THE MECHANISMS BY WHICH APOPTOTIC NEURONS IN THE DEVELOPING DORSAL ROOT GANGLIA ARE ENGULFED

By

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
Arf6	ADP-ribosylation factor 6
BAI1	Brain-specific angiogenesis inhibitor-1
BFABP	Brain fatty-acid binding protein
Ced	Cell death abnormal
CHO	Chinese hamster ovarian cells
CMV	Cytomegalovirus
CNS	Central nervous system
CRT	Calreticulin
dCed-6	Drosophila Ced-6
DMEM	Dulbecco's Modified Eagle Medium
DRG	Dorsal root ganglia
E13.5	Embryonic day 13.5
ECD	Extracellular domain
EGF-like	Epidermal growth factor-like
EMI	EMILIN domain
ER	Endoplasmic Reticulum
FBS	Fetal bovine serum
FcγR	Fc fragment of IgG receptor
Gas6	Growth arrest-specific 6
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GGF	Glial growth factor
Gulp	Engulfment adapter PTB-containing
HEK293	Human embryonic kidney 293
ICD	Intracullular domain
IP	Immunoprecipitation
ITAM	Immuno-receptor tyrosine-based activation motif
Jedi-1	Jagged and Delta-1
LAMP	Lysosomal-associated membrane protein
LRP	Low density lipoprotein receptor-related protein
MEGF10-	Multiple EGF-like domains 10-12
meGFP	Membrane bound GFP
MFG-E8	Milk fat globule EGF-factor 8
mRNA	messenger RNA
NGF	Nerve growth factor
NT3	Neurotrophin-3
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEAR1	Platelet endothelial aggregation receptor 1
PI	Propidium iodide

PI3K	Phosphoinisitide 3 kinase
PLCγ	Phospholipase Cγ
PLOSL	Polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy
PNS	Peripheral nervous system
PS	Phosphatidylserine
PTB	Phosphotyrosine binding domain
RAGE	Receptor for advanced glycation endproducts
RT-PCR	Reverse transcription-PCR
SEM	Standerd error of mean
SFK	Src Family kinase
SGC	Satellite Glial Cell
SH2	Src homology 2 domain
shRNA	Short hairpin ribonucleic acid
Simu	Six microns under
SLE	Systemic lupus erytheromatosus
SNP	Small nucleotide polymorphism
Src	Rous sarcoma oncogene
Syk	Spleen tyrosine kinase
TAM	Tyro-Axl-Mer kinases
TIM4	T cell immunoglobulin mucin 4
TREM2	Triggering receptor expressed on myeloid cells–2
TTR-52	Transthyretin-like protein
TuJ1	Neuronal class III beta-tubulin
Tulp1	Tubby-related protein 1
Zap-70	Zeta-chain associated protein kinase 70

CHAPTER

INTRODUCTION

Overview

The capacity of cells to internalize foreign particles as well as other cells has been studied since the late 1800s when Ilya Metchnikov first observed the phenomenon. Metchnikov observed starfish larvae internalizing microorganisms, and he also determined that white blood cells could ingest bacteria. He named these white blood cells "phagocytes", derived from the Greek "phagein" meaning to devour. Since Metchnikov's early observations, the process of engulfment of both pathogens and apoptotic cells by specialized phagocytic cells has been extensively studied.

Within this chapter, I introduce the importance of rapidly and efficiently engulfing apoptotic cells, a specialized form of phagocytosis known as either engulfment or efferocytosis (derived from the Latin efferre "to take to the grave"). My focus will be on removal of apoptotic cells in the nervous system, and I will introduce a number of signaling receptors and molecules involved in the process of efferocytosis. Most of this chapter contains information specific to the central nervous system (CNS) because relatively little is known about engulfment of apoptotic cells in the peripheral nervous system (PNS). Chapters II, III, and IV describe our observations in the developing dorsal root ganglia (DRG)wherein we elucidate the signaling mechanisms used by the engulfment receptors Jedi-1 and MEGF10. Chapters V and VI detail the work we have done to characterize the phenotypes of jedi-1 -/- mice, and the final chapter discusses the importance and potential implications of my dissertation research.

More than waste removal: The vital importance of engulfment

The rapid and efficient clearance of apoptotic cells is essential, as aberrantly persistent cell corpses can undergo secondary necrosis, a process in which membrane integrity is lost and intracellular contents are exposed to neighboring cells (Savill et al., 2002). Improper removal of apoptotic cells is thought to trigger an inflammatory response, and secondary necrosis is also thought to activate the immune system and potentially lead to autoimmunity, such as systemic lupus erythematosus (SLE) (Janko et al., 2008). Interestingly, mice deficient in engulfment proteins such as SCARF-1, MerTK, or MFG-E8 (described in this chapter) are observed to phenocopy many of the hallmark symptoms of SLE (Ramirez-Ortiz et al., 2013; Scott et al., 2001; Hanayama et al., 2006). In addition to SLE, mutations in components of engulfment pathways have been implicated in numerous diseases. Since apoptotic cell removal is essential throughout the body, diseases as varied as neurodegenerative disorders, cancer, respiratory diseases, and atherosclerosis have been linked to defects in engulfment proteins, as described in a recent review (Elliot and Ravichandran, 2010).

Amateur Phagocytes

The primary job of professional phagocytes (such as macrophages, microglia, and dendritic cells) is to engulf pathogens or apoptotic cells; however, so-called amateur phagocytes also have the ability to internalize cellular debris as well as apoptotic cells. Due to the fact that these cells perform other roles than apoptotic cell recognition and clearance, they have been named “amateur” or “non-professional” phagocytes. Engulfment by amateur phagocytes has been discovered in many regions of the body, including during spermatogenesis (Lu et al., 1999), mammary gland involution (Monks and Henson, 2009), and engulfment of photoreceptor outer segments by retinal pigmented epithelial cells (Kevany and Palczewski, 2010).

While professional phagocytes have been extensively studied for many years, the notion that other cell types may act as non-professional phagocytes to engulf neighboring apoptotic cell corpses is a comparatively recent idea. Many questions exist about the definition of amateur phagocytes and how they act as non-professional efferocytes. For example, do they utilize the same engulfment receptors and signaling pathways as professional phagocytes? A complex network of molecules is implicated in each of the sequential steps of efferocytosis. First, apoptotic cells must lose the "don't-eat-me" signals that are displayed on the surface of healthy cells, and then they must expose "find-me" and "eat-me" signals that are subsequently recognized by receptors and adapter proteins on the phagocytic cell. After initial recognition of the apoptotic cell, the efferocyte undergoes cytoskeletal rearrangement and membrane surface area increases. This rearrangement of the cell to form a phagocytic cup is followed by internalization of the prey and phagosome maturation that eventually leads to lysosomal degradation of the cell corpse and recycling of internalized cellular components.

Engulfment in the Nervous System

Roles for both professional and amateur phagocytes have been characterized in the nervous system. In the course of development as well as after injury, apoptotic neurons must be cleared. During development of the mammalian nervous system, approximately 50% of the neurons that are generated undergo apoptosis as part of a normal pruning process. Only the neurons that establish proper connections with their target tissues will survive, while neurons that fail to reach their target and gain trophic factors will die.

Microglia

The classic mammalian professional phagocytes are macrophages, neutrophils, and dendritic cells; however, the blood brain barrier of the central nervous system blocks the infiltration of these types of cells under normal conditions. Microglia, a type of tissue resident macrophage, are the sole professional phagocyte found in the healthy brain and spinal cord. The origin of microglia has been debated for over a century (Ransohoff and Cardona, 2010), but recent studies have shown that microglia arise from early progenitors in the embryonic yolk sac which seed the brain and appear to persist there into adulthood (Ginhoux et al., 2013). Microglia are classified into different subpopulations based upon whether they are resting or activated (M2 or M1, respectively). M2 microglia are termed "resting" microglia, but this nomenclature does not capture the fact that these microglia are continuously surveying their environment (Nimmerjahn et al., 2005). M1 microglia are described as "active" microglia, and they are called so because they can release cytotoxic and inflammatory mediators and activate T cells in response to pathogens or other insults. Microglia are generally described as scavenging cells that identify and remove apoptotic cells and pathogens in the CNS, but new research suggests that they also play a role in neurogenesis (Shigemoto-Mogami et al., 2014) as well as synapse pruning and modulation (Paolicelli et al., 2011; Wake et al., 2009).

Microglia also affect the health and viability of neurons by releasing pro- or anti-inflammatory cues. Many extracellular signals are able to activate microglia, including chemokines, cytokines, pathogens, and even misfolded proteins. Toll-like receptors (TLRs) are expressed by microglia, and these TLRs are able to recognize pathogen associated molecular patterns (PAMPS) from bacteria, fungi, and viruses. In the course of bacterial infections, TLR4 promotes transcription of inflammatory genes (Trudler et al., 2010). Microglial activation promotes a change from a resting or "ramified" shape to an active or "amoeboid" shape characterized by increased proliferation and

migration. Activated microglia also produce neurotrophic factors and anti-inflammatory signals that can promote neuronal viability and combat invading pathogens. Microglia have been implicated in promoting neuronal apoptosis under certain developmental and pathological conditions. For instance, microglia in the developing retina secrete NGF and mediate neuronal apoptosis through the p75 neurotrophin receptor (Frade and Barde, 1998; Wakselman et al., 2008). The mechanism by which the neurons are targeted is unknown. Many neurodegenerative diseases have also been linked to activated microglia (Block et al., 2007).

The type of material engulfed by microglia directly affects the response of the microglia. When a microglial cell engulfs apoptotic cell debris, it will release anti-inflammatory cytokines. However, if a microglial cell engulfs a pathogen the response is pro-inflammatory (Napoli and Neumann, 2009). Neuroinflammation is typical in many neurological disorders. Whether microglia participate in Alzheimer's Disease (AD) progression is an important area of research (Cameron and Landreth, 2010). Microglia are known to express receptors that bind and internalize Aβ, and studies suggest that microglia may become chronically activated in AD (Lee et al., 2010). It has also been hypothesized that Parkinson's disease results from increased ROS production that leads to increased microglial activation and cytokine release resulting in neuronal death (Long-Smith et al., 2009). In addition to their role in AD and Parkinson's disease, microglia are implicated in neuropathic pain (Smith, 2010), Rett syndrome (Maezawa and Jin, 2010) and psychiatric disorders (Bayer et al., 1999; Chen et al., 2010). One group suggested that microglia may also be linked to obsessive-compulsive behavior (Chen et al., 2010). They discovered that mice with conditional deletion of Hoxb8 in microglia exhibited increased grooming severe enough to induce hair loss and skin wounds. They were able to rescue the phenotype using a bone marrow transplant with wild-type cells, suggesting that the phenotype was directly related to microglial function. The means by which microglia affected grooming behavior in the mice is unknown, but it could be hypothesized that the phenotype is due to changes in

neuronal engulfment or synapse pruning mediated by microglia.

Astrocytes

Astrocytes, an important glial cell type in the CNS, also aid in removal of debris after injury (Aldskogius et al., 1999). It has been observed in the gerbil brain that after induction of ischemia dead neurons are disassembled by astrocytes and subsequently engulfed by both microglia and astrocytes (Ito et al., 2007). A study of traumatic brain injury in mice revealed that astrocytes play a major role in engulfing apoptotic cell corpses (Loov et al., 2012). Interestingly, astrocytes have also been shown to play a role in synaptic pruning. This process involved the cooperation of two engulfment receptors, MEGF10 and MerTK (Chung et al., 2013). Astrocytes have also been observed to be capable of engulfing microspheres and cell debris in culture (Sokolowski et al., 2010). While microglia have been classically thought to be the primary phagocyte of the CNS, new information is suggesting that astrocytes play a very important phagocytic role. Future studies are required to determine the role of astrocytes in engulfment, and the influence of astrocytes on neuronal degeneration and inflammation also remains to be elucidated.

Neural Precursor Cells

Recent research has added a new amateur phagocyte to the CNS repertoire, the neural progenitor cell (NPC). Programmed cell death of neural progenitor cells occurs in the postnatal subventricular zone (SVZ) of the lateral ventricles and subgranular zone (SGZ) of the hippocampal dentate gyrus in order to limit the numbers of proliferating cells (Blaschke et al., 1996). Approximately 50% of the neurons born in the SGZ and SVZ will undergo apoptosis within the first month after they are born (Biebl et al., 2000; Petreanu and Alvarez-Buylla, 2002). Recent research from the Kipnis lab identified neural precursor cells in the subventricular zone and the subgranular zone of the

hippocampus as cells that have the ability to phagocytose their apoptotic neighbors. This engulfment was dependent on phosphatidylserine and the engulfment adapter protein ELMO, although the receptor required was not identified. When efferocytosis by NPCs was disrupted genetically (ELMO -/- mice) or by blocking phosphatidylserine (Annexin V), a marked decrease in neurogenesis was observed (Lu et al., 2011). The effect of inefficient phagocytosis of apoptotic cells on neurogenesis suggests that elucidation of the mechanism of engulfment by NPCs could lead to insights into the etiology of pathologies linked to changes in adult neurogenesis.

Engulfment Signals

Don't-eat-me signals

Healthy cells present signals that prevent their engulfment by phagocytes, known as "don't-eat-me signals", and these signals are down regulated by dying cells (Grimsley and Ravichandran, 2003). Relatively few don't-eat-me signals have been experimentally identified. For example, CD47 binds to its receptor CD47-signal regulatory protein α (SIRPα) on macrophages and blocks the engulfment process (Oldenborg et al., 2000). Tumor cells are known to upregulate CD47, and this is now being studied as a potential new target for cancer treatment (Willingham et al., 2012).

Come-eat-me signals

In parallel with down regulation of "don't-eat-me" signals, another important stage of engulfment involves professional phagocytes sensing a cell corpse by recognizing "come-eat-me" or "find-me" signals. These signals promote chemotaxis of efferocytes toward the apoptotic cell (Munoz et al., 2010). Once the apoptotic cell has been detected, the phagocyte will recognize "eat-me" signals, bind to the cell, internalize it, and degrade it. After injury, neurons as well as glial cells produce numerous signals to

attract microglia to the region of damage. One of the best-described family of signals for promoting microglial migration are chemokines, and chemokines are released as a result of many neuronal pathologies. For instance, CXCL10 expression was increased in hippocampal neurons after lesion, and this promoted migration of microglia to clear resulting debris. This migration was dependent on the CXCR3 receptor, which recognizes CXCL10 (Rappert et al., 2004).

Damaged neurons will also release glutamate under conditions such as excitotoxic injury. Microglia express both metabotropic and ionotropic glutamate receptors, and microglial chemotaxis has been linked to AMPA and metabotropic receptors. For example, in spinal cord slices perfused with glutamate there was enhanced microglial migration and membrane ruffling. The effect was blocked by metabotropic or AMPA glutamate receptor antagonists (Liu et al., 2009). This study suggests that glutamate release by damaged neurons may act as yet another signal to promote microglial chemotaxis.

In response to damage or insult, neurons also release purines (such as ATP and UTP) that stimulate microglia and promote chemotaxis. At first, purines were considered to act principally as "come-eat-me" signals. Several groups have shown that ATP is released in areas of neuronal damage to promote chemotaxis of microglia through the P2Y12 receptor, a receptor specific to microglia in the CNS (Davalos et al., 2005; Haynes et al., 2006; Honda et al., 2001). Interestingly, after activation, microglia also ramp up expression of other purinergic receptors. For instance, after kainic acid-induced injury in hippocampal neurons, Koizumi et al. determined that injured neurons release UDP and microglia upregulate the P2Y6 receptor. UDP enhanced the phagocytosis of latex beads by microglia, and this increased phagocytosis was abrogated by a P2Y6 antagonist. Interestingly, UDP did not promote migration of microglia. These findings led to the hypothesis that neurons may use ATP as the "come-eat-me" signal to promote chemotaxis, and UDP could act as a localized "eat-me" signal (Koizumi et al.,

2007). Additional factors (ROS, cytokines, and prostaglandins) have been identified as come-eat-me signals (Yenari et al., 2010). It is likely that additional come-eat-me signals will be discovered in the context of CNS cell death.

Eat-me signals and their receptors

Apoptotic cells expose "eat-me" signals that activate engulfment receptors and promote efferocytosis. One of the best-known eat-me signals is phosphatidylserine (PS), a membrane lipid that is flipped to the outer membrane of apoptotic cells during apoptosis (McEvoy et al., 1986). The first clue that PS was an eat-me signal was a study that found liposomes made of PS were able to decrease the ability of macrophages to efferocytose dead lymphocytes (Fadok et al., 1992). PS is the most studied eat-me signal and is used to promote engulfment in organisms as varied as flies, nematodes, and mammals (Kinchen and Ravichandran, 2007). Interestingly, there are multiple engulfment receptors that recognize the eat-me signal PS either directly or through various bridging molecules. The reason for evolution of multiple PS binding receptors is unknown, but the multitude of receptors that can recognize PS suggests that it is a very important signal of cell death.

Fadok et al identified the first protein thought to act as a receptor for PS, and they named it PSR (Fadok et al., 2000). However, the validity of their conclusions was later debated because PSR was determined to localize to the nucleus. PSR was renamed Jumonji domain containing gene 6 (Jmjd6) and grouped into the family of Jumonji domain containing chromatin-remodeling proteins (Schlegel and Williamson, 2007; Wolf et al., 2007). Much controversy still exists about the actual function of PSR, but since then other receptors that recognize PS have been discovered.

Nagata et al identified two PS receptors through the use of an antibody that blocks engulfment by macrophages (Miyanishi et al., 2007). The antibody was binding to T cell

immunoglobulin mucin 4 (TIM-4), and when they screened other TIM proteins they found that TIM-1 also interacted with PS and promoted engulfment. In the same year, Kobayashi et al published their finding that TIM-4 is a PS receptor. They chose to study the TIM proteins because TIM proteins had been linked to autoimmunity (Kuchroo et al., 2006). Their results showed that TIM-4 is required for macrophage engulfment and that it binds PS specifically. Santiago et al solved the crystal structure of TIM-4 bound to PS, and they found that TIM-4 binds to PS at a metal-ion-dependent site in the extracellular immunoglobulin domain (Santiago et al., 2007). In vivo studies showed that TIM-4 -/- mice had defective cell corpse clearance and systemic autoimmunity including hyperactive T and B cells as well as increased auto-antibodies (Rodriguez-Manzanet et al., 2010). Although the importance of TIM-4 in efferocytosis has been confirmed by several groups, evidence suggests that it does not promote signaling directly. A truncated mutant of TIM-4 lacking the intracellular domain was able to promote engulfment (Park et al., 2009); therefore, TIM-4 may act as a tethering molecule.

