP2-/-
Bone marrow from donor mice
WT → WT
WT→ cIAP2-/-
cIAP2-/- → WT
cIAP2-/- → cIAP2-/-

Figure 4- 13:Experimental scheme for reconstitution of bone marrow in wildtype and cIAP2$^{-/-}$ mice.

Schematic representation of bone marrow reconstitution. Naïve wildtype and cIAP2$^{-/-}$ mice were irradiated to eliminate their bone marrow, which was subsequently replaced with either wildtype or cIAP2 deficient bone marrow.


(A)
Clinical Score
Days after second immunization
cIAP2-/- (BM) > cIAP2-/- (n=6)
cIAP2-/- (BM) > WT (n=11)
WT (BM) > cIAP2-/- (n=9)
WT (BM) > WT (n=4)

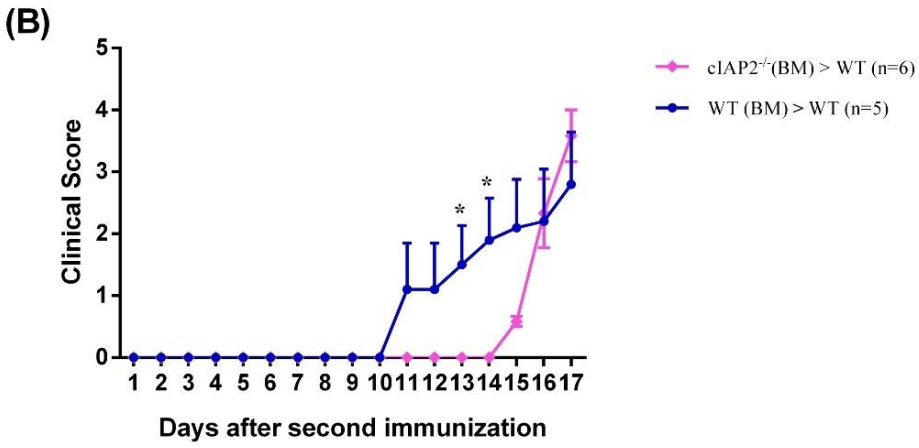

(B)
Clinical Score
Days after second immunization
cIAP2-/-(BM) > WT (n=6)
WT (BM) > WT (n=5)

Figure 4- 14: The severity of EAE is bone marrow independent.

Mean clinical score of EAE in the bone marrow of reconstituted mice (A) up to day 12 after second immunization and (B) extended for 18 days after second immunization.

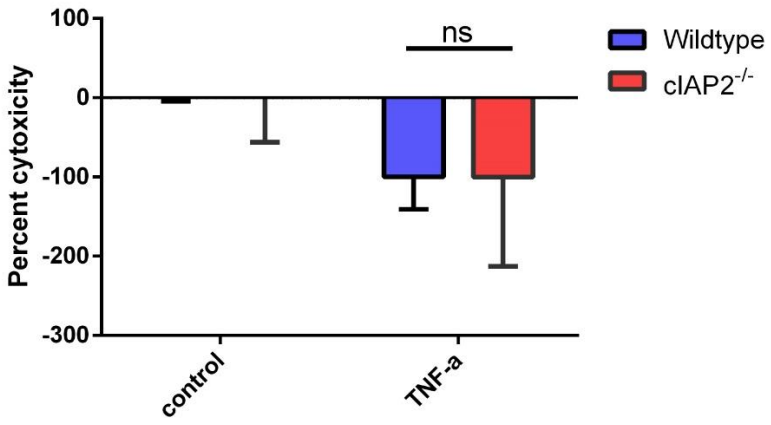


Figure 4- 15: Survival of macrophages upon TNF-α stimulation is not affected by cIAP2.

BMDMs were treated with TNF-α for 24 hours and cell death was quantified by LDH assay

*p <0.05, Two-way ANOVA; Error bars = SD.

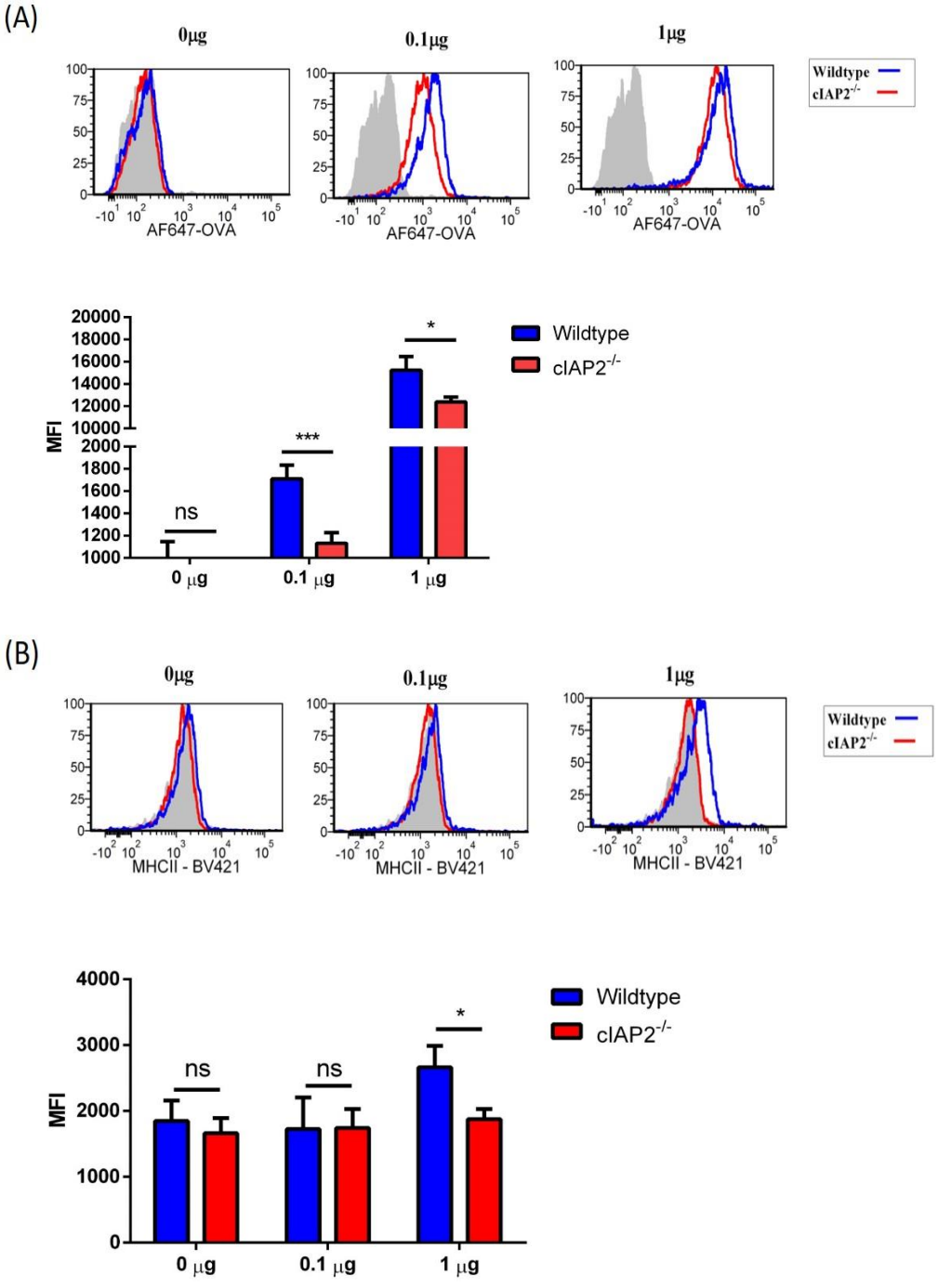

(A)
0μg
0.1μg
1μg
Wildtype
cIAP2-/-
AF647-OVA
MFI
ns
***
*
0 μg
0.1 μg
1 μg
(B)
MHCII - BV421

Figure 4- 16: cIAP2 is required for optimal antigen presentation.

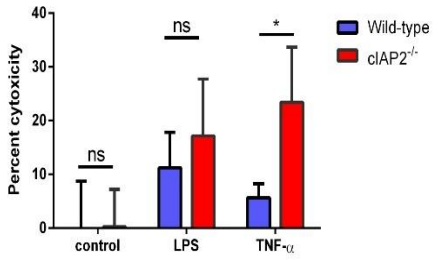
Wildtype and cIAP2$^{-/-}$ BMDMs were treated with IFNγ and AF647 OVA antigen at different concentration (as indicated). The mean fluorescence intensity for (A) antigen uptake and (B) MHC II expression is shown by representative histogram (blue line represents the wildtype BMDM with IFNγ and OVA; red line represents cIAP2$^{-/-}$ BMDM with IFNγ and OVA; grey region represents wildtype macrophages with no IFNγ treatment). Graphical representation from triplicate experiments. *$p<0.05$, Student T. test Error bar=S.D for were quantified with different.

4.2.5 cIAP2 is required for the survival of glial cells during neuroinflammation.

Activation of cells during inflammation, causes the upregulation of a multitude of genes, including production of inflammatory mediators, cell surface markers and cell survival proteins. Previous studies have shown that cIAP2, a critical survival protein is abundantly expressed during inflammation. Upon activation of TNF-R1 receptor, cIAP2/cIAP2/TRAF2, adds K63 linked ubiquitin chains to RIPK1, initiating the pro-survival NF-kB signaling pathway. It has been also shown that absence of cIAP2 can activate caspase- 8-dependent (apoptosis) or RIPK3-dependent (necroptosis) processes. (Hisahara et al., 2001, 2003; Ofengeim et al., 2015)

Kavanagh et al previously reported that the downregulation of cIAP2 causes apoptosis-mediated death of LPS treated-BV2 microglia cell line(Kavanagh et al., 2014b).. To test if primary cells are also sensitive to cell death, we treated mixed glial cells (astrocytes, microglia and oligodendrocytes) with TNF-α, which is abundantly expressed during neuroinflammation. Also, as a control we also used LPS. We observed a significant increase in glial cell death in the absence of cIAP2 when treated with TNF-α but not LPS (Figure 4-17 A). No difference in expression of cytokine was observed in TNFα treated microglia (Figure 4-17 B).

(A)


Percent cytoxicity
40
30
20
10
0
ns
ns
*
control
LPS
TNF-α
Wild-type
cIAP2-/-

(B)

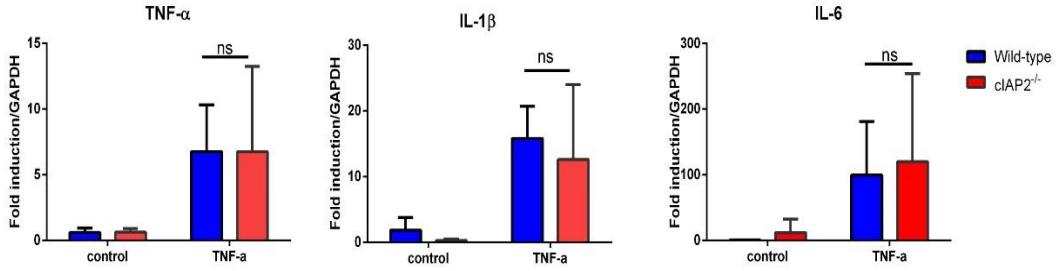

TNF-α
IL-1β
IL-6
Fold induction/GAPDH
ns
control
TNF-a
Wild-type
cIAP2-/-

Figure 4- 17: cIAP2 protects the glial cells from TNF-α mediated cell death

(A) Mixed glial cells from the wildtype and cAIP2$^{-/-}$ mice were treated with LPS (100 ng/ml) and TNF-α (30 ng/ml) for 24 hours. Cell supernatant was quantified for LDH release. *p<0.05, Student t. test. Error bar=S.D. (B) Wildtype and cIAP2$^{-/-}$ microglia were treated with TNF-α (30 ng/ml) for 4hours and analyzed for expression of TNFα, IL-1β and IL-6 by real time PCR, *p<0.05, two-way ANOVA. Error bar = S.D

4.2.6 cIAP2 regulates death of oligodendrocytes and activation of M1 proinflammatory microglia by restricting Caspase-8 activity.

In MS, demyelination is accompanied by death of oligodendrocytes. Loss of oligodendrocytes in MS can be elicited by Caspase- 8-mediated apoptosis(Gu et al., 1999) or RIPK3 dependent necroptosis.(Ofengeim et al., 2015) The death of oligodendrocytes consequently results in failure of remyelination of axons during MS and EAE. Death of oligodendrocytes can initiate a series of responses including activation of microglia.

We found that EAE-induced cIAP2$^{-/-}$ mice demonstrated significantly higher number of TUNEL positive cells as compared to the wildtype mice. (Figure 4.18). To confirm if the dead cells were oligodendrocytes, we performed co-immunostaining for CC1 (oligodendrocyte marker) and TUNEL. Our data showed that in comparison to wildtype mice, cIAP2$^{-/-}$ mice demonstrated a significant increase in the number of dying oligodendrocytes at the white matter of spinal cords (Figure 4.19).

Ofengiem *et al* reported that during EAE, oligodendrocytes die due to necroptosis. This study demonstrated that MS patients have higher expression of RIPK1 and RIPK3, which are critical molecules that initiate necroptosis. It has been well documented in multiple cell types that lack of cIAP2 restricts cell survival and allows the phosphorylation of RIPK1 and activation of RIPK3 dependent (necroptosis) cell death.(Dagenais et al., 2016; Rodrigue-Gervais et al., 2014) Therefore, to examine the possible role of necroptosis dependent death of oligodendrocytes contributing to the severity of disease in cIAP2$^{-/-}$mice,

we probed for expression of RIPK1 and RIPK3 in the lysate of lumbar tissue from naïve and EAE mice. There was no difference in the expression of both RIPK1 and RIPK3 proteins in the naïve mice. Additionally, the expression of RIPK3 remained inconsistent during EAE in both wildtype and cIAP2$^{-/-}$ mice (Figure 4-20). This confirmed that exacerbation of symptoms associated with EAE in cIAP2 $^{-/-}$ mice is independent of necroptosis.