In addition to TIM-4, Brain-specific angiogenisis inhibitor 1 (BAI1) was also identified as a PS receptor, although it binds PS using thrompospondin repeats in its extracellular domain (Park et al., 2007). Interestingly, BAI1 is highly expressed by both neurons and astrocytes but is expressed at low levels in microglia (Sokolowski et al., 2011). The authors hypothesized that microglia likely use other engulfment receptors, or perhaps BAI1 may be upregulated in injury conditions. The most recently discovered PS receptors are Stabilin-2 and RAGE (Receptor for advanced glycatino end-products), but neither of these receptors have been implicated in engulfment in the CNS or PNS (Falkowski et al., 2003; He et al., 2011; Kim et al., 2010; Park et al., 2008a; Park et al., 2008c). RAGE plays a role in neutrophil clearance by macrophages, and it exists in transmembrane (mRAGE) and soluble (sRAGE) forms. Both forms can bind PS, and sRAGE can bind PS and block recognition by mRAGE.

Bridging molecules

Efferocytes can also utilize secreted bridging molecules to recognize PS. These bridging molecules enable the phagocyte to recognize its prey by binding to PS on the apoptotic cell and to a receptor on the membrane of the phagocyte. A newly discovered engulfment receptor, SCARF-1 (scavenger receptor expressed by endothelial cells), binds to apoptotic cells through interaction with complement component 1q (C1q). C1q links SCARF-1 to PS, and this is important for engulfment by macrophages, dendritic cells, and endothelial cells. SCARF-1 knockout mice developed symptoms of autoimmunity including glomerulonephritis and serum autoantibodies (Ramirez-Ortiz et al., 2013).

Milk fat globule-EGF-factor 8 (MFG-E8) is one of the most thoroughly studied bridging molecules. MFG-E8 is known to contribute to engulfment in the brain as well as other regions of the body (Fuller and Van Eldik, 2008; Raymond et al., 2009). Phagocytic cells such as macrophages and microglia produce and secrete MFG-E8, and this protein binds to PS on dying cells. MFG-E8 acts as a tether beween PS and αvβ3/5 integrins on the efferocyte (Hanayama et al., 2006). MFG-E8 -/- macrophages retain the ability to associate with apoptotic cells, but the process of internalization of cell corpses is diminished. As is commonly the case, knocking out MFG-E8 does not completely abrogate engulfment by primary phagocytes, likely because these specialized efferocytes express a range of engulfment receptors that are able to compensate for the loss of MFG-E8. MFG-E8 has been implicated in phagocytosis by both professional and amateur phagocytes in the process of mammary gland involution (Hanayama and Nagata, 2005).

The TAM kinase family utilizes several different secreted proteins as adapters to PS: Protein S, Growth arrest-specific gene 6 (Gas6), Tubby, and Tubby-like protein 1 (Tulp1) (Caberoy et al., 2010; Rothlin and Lemke, 2010). The receptor tyrosine kinases Tyro3, Axl, and MerTK are the three members of the TAM family. Gas-6 (which binds all 3 TAM

receptors) and Protein S (which binds MerTK and Tyro-3) can act as recognition bridges between phosphatidylserine-containing membranes and TAM receptors while also stimulating activation of TAM receptors, and this bridging is important for binding of phagocytes to apoptotic cells and TAM activation (Ishimoto et al., 2000, Nagata et al., 1996). TAM receptors and ligands (Gas6 and Protein S) are expressed in the immune, reproductive, vascular, and nervous systems. The physiological roles of TAM receptors were mostly determined by examination of mice lacking Axl, Tyro-3, or Mer. Even mice lacking all three receptors are phenotypically normal until about 3 weeks of age when they develop severe degenerative phenotypes: blindness due to death of photoreceptors (Gal et al., 2000), loss of germ cells in the testes (Lu et al., 1999), and severe autoimmunity (Lu and Lemke, 2001). Photoreceptor death is due to inefficient clearance of shed photoreceptor outer segments by the phagocytic retinal pigment epithelial cells, and germ cell loss is due to the inability of Sertoli cells to engulf apoptotic germ cells and cellular contents extruded during sperm maturation (Lemke and Rothlin, 2008). Autoimmunity likely stems from inefficient clearance of apoptotic cells and membranes in general in these animals, and cells responsible for clearance by the immune system (macrophages and dendritic cells) show decreased phagocytic activity when their respective TAM receptors are absent (Seitz et al., 2007).

In the nervous sytem, the TAM receptors have been implicated as important phagocytic receptors. Microglia express Axl and Mer, and stimulation of microglia with Gas6 (a ligand of Mer and Axl) enhances phagocytic activity (Grommes et al., 2008). Several studies suggest that Gas6/Axl signaling may play an important role in microglial clearance of apoptotic cells in the CNS: Axl and Gas6 expression increases during demyelinating injury in the CNS, but the significance of this is unknown (Binder et al., 2008). Recombinant Gas-6 injection into the brains of cuprizone demyelinated mice led to enhanced clearance of apoptotic debris, but no mechanism was proposed (Tsiperson et al., 2010). Axl -/- mice showed delayed phagocytosis of debris after demyelinating injury (Hoehn et al., 2008), and this could be due to loss of Axl-mediated phagocytic

signaling and/or loss of Axl-mediated enhancement of Mer signaling. In addition to playing a role in clearance of apoptotic cells, MerTK plays a role in synapse elimination in the CNS (Chung et al., 2013). Evidence clearly suggests that the TAM receptors should be studied further in the context of microglial and astrocyte phagocytosis and synapse modulation.

PS-Independent and Orphan Engulfment Receptors

Many engulfment receptors recognize signals other than PS. ATP Binding Cassette sub-family A 1 (ABCA1) was initially studied in macrophage clearance of apoptotic cells in inderdigital webs. The use of antibodies generated against ABCA1 significantly decreased the phagocytic capability of macrophages to clear apoptotic cell corpses (Luciani and Chimini, 1996). The role of ABCA1 in the nervous system has not been elucidated, although it is expressed in microglia, neurons, and astrocytes (Kim et al., 2006). A related protein, ABCA7, is also an engulfment receptor used by macrophages (Jehle et al., 2006). The function of ABCA1 and ABCA7 is to modulate lipid transport across the cell membrane (Landry et al., 2006; Wang et al., 2003; Wang and Tall, 2003), but how this function is important for engulfment is not clear. These are not the translocases for flipping PS to the extracellular leaflet of the membrane, rather they are known to efflux cholesterol and other phospholipids. One idea is that the ability of these proteins to regulate the efflux of cholesterol and phospholipids could promote receptor mobility in the context of apoptotic cell recognition and clearance (Hamon et al., 2006).

In addition to exposure of PS during apoptosis, some intracellular molecules are exposed as eat-me signals. The engulfment receptor CD91/Low-density lipoprotein receptor related protein-1 (LRP-1) binds to Calreticulin (CRT), a calcium binding protein that acts as a chaperone in the ER in healthy cells (Gardai et al., 2005; Martins et al., 2010). After binding of LRP-1 to CRT, LRP-1 promotes engulfment of apoptotic cells by regulating Rac activation and lipid homeostasis (Gardai et al., 2005; Kinchen et al., 2005;

Kiss et al., 2006; Su et al., 2002). The mechanism by which CRT is exposed during apoptosis is unclear, but one hypothesis is that CRT may translocate to the cell membrane before blebbing occurs (Obeid et al., 2007).

An example of an orphan engulfment receptor is Triggering receptor expressed on myeloid cells-2 (TREM2). TREM2 is vital for clearing apoptotic neurons in the CNS and also for inhibiting inflammation (Takahashi et al., 2005). TREM2 is expressed in microglia, osteoclasts, and dendritic cells (Colonna, 2003), and its unidentified ligand seems to be present in all apoptotic cells (Hsieh et al., 2009). TREM2 also has implications for human diseases such as Nasu-Hakola disease, a fatal genetic neurodegenerative disease with symptoms including early onset dementia, cerebral atrophy, microglial activation, demyelination, and bone cysts. One hypothesis is that the phagocytic deficiency of microglia lacking TREM2 may contribute to the disease, but the mechanism is unclear at this time (Thrash et al., 2009).

Engulfment Molecules of the Innate Immune System

The function of the Innate Immune System is to respond to and eliminate foreign pathogens, but overlap between this system and apoptotic cell engulfment is now well established. For example, the complement system plays a role in apoptotic cell removal as well as synapse elimination. Complement receptor 3 (CR3/CD11b/Mac1) is expressed by microglia (Fiske and Brunjes, 2000; Gasque et al., 1998), and C1q, a secreted complement protein, is able to bind PS and label apoptotic cells for engulfment (Paidassi et al., 2008). In 2007, Stephens et al showed that C1q and CR3 were involved in microglial regulation of synaptic remodeling in the retinogeniculate pathway. In that system, C1q and CR3 were important for marking and clearing unwanted synapses during development (Stevens et al., 2007). In development and after injury, neurons produce C1q to tag synapses for removal by microglia. Mice lacking the A chain of C1q have abberant refinement of the retinogeniculate circuitry and increased innervation of

the retina by neurons in the lateral geniculate nucleus (Stevens et al., 2007). C1q knock-out mice also exhibit an autoimmune phenotype (Botto, 1998), and the gene most implicated in SLE susceptibility is C1q (Trendelenburg, 2005). This evidence suggests that the role of the complement system in synapse elimination as well as in efferocytosis is an important area of future research and a potential target for treating autoimmune diseases.

Other examples of innate immune receptors that promote apoptotic cell engulfment include CD14 (Devitt et al., 1998; Gregory et al., 1998; Moffatt et al., 1999; Schlegel et al., 1999) and Scavenger Receptors (Husemann et al., 2002; Silverstein and Febbraio 2009). Interestingly, a few engulfment receptors such as Trem2 (Daws et al., 2003; N'Diaye et al., 2009) and BAI-1 (Das et al., 2011) are able to recognize both pathogens and apoptotic cells. Future research may reveal more overlap between the receptors involved in pathogen recognition and apoptotic cell recognition.

Evolutionarily Conserved Pathways for Efferocytosis

Professional phagocytes such as macrophages, microglia, and dendritic cells possess a vast repertoire of engulfment receptors able to recognize a variety of eat-me signals. Whether amateur phagocytes utilize the same or different receptors has not been well established, but new studies suggest that many of the same mechanisms may be conserved in efferocytosis by amateur phagocytes.

C. elegans

Due to the fact that the nematode *C. elegans* lacks professional phagocytes, it is a useful model for studying amateur phagocytosis. The process of cell death in C. *elegans* has also been well characterized. Each worm is composed of 1090 cells, and 131 of these cells undergo apoptosis during development (Kimble and Hirsh, 1979; Sulston and

Horvitz, 1977; Sulston et al., 1983) and are recognized and removed by neighboring cells. The Horvitz group used a combination of genetic and molecular experiments to identify two signaling pathways involved in apoptotic cell clearance. The members of these pathways belong to the cell death abnormal (ced) genes, and alterations in these genes resulted in abnormally persisting apoptotic cell corpses in worms.

One of the identified engulfment pathways in *C. elegans* is composed of ced-2, ced-5, ced-10, and ced-12. Ced-2, ced-5, and ced-7 interact to form a complex (Wu et al., 2001; Zhou et al., 2001a), and the mammalian homologs of these proteins (CrkII, Dock180, and ELMO respectively) also form a complex (Figure 1.1). Dock180 and ELMO form a complex to act as a guanine nucleotide exchange factor for Rac1 (Brugnera et al., 2002), and CrkII acts as an adapter protein through its SH2-SH3 domains. The final signaling result of formation of the ced2/ced5/ced12 complex is Ced-10 (Rac1) activation and cytoskeletal rearrangement (Kinchen et al., 2005; Tosello-Trampont et al., 2007). In mammals, this pathway is conserved and has been linked to signaling downstream of the integrin/MFG-E8 complex (Akakura et al., 2004) and BAI1 (Park et al., 2007).

The other pathway of efferocytosis in *C. elegans* contains ced-1, ced-6, and ced-7 (Figure 1.1). Ced-1 is a single pass transmembrane protein on engulfing cells that binds to PS through the bridging molecule TTR-52 (Rutkowski and Gartner, 2010). Mammalian proteins which are highly homologous to ced-1 include MEGF-10, -11, and -12. These genes code for single pass transmembrane proteins and are named for the multiple EGF-like repeats in their extracellular domains. MEGF-12 (also known as Jedi-1 or PEAR-1) and MEGF-10 are characterized in this dissertation. Ced-6, an adapter protein known to be important for engulfment, binds to the intracellular domain of Ced-1 at an NPXY motif. The phosphotyrosine binding (PTB) domain of GULP is required for this interaction (Zhou et al., 2001; Su et al., 2002). The homolog of Ced-6, GULP, interacts with the mammalian engulfment receptors Stabilin-2 (Park et al. 2008b) and LRP-1 (Kiss et al., 2006) to promote engulfment. The final member of this pathway is Ced-7, a

protein expressed on both engulfing and apoptotic cells. Ced-7 is the homolog of ABCA1, described previously. The mechanism by which this pathway signals has not been clearly elucidated, although it is possible that it promotes Ced-10 (the homolog of mammalian Rac1) activation (Kinchen et al., 2005).

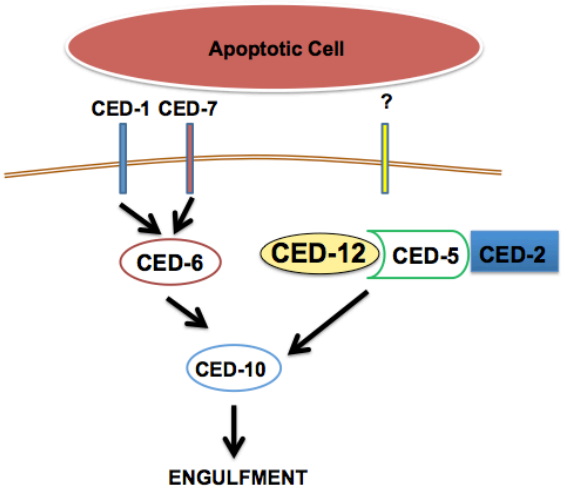


Figure 1.1 Two pathways of engulfment have been discovered in C. elegans. The engulfment receptor CED-1 signals through the adapter protein CED-6 and CED-7 to promote CED-10 activation and engulfment. Downstream of an unknown stimulus, the complex of CED-12, CED-5, and CED-2 promotes CED-10 activation and engulfment.

Drosophila

Drosophila is a model system that possesses both professional and amateur phagocytes. Macrophages engulf apoptotic cells during development except for in the CNS, where glia act as a barrier around the ventral nerve cord and block their entry (Schwabe et al., 2005). Glia, therefore, act as the efferocytes that engulf apoptotic neurons during development (Kurant et al., 2008). The receptor used by glial cells in engulfment of apoptotic neurons is known as Draper, and it is a structurally similar homolog to Ced-1 in *C. elegans* (Awasaki and Ito, 2004; Fuentes-Medel et al., 2009; Hoopfer et al., 2006; MacDonald et al., 2006). The name "Draper" derives from the French name for a

fashion designer, specifically one who drapes clothing or fabric over a dress form.

While Ced-1 recognizes PS through a bridging protein, Draper-mediated engulfment is PS independent (Manaka et al., 2004). Both Draper and six-microns-under (Simu) are homologous to Ced-1, being predicted to be single transmembrane domain proteins containing EGF repeats in their extracellular domains. The protein Six-microns under (Simu) is in the same pathway as Draper genetically (Kurant et al., 2008), and it is expressed on glia. Simu is thought to play a tethering role because a mutant of Simu that lacks the transmembrane and intracellular domain can rescue Simu null mutant fly engulfment (Kurant et al., 2008). The findings of Kuraishi et al suggest that Pretaporter, an ER protein, translocates to the membrane of apoptotic cells and is a potential ligand for Draper (Kuraishi et al., 2009).

Draper, like its homolog Ced-1, interacts with the engulfment adapter protein dCed-6 (Drosophila Ced-6), and this interaction is essential for the clearance of cell corpses. How Draper and dCed6 signal to promote engulfment is unknown. Draper also signals through an Immunoreceptor Tyrosine-based Activation Motif (ITAM) located in its extraceullar domain which is phosphorylated by Src42A and subsequently bound by the non-receptor tyrosine kinase Shark (Ziegenfuss et al., 2008). ITAM motifs are found in several mammalian immune receptors such as the T and B cell receptors (Underhill and Goodridge, 2007). The downstream signaling of Draper and Shark are unknown at this time.

Engulfment in the Peripheral Nervous System of Mammals

While engulfment of apoptotic neurons has been well studied in the CNS, relatively little is known about how apoptotic cell corpses in the PNS are cleared. Schwann cells (SCs) are a type of glial cell known to myelinate and ensheath axons, but they are also amateur phagocytes. In 2004, Bishop et al. determined that Schwann cells play a role in

developmental axon pruning using mice with fluorescently labeled motor neurons (Bishop et al., 2004). Schwann cells are also known to play a role in engulfment of myelin debris after nerve injury. Soon after a nerve insult such as a nerve crush or axotomy, SCs dedifferentiate to engulf myelin debris until macrophages later invade to aid in the clearance process (Fernandez-Valle et al., 1995). Efficient removal of myelin debris is important for reinnervation after injury because myelin debris contains proteins that discourage neurite outgrowth (Brushart et al., 1998; Mears et al., 2003; Schafer et al., 1996; Vargas et al., 2010). In the context of development and injury, Schwann cells are known to be active amateur phagocytes.