Increased death of oligodendrocytes contributes to the activation of microglia. Microglia can phagocytose toxic cellular debris and release anti-inflammatory factors to resolve inflammation. However, microglia can also become hyperreactive and induce the expression of high levels of proinflammatory cytokines, such as TNF-α, nitric oxide and superoxide, and induce apoptosis of oligodendrocytes (Huizinga et al., 2012; Zajicek et al., 1992)

Strikingly, we observed a significant increase in the number of activated and phagocytotic microglia/macrophages recruited at the lesion of EAE induced cIAP2$^{-/-}$ mice, compared to their wildtype littermate (Figure 4-21 A).We investigated if these activated microglia/macrophages are hyperactivated in cIAP2$^{-/-}$ mice by probing for M1 (proinflammatory) and M2 (anti-inflammatory) markers of the myeloid cells Our data revealed that during EAE, the microglia lacking cIAP2 exhibited upregulation of M1 markers, such as iNos2, CD86 and CD14, while the expression of M2 specific markers, such as IL-10 or CD163, was not influenced by cIAP2. Interestingly, Xu et al. reported that the M2 specific marker, arginase is increased during acute EAE in spinal cords(Xu et al.,

2003). Similarly, we found that the expression of arginase was upregulated in cIAP2$^{-/-}$ mice and correlated with severe disease score. (Figure 4-21 B).

Since, our data revealed that enhanced death of oligodendrocytes was not meditated by necroptosis, we explored the role of caspase-8, a critical regulator of apoptosis but also can regulate several mechanisms leading to death associated inflammation. We quantified Caspase-8 activity in the lumbar of EAE induced mice. We found that Caspase-8 activity was increased in EAE mice compared to that of naïve mice. Interestingly, absence of cIAP2 during EAE increased Caspase- 8 activity significantly (Figure 4-22. A)

Although Caspase-8 plays an important role in apoptosis, it has been reported that it has also has non-apoptotic role, in the activation of microglia in response to different stimuli (Burguillos et al., 2011; Viceconte et al., 2015). Hence, we explored if cIAP2 deficiency can activate Caspase-8 in myeloid cells and modulate its activation. To our surprise, we found that nearly 85% of activated microglia /macrophages were caspase-8 positive in spinal cord of cIAP2$^{-/-}$ mice during EAE. This was in striking contrast to the wildtype mice, only 34% of microglia/macrophages were Caspase-8 positive. In summary, this study reveals a previously unrecognized mechanism where cIAP2 regulates inflammatory immune responses during EAE. Upregulation of cIAP2 during EAE induced inflammation can repress Caspase-8 activation, resulting in dampening activation of hyperactivated microglia/macrophages. Attenuation of microglia/macrophage activation inhibits the production of a cytokine storm in the CNS, promoting the survival of oligodendrocytes and the remyelination of axons.

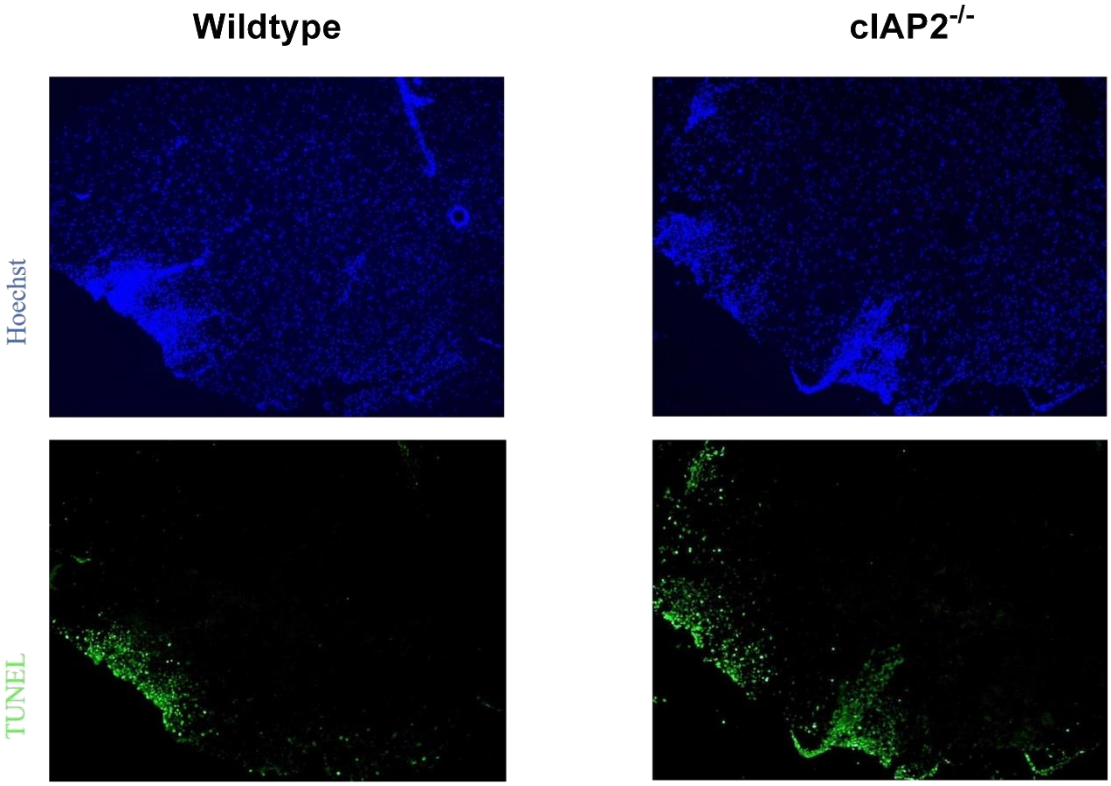
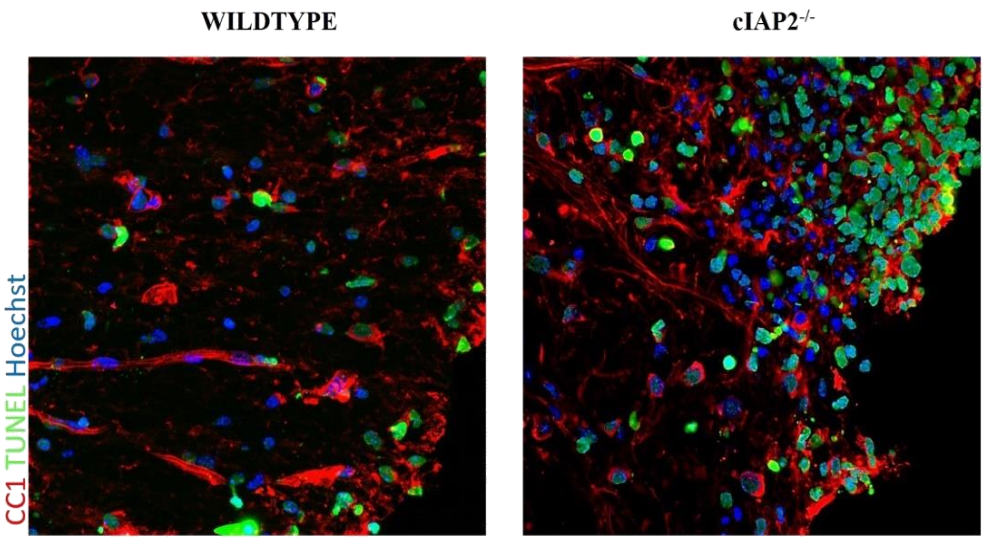

Wildtype
cIAP2-/-
Hoechst
TUNEL

Figure 4- 18 Increased cell death in the spinal cord of cIAP2 deficient mice during EAE.

Analysis of cell death in wildtype and cIAP2$^{-/-}$ mice during EAE using TUNEL staining.

Hoechst was used to label cell nuclei.

(A)


WILDTYPE
cIAP2-/-
CC1 TUNEL Hoechst

(B)

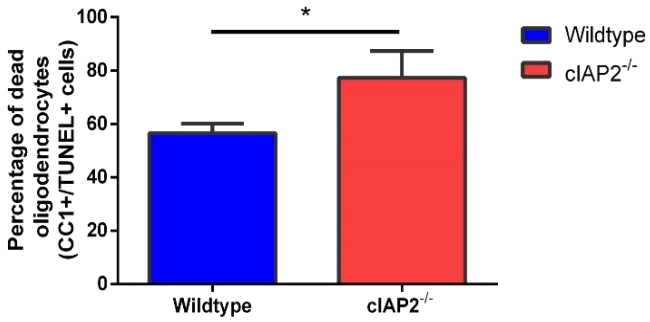

Percentage of dead oligodendrocytes (CC1+/TUNEL+ cells)
100
80
60
40
20
0
*
Wildtype
cIAP2-/-
Wildtype
cIAP2-/-

Figure 4- 19 Increased oligodendrocyte cell death in the spinal cord of the cIAP2$^{-/-}$ mice.

(A) Immunofluorescence staining of oligodendrocytes using anti- CC1 and TUNEL in the lumbar tissue. and Hoechst was used for staining nuclei (B) Graphical representation of percentage of dead oligodendrocytes (CC1 and TUNEL double positive cells) in EAE induced wildtype and cIAP2$^{-/-}$ mice from three different sections; **p>0.01, ***p>0.001 vs wildtype EAE: Student T. test; error bars = SEM.

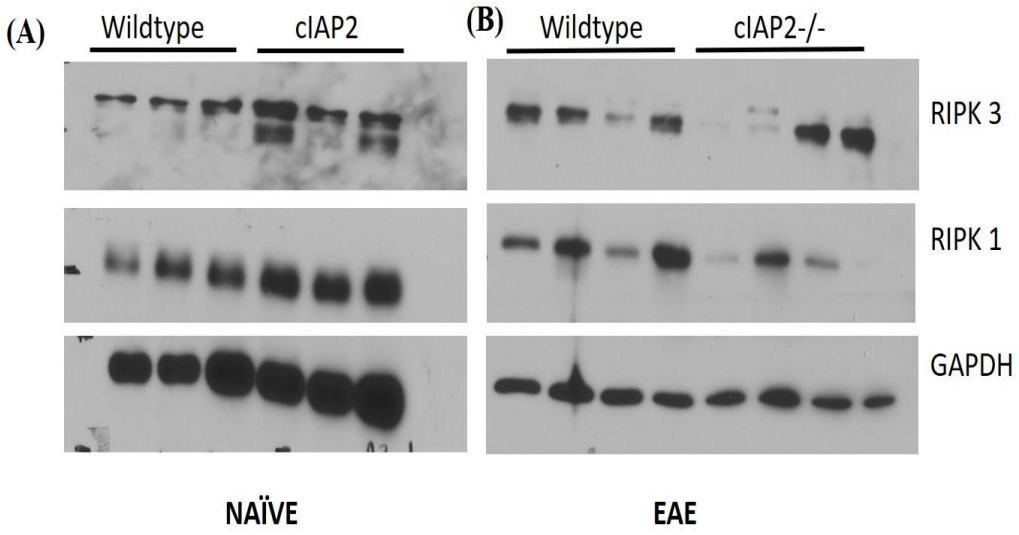

(A)
Wildtype
cIAP2
(B)
Wildtype
cIAP2-/-
RIPK 3
RIPK 1
GAPDH
NAÏVE
EAE

Figure 4- 20 EAE-induced cell death in cIAP2 deficient mice is independent of necroptosis.

Western blot for RIPK3 and RIPK1 from lumbar tissue of the (A) naïve and (B) EAE induced mice.