The question of what cell type promotes clearance of apoptotic cell corpses in the PNS remained unknown until recently. As is the case in the CNS, massive neuronal apoptosis occurs in the PNS (Hamburger and Levi-Montalcini, 1949). Our lab identified satellite glial cell (SGC) precursor cells as the primary phagocyte of apoptotic neurons during development, and the Carter lab also identified the engulfment receptors Jedi-1 and MEGF10 as essential players in this process (Wu et al., 2009).

Conclusion

Based on current evidence, it is clear that both amateur and professional efferocytes are essential for removing apoptotic cells and debris in the nervous sytem. While different cell types express different engulfment receptors, it appears that many professional and amateur phagocytes utilize common receptors to signal engulfment.

Differences between phagocytosis by amateur and professional phagocytes have not been well studied. One example of such a study involved comparison between the rate of efferocytosis of apoptotic cerebellar granular neurons by microglia and amateur phagocytes (Parnaik et al., 2000). They discovered that microglia engulf apoptotic neurons within a few minutes of incubation with prey, while amateur phagocytes (baby

hamster kidney fibroblasts and lens epithelial cells) required hours of contact with apoptotic cells prior to engulfment. The receptors and signaling molecules that control the differences in professional and amateur efferocytosis remain unclear. One hypothesis is that professional phagocytes are more effective in engulfment due to high expression of a wide variety of engulfment receptors, whereas amateur phagocytes may express fewer receptors. However, the exact reason for differences in phagocytic capability between different cell types has not been determined.

Efferocytosis is a highly complex process, as evidenced by the extensive variety of molecules that are able to recognize eat-me signals, and it is possible that more molecules that regulate engulfment may be discovered. We do not know why different cell types and tissues express different engulfment machinery, but most phagocytes utilize more than one engulfment receptor or bridging molecule. This redundancy of engulfment receptors may be a safety measure to ensure that all apoptotic cells are recognized and rapidly cleared. Amateur phagocytes themselves may be a back-up system for situations in which professional efferocytes are not present. Perhaps amateur phagocytosis may be a mechanism to prevent a greater immune response and to prevent release of inflammatory signals to recruit professional phagocytes. Due to the fact that professional phagocytes are only found in higher organisms, it is possible that they evolved due to the fact that tissues were too complex for amateur phagocytes alone.

Aims of Dissertation

During development, approximately half of developing neurons in the peripheral nervous system undergo apoptosis as part of a normal pruning process. These corpses need to be cleared in order to prevent an inflammatory response and the possible development of autoimmunity. We have recently shown that apoptotic neurons in the developing mammalian dorsal root ganglia are cleared by satellite glial cell (SGC)

precursors; moreover, Jedi and MEGF10 have been identified as engulfment receptors on the membranes of SGC precursors involved in phagocytosis. The primary goal of this research was to determine the signaling pathways involved in the engulfment of dead neurons. Due to the high level of homology between Jedi-1 and the *C. elegans* and *Drosophila* engulfment receptors Ced-1 and Draper, I hypothesized that Jedi-1 and MEGF10 would utilize similar signaling pathways. I also hypothesized that Jedi-1 knockout mice would have an autoimmune phenotype due to defective apoptotic cell clearance, and I have begun the characterization of these Jedi-1 null mice. To test these hypotheses I proposed the following three aims:

Aim 1: To determine if SFKs phosphorylate Jedi ITAM motifs to promote engulfment of apoptotic cells.

Binding of Shark to Draper requires phosphorylation of the Immunoreceptor Tyrosine-Based Activation Motif (ITAM) of Draper by the kinase Src42A, a mammalian SFK homolog. Therefore, I will determine whether SFKs phosphorylate the conserved ITAMs in Jedi-1 and MEGF10 to promote engulfment.

Aim 2: To determine whether Jedi-1 signals through interaction with GULP and clathrin.

The homologs of GULP, dCed-6 and Ced-6, bind to Draper and Ced-1, respectively. The interaction occurs between the PTB domain of Ced-6 and the NPXY motif of Draper or Ced-1, and this interaction is essential for engulfment. GULP has recently been identified as a clathrin adapter protein in yolk endocytosis, and GULP is also known to interact with clathrin in neurons. Although clathrin has not been identified as an engulfment protein in mammalian systems due to the size limitation of clathrin coated vesicles, a recent study suggests that clathrin can promote internalization of large bacteria by acting as a scaffold for actin polymerization (Bonazzi et al., 2011).

Therefore, I hypothesized that Jedi-1 interacts with GULP, and I proposed that GULP promotes engulfment by recruiting clathrin to promote actin polymerization and engulfment.

Aim 3: To determine whether Jedi is necessary for neuronal corpse engulfment *in vivo*.

Many mouse models lacking major engulfment receptors or bridging molecules develop symptoms of autoimmunity due to defective clearance of apoptotic cells. Many of the symptoms are similar to those of SLE patients: serum autoantibodies, glomerulonephritis, and skin phenotypes. I hypothesized that Jedi-1 knockout mice would have autoimmune phenotypes due to defective clearance of apoptotic neuron corpses in the dorsal root ganglia.

CHAPTER II

SRC FAMILY KINASES ARE REQUIRED FOR JEDI-1- and MEGF10-MEDIATED ENGULFMENT

Introduction

The novel engulfment receptor Jedi-1 and the previously identified engulfment receptor MEGF10 are required for clearance of apoptotic neurons in the mouse dorsal root ganglia during development (Wu et al., 2009). The ligands of Jedi-1 and MEGF10 remain unknown, and the signaling mechanisms of Jedi-1 and MEGF10 have not been characterized. Our data support a mechanism where Jedi-1 and MEGF10 signal through parallel pathways to promote activation of Syk, a non-receptor tyrosine kinase (Scheib et al., 2012). The ITAM motifs of Jedi-1 and MEGF10 are the interaction site of Syk, and the phosphorylation of these ITAM motifs is required for Syk binding (Scheib et al., 2012). Syk family kinases (Syk and Zap-70) are known to interact with the ITAMs of multiple immune receptors . Upon binding of an antigen to an immune receptor, ITAMs become phosphorylated by Src Family Kinases (SFKs), and Syk or Zap-70 subsequently binds the phosphorylated ITAMs (Underhill and Goodridge, 2007).

Src family kinases have been well studied in many contexts, and they play a role in many types of signaling besides phagocytosis. There are 9 non-receptor tyrosine kinases in the SFK group: Src, Fgr, Fyn, Yes, Lck, Lyn, Blk, Hck, and Frk. Each SFK contains a SH2, SH3, and tyrosine kinase domain. Although multiple mechanisms exist to promote activation of various SFKS, all of the methods induce structural changes to promote exposure of the active site of the kinase domain. While it has been established that SFKs induce phagocytosis by phosphorylating ITAMs of immune receptors, there is a paucity of information about how the SFKs are activated to promote this process. Dimer formation is induced when certain immune receptors bind their antigens, and this structural change permits phosphorylation of ITAMs by SFKs.

The engulfment receptor in *Drosophila*, Draper, is phosphorylated by Src42A at its ITAM tyrosines, where it then interacts with the nonreceptor tyrosine kinase Shark, the homolog of Syk (Ziegenfuss et al., 2008). Each of these signaling events was required for Draper-mediated engulfment. Based on the homology of Jedi-1 and MEGF10 with Draper in addition to the discovery of signaling between the ITAM of Draper and Shark, we hypothesized that SFKs phosphorylate the ITAM tyrosines of Jedi-1 and MEGF10 to promote binding of Syk and engulfment.

Experimental Procedures

DNA constructs

Jedi-GFP and MEGF10-GFP construction was previously described (Wu et al., 2009). Jedi-GFP and MEGF10-GFP mutants were obtained by site-directed mutagenesis with PfuUltra HF (Stratagene) PCR and Dpn1 (New England Biolabs) digestion. Syk-myc, Zap-70-myc and Lck-F505 were kind gifts from Dr. Arthur Weiss (University of California, San Francisco) and Src-F529 was generously provided by Dr. Steven Hanks (Vanderbilt University, Nashville). Fyn and Lyn were kind gifts from Dr. Clifford Lowell (University of California, San Francisco), and Yes was purchased from Addgene (Plasmid #18067).

Cell culture and transfection

HEK293 cells and HeLa cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS. Transfections of the cells were carried out with Lipofectamine 2000 (Invitrogen), per manufacturer's recommendations. Stably transfected cells were selected with 4 mg/ml G418.

Immunoprecipitation and Western blot analysis

Transfected cells were harvested in 500 L NP-40 lysis buffer [25 mM Tris cocktail tablet (Roche)]. Jedi-GFP and mutants were immunoprecipitated with anti-GFP (Roche) and Protein A Sepharose (Invitrogen). Western blot analysis was performed using primary antibodies to the myc-tag (1:1000, 9B11, Cell Signaling), phospho-Syk (1:1000, Y525/Y526, Cell Signaling), phosphoTyrosine (1:1000, PY99, Santa Cruz) or alpha-tubulin (1:1000, Calbiochem).

RT-PCR and DRG satellite glial precursor isolation

Reverse transcription, PCR and the isolation of satellite glial cell precursors from E13.5 DRG from CD1 mice (Charles River) was performed as previously described (Wu et al., 2009). Primer sequences used to detect SFK mRNA have been described (Meyn et al., 2005).

Engulfment assays

DRG co-cultures and engulfment of apoptotic neuron assays were performed as described by Wu et al (2009), except cells were cultured in the presence of NGF for four days before NGF withdrawal. In brief, DRG from E13.5 CD1 mouse embryos were dissociated and 50,000 cells plated onto a glass, collagen coated coverslip in 1:1 Ultraculture (BioWhittaker): Neural Basal medium (Invitrogen), with 3% fetal bovine serum and N2 and B27 supplements plus 50 ng/ml NGF. The cells were transfected using Effectene (Qiagen) and the transfected cells were detected using anti-GFP (1:500, Abcam) or anti-myc-tag (1:1000, 9B11, Cell Signaling) and anti-mouse labeled with Alexa 488 (1:400). The nuclei were detected using TOPRO-3 (Life Technologies). Photomicrographs of z-stacks were taken using a Zeiss LSM 510 inverted confocal microscope (Cell Imaging Shared Resource at Vanderbilt University Medical Center) and

at least 50 cells were analyzed for each experiment. Any cell with an internalized TOPRO positive signal, other than its own nucleus, was counted as having phagocytosed an apoptotic body.

For the microsphere engulfment assay, 300,000 HeLa cells were plated on 35 mm tissue culture plates in DMEM 10% FBS. The following day, cells were transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen). After 24 hours, 50,000 of these cells were plated per well on collagen coated 8-well chamber slides. The next day, 2 m carboxylate-modified fluorescent microsphere beads (F-8826, Invitrogen) in PBS with 1 mg/ml BSA were incubated with the cells at 37°C for two hours then the unbound microspheres were removed by PBS rinses and the cells fixed in 10% Formalin. The cells were then immunostained, and imaged with Zeiss LSM 510 inverted confocal microscope. The percentage of transfected cells that had engulfed one or more microspheres was determined for each condition.

To inhibit Src Family Kinases, PP2 (Calbiochem) was added to the cells. In the microsphere engulfment assay depicted in Figure 2.2, the inhibitor was added to the cell culture media 1 hour before adding microspheres. For engulfment assays with DRG co-cultures, inhibitors were added 24 hours after transfection and NGF withdrawal, and the co-cultures were fixed 24 hours later.

Results

Src Family Kinases phosphorylate the ITAMs of Jedi-1 and MEGF10

Syk family kinases bind to ITAMs through interaction of the SH2 domain of the kinase and phosphorylated ITAM tyrosines downstream of activation of immune receptors such as the T- and B-cell receptors (Geahlen, 2009; Love and Hayes, 2010). Due to the engulfment signaling pathway in Drosophila in which Src42A phosphorylates the ITAM motif of Draper, we decided to screen for SFKs that could phosphorylate Jedi-1 and/or

MEGF10 in glia of E13.5 mouse DRG, the time at which massive neuronal death occurs during development. RT-PCR of glial precursors showed that the glia expressed four members of the Src family: Src, Fyn, Lyn, and Yes (data not shown). When any of these four kinases were co-expressed with Jedi-1-GFP in HEK293 cells, Jedi-1-GFP was tyrosine phosphorylated (Figure 2.1). These 4 kinases were also able to promote phosphorylation of MEGF10-GFP in HEK293 cells (Figure 2.1). Although Syk is also a tyrosine kinase, we found that overexpression of Syk did not increase phosphorylation of Jedi-1 suggesting that only SFKs have a role in ITAM phosphorylation (Figure 2.1).

In order to determine whether the ITAM motif tyrosines of Jedi-1 and MEGF10 were the site of phosphorylation by SFKs, Fyn was co-expressed with Jedi-1-GFP or MEGF10 or the ITAM tyrosine mutants. We found that mutation of tyrosines in either ITAM of Jedi-1, and mutation of all four tyrosines of the ITAM motifs prevented tyrosine phosphorylation of Jedi-1 (Figure 2.1). Mutation of the first ITAM tyrosine of MEGF10 to phenylalanine also decreased phosphorylation of MEGF10 by Fyn (Figure 2.1).

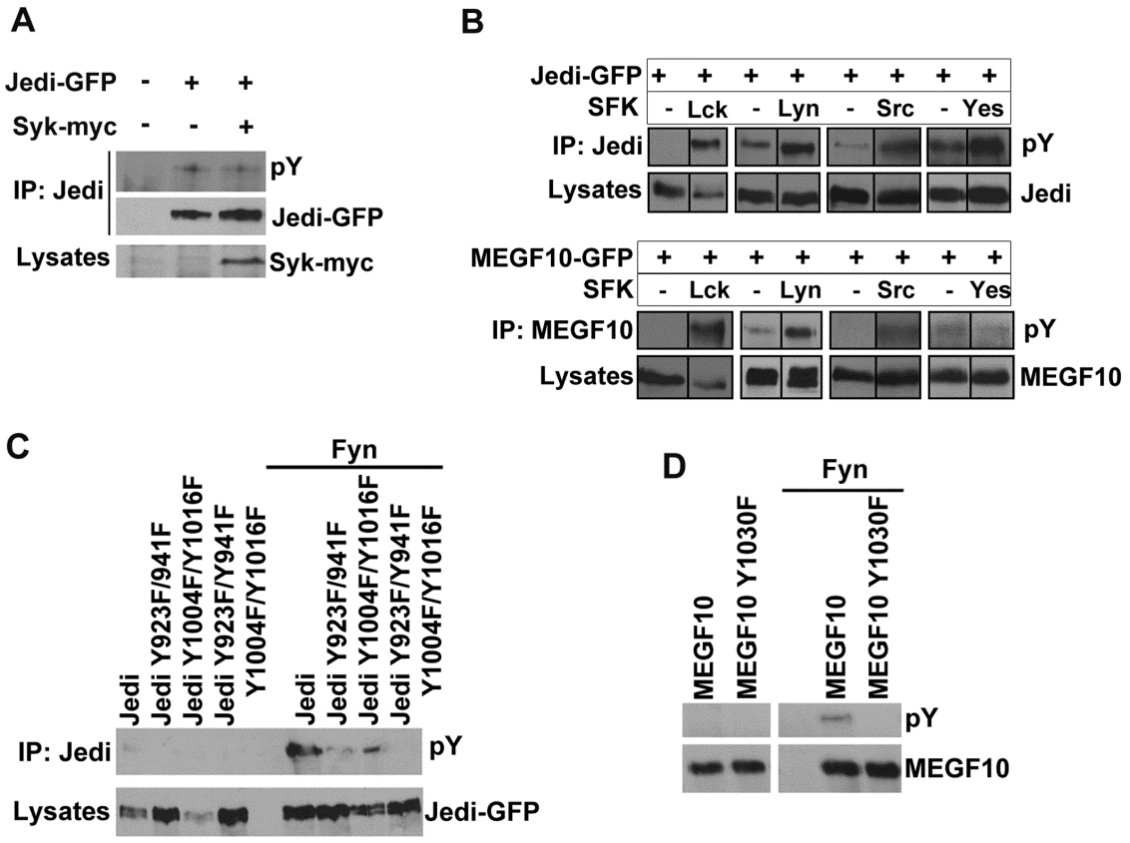


Figure 2.1 Some SFKs phosphorylate Jedi-1 and MEGF10 at ITAM tyrosines. A, HEK293 cells stably expressing Jedi-GFP were untransfected or transfected with Syk-myc. Jedi-GFP immunoprecipitates were immunoblotted with anti-phosphoTyrosine, anti-GFP and anti-myc. B, HEK293 cells were transfected with Jedi-GFP or MEGF10-GFP and Lck, Lyn, Src, and Yes. Jedi-GFP or MEGF10-GFP were immunoprecipitated with anti-GFP and immunoblotted with anti-phophoTyrosine. C and D, HEK293 cells expressing WT or mutant Jedi-GFP, or WT or mutant MEGF10-GFP, were transfected with Fyn. Jedi-GFP or MEGF10-GFP was immunoprecipitated and the precipitates were immunoblotted with anti-phosphoTyrosine and anti-GFP.

Jedi-1 and MEGF10-mediated engulfment requires SFKs

After determining that Jedi-1 and MEGF10 are phosphorylated by SFKs at ITAM motifs, we wanted to determine the importance of SFKs in engulfment by glial cells. Satellite glial cells were transfected with GFP or Fyn in DRG co-cultures, and neuronal apoptosis was induced by removal of NGF from the culture media. Confocal microscopy was used to quantify the percent of transfected glial cells that had internalized at least one apoptotic body (Figure 2.2). Over expression of Fyn increased engulfment by glial cells in comparison to cells transfected with GFP control ($p=0.0053$, $n=3$). When SFK activity was blocked upon addition of the inhibitor PP2 (which inhibits all SFKs), engulfment was significantly reduced in comparison to cells treated with vehicle only (Figure 2.2) ($p=0.0083$, $n=3$). These results suggest that SFK activity is important for engulfment of apoptotic cell corpses by satellite glial cell precursors. The role of SFKs in Jedi-1 or MEGF10-mediated engulfment was also tested in a microsphere engulfment assay. HeLa cells transfected with Jedi-1-GFP or MEGF10-GFP were treated with the SFK inhibitor PP2, and engulfment of microspheres was drastically reduced ($p=0.016$; $n=5$; $p=0.024$, $n=3$, respectively) (Figure 2.3).

The results of these 2 different assays of engulfment suggest a model in which the ITAM motifs of Jedi-1 and MEGF10 are phosphorylated by SFKs. Additional studies performed in our lab suggest that phosphorylation of the ITAM motifs by SFKs promotes the interaction of Syk with Jedi-1 and MEGF10 (Scheib et al., 2012). The signaling pathway used by the engulfment receptor Draper appears to be highly conserved in mammalian cells.

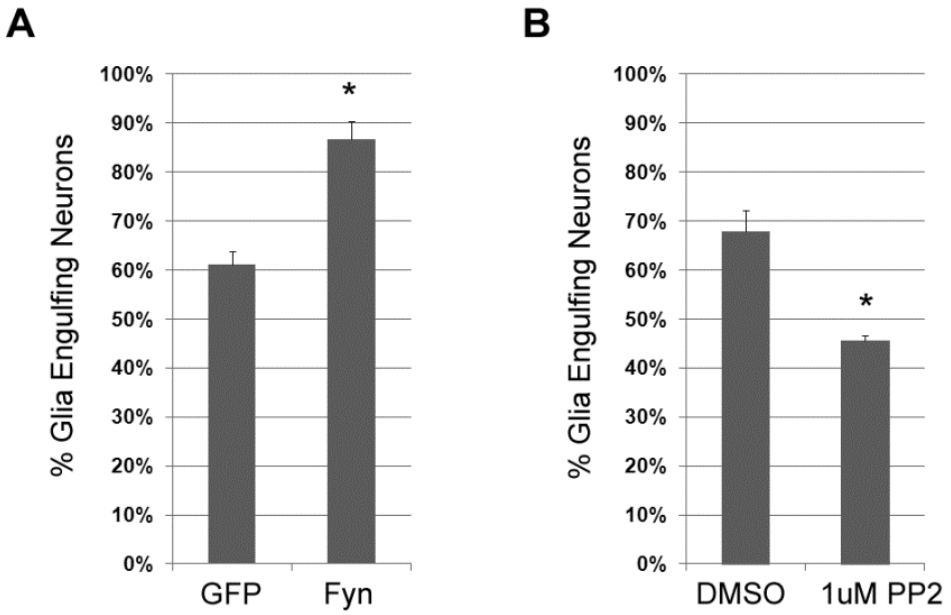


Figure 2.2 SFKs are involved in Jedi-1-mediated engulfment of neurons. A, Co-cultures of DRG neurons and glial precursors were transfected with GFP alone or GFP and Fyn or ,B, with GFP and treated for 24 hr with DMSO or 1 M PP2. The cultures were imaged by confocal microscopy and the percentage of transfected glia that had engulfed an apoptotic fragment was quantified (*, $p<0.01$, $n=3$). The assay in which GFP and Fyn were transfected into glial cells was performed by Jami Scheib.

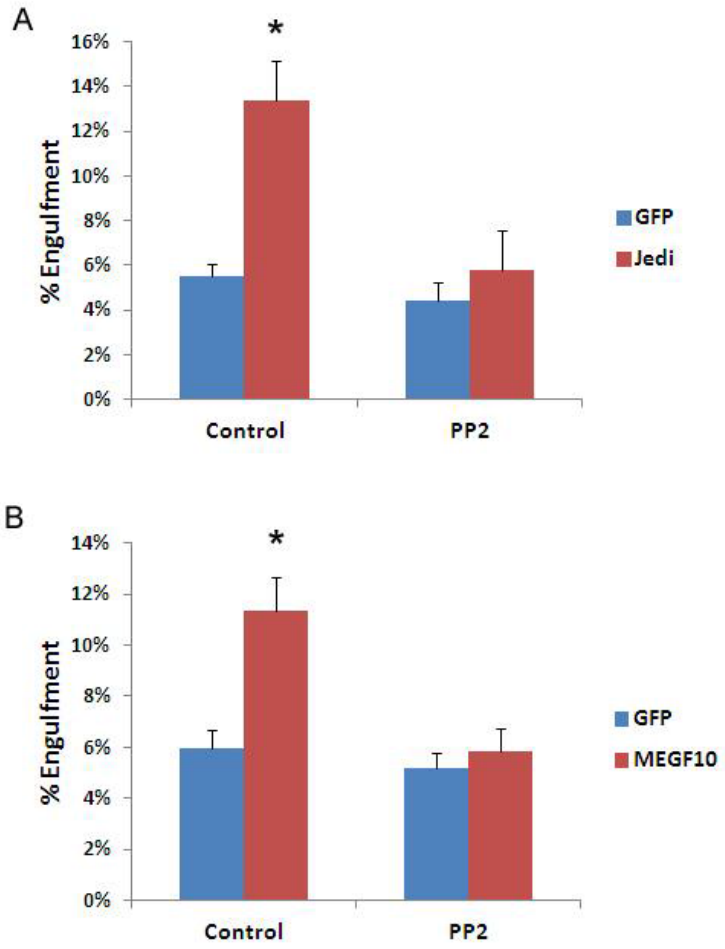


Figure 2.3 SFKs are required for Jedi-1 or MEGF10-mediated engulfment. HeLa cells were transfected with (A) GFP or Jedi-GFP, or (B) MEGF10-GFP, and after 48 hrs, 1 M PP2 was added to the HeLa cells 1 hr before addition of microspheres for 2 hr. Cells were rinsed, fixed, and engulfment of microspheres was detected by confocal microscopy and quantified. PP2 significantly decreased the engulfment mediated by Jedi ($p=0.016$, $n=5$) or MEGF10 ($p=0.024$, $n=3$).

Discussion

The mammalian engulfment receptors Jedi-1 and MEGF10 promote clearance of apoptotic neuron corpses in the developing mouse DRG (Wu et al., 2009). Although the ligand(s) of Jedi-1 and MEGF10 are unknown, they contain conserved signaling motifs (such as ITAMs) in common with the homologous engulfment receptor, Draper. Here, we have verified that the signaling mechanisms of MEGF10 and Jedi-1 are similar to the engulfment receptor Draper and involve signaling through SFKs.

In the Scheib et al paper from our lab in 2012, we showed that Jedi-1 and MEGF10 interact with Syk. The *Drosophila* homolog of Syk, Shark, binds to Draper at the ITAM motif (Ziegenfuss et al., 2008). This interaction was mediated by Src42A phosphorylation of Draper's ITAM tyrosines. In Chapter II I demonstrated that overexpression of Src family kinases such as Lck, Lyn, Fyn, and Src can induce phosphorylation of Jedi-1 and MEGF10. SFKs are believed in most cases to directly phosphorylate the tyrosines of ITAM motifs, but the possibility that SFKs indirectly induce phosphorylation of Jedi-1 and MEGF10 has not yet been addressed.

Human Jedi-1 is also called Platelet Endothelial Aggregation Receptor-1 (PEAR1) due to its identification in a screen for proteins that become phosphorylated upon platelet activation. Nanda et al determined that PEAR1 is phosphorylated on the first tyrosine in the first ITAM motif (mouse Y923), and this phosphorylation was blocked by an inhibitor of 2β3 integrin (Nanda et al., 2005). Integrins are known to play a role in engulfment (Hanayama et al., 2002; Hsu and Wu, 2010; Akakura et al., 2004). Src family kinases are known to act downstream of these receptors (Abram and Lowell, 2007). Therefore, it may be the case that integrins signal with Jedi-1 and MEGF10 to promote SFK activation and phosphorylation of the ITAM motifs during engulfment to enhance Syk binding.

SFK phosphorylation of ITAMs in receptors such as the B-cell receptor enhances Syk binding (Underhill and Goodridge, 2007), but the signal that promotes SFK activation and subsequent ITAM phosphorylation are unknown. Structural studies of the T cell receptor identified key tyrosines within the CD3 subunit that are usually concealed within the lipid bilayer due to basic residues near the membrane. When ligand binds to the T-cell receptor, the receptor complex is formed and shifts the CD3 ITAM away from the membrane into a position where it can be accessed by SFKs for phosphorylation (Xu et al., 2008). Whether the structure of Jedi-1 or MEGF10 similarly conceals ITAM motifs until ligand engagement is unknown at this time.

CHAPTER III

JEDI-1 SIGNALS THROUGH THE ADAPTER PROTEIN GULP

Introduction

Apoptosis is a normal part of development for all multicellular organisms as it is a means of eliminating unnecessary or defective cells, establishing proper cell numbers, and sculpting tissues. We previously reported that glial cell precursors act as "non-professional" phagocytes to clear the heavy burden of apoptotic neurons during the development of the peripheral nervous system (Wu et al., 2009). This phagocytosis is mediated, in part, through the engulfment receptor Jedi-1 (Wu et al., 2009), a mammalian homolog of the *Drosophila melanogaster* receptor Draper, and the *Caenorhabditis elegans* receptor CED-1. Jedi-1 and Draper signal engulfment through recruitment of the tyrosine kinase Syk (Scheib, Sullivan, & Carter, 2012) or Shark (Ziegenfuss et al., 2008), respectively, which binds to an immunoreceptor tyrosine-based activation motif (ITAM) within the intracellular domain of the receptor.

Draper (Awasaki et al., 2006) and CED-1 (Zhou, Hartwieg, & Horvitz, 2001);((Su et al., 2002) mediated engulfment also depends on the adaptor protein CED-6, which binds to the NPXY motif in the receptor's intracellular domain. CED-6 binds to CED-1 via the NPXY motif in CED-1 and the phosphotyrosine binding (PTB) domain of CED-6 (Su et al., 2002). The intracellular domain of Draper was also shown to bind to a portion of dCED-6 containing its PTB (Awasaki et al., 2006). Jedi-1, like CED-1 and Draper, contains an NPXY motif, but the importance of this motif in Jedi-1 signaling has not been established. Due to the high level of conservation in the protein domains and functional roles of Jedi-1 and its homologues, we predicted that GULP (CED-6) would also bind to Jedi-1 at the NPXY motif and that this interaction would be essential for engulfment signaling. The other mammalian homolog of Draper and Ced-1, MEGF10, has been

shown to interact with the mammalian homolog of CED-6, GULP, at the NPXY motif (Hamon et al., 2006). The mammalian homolog of CED-6, GULP, has been shown to bind to the NPXY motif of other mammalian engulfment receptors, including stabilin-1 (Park, Kim, Kang, & Kim, 2010), stabilin-2 (Park et al., 2008), LRP-1 (Su et al., 2002), and Class B Scavenger Receptor Type I (SR-B1) (Osada et al., 2009). In the case of each of the listed receptors, GULP was shown to be essential for engulfment of apoptotic cells; however, the signaling pathways downstream of GULP are poorly defined.

Several groups have begun to define signaling downstream of GULP. In the case of SR-B1, the investigators showed that when macrophages were exposed to apoptotic cells, there was increased MAPK phosphorylation and increased Rac1-GTP levels (Osada et al., 2009). When GULP was knocked down MAPK phosphorylation and Rac1 activation were reduced, and there was decreased engulfment. In *C. elegans*, both CED-1 and CED-6/GULP are necessary for recruitment of the GTPase Dynamin-1 to phagosomes (Yu et al., 2006). Ma et al recently demonstrated that GULP sequesters ACAP1 (a GTPase activating protein) and therefore acts as a positive regulator of Arf6, a GTPase implicated in promoting positive membrane curvature required for endocytosis. Knock-down of GULP led to reduced Arf6-GTP levels, and over expression of GULP resulted in an Arf6-GTP increase (Ma et al., 2007). Whether the ability of GULP to positively regulate Arf6 occurs in response to the activation of any receptors remains an open question as does its potential role in the engulfment process.

In this chapter, I report that GULP binds to the NPXY motif of Jedi-1, and this interaction was essential for engulfment signaling. The NPXY motif of Jedi-1 was essential for engulfment of fluorescent microspheres by transfected HeLa cells in a recombinant engulfment system. The NPXY motif was also essential for engulfment of apoptotic neurons by primary satellite glial cells. I found that GULP is also essential for engulfment downstream of Jedi-1. The NPXY motif of Jedi-1 was required for receptor internalization after stimulation with beads. I also found that GULP was essential for

internalization of Jedi-1 during engulfment. These results identify GULP as an essential signaling mediator downstream of Jedi-1 during engulfment of apoptotic cells.

Experimental Procedures

DNA constructs

Jedi-1 was cloned into the pEGFP or pFlag plasmid as described previously (Wu et al., 2009). Jedi-1-GFP mutants were obtained by site-directed mutagenesis with PfuUltra HF (Stratagene) PCR, and Dpn1 (New England BioLabs) digestion. GULP was expressed in the pEBB triple-HA vector. To make the GULP shRNA targeting both mouse and human GULP mRNA, the synthetic oligonucleotide 5'-GATCCGGAACAGAAGTTGTGAGAGATGTTCAAGAGACATCTCTCACAACTTCTGTTCCTTTTTACGCGTG. -3' and the reverse compliment were annealed and ligated into pSIREN-RetroQ-ZsGreen (Clontech). The scrambled shRNA was in the same vector.

Cell culture and transfection

HeLa cells and mouse embryonic fibroblasts (MEFs) were grown in DMEM with 10% FBS in 5% CO_2. Transfections were carried out using Lipofectamine 2000 (Invitrogen) per the manufacturer's instructions. MEF cell lines were described previously (Ma, Nie, Luo, Casanova, & Ravichandran, 2007). Briefly, an siRNA against GULP was designed to target nucleotides 125 to 145 (from the starting codon) with staggered ends to form BamH I and Hind III restriction sites that would allow subsequent subcloning into the pSilencer2.1-U6 puro plasmid (Ambion). Mouse embryonic fibroblasts (MEF-1) were transfected with the above plasmid and selected with 6 μg/ml of puromycin for stable GULP knockdown clones. The control clone was transfected with a control plasmid (pApuro) and selected under the same conditions.

Immunoprecipitation and Western blot analysis

Transfected cells were harvested in 500 µl of NP-40 lysis buffer [25 mM Tris, 137 mM NaCl, 2.7 mM KCl, 1% NP-40, 10% glycerol, 1 mM Na_3VO_4, Complete Mini EDTA-free Protease Inhibitor Cocktail tablet (Roche)]. Jedi-1-GFP and mutants were immunoprecipitated with anti-GFP (Roche) and Protein A Sepharose (Invitrogen). GST-GULP was pulled down using glutathione Sepharose beads (Amersham Biosciences). HA-GULP was immunoprecipitated with a monoclonal HA antibody (Covance). Endogenous Jedi-1 was immunoprecipitated using a polyclonal Jedi-1 antibody previously described (Scheib et al., 2012). After separation of the proteins by SDS-PAGE, Western blot analysis was performed using primary antibodies to the HA-tag (1:3000; Covance), GFP (1:1000; Roche), GULP (1:1000; Abcam), endogenous Jedi (1:1000), or α-tubulin (1:1000; Calbiochem).

Biotinylation

HeLa cells or MEFs were transfected with wild type or APXA mutant Jedi-GFP. After 24-48 hours, the cells were placed on ice and surface proteins were biotinylated with EZ-Link Sulfo-NHS-SS-Biotin (Pierce). The biotinylation of surface proteins was reversed using 50 mM DTT at various time points after stimulation with 2 µm carboxylate-modified latex beads (Invitrogen) and warming to 37C. Internalized biotinylated Jedi-1-GFP or mutant Jedi-1-GFP was pulled down with Avidin agarose beads (Pierce) and detected by immunoblotting with a GFP antibody (Roche).

Engulfment assays

Dorsal root ganglia (DRG) co-cultures and engulfment of apoptotic neuron assays were performed as described by Scheib et al. (2012). In brief, DRG from E13.5 CD1 mouse embryos of either sex were dissociated, and 50,000 cells were plated onto a glass, collagen-coated coverslip in 1:1 UltraCULTURE (BioWhittaker):Neuralbasal medium (Invitrogen) with 3% fetal bovine serum and N2 and B27 supplements plus 50 ng/ml NGF. The glial cells were transfected using Effectene (Qiagen) and after 2 days the NGF was removed to induce apoptosis. The co-cultures were then fixed in 4% paraformaldehyde and the transfected cells were detected using anti-GFP (1:500, Abcam) and anti-chicken labeled with Alexa Fluor 488 (1:500). The nuclei were detected using TOPRO-3 (Life Technologies). Photomicrographs of z-stacks were taken using a Zeiss LSM 510 inverted confocal microscope (Cell Imaging Shared Resource at Vanderbilt University Medical Center, Nashville, TN), and at least 50 cells were analyzed for each experiment. Any cell with an internalized TOPRO-positive signal, other than its own nucleus, was counted as having phagocytosed an apoptotic body.

For the microsphere engulfment assay, 300,000 HeLa cells or MEFs were plated on 35 mm tissue culture plates. The following day, cells were transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen). After 24 hours for expression or 48 hours for shRNA knock down, 50,000 of these cells were plated per well on collagen-coated 8-well chamber slides. The next day, 2 μm carboxylate-modified fluorescent polystyrene microspheres (Invitrogen) or non-fluorescent carboxylate-modified latex microspheres (Invitrogen) in PBS with 1 mg/ml BSA were incubated with the cells at 37°C for 2 hours, then the unbound microspheres were removed by PBS rinses and the cells were fixed in 10% formalin. The cells were then immunostained and imaged with a Zeiss LSM 510 inverted confocal microscope. The percentage of transfected cells that had engulfed one or more microspheres was determined for each condition.