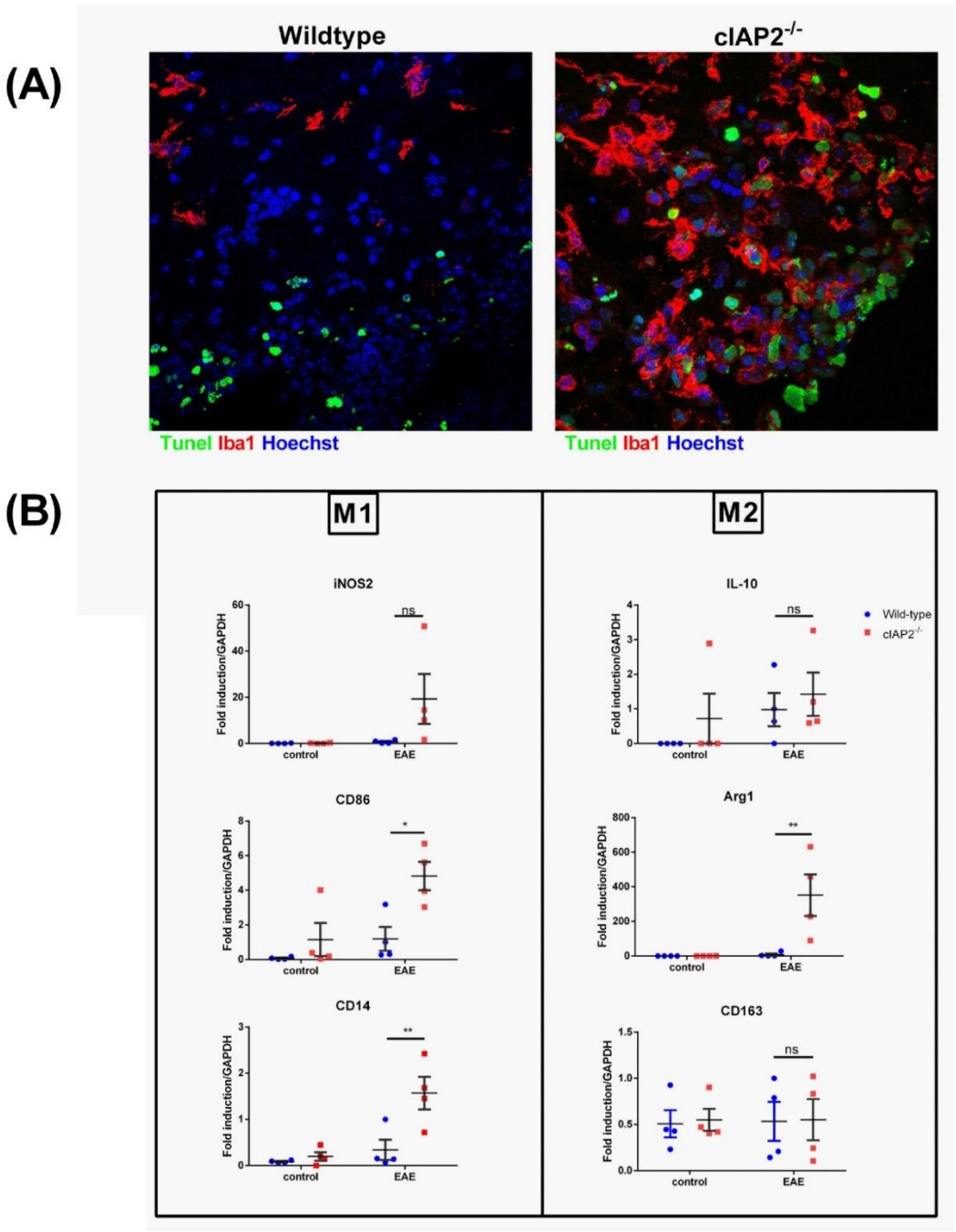
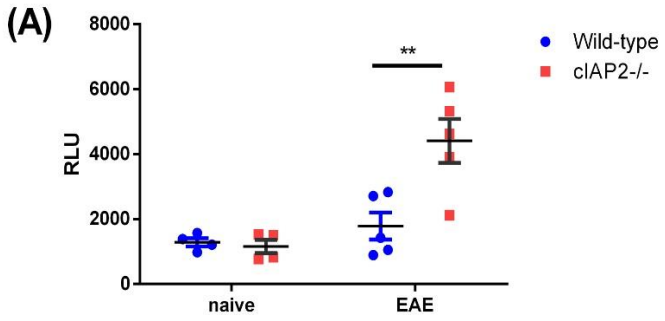
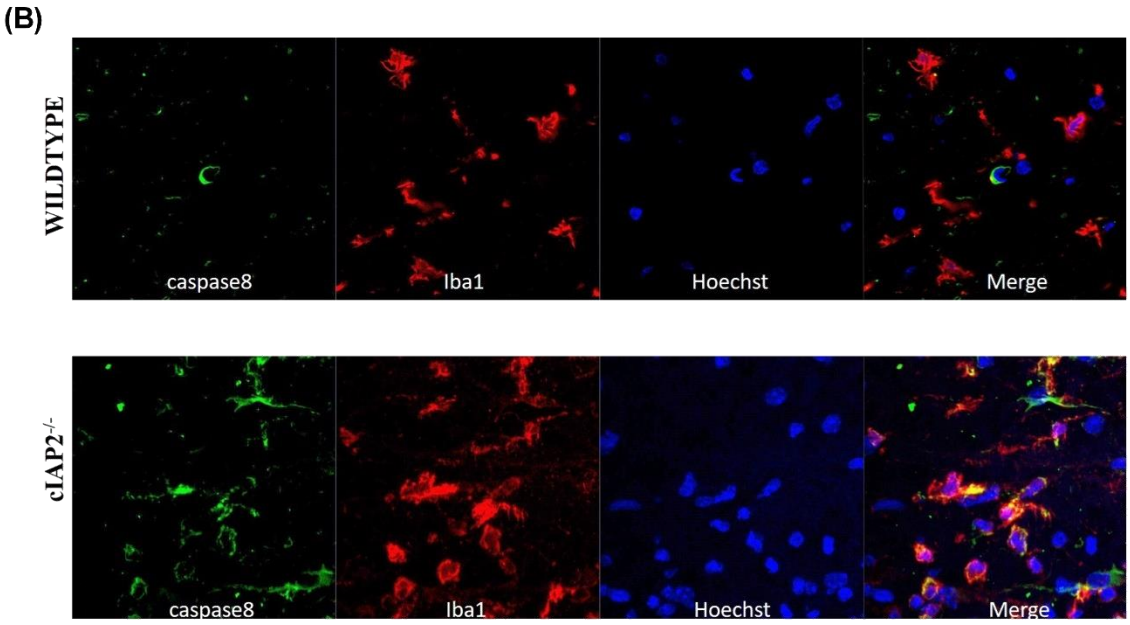

(A)
Wildtype
cIAP2-/-
Tunel Iba1 Hoechst
Tunel Iba1 Hoechst
(B)
M1
M2
iNOS2
IL-10
CD86
Arg1
CD14
CD163
Fold induction/GAPDH
control
EAE
ns
*
**
Wild-type
cIAP2-/-

Figure 4- 21: Lack of cIAP2 increases M1 phagocytotic microglia in the CNS.

(A) Immunostaining of lumbar tissue of EAE induced wildtype and cIAP2$^{-/-}$ mice using anti-Iba1 and TUNEL. (B) Real time PCR analysis of the expression of M1 and M2 markers in the lumbar region of the spinal cord of control mice (without MOG treatment) and EAE induced mice 12 days after second MOG immunization. Each dot represents a mouse. *$p<0.05$, **$p<0.01$, two-way ANOVA. Error bar=SEM.


(A)
8000
6000
4000
2000
0
RLU
**
naive
EAE
Wild-type
cIAP2-/-


(B)
WILDTYPE
caspase8
Iba1
Hoechst
Merge
cIAP2-/-
caspase8
Iba1
Hoechst
Merge

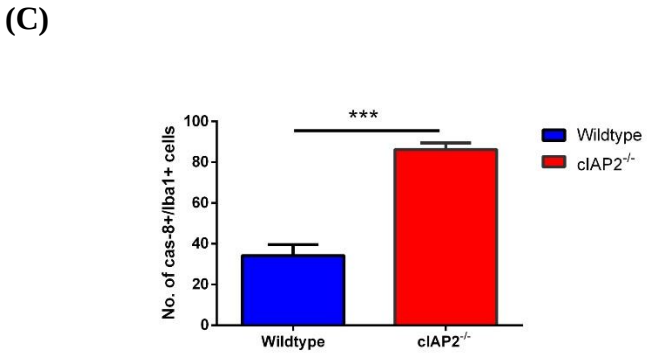

(C)
No. of cas-8+/Iba1+ cells
100
80
60
40
20
0
***
Wildtype
cIAP2-/-
Wildtype
cIAP2-/-

Figure 4- 22: Increased expression of Caspase-8 in the myeloid cells in absence of cIAP2.

(A) Caspase-8 specific activity was assessed in 50 μg of lumber tissue of the naïve and EAE wildtype and cIAP2$^{-/-}$ mice. (B) Immunofluorescence staining of lumbar tissue using anti-Caspase-8 and anti-Iba1 antibody. (C) Graphical representation of Iba1 and Caspase-8 double positive cells from three different sections; **p>0.01, ***p>0.001 vs wildtype EAE: Student T. test; Error bars = SEM.

4.3 Chapter summary

cIAP2 is an E3 ligase that plays an essential role during inflammation by promoting pro-survival and inflammatory signaling pathways(Beug et al., 2012; Sharma et al., 2017). Based on previous literature and our recent work implicating cIAP2 as a positive regulator of innate immunity-induced neuroinflammation (chapter 3), we hypothesized that cIAP2 might a play similar role in response to adaptive immunity.