Results

GULP interacts with the NPXY motif of Jedi-1

Previous studies demonstrated that CED-6/GULP binds to the NPXY motif of CED-1 (Su et al., 2002) and Draper (Awasaki et al., 2006), as well as a number of mammalian receptors involved in phagocytosis (Hamon et al., 2006; Park et al., 2008; Park et al., 2010; Su et al., 2002). Therefore, we investigated whether Jedi-1 also associates with GULP. Using lysates from mouse dorsal root ganglia and spinal cord at embryonic day 13.5, a time at which apoptotic cell death of neurons occurs during development (Farinas, Yoshida, Backus, & Reichardt, 1996), we found that endogenous GULP co-immunoprecipitated with Jedi-1 (Figure 3.1). To determine whether the NPXY motif of Jedi-1 was required for GULP binding, we generated a Jedi-1 construct with the NPXY motif (^{788}NPSY) mutated to APXA. Wild-type Jedi-GFP or APXA mutant Jedi-GFP were transiently transfected into mouse embryonic fibroblasts (MEFs) or MEFs that stably express GST-GULP. Upon pull down of GST-GULP, an interaction with wild-type Jedi-1 but not the APXA mutant was observed by Western blot (Figure 3.1). We also tested a Jedi-1 construct with the tyrosine residues (923, 941, 1004, 1016) in the ITAM domains, where Syk binds (Scheib et al., 2012), mutated to phenylalanine to determine if this domain was required for association with GULP. In contrast to the NPXY sequence in Jedi-1, mutation of the ITAM motifs did not affect interaction with GULP (Figure 3.1).

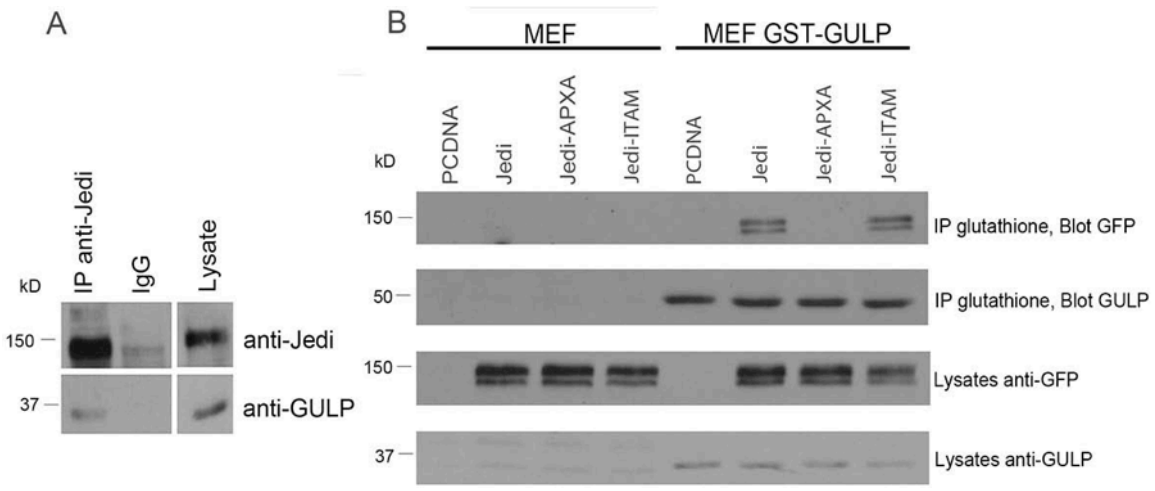


Figure 3.1. Jedi-1 interacts with GULP through its NPXY motif. (A) Endogenous Jedi was immunoprecipitated from lysates of E13.5 dorsal root ganglia and spinal cord, and the proteins were separated by SDS-PAGE and immunoblotted with an antibody to Jedi-1 or CED-6 (GULP). A representative blot of 3 experiments is depicted. (B) Jedi-1-GFP, Jedi-1-GFP with the NPXY motif mutated to APXA or the 4 ITAM tyrosines mutated to phenylalanine were transiently transfected into control MEF cells or MEF cells stably expressing GST-GULP. GST-GULP was pulled down with glutathione beads, and anti-GFP was used to detect co-immunoprecipitation of Jedi-1 by Western blot (n=3).

The NPXY motif of Jedi-1 is required for engulfment

To assess the functional significance of the NPXY motif during phagocytosis, a microsphere engulfment assay using HeLa cells was utilized. GFP-tagged wild type Jedi-1 or Jedi-1 with the NPXY domain mutated (either to APXA or NPXF) was expressed in HeLa cells, and cells were exposed to 2 µm carboxylate-modified fluorescent microspheres, which mimic certain features of apoptotic cells, for 2 hours. Uptake of microspheres was analyzed by confocal microscopy (Figure 3.2), and the percentage of transfected cells with at least one microsphere fully engulfed (as determined based on a confocal z-stack), was determined. In comparison to GFP transfected control cells, Jedi-1 expression significantly increased the engulfment of microspheres (Figure 3.2). We also calculated the phagocytic index (PI) of the HeLa cells using this formula: PI=(total number of engulfed beads/total number of counted cells) × (number of cells containing

engulfed beads/total number of counted cells) × 100. GFP transfected HeLa cells had a phagocytic index of 0.49, while Jedi-GFP transfected cells had a phagocytic index of 15.70. As previously observed (Scheib et al., 2012), Jedi-GFP transfected cells typically engulf no more than 1 to 3 beads. However, neither of the NPXY mutants exhibited any engulfment capability (Figure 3.2). These findings indicate that the NPXY motif is essential for Jedi-mediated engulfment of microspheres, consistent with its requirement for GULP binding.

To determine the importance of the NPXY motif in Jedi-1 mediated engulfment of apoptotic neurons, sensory neurons and glial precursors from E13.5 mouse dorsal root ganglia (DRG) were co-cultured and wild-type Jedi-1-GFP or APXA mutant Jedi-1-GFP was transfected into the glial precursor cells. Nerve growth factor (NGF), initially added to promote neuronal survival, was then removed to induce neuronal apoptosis and after 2 days, confocal microscopy was used to determine the percentage of GFP-positive glial cells that were engulfing at least one apoptotic body. In accordance with our previous results (Scheib et al., 2012; Wu et al., 2009), overexpression of Jedi-1 in glial precursor cells increased the engulfment of dead neurons. In contrast, expression of the APXA mutant Jedi-1 did not increase engulfment above basal levels, demonstrating an essential role for the NPXY motif in apoptotic cell clearance (Figure 3.2).

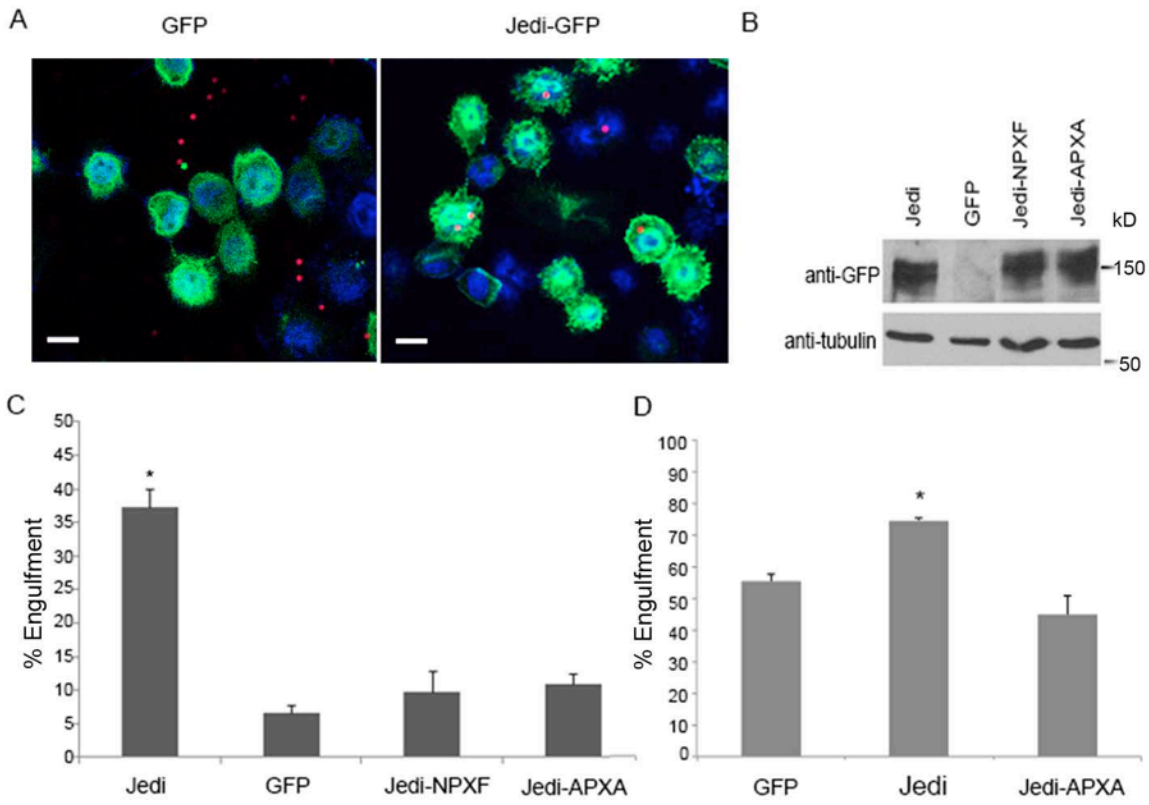


Figure 3.2. **The NPXY motif of Jedi-1 is required for engulfment.** Jedi-1-GFP, Jedi-1-GFP with the NPXY mutated to NPXF or APXA, or GFP only was transfected into HeLa cells and 2 days later, fluorescent microspheres were added for 2 hrs before rinsing and fixing the cells. The cells were imaged by confocal microscopy to ensure complete internalization. (A) Confocal images are shown with Jedi-1-GFP or GFP expressing cells (green) with microspheres (red). Cell nuclei are shown in blue. Scale bars are 10 mm. (B) Expression of Jedi-1-GFP and mutant Jedi-1-GFP was confirmed by immunoblotting. (C) The percentage of transfected HeLa cells engulfing at least one microsphere was quantified (a Student's t-test revealed p=0.00015 for Jedi-1 relative to GFP, p=0.0003 for Jedi-1 NPXF and p=0.0004 for Jedi-1 APXA relative to wild type Jedi-1, n = 3). (D) GFP, Jedi-1-GFP, or APXA mutant Jedi-1-GFP was transfected into glial cells in co-cultures of E13.5 DRG neurons and glia in the presence of NGF. NGF was withdrawn to induce neuronal death and 48 hrs later the cultures were fixed, immunostained with anti-GFP and nuclei labeled with TO-PRO3. The percentage of transfected glia (GFP positive) engulfing at least one apoptotic body (based on condensed TO-PRO3 staining) was quantified by confocal analysis (a Student's t-test revealed p=0.002 for Jedi-1 relative to GFP, p= 0.007 for Jedi-1-APXA relative to wild type Jedi-1, n=3).

GULP is essential for Jedi-1-mediated engulfment

The requirement for GULP in Jedi-1-mediated engulfment was investigated by transfecting Jedi-1 or the APXA mutant of Jedi-1 into control MEFs, MEFs with GULP constitutively knocked down, or MEFs with GULP knocked down but stably transfected with a knock down resistant GST-GULP construct. The transfected cells were incubated with carboxylated fluorescent microspheres and evaluated by confocal microscopy to determine the percentage of cells that had engulfed the spheres. As observed in HeLa cells, expression of the APXA mutant of Jedi-1 did not increase engulfment above the GFP control. Notably, knock down of GULP in cells expressing wild type Jedi-1 significantly decreased the engulfment of spheres, consistent with an essential role for GULP in Jedi-1 signaling, and this decrease in engulfment could be rescued by expression of GST-GULP (Figure 3.3).

To determine whether GULP is required for phagocytosis of apoptotic neurons by glial cells, which depends on endogenous Jedi-1 (Wu et al., 2009), we knocked down GULP in glial cells co-cultured with DRG neurons. Following NGF removal, the percentage of transfected glia that engulfed an apoptotic body was quantified. We found that GULP knock down significantly reduced engulfment of the apoptotic neurons by 43% in comparison to cells transfected with GFP (Figure 3.4). In addition we calculated the phagocytic index (PI) of the glial cells. Control shRNA transfected glial cells had a phagocytic index of 44.64, while GULP shRNA transfected cells had a phagocytic index of 12.24. This is consistent with previous studies (Scheib et al., 2012) in which we have observed that glial cells typically engulf between 1 and 3 apoptotic cell fragments.

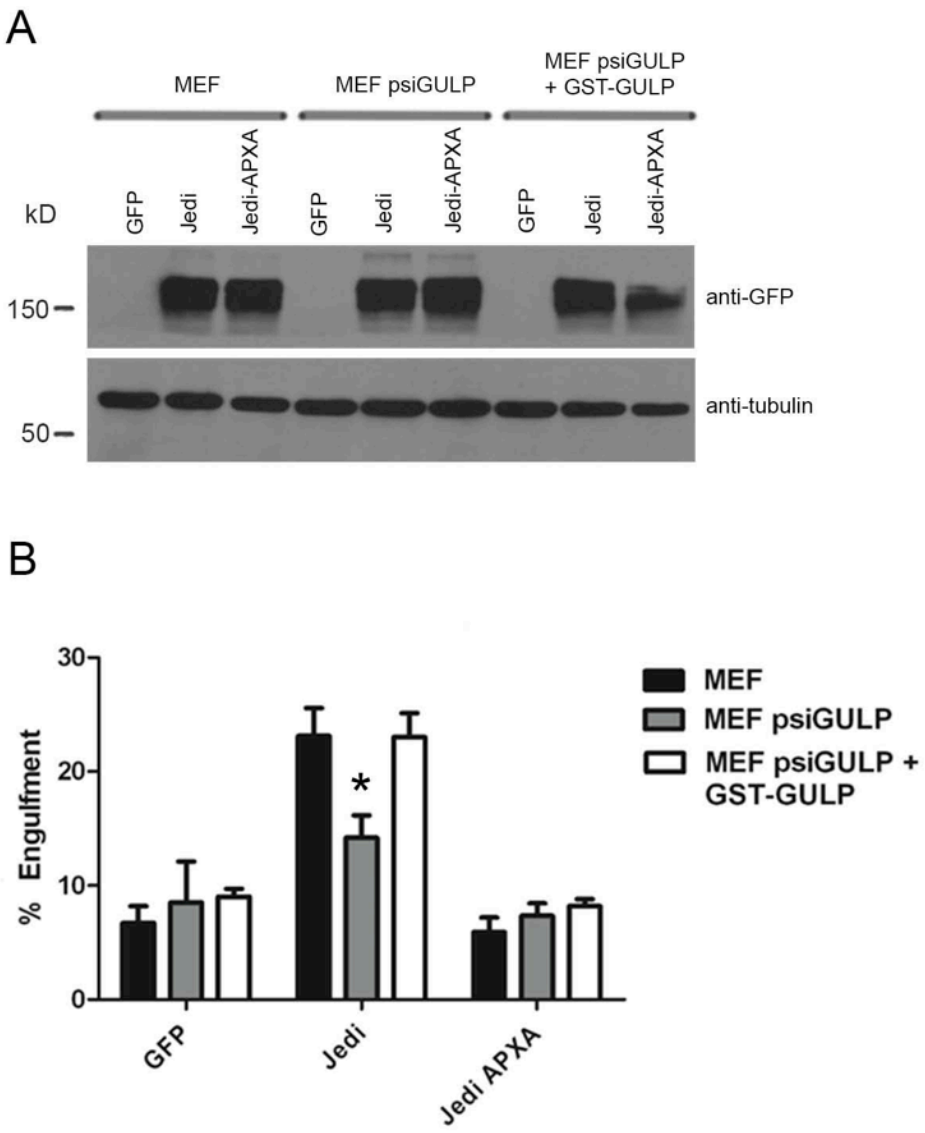


Figure 3.3. **GULP is required for Jedi-1-mediated engulfment.** Control MEFs, MEFs with GULP stably knocked down (psiGULP), or MEFs with endogenous GULP silenced but stably transfected with a knock down resistant GULP (psiGULP+GST-GULP), were transfected with GFP, Jedi-1-GFP, or the NPXY/APXA mutant of Jedi-GFP. (A) The cells were lysed, and the proteins were separated by SDS-PAGE and Western blotted for GFP (to detect Jedi-1) or tubulin. (B) Fluorescent microspheres were added to the cells and after 2 hrs the cells were fixed and engulfment of microspheres was analyzed by confocal microscopy and quantified to determine the percentage of transfected cells (GFP positive) engulfing at least one microsphere (by 2-way ANOVA with a Bonferroni post-hoc analysis $P<0.001$ for MEFs with wild type Jedi-1 relative to MEFs with GULP knockdown expressing wild type Jedi-1, $P<0.001$ for MEFs with GULP knocked down with wild type Jedi-1 relative to MEFs with GULP knocked down and rescue with GST-GULP with wild type Jedi).