To our surprise, we found that mice deficient in cIAP2 exhibited severe symptoms of EAE. These mice exhibited increased levels of cytokines and chemokines in their CNS, which led to the recruitment of CD4+ T cells. Moreover, cIAP2$^{-/-}$ mice demonstrated more CD4+ T cell recruitment when compared to wildtype. Along with extreme demyelination, these cIAP2 deficient mice exhibited astrogliosis and myeloid cell proliferation. Our data showed that the absence of cIAP2 significantly increased the proliferation of myeloid cells in the spinal cord as well as in the brain. Most of these myeloid cells were MiDM and were proinflammatory (M1) in nature. Also, reconstitution of bone marrow in both wildtype and cIAP2 deficient mice revealed that resident brain cells were responsible for the exacerbated symptoms of EAE.

It has been reported that during inflammation, lack of cIAP2 results in phosphorylation of RIPK1, which consequently directs the affected cells to die by Caspase-8-dependent apoptosis or RIPK3-dependent necroptosis. Therefore, we investigated if the EAE induced in cIAP2$^{-/-}$ mice displayed higher incidence of cell death than wildtype. Indeed, a majority of cIAP2 deficient oligodendrocytes were dead during EAE induced inflammation in the

CNS. RIPK1 and RIPK3 aggregate in the CNS during necroptosis, we ruled out the possibility of necroptosis since the expression of RIPK1 and RIPK3 did not corelate with the disease score. Rather the expression was dependent on the mice gender. Thus, we explored if Caspase-8 mediated death in EAE mice. We observed higher levels of Caspase-8 activity in the lumbar region of the EAE induced cIAP2$^{-/-}$ mice than that of EAE induced wild type mice. This suggests that oligodendrocyte death is mediated by apoptosis.

It has previously been demonstrated that Caspase-8 can also exhibit a non-apoptotic role in microglia. Studies report that Caspase-8 can modulate microglia activity and induce neurotoxicity. Hence, we explored if cIAP2 plays a role in the expression of Caspase-8 and modulates the activation of microglia in the CNS. Our data demonstrated that 85% of microglia deficient of cIAP2 were Caspase-8 positive. In contrast, only 35% of wildtype microglia were Caspase-8 positive. Recent studies of dendritic cells and macrophages demonstrated that Caspase-8 can contributes to inflammation by two mechanisms. It can be either by directly cleaving pro IL-1β to produce active IL-1β that fuels inflammation or by activating a NLRP3 inflammasome that also promotes cleavage pro IL-1β to produce active IL-1β. Here, we identified a novel mechanism by which cIAP2 regulates Caspase-8 to activate microglia.

Further studies are required to understand cIAP2 dependent activation of Caspase-8 in microglia.

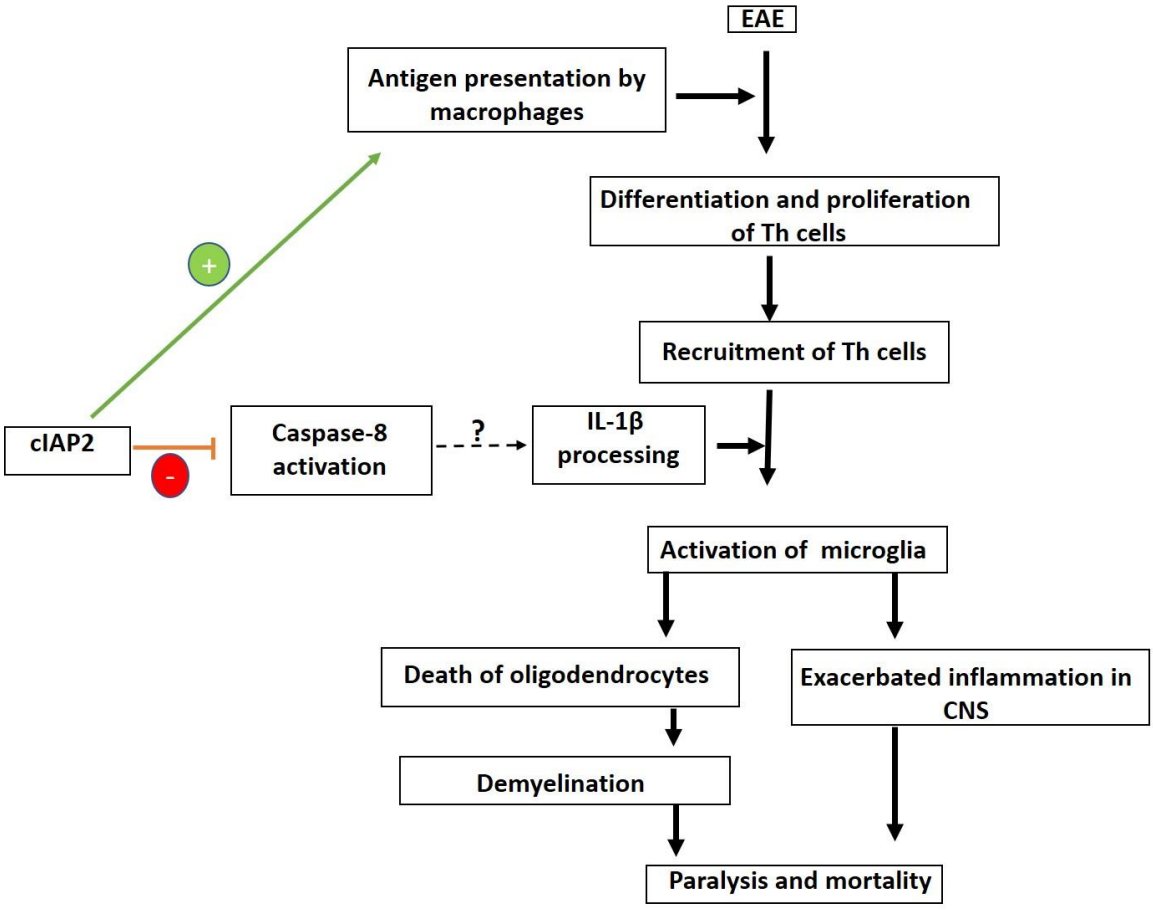

EAE
Antigen presentation by macrophages
Differentiation and proliferation of Th cells
Recruitment of Th cells
+
cIAP2
-
Caspase-8 activation
?
IL-1β processing
Activation of microglia
Death of oligodendrocytes
Exacerbated inflammation in CNS
Demyelination
Paralysis and mortality

Figure 4- 23 Role of cIAP2 in neuroinflammation in response to adaptive immune response induced by EAE.

cIAP2 in APC-like macrophages are required for optimal presentation of antigens (MOG) to T cells which allows its differentiation as well as proliferation. These activated Th cells are recruited to the CNS, causing activation of microglia and the secretion of cytokines and chemokines. cIAP2 in microglial cells suppress the activation of Caspase-8, consequently repressing M1 microglia dependent inflammation and promoting survival of oligodendrocytes for the remyelination of axons.

Chapter 5

Discussion

During an inflammation, a cell displays well-orchestrated activation of multiple signaling pathways. A fine balance of these pathways decides the fate of a cell to survive or die, leading to a state of homeostasis and the resolution of inflammation. Among the plethora of proteins activated during inflammation, cIAP2 is activated to regulate the production of cytokines, proliferation, and cell survival.