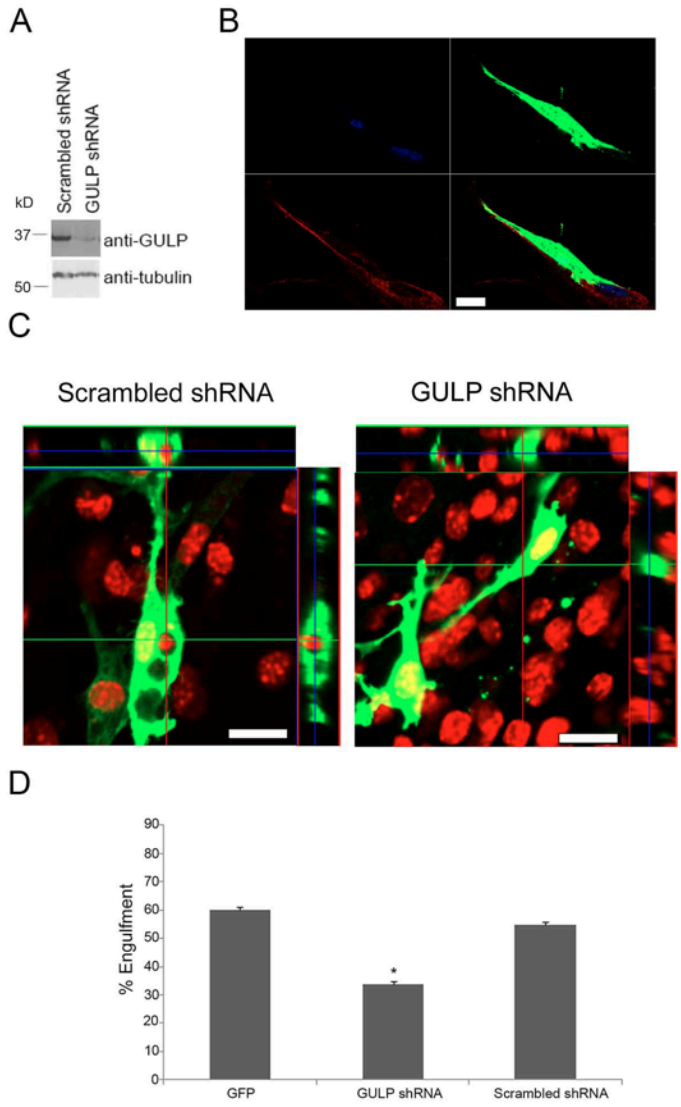


Figure 3.4. **Knock down of GULP reduces the engulfment of neurons by glial precursors**. (A) Non-targeting or GULP shRNA was transfected into 3T3 cells. Lysates were subjected to Western blotting with antibodies to GULP and tubulin. (B) Knockdown of GULP was also verified by immunostaining glial cell precursors transfected with GULP shRNA for GFP to identify transfected cells (green) and GULP (red). Nuclei were stained with TO-PRO3. (C) Representative confocal images of co-cultures of E13.5 DRG neurons and glial precursor cells transfected with non-targeting or GULP shRNA (co-expressing GFP). On the day of transfection, neuronal death was induced by removing NGF. After 48 hrs, the cultures were fixed, immunostained with anti-GFP and nuclei labeled with TO-PRO3. Transfected glia are depicted in green and nuclei in red. Scale bars are 20 mm. The crosshair in the orthogonal view of the cell transfected with scrambled shRNA is positioned on an engulfed apoptotic nuclear fragment while the crosshair in the GULP shRNA transfected image is positioned on the cell body of a glial cell containing no

apoptotic nuclear fragments. (The rectangle on top of the images depicts the x-z plane and to the right is the y-z plane.) (D) Images from transfected co-cultures were analyzed by confocal microscopy. The percentage of transfected glia engulfing TO-PRO3 stained apoptotic nuclear fragments was quantified (p=0.0005 for GULP shRNA relative to GFP only by Student's t-test, n=3).

GULP is required for Jedi-1 internalization

Phagocytosis is a complex, multistep process involving recognition of the body to be engulfed, binding, internalization, maturation of the phagosome and eventual lysosomal degradation of the engulfed material. Since NPXY motifs are often involved in the internalization of cell surface proteins (Bonifacino & Traub, 2003), we hypothesized that the NPXY motif in Jedi-1 and the association with GULP are required for the internalization process. To monitor internalization of Jedi-1 in response to exposure to the microspheres, we used a reversible biotinylation system in which surface proteins were biotinylated. Following the addition of the microspheres for various times, the surface bound biotin was removed by treatment with the reducing agent DTT. The internalized, biotinylated proteins were then detected by avidin pull down and Western blotting. We found that within 30 min of exposing HeLa cells that express Jedi-1 to the microspheres, internalized Jedi-1 could be detected; however, there was only marginal internalization of the APXA mutant detected, even after 90 min (Figure 3.5). These results indicate that the NPXY motif is essential for internalization of Jedi-1 in response to stimulation with carboxylated microspheres.

Since the NPXY motif in Jedi-1 is required for association with GULP, we sought to directly investigate whether GULP is necessary for Jedi-1 internalization using the same reversible biotinylation assay with control MEFs or MEFs with GULP knocked down. The MEFs were transfected with Jedi-1 and exposed to microspheres for 90 min. Jedi-1 was internalized in the microsphere-exposed control MEFs, but no internalization was

detected in the cells with GULP knocked down (Figure 3.5), indicating that GULP is required for Jedi-1 internalization.

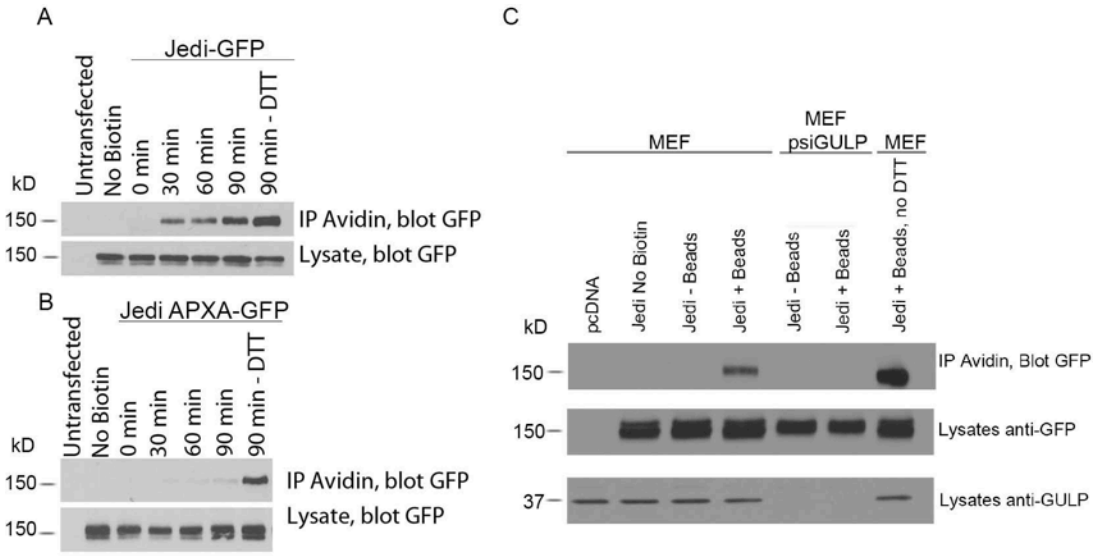


Figure 3.5. **The NPXY motif and GULP are required for internalization of Jedi-1.** Time-course for internalization of Jedi-1-GFP (A) or Jedi-1-GFP APXA mutant (B) following addition of microspheres. HeLa cells were transfected with wild type or APXA mutant Jedi-1-GFP. Surface proteins were biotinylated with EZ-Link Sulfo-NHS-SS-Biotin at 4°C, then exposed to fluorescent microspheres and warmed to 37°C for the time indicated. The biotinylation of surface proteins was then reversed using the reducing agent DTT. The cells were lysed and internalized, biotinylated Jedi-1 or mutant Jedi-1 was pulled down with avidin-conjugated agarose beads and detected by immunoblotting with a GFP antibody. Total levels of Jedi-1 are shown in lysates (n=3). (C) Control MEFs or MEFs with GULP knocked down (MEF psiGULP) were transfected with Jedi-GFP. The cells were then exposed to microspheres or left untreated for 90 min and the internalized Jedi-1 was detected as in (A) and (B). Levels of Jedi-1-GFP and GULP are shown in the lysates.

Discussion

Recognition and removal of apoptotic cells generated in the course of development is essential to prevent progression into secondary necrosis and exposure of intracellular contents that have the potential to be immunogenic or toxic. Indeed, there is evidence that suppressing the timely clearance of cell corpses results in autoimmune phenotypes in mouse models (Elliott & Ravichandran, 2010; Nagata, Hanayama, & Kawane, 2010). Currently, relatively little is known about mechanisms of neuronal corpse removal, especially in the PNS. We recently demonstrated that glial precursors in the developing dorsal root ganglia (DRG) are the primary phagocytes responsible for clearing apoptotic sensory neurons (Wu et al., 2009). In addition, we identified MEGF10 and Jedi-1 as engulfment receptors expressed by the glial precursors and required for phagocytosis of the dead neurons. MEGF10 and Jedi-1 are both mammalian homologs of the *Drosophila melanogaster* receptor Draper, and the *Caenorhabditis elegans* receptor CED-1, which are known to signal at least in part via recruitment of the adapter protein CED-6 (GULP in mammals) (Awasaki et al., 2006; Su et al., 2002). Several mammalian engulfment receptors, including stabilin-1 (Park et al., 2010), stabilin-2 (Park et al., 2008), LRP-1 (Su et al., 2002), and MEGF10 (Hamon et al., 2006) have been reported to interact with GULP; however, the role of GULP in the phagocytic process was largely unknown. Here, we demonstrate that Jedi-1 interacts with GULP, and this interaction is required for Jedi-1-mediated phagocytosis.

CED6/GULP is an adaptor protein without catalytic activity that was first identified in a screen for genes regulating the clearance of apoptotic cells in *C. elegans* (Ellis, Jacobson, & Horvitz, 1991). It was subsequently shown to bind to the nematode engulfment receptor CED-1 (Su et al., 2002) and the fly receptor Draper (Awasaki et al., 2006); however, only recently has its functional role in the phagocytic process been considered. Osada and colleagues suggested that GULP is involved in activation of Rac by the Scavenger Receptor B1 (SR-B1) (Osada, Sunatani, Kim, Nakanishi, & Shiratsuchi,

2009). They demonstrated that upon exposure of a macrophage cell line to phosphatidylserine (PS) containing liposomes there was an increase in GTP-bound Rac. In addition, PS treatment increased p38 and ERK phosphorylation, which was reduced by silencing GULP. Addition of p38 and ERK inhibitors prevented Rac activation by PS; however, GULP was not directly linked to Rac activation (Osada et al., 2009). CED-6 was also suggested to function upstream of CED-10, the worm homolog of Rac, based on an epistatic relationship in genetic studies. Moreover, both CED-1 and CED-6 were required for the accumulation of "actin halos" around the engulfed apoptotic cells (Kinchen et al., 2005); however, the mechanism underlying the actin recruitment was not addressed.

Our results demonstrate that GULP facilitates engulfment through interaction with Jedi-1 (Figure 3.1). CED-6 associates with CED-1 (Su et al., 2002) and Draper (Awasaki et al., 2006) through an interaction between the phosphotyrosine binding (PTB) domain of CED-6/GULP and an NPXY motif in the intracellular domain of the receptors. Similarly, we found that the NPXY domain in Jedi-1 was required for binding to GULP. Mutation of the NPXY in Jedi-1 or knock down of GULP prevented Jedi-mediated engulfment, indicating that Jedi-1 interaction with GULP is essential for this process (Figure 3.2). NPXY motifs are known to play a role in the internalization of proteins from the plasma membrane through clathrin-coated vesicles (Bonazzi et al., 2011). Furthermore, clathrin and AP-2 directly bind to peptides containing an FXNPXY sequence (Boll et al., 2002; Kibbey, Rizo, Gierasch, & Anderson, 1998), but increasing evidence suggests that this motif is actually recognized by PTB-containing proteins, such as Numb, ARH, Dab1, and Dab2 which work with AP-2 to promote clathrin recruitment and assembly (Traub, 2003); therefore GULP could function as such an adapter. We found that Jedi-1 internalization in response to addition of microspheres was GULP dependent (Figure 3.5). Taken together, these data suggest that Jedi-1 mediated engulfment and receptor internalization are dependent upon interaction with GULP at the NPXY motif of Jedi-1.

CHAPTER IV

GULP RECRUITS CLATHRIN TO PROMOTE ACTIN REARRANGEMENT AND ENGULFMENT

Introduction

Although many groups have identified GULP as an important adapter protein involved in engulfment, little is known about how GULP signals to promote engulfment. We found that GULP signals through a non-canonical clathrin-dependent process to promote engulfment. Previously, clathrin mediated endocytosis was considered a completely independent process from phagocytosis, largely due to the size limitation of the well characterized clathrin-coated vesicles (CCVs), which would be too small for phagocytosis of large particles. However, recent findings have begun to overturn this dogma. Clathrin was recently reported to act as a scaffold for actin remodeling, which was required for the internalization of large cargo such as bacteria (Bonazzi et al., 2011). This scaffolding function of clathrin was dependent on a PTB domain containing adapter protein, Dab2, and resulted in the recruitment of myosin and actin polymerization. Other groups have shown that clathrin plays a role in internalization of certain viruses, bacteria, and large latex beads (Aggeler and Werb, 1982; Ehrlich et al., 2004; Rust et al., 2004; Veiga and Cossart, 2005).

Several lines of evidence support a rationale for evaluating the role of clathrin in engulfment of apoptotic cells downstream of Jedi-1. Jedi-1 contains an NPXY motif, and NPXY motifs are known to allow internalization of proteins from the plasma membrane, typically through clathrin-coated vesicles (Bonifacino and Traub, 2003). Some studies have indicated that clathrin and AP-2 (a clathrin adapter protein) directly bind peptides containing the FXNPXY sequence (Boll et al., 2002; Kibbey et al., 1998), but increasing evidence suggests that this motif is actually recognized by PTB-containing proteins, such

as Numb, ARH, Dab1, and Dab2 which work with AP-2 to promote clathrin recruitment and assembly (Traub, 2003). Ectopically expressed MEGF10 (another potential mammalian homolog of CED-1) in HEK293 cells was recently reported to interact with assembly protein complex 2 medium chain (AP50), a component of clathrin coated pits, as well as AP-2, a protein involved in clathrin-mediated endocytosis (Suzuki and Nakayama, 2007). The functional significance of this interaction was not evaluated. GULP, like many clathrin adaptor proteins, contains a PTB domain and associates with receptors through NPXY domains. GULP was shown to bind clathrin by yeast-2-hybrid and GST-pulldown assays, and it also colocalized with clathrin in neural cells (Martins-Silva, 2006), but the functional significance of this interaction was not investigated.

Recently, CED-6 was reported to act as a clathrin adapter protein necessary for yolk uptake in Drosophila egg chambers (Jha, Watkins, & Traub, 2012). CED-6 associated with the vitellogenin receptor Yolkless and was also found to bind to clathrin heavy chain (CHC), thereby facilitating clathrin-dependent endocytosis. GULP was also shown to be a positive regulator of Arf6, a GTPase implicated in promoting clathrin-mediated endocytosis by recruiting AP-2 to the cell membrane (Ma et al., 2007). Despite these observations, clathrin is generally not considered to contribute to phagocytosis due to the size limitation of clathrin-coated vesicles. However, clathrin was recently reported to act as a scaffold for actin remodeling, which was required for the internalization of pathogenic bacteria (Bonazzi et al., 2011). Bacterial adhesion to the host cell induced the phosphorylation of CHC, which was required for the recruitment of actin. The formation of this clathrin-actin network was necessary for bacterial internalization. Similarly, clathrin has been implicated in the internalization of certain large viruses (Ehrlich et al., 2004; Rust, Lakadamyali, Zhang, & Zhuang, 2004)and fungi (Moreno-Ruiz et al., 2009). Taken together, these results led us to hypothesize that Jedi-1 mediates engulfment through the recruitment of GULP, which then associates with clathrin to facilitate phagocytosis. Based on the numerous lines of evidence which suggest potential signaling through GULP and clathrin, we proposed that GULP binds to the

NPXY motif of Jedi-1 and promotes recruitment of phosphorylated clathrin heavy chain (CHC) and actin.

Experimental Procedures

DNA constructs

Jedi-1 was cloned into the pEGFP or pFlag plasmid as described previously (Wu et al., 2009). Jedi-1-GFP mutants were obtained by site-directed mutagenesis with PfuUltra HF (Stratagene) PCR, and Dpn1 (New England BioLabs) digestion. The GULP deletion mutant was created by mutation of residue R324 to create a new stop codon using the same reagents used to create the Jedi-1 mutants, and GULP was expressed in the pEBB triple-HA vector. siRNA targeting the CHC17 target sequence: 5'-AAG CAA TGA GCT GTT TGA AGA and control non-targeting siRNA were purchased from Qiagen. Wild-type or phospho-mutant CHC-GFP rescue constructs resistant to CHC17 siRNA (pcDNA3.1/Zeo-siRNA(AS)resCHC17WT-GFP and pcDNA3.1/Zeo-siRNA(AS)resCHC17&Y1477F+Y1487F-GFP) were previously described (Bonazzi et al., 2011). The clathrin heavy chain (CHC) shRNA (pBrain-GFP-CHC4), which targets both mouse and human CHC and control shRNA, which targets rat CHC (pBrain-GFP-CHC1) were kindly provided by Dr. Stephen Royle (University of Liverpool).

Cell culture and transfection

HeLa cells and mouse embryonic fibroblasts (MEFs) were grown in DMEM with 10% FBS in 5% CO_2. Transfections were carried out using Lipofectamine 2000 (Invitrogen) per the manufacturer's instructions. MEF cell lines were described previously (Ma et al., 2007). Briefly, an siRNA against GULP was designed to target nucleotides 125 to 145 (from the starting codon) with staggered ends to form BamH I and Hind III restriction sites that would allow subsequent subcloning into the pSilencer2.1-U6 puro plasmid (Ambion).

Mouse embryonic fibroblasts (MEF-1) were transfected with the above plasmid and selected with 6 μg/ml of puromycin for stable GULP knockdown clones. The control clone was transfected with a control plasmid (pApuro) and selected under the same conditions. For siRNA transfections, HeLa cells were transfected with HiPerfect (Qiagen) according to the manufacturer's instructions. Cells were assessed for siRNA efficacy 72 hours after transfection by Western blotting. For CHC rescue experiments, cells were transfected with CHC rescue constructs 48 hours after siRNA transfection using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Immunoprecipitation and Western blot analysis

Transfected cells were harvested in 500 μl of NP-40 lysis buffer [25 mM Tris, 137 mM NaCl, 2.7 mM KCl, 1% NP-40, 10% glycerol, 1 mM Na_3VO_4, Complete Mini EDTA-free Protease Inhibitor Cocktail tablet (Roche)]. Jedi-1-GFP and mutants were immunoprecipitated with anti-GFP (Roche) and Protein A Sepharose (Invitrogen). GST-GULP was pulled down using glutathione Sepharose beads (Amersham Biosciences). Clathrin heavy chain was immunoprecipitated using the X-22 antibody (Abcam). HA-GULP was immunoprecipitated with a monoclonal HA antibody (Covance). Endogenous Jedi-1 was immunoprecipitated using a polyclonal Jedi-1 antibody previously described (Scheib et al., 2012). After separation of the proteins by SDS-PAGE, Western blot analysis was performed using primary antibodies to the HA-tag (1:3000; Covance), GFP (1:1000; Roche), GULP (1:1000; Abcam), clathrin heavy chain (1:1000; Santa Cruz Biotechnology), anti-FLAG (1:1000; Covance), anti-Phospho-CHC (1:1000; developed by Frances Brodsky, (Bonazzi et al., 2011)) endogenous Jedi (1:1000) or α-tubulin (1:1000; Calbiochem).

Engulfment assays

Dorsal root ganglia (DRG) co-cultures and engulfment of apoptotic neuron assays were performed as described by Scheib et al., 2012. In brief, DRG from E13.5 CD1 mouse embryos of either sex were dissociated, and 50,000 cells were plated onto a glass, collagen-coated coverslip in 1:1 UltraCULTURE (BioWhittaker):Neuralbasal medium (Invitrogen) with 3% fetal bovine serum and N2 and B27 supplements plus 50 ng/ml NGF. The glial cells were transfected using Effectene (Qiagen) and after 2 days the NGF was removed to induce apoptosis. The co-cultures were then fixed in 4% paraformaldehyde and the transfected cells were detected using anti-GFP (1:500, Abcam) and anti-mouse labeled with Alexa Fluor 488 (1:400). The nuclei were detected using TOPRO-3 (Life Technologies). Photomicrographs of z-stacks were taken using a Zeiss LSM 510 inverted confocal microscope (Cell Imaging Shared Resource at Vanderbilt University Medical Center, Nashville, TN), and at least 50 cells were analyzed for each experiment. Any cell with an internalized TOPRO-positive signal, other than its own nucleus, was counted as having phagocytosed an apoptotic body.

For the microsphere engulfment assay, 300,000 HeLa cells or MEFs were plated on 35 mm tissue culture plates. The following day, cells were transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen). After 24 hours for expression or 48 hours for shRNA knock down, 50,000 of these cells were plated per well on collagen-coated 8-well chamber slides. The next day, 2 µm carboxylate-modified fluorescent polystyrene microspheres (Invitrogen) or non-fluorescent carboxylate-modified latex microspheres (Invitrogen) in PBS with 1 mg/ml BSA were incubated with the cells at 37°C for 2 hours, then the unbound microspheres were removed by PBS rinses and the cells were fixed in 10% formalin. The cells were then immunostained and imaged with a Zeiss LSM 510 inverted confocal microscope. The percentage of transfected cells that had engulfed one or more microspheres was determined for each condition. Microspheres were scored as being positive for actin or phospho-CHC accumulation if the staining was within 0.5 micrometers of the microsphere and surrounded at least

50% of it, using ImageJ. Actin or phospho-CHC positive microspheres were placed into subcategories based on whether they were distal (>10 micrometers) from the closest edge of the nucleus (based on DAPI staining) or proximal (<10 micrometers) to the nucleus using ImageJ.

Immunostaining

MEFs or MEFs with GULP knocked down were transfected with Jedi-1-GFP and plated using the same protocol as the microsphere engulfment assay. After microsphere incubation and fixation in 10% formalin, cells were stained for Jedi-GFP (as in the engulfment assay) and phospho-CHC (primary anti-phospho-CHC 1:1000, secondary anti-mouse Alexa-647, 1:500 (Invitrogen)). For actin localization studies, MEFs were transfected with Jedi-1-FLAG (primary anti-FLAG 1:500 (Sigma), secondary anti-mouse Alexa-647 1:500(Invitrogen)) and incubated with Alexa-488 phalloidin (1:500) (Invitrogen). For actin and total clathrin localization studies, Jedi-1 transfected MEF cells were stained for total clathrin using the X-22 antibody(1:500) (Abcam) and incubated with secondary anti-mouse Alexa-647 (Invitrogen) along with phalloidin (1:500)(Invitrogen).

Results

GULP association with clathrin is required for Jedi-mediated engulfment

Increasing evidence suggests that NPXY motifs are recognized by phosphotyrosine binding domain (PTB)-containing proteins, such as Numb, ARH, Dab1, and Dab2, which work with AP-2 to promote clathrin recruitment and assembly (Traub, 2003). Furthermore, dCED-6 (which has a PTB domain) was shown to be a clathrin adapter protein in *Drosophila* required for yolk endocytosis (Jha et al., 2012). These authors also demonstrated that mammalian GULP could bind clathrin heavy chain (CHC). Therefore, we hypothesized that GULP may be an essential adapter protein to promote Jedi-1-mediated phagocytosis by recruiting clathrin. By transfecting HA-GULP into HeLa cells, we confirmed the interaction with CHC by co-immunoprecipitation (Figure 4.1). The interaction between CED-6 and CHC required the C-terminal portion of CED-6, outside of the PTB domain, where there is a conserved DLF sequence required for CHC binding (Jha et al., 2012). GULP also contains a DLF triplet in its C-terminal region and deletion of the C-terminal 71 amino acids, including this sequence, abolished the association with CHC (Figure 4.1). Importantly, deletion of the C-terminal domain did not disrupt GULP binding to Jedi-1 (Figure 4.1), thereby allowing utilization of this mutant to explore the functional importance of GULP association with CHC.

To determine whether the interaction between GULP and CHC was necessary for Jedi-1-mediated engulfment, we expressed the C-terminal deletion mutant of GULP in MEFs with GULP constitutively knocked down and measured Jedi-1-mediated phagocytosis of microspheres. Control MEFs or MEFs with GULP knocked down were transfected with GFP or Jedi-1-GFP with or without HA-GULP or the C-terminal GULP mutant. Full-length GULP rescued the engulfment; however, the clathrin-binding deficient mutant of GULP did not (Figure 4.1).

Clathrin is required for Jedi-1-mediated engulfment

To further explore the requirement for clathrin in Jedi-1-mediated engulfment, we assessed the effects of knocking down CHC. HeLa cells expressing Jedi-1 were transfected with shRNA to CHC, and the engulfment of microspheres was analyzed. There was a significant 39.9% reduction in engulfment in cells with reduced levels of CHC (Figure 4.2). Similarly, silencing CHC in glial cells reduced their ability to phagocytose dead neurons in co-cultures of DRG cells (Figure 4.2).

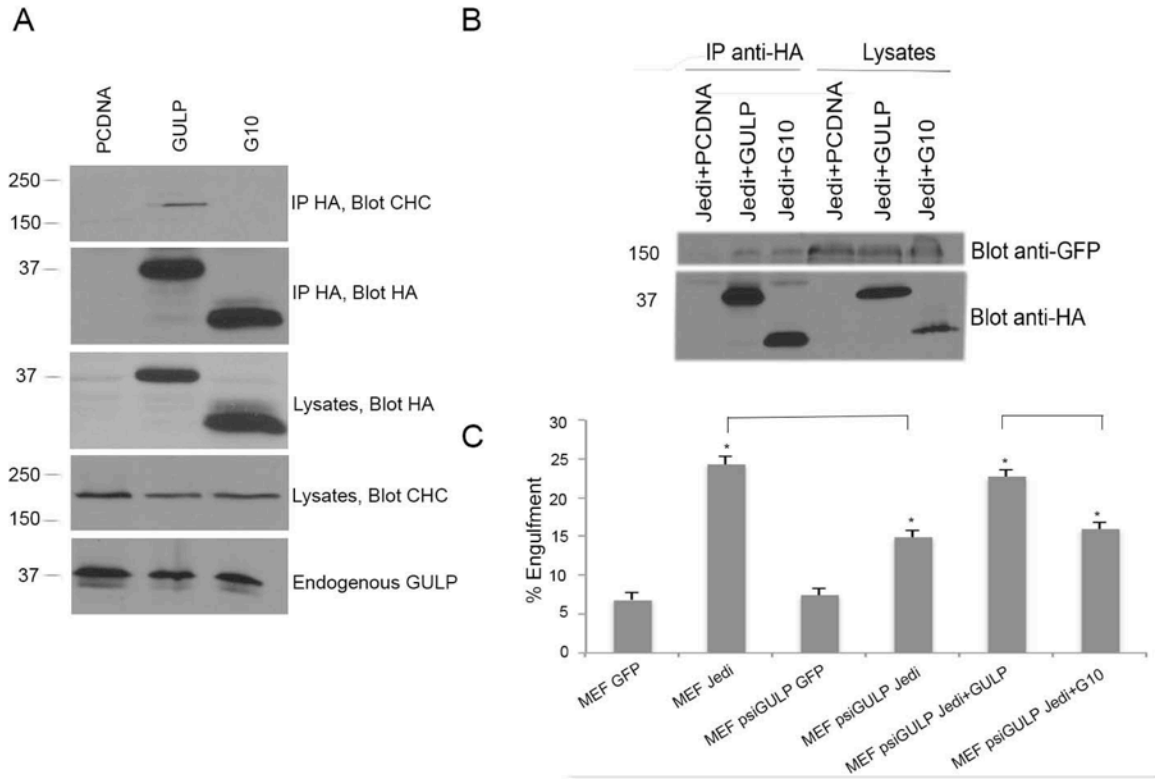


Figure 4.1. **GULP interacts with clathrin, and this interaction is required for Jedi-1 mediated engulfment.** (A) HeLa cells were transfected with pcDNA3, HA-GULP or HA-GULP lacking the C-terminal 71 amino acids (G10). After 2 days, the cells were lysed, immunoprecipitated with HA antibody and subjected to Western blotting with antibodies to HA or clathrin heavy chain (CHC). Endogenous GULP levels were analyzed by immunodepleting the lysates of HA-GULP using 2 subsequent rounds of immunoprecipitation with HA antibody and blotting the supernatant lysate with an antibody that recognizes endogenous GULP. A representative blot is depicted (n=3). (B) HeLa cells transfected with Jedi-1-GFP and pcDNA3, HA-GULP or HA-G10 were lysed and immunoprecipitated with an antibody to HA and blotted with an antibody to GFP to detect co-immunoprecipitated Jedi-1. The lysates were subjected to Western blotting using antibodies to HA (to detect GULP) or GFP (to detect Jedi-1). (C) Control MEFs or MEFs with GULP constitutively knocked down (MEF psiGULP) were transfected with GFP, Jedi-1-GFP, or Jedi-1-GFP and HA-GULP or HA-G10. Two days later, microspheres were added for 2 hrs. The cells were then fixed and the engulfment of the microspheres was quantified by confocal microscopy ($p=0.0001$ for MEFs with GFP relative to MEFs expressing Jedi-1, $p=0.0009$ for MEFs expressing Jedi-1 relative to MEFs expressing Jedi-1 with GULP knocked down, $p=0.0009$ for MEFs expressing Jedi-1 with GULP knocked down and resistant GULP added back relative to MEFs expressing Jedi-1 with GULP knocked down and transfected with G10 by Student's t-test).

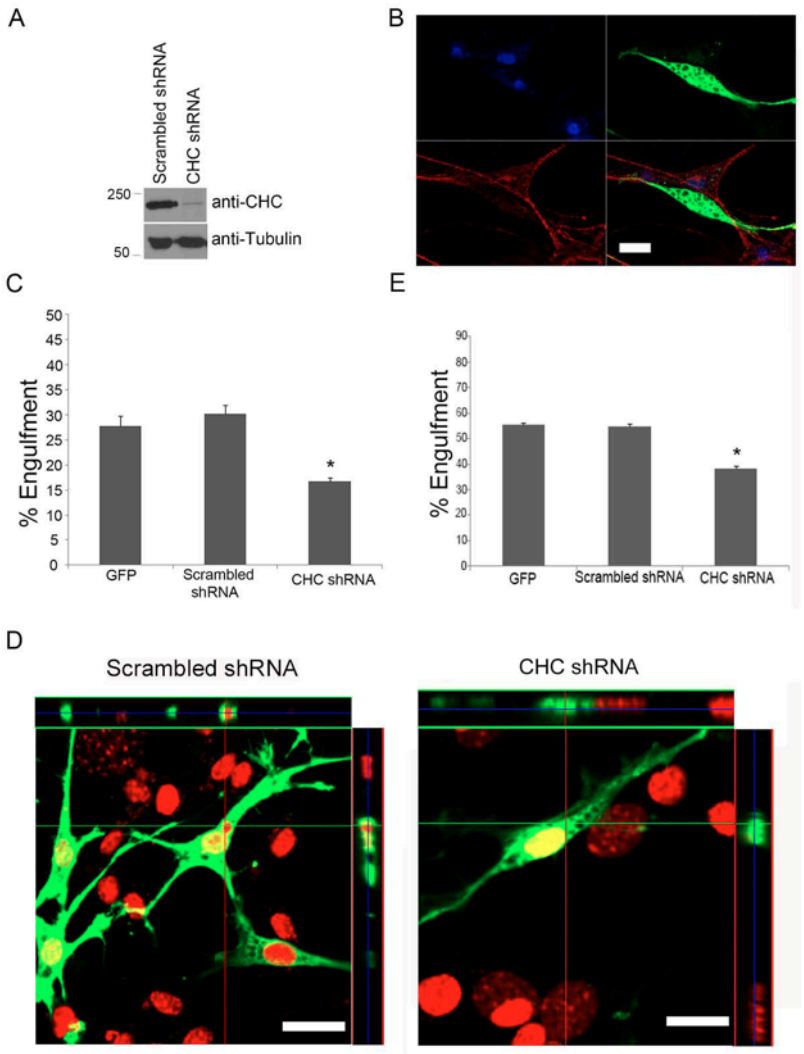


Figure 4.2. **Clathrin is required for Jedi-1-mediated engulfment.** (A) Non-targeting or clathrin heavy chain (CHC) shRNA were transfected into HeLa cells. Lysates were subjected to Western blotting with antibodies to CHC and tubulin. (B) Knockdown of CHC was also verified by immunostaining glial cell precursors transfected with CHC shRNA for GFP to identify transfected cells (green) and CHC (red). Nuclei were stained with TO-PRO3. (C) HeLa cells were co-transfected with Jedi-1-FLAG and GFP, non-targeting shRNA or clathrin heavy chain (CHC) shRNA that co-expresses GFP. After 48 hrs, fluorescent microspheres were incubated with the HeLa cells for 2 hrs prior to rinsing and fixation. The percentage of GFP positive cells that had engulfed one or more spheres was determined by confocal microscopy (n=3, p=0.006 for CHC shRNA relative to Jedi-1-FLAG only control by Student's t-test). (D) Confocal images depicting co-cultures of E13.5 neurons and glial precursor cells transfected with GFP or CHC shRNA co-expressing GFP. Neuronal death was induced by removal of NGF and after 48 hrs the

co-cultures were fixed and immunostained with antibodies to GFP and nuclei were labeled with TO-PRO3. The crosshair in the orthogonal view of the cell transfected with scrambled shRNA is positioned on an engulfed apoptotic nuclear fragment while the crosshair in the CHC shRNA transfected image is positioned on the cell body of a glial cell containing no apoptotic nuclear fragments. Scale bars are 20 mm. (E) Images from transfected co-cultures were analyzed by confocal microscopy, and the percentage of GFP positive glia that were engulfing apoptotic nuclear fragments was quantified (n=3, p=0.0002 for CHC shRNA relative to non-targeting control by Student's t-test).

Jedi-1 co-localizes with phosphorylated clathrin and actin during engulfment

It was previously reported that CHC becomes tyrosine phosphorylated and accumulates around sites of bacterial adhesion. This phosphorylation promoted internalization by acting as a scaffold for actin polymerization (Bonazzi et al., 2011). After discovering that CHC was required for Jedi-1-mediated engulfment, we examined its phosphorylation status during Jedi-1-mediated phagocytosis using a phospho-CHC antibody (Bonazzi et al., 2011)(Figure 4.3). MEFs expressing Jedi-1 were exposed to microspheres and after 2 hours, we observed a significant increase in phospho-CHC (Figure 4.3). However, when GULP was knocked down, there was a marked decrease in the level of CHC phosphorylation. Re-expression of a knockdown resistant GULP rescued the inducible phosphorylation of CHC. The results were confirmed by immunoprecipitating CHC and blotting for total phospho-tyrosine (Figure 4.3).