Previously, it has been reported that cIAP2$^{-/-}$ macrophage cells are highly susceptible to apoptosis in an LPS-induced proinflammatory environment. This contributes to the attenuation of systemic inflammation (Conte et al., 2006). In chapter 3, our studies demonstrated that cIAP2$^{-/-}$ mice exhibit a robust resistance to systemically administered LPS. We observed a significantly reduced level of TNF-α expression by Kupffer cells (liver residential macrophages) in the liver of cIAP2$^{-/-}$ mice as compared to that wildtype mice. Peripheral TNF-α can cross the blood brain barrier and activate glial cells such as microglia and astrocytes in the CNS. As expected, due to limited amounts of TNF-α available in the peripheral of cIAP2$^{-/-}$ mice, we detected decreased glial cell activation and decreased expression of proinflammatory cytokines in the CNS. This confirms that cIAP2 in macrophages is critical for maintaining a normal innate immune response-induced neuroinflammation. In chapter 4, we also found that cIAP2 plays a crucial role in antigen presenting cells (APC), including macrophages. Absence of cIAP2 reduced the recognition of antigens by macrophages and ablated the expression of major histocompatibility complex (MHC) molecules II on macrophages. Consequently, this attenuated antigen

presenting potential of macrophages, confirming that cIAP2 is required for the innate immune system to mediate neuroinflammation.

Antigen presentation is a critical process essential for T cell immune responses. APCs, such as macrophages or dendritic cells, internalize exogenous antigens and degrade them into oligopeptide. Subsequently, MHC antigen peptides are transported to the plasma membrane and the processed antigen is presented to the helper T cells in the lymph node.

NF-kB activation plays an important role in antigen presentation and T cell activation by APCs. It is required for expression of transporter associated with antigen processing 1 (TAP1)(Marqués et al., 2004), MHC (Israël et al., 1989) and co-stimulatory molecules such as CD40 and CD86 (Hinz et al., 2001). Studies of dendritic cells have shown that antigen presentation is dependent on NF-κB activation and can coordinate different aspects of antigen-presenting function, including MHC expression, co-stimulatory molecule expression and cytokine production (Yoshimura et al., 2001). Since cIAP2 is known to play a significant role in activation of NF-κB pathway, we can speculate that in the absence of cIAP2, the activation of NF-κB pathway is attenuated, which could lead to limited antigen uptake and MHC class II expression on BMDM.

cIAP2 has been extensively studied as a critical player that promotes the NF-kB pathway activation, the MAP kinase pathway and inflammasome activation. Based on previous literature and our recent work implicating cIAP2 as a positive regulator of innate immunity-induced neuroinflammation, we postulated cIAP2 might a play similar role in response to adaptive immunity

On the contrary, cIAP2 played an opposite role in adaptive immune driven neuroinflammation. Lack of cIAP2 in the glial cells contributed to severe neuroinflammation induced during EAE. Absence of cIAP2 led to elevated expression of cytokines and chemokines that significantly increased the recruitment of immune cells in the CNS. This infiltration of immune cells led to the activation of glial cells, such as microglia and astrocytes, initiating a second wave of inflammation in the CNS. Since inflammation in the CNS of the cIAP2$^{-/-}$ mice was much higher compared to that of wildtype mice, it resulted in increased death of oligodendrocytes followed by extensive demyelination. Interestingly, we found that 85% of M1 activated MiDM in the CNS, expressed Caspase-8 while only 35% of wildtype MiDM were Caspase-8 positive.

Our studies revealed a previously unrecognized mechanism by which cIAP2 in MiDM specific regulates inflammatory immune responses during EAE. cIAP2 can repress Caspase-8 activation in MiDM, which inhibits cytokine storm in the CNS and allows for the survival of oligodendrocytes.

Caspase-8 is an initiator caspase- that plays a central role in apoptosis. Several recent studies have identified non-apoptotic roles for Caspase-8(Lemmers et al., 2007; Maelfait and Beyaert, 2008). Caspase-8 can directly cleave pro-IL-1β in response to CD95L induced signaling (Bossaller et al., 2012), TLR3/TLR 4 signaling (Maelfait et al., 2008) or during fungal infections (Gringhuis et al., 2012), independently of caspase-1 inflammasome. Other studies of macrophages and dendritic cells have shown that caspase- 8 is required for priming and activation of NLRP3 inflammasomes (Gurung et al., 2014).

Additionally, it has been reported that caspase-8 can activate microglia. Further, the inhibition of the caspase- signaling can prevent microglia mediated neurodegeneration (Burguillos et al., 2011; Viceconte et al., 2015). Together, this suggest that cIAP2 mediated repression of Caspase-8 is necessary for limiting neuroinflammation during EAE and MS. Further investigations are required to determine if the absence of cIAP2 mediates IL-1β processing or NLRP3 inflammasome formation in the microglia.

Physical interaction between cIAP2 and Caspase-8 has not been identified. However, it has been shown that TRAF2 can tag Caspase-8 with K48 linked ubiquitin chain which can lead to its proteasomal degradation (Gonzalvez et al., 2012). Crystal structure studies of TRAF2 show that it can directly interact with the BIR1 domain of cIAP2 and form a complex(Zheng et al., 2010) It would be interesting to investigate if Caspase-8 is K48 polyubiquitinated by cIAP2/TRAF2 complex in activated microglia.

It has been determined that cIAP2 plays a crucial role downstream of TNF-R1 activation. Upon stimulation with TNF-α, TNF-R1 receptors recruits TRADD, FADD, RIPK1, cIAP1, cIAP2 and TRAF2. These E3 ligases form a complex which then polyubiquitinate RIPK1. This allows the recruitment of TAK/TAB1/TAK2 and IKKα/IKKβ/IKKγ complexes, and consequent activation of NF-κB. In absence of cIAP2, RIPK1 is phosphorylated and forms a complex with either Caspase-8 or RIPK3 to induce cell death by apoptosis or necroptosis, respectively.

In spinal cord tissue from EAE induced cIAP2$^{-/-}$ mice, we found that about 78% of oligodendrocytes were dying while only 50% were dead in the wildtype mice. This

suggests that in the absence of cIAP2, a large population of oligodendrocytes die either by Caspase-8 mediated apoptosis or RIPK3 mediated necroptosis. Ofegiem *et al*. reported that during EAE, oligodendrocytes die due to necroptosis during neuroinflammation. Since RIPK1 and RIPK3 aggregate in the CNS during necroptosis(Li et al., 2012; Ofengeim et al., 2015), we examined the level of expression of these proteins in the lumbar region of EAE induced mice. However, we found that the expression of RIPK3 was inconsistent and were sex dependent. Also, it has been reported through personal communication from Dr. Lukens, UVA (unpublished data) that $RIPK3^{-/-}$ mice did not show any difference in symptoms of EAE as compared to that of wildtype mice. This suggests that absence of cIAP2 might not induce necroptosis induced neuroinflammation during EAE.

Smac mimetics are cIAP2 anatagonistis that cause K48 linked autoubiquitination of cIAP2 leading to its degradation. As mentioned earlier, currently there are five Smac mimetics that are evaluated in clinical trials as cancer therapeutics in phase I/II.

Although the administration of smac mimetics has proven to be well-tolerated and successful in killing tumor cells, however, during the first in-human phase I with smac mimetics, LCL161, given to patients with advanced stages of solid tumors reported a dose-limiting toxicity also known as cytokine release syndrome (Infante et al., 2014)

Some *invitro* studies have reported that smac mimetic–mediated depletion of cIAPs protein leads to activation of caspase- 8 to trigger formation of a complex of ripoptosome

cytosolic cell death complex containing RIPK1, FADD, and caspase-8 which facilitates apoptosis of cells. (Allensworth et al., 2013; McComb et al., 2016)

Some other group have also elucidated that the loss of cIAPs modulate tumor associated macrophages. M2-antiinflammatory macrophages were converted to M1-proinflammatory macrophages that secrete cytokines and chemokines that are antitumor in response (Lecis et al., 2013).

Also based on our data, we conclude that absence of cIAP2 led to deleterious effect in mice with EAE. Therefore, the patients predisposed to neurodegeneration disease such as MS must reconsider using Smac mimetics as a drug of choice for chemotherapy.

Also, utilization of this drug might prove beneficial in cases of glioblastoma multiforme (Beug et al., 2017). However, during the process of treating tumors, Smac mimetics might activate microglia and tumor associated macrophages and disturb the homeostasis in the CNS. Therefore, it is important to further study the role of Smac mimetics during neuroinflammation before confirming its potential as a treatment for cancer.

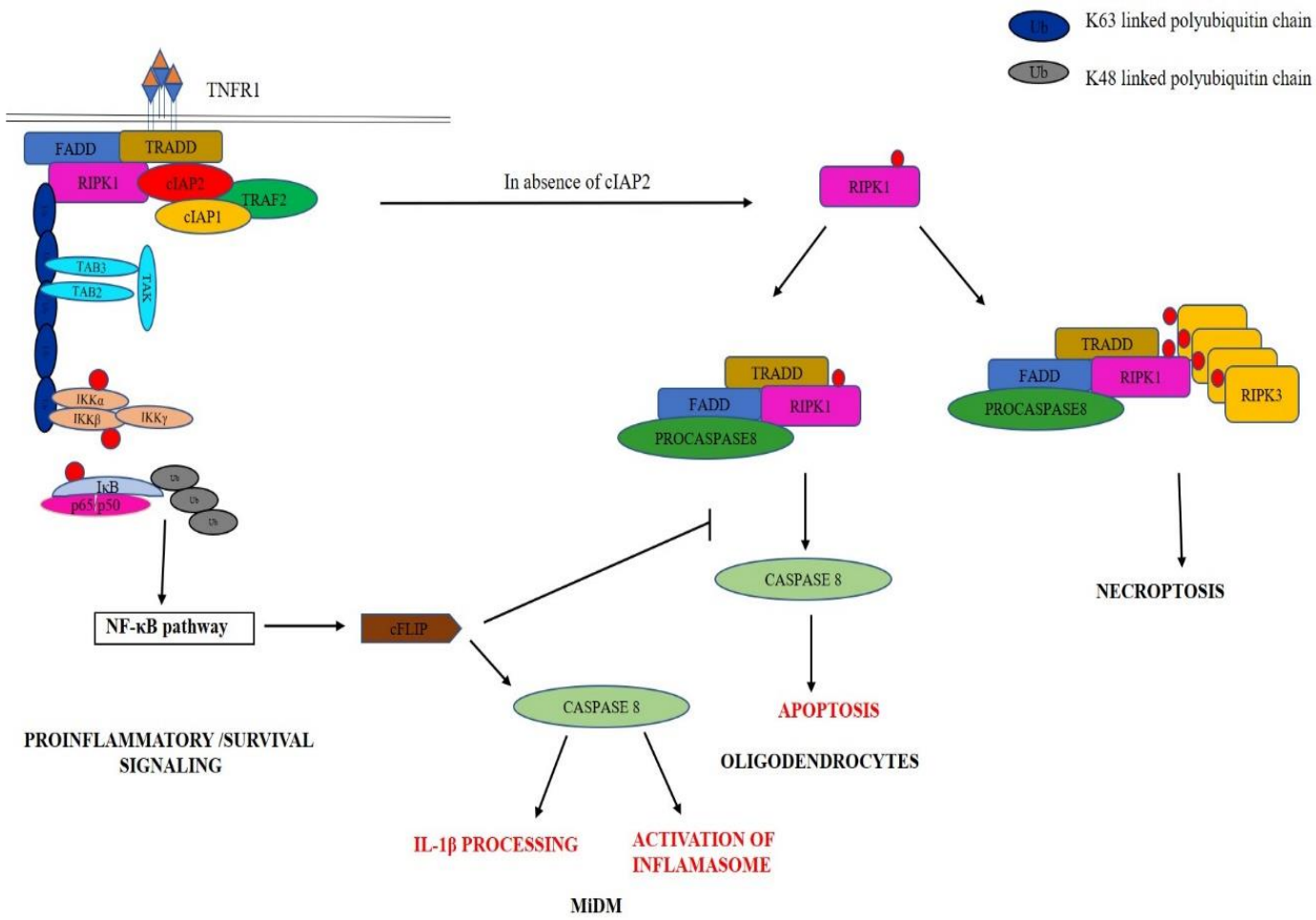

K63 linked polyubiquitin chain
K48 linked polyubiquitin chain
TNFR1
FADD
TRADD
RIPK1
cIAP2
TRAF2
cIAP1
TAB3
TAB2
TAK
IKKα
IKKβ
IKKγ
IκB
p65/p50
Ub
In absence of cIAP2
RIPK1
TRADD
FADD
RIPK1
PROCASPASE8
TRADD
FADD
RIPK1
RIPK3
PROCASPASE8
NF-κB pathway
cFLIP
CASPASE 8
NECROPTOSIS
CASPASE 8
APOPTOSIS
PROINFLAMMATORY /SURVIVAL SIGNALING
OLIGODENDROCYTES
IL-1β PROCESSING
ACTIVATION OF INFLAMASOME
MiDM

Figure 5- 2: Proposed model of cIAP2 mediated neuroinflammation.

Upon stimulation with TNF-α, TNF-R1 receptors recruits TRADD, RIPK1, cIAP1, cIAP2 and TRAF2. These E3 ligase can K63 polyubiquitinate RIPK1 and consequently recruitment of the TAK/TAB1/TAK2 and IKKα/IKKβ/IKKγ complexes, and consequent activation of NF-κB, respectively. In absence of cIAP2, RIPK1 is phosphorylated and form a complex with caspase- 8 induce cell death by apoptosis in oligodendrocytes. Absence of cIAP2 can also activate caspase- 8 to directly cleave pro IL-1β in a caspase-1 independent pathway or activate inflammasome in Microglia derived macrophages (MiDM).

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- Honor Society of Alpha Epsilon Lambda award and nominated membership for Outstanding Scholarly Achievement, 2014-2015
- C.C. Clayton Award, Academic excellence, School of Medicine, Virginia Commonwealth University, 2012-2013
- Inducted member of Phi Kappa Phi Honor Society- Doctoral, VCU chapter, 2012-2013
- "Undergraduate Merit Scholarship" Shivaji University for two years 2003-2004 and 2005-2006

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- Bhardwaj R., Yester J., Singh S., **Biswas DD.,** Surace M., Hauser K., Kordula T. Sterile inflammation-associated expression of YKL-40 is regulated by RelB/p50 complexes. *Journal of Immunology,* January 2015
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