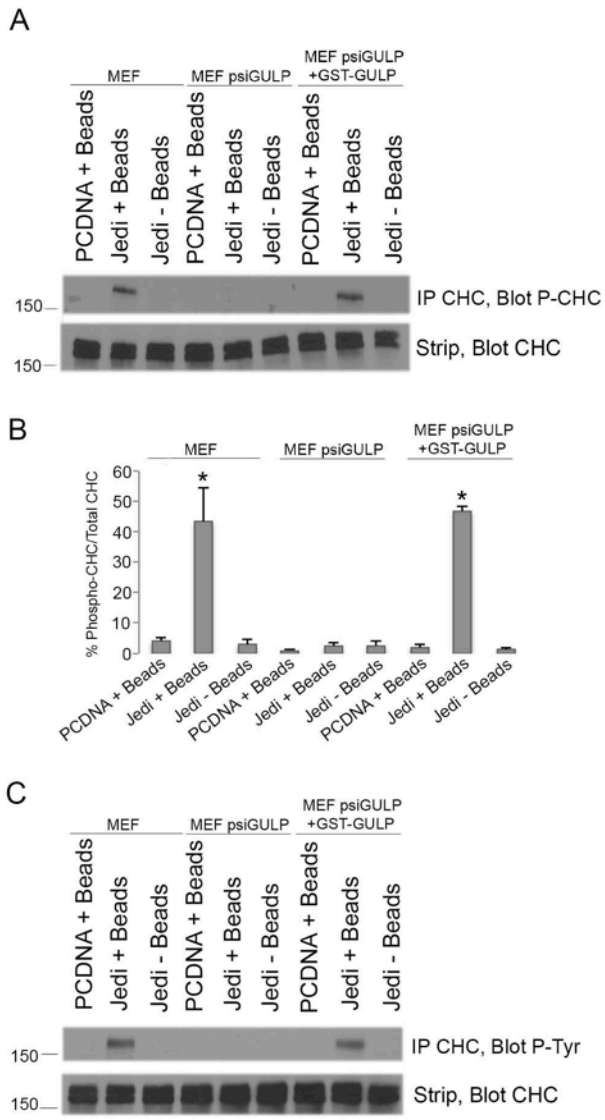
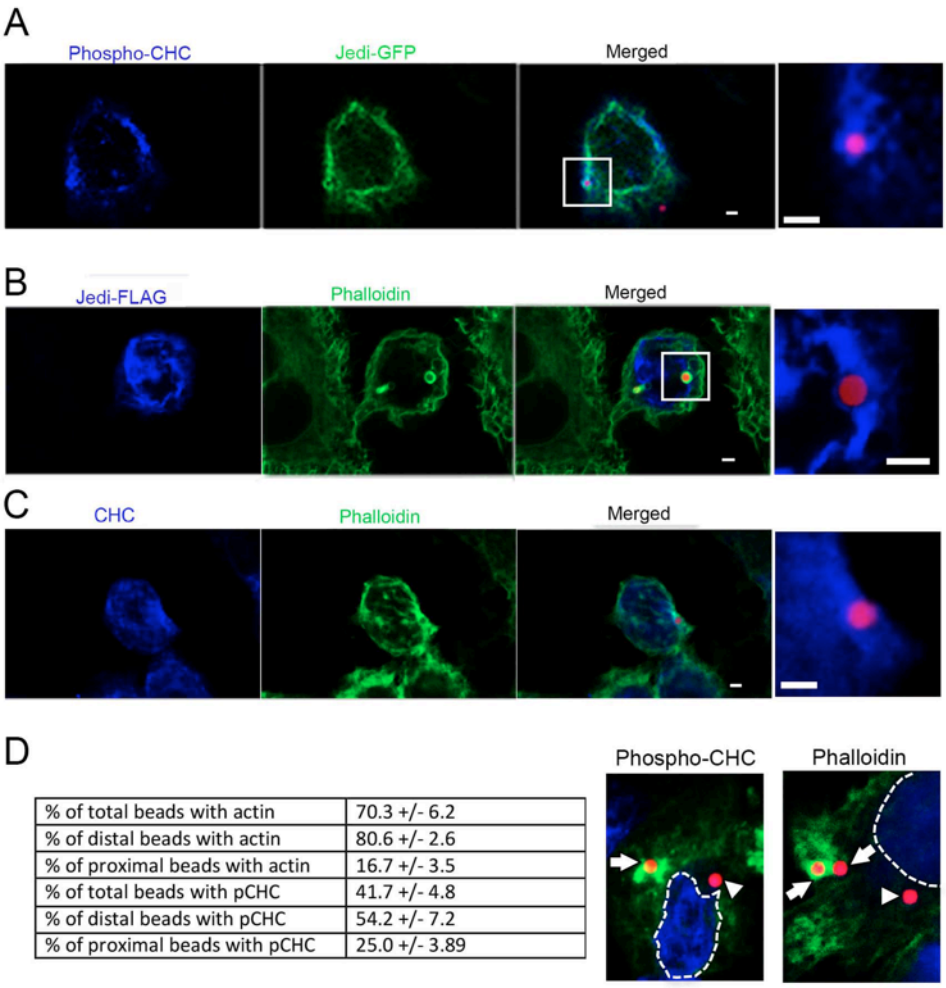


Figure 4.3. **CHC is phosphorylated during Jedi-1-mediated engulfment in a GULP-dependent manner.** Control MEFs, MEFs with GULP stably knocked down (psiGULP), or MEFs with endogenous GULP silenced but stably transfected with a knock down resistant GST-GULP (psiGULP+GST-GULP) were transfected with pcDNA3 or Jedi-1-GFP and then treated with or without fluorsescent microspheres for 90 min. (A) The cells were lysed and immunoprecipitated with anti-CHC. The protein was subjected to Western blotting using anti-phospho-CHC and total immunoprecipitated CHC (n=3). (B) Densitometry of phospho-CHC divided by the total CHC signal is shown in the graph for n=3 experiments. (n=3 p=.02 for MEFs transfected with Jedi-1 with bead stimulation relative to Jedi transfected MEFs with no bead stimulation, and p=.0009 for MEFs with GULP knockdown and GST-GULP rescue with Jedi-1 and bead stimulation relative to MEFs with GULP knockdown and GST-GULP rescue with Jedi-1 and no bead stimulation by Student's t-test. (C) The experiment in (A) was repeated but total CHC was immunoprecipitated from the lysates and Western blotted for phospho-tyrosine (n=3).

To determine whether phospho-CHC and actin accumulated around engulfed microspheres, we assessed their localization in MEFs expressing Jedi-1 during phagocytosis. After confirming the specificity of the phospho-CHC antibody for immunostaining (data not shown), we found that Jedi-1 and phospho-CHC co-localized around internalized microspheres (Figure 4.4). Bonazzi and colleagues found that phosphorylation of clathrin during bacterial internalization promoted recruitment of an actin scaffold (Bonazzi et al., 2011). We found that in MEFs transfected with Jedi-1, both Jedi-1 and actin accumulated around internalized microspheres (Figure 4.4). We also observed colocalization of total clathrin heavy chain and actin around microspheres in Jedi-1 expressing MEF cells, although total CHC was more broadly distributed than phospho-CHC, as would be expected due to the many roles clathrin has in the cell (Figure 4.4). To quantify the localization of phospho-CHC and actin around the microspheres, we scored the percentage of fully internalized microspheres that showed positive staining within 0.5 microns and surrounding at least 50% of the sphere. We observed that 70.3 +/- 6.2% of engulfed microspheres were actin positive and 41.7 +/- 4.8% were phospho-CHC positive (Figure 4.4). Since we hypothesized that actin and phospho-CHC were necessary for the internalization step of engulfment, we also divided the population of internalized beads into those distal (> 10 mm) to the nucleus, which were closer to the cell surface, and those proximal (< 10 mm). The majority of internalized microspheres that were positive for actin or phospho-CHC were localized distally, suggesting that the accumulation of actin and phospho-CHC occurs during early engulfment but dissipates as the phagosome matures and progresses deeper into the cell.



% of total beads with actin	70.3 +/- 6.2
% of distal beads with actin	80.6 +/- 2.6
% of proximal beads with actin	16.7 +/- 3.5
% of total beads with pCHC	41.7 +/- 4.8
% of distal beads with pCHC	54.2 +/- 7.2
% of proximal beads with pCHC	25.0 +/- 3.89

Figure 4.4. **Phospho-CHC and actin co-localize with Jedi-1 and accumulate around engulfed microspheres.** (A) MEFs transfected with Jedi-1-GFP (green) and treated with fluorescent microspheres (red) for 2 hrs were fixed and immunostained with anti-phospho-CHC (blue). The cells were imaged using confocal microscopy to ensure full internalization of microspheres. Note the accumulation of Jedi-1 and phospho-CHC around the internalized microsphere. (B) MEFs transfected with Jedi-1-FLAG (blue) were incubated with fluorescent microspheres, then fixed and stained with anti-FLAG and Alexa-488 phalloidin (green) to label actin. Note the ring of actin and Jedi-1-FLAG around the internalized microsphere. (C) Jedi-1 transfected MEF cells were incubated with fluorescent microspheres (red), then fixed and stained with an antibody recognizing all clathrin heavy chain and phalloidin. Note the accumulation of phalloidin and CHC near the internalized microsphere. All scale bars are 5 mm. The regions boxed in white in A-C are enlarged and shown to the right of the merged image (only the blue and red channels are shown). (D) Table representing results of quantification of accumulation of actin or phospho-CHC within 0.5 micrometers of internalized beads and surrounding them by at least 50%. Distal beads are beads greater than 10 mm from the nearest edge of the nucleus, while proximal beads are less than 10 mm from the nucleus.

Clathrin is required for recruitment actin to the phagocytic cup

After finding that clathrin is required for Jedi-1-mediated engulfment, we wanted to determine whether CHC plays a role in recruitment of Jedi-1 and/or actin to the phagocytic cup. We performed a kinetic analysis of localization of Jedi-GFP and actin using a microsphere engulfment assay (Figure 4.5). We found that in early stages of engulfment, Jedi-GFP accumulates near microspheres in contact with the cells, even when CHC has been knocked down. However, recruitment of actin was delayed or markedly decreased when CHC was silenced. This suggests that the inability of Jedi-1 expressing cells to internalize microspheres when CHC is knocked down could be due to a defect in actin localization. Jedi-1 could be observed accumulating near beads in contact with the surface of the cells, even in the absence of clathrin (e.g. notice the 45 min time point in Figure 4.5), but complete phagocytic cups were not formed. These results were consistent with the results of Bonazzi et al. and suggest that CHC mediates actin scaffolding during engulfment.

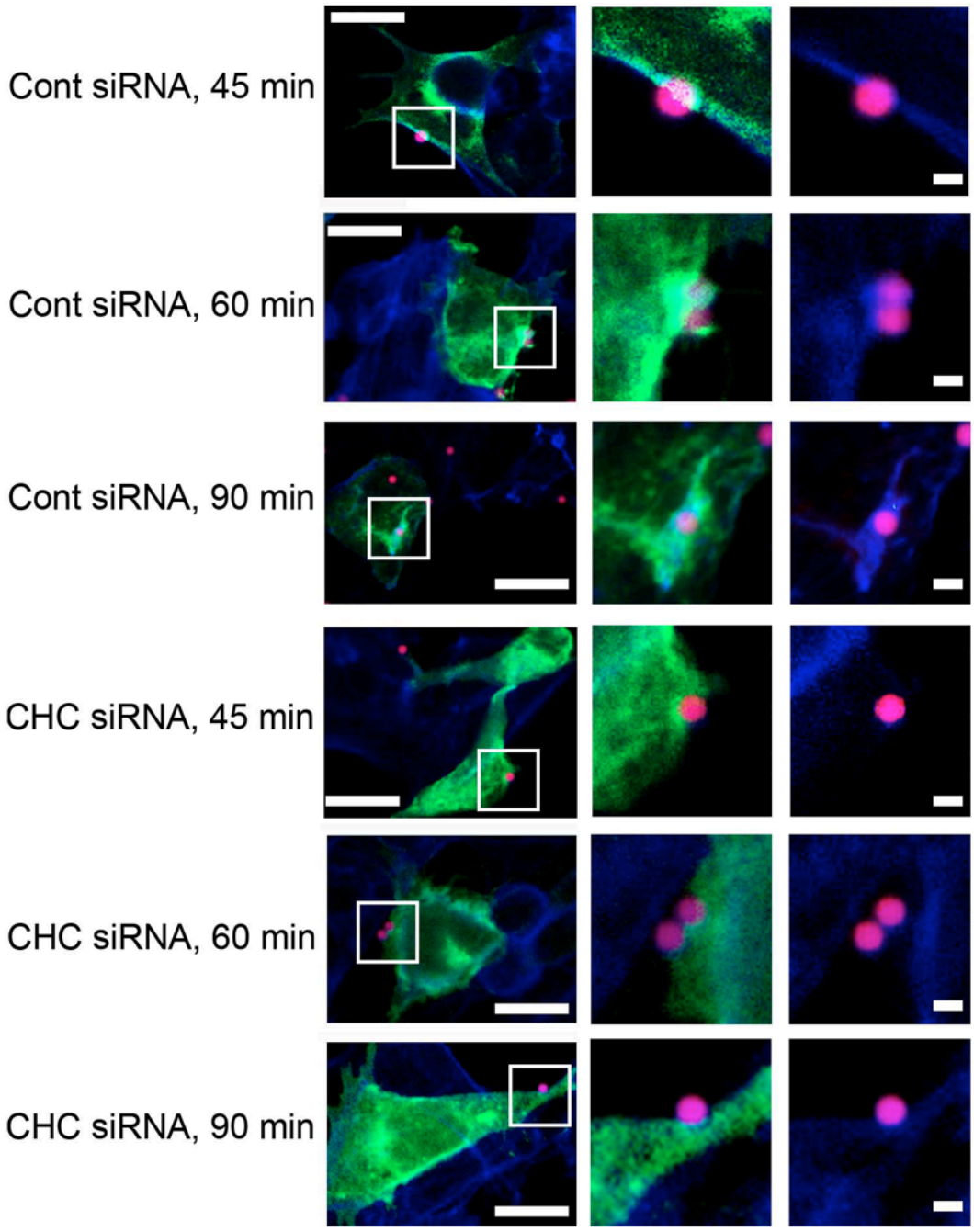


Figure 4.5. **Clathrin is required for recruitment of actin to the phagocytic cup.** HeLa cells were transfected with Jedi-GFP and control siRNA or CHC siRNA. Cells were incubated for 45, 60, or 90 minutes with fluorescent microspheres. Localization of Jedi-GFP (green) and actin (blue) was analyzed using confocal microscopy. Scale bars in the low magnification images are 5 mM, and scale bars in the enlarged images are 2 mM. The regions boxed in white are enlarged and shown to the right of the original image. In the far right panel, only the blue and red channels are shown. Note that beads are still in contact with Jedi-GFP even with CHC siRNA treatment, but the recruitment of actin is delayed and less robust. Representative images are shown from 3 experiments.

Phosphorylation of clathrin is required for engulfment

To test the importance of clathrin phosphorylation in engulfment, we knocked down endogenous CHC in Jedi-1 transfected HeLa cells and rescued with either siRNA resistant WT-CHC-GFP or a phospho-mutant CHC (CHC-Y1477F/Y1487F-GFP) in a microsphere engulfment assay. The phosphorylation of CHC at Y1477 and Y1487 was previously shown to be required for internalization of bacteria (Bonazzi et al., 2011). The WT-CHC-GFP was able to rescue the defect in Jedi-mediated engulfment observed after knocking down endogenous CHC; however, the phospho-mutant CHC-GFP did not provide any significant rescue (Figure 4.6). These results indicate that Jedi-1-mediated engulfment requires recruitment and phosphorylation of CHC.

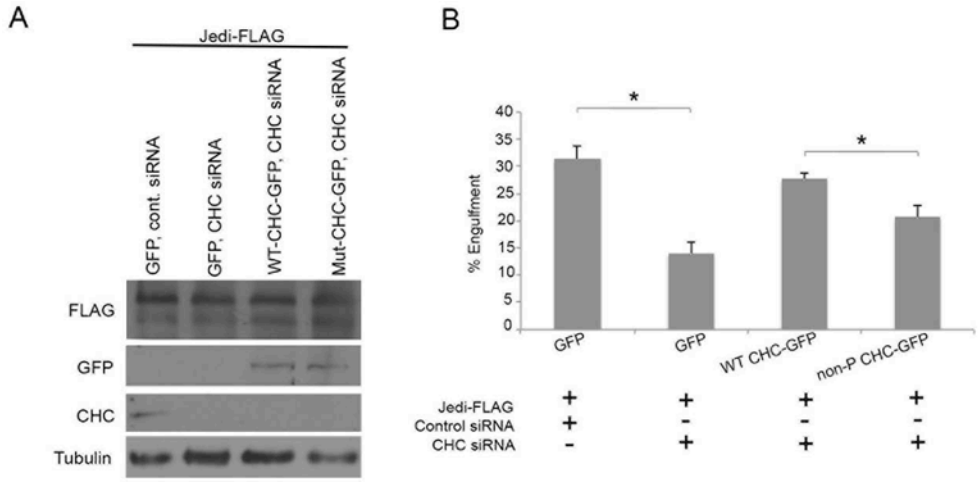


Figure 4.6. **Phospho-CHC is required for Jedi-1-mediated engulfment of microspheres.** (A) HeLa cells were transfected with Jedi-FLAG, control or CHC targeting siRNA, and GFP, WT-CHC-GFP, or phospho-mutant-CHC-GFP and verified for expression and knock-down by immunoblotting. WT-CHC-GFP and phospho-mutant-CHC-GFP are siRNA resistant. (B) Cells were plated for a microsphere engulfment assay, and the number of GFP/FLAG double positive cells engulfing at least one microsphere was determined (n=3, p=0.006 for Jedi-FLAG control siRNA versus Jedi-FLAG with CHC siRNA, p=0.04 for Jedi-FLAG CHC siRNA WT-CHC-GFP vs Jedi-FLAG CHC siRNA phospho-mutant CHC-GFP transfected cells by Student's t-test.

Discussion

Programmed cell death is a natural part of the development of the mammalian nervous system, necessary for establishing proper cell numbers and connections. The neuronal corpses must be efficiently removed in order to avoid an immune system response, which can induce inflammation and autoimmunity. An assortment of autoimmune diseases is associated with a failure to properly clear dead cells (Krysko et al., 2006). However, the molecular mechanisms underlying this phagocytic process have not been well characterized, particularly in the nervous system. We identified MEGF10 and Jedi-1 as engulfment receptors expressed by the glial precursors and required for phagocytosis of dead neurons. MEGF10 and Jedi-1 are both mammalian homologs of the *Drosophila melanogaster* receptor Draper, and the *Caenorhabditis elegans* receptor CED-1, which are known to signal at least in part via recruitment of the adapter protein CED-6 (GULP in mammals) (Awasaki et al., 2006; Su et al., 2002). Here, we demonstrate that Jedi-1 interacts with GULP, resulting in the phosphorylation and recruitment of clathrin, which is required for actin rearrangement and engulfment.

Our results demonstrate that GULP facilitates engulfment through interaction with clathrin. Several previous findings suggested a role for clathrin in Jedi-1/GULP -mediated phagocytosis. First, GULP was shown to sequester ACAP1 (a GTPase activating protein) and thereby act as a positive regulator of Arf6, a GTPase implicated in promoting clathrin-mediated endocytosis (Ma et al., 2007). The regulation of Arf6 by GULP was assessed during cell migration; however Arf6 is known to act in a multitude of cellular functions, including cell migration, cell adhesion, endocytosis and phagocytosis (Casanova, 2007; Donaldson & Honda, 2005). Second, CED-6 associates with CED-1 (Su et al., 2002) and Draper (Awasaki et al., 2006) through an interaction between the phosphotyrosine binding (PTB) domain of CED-6/GULP and an NPXY motif in the intracellular domain of the receptors. Similarly, we found that the NPXY domain in Jedi-1 was required for binding to GULP. Mutation of the NPXY motif in Jedi-1 or knock down

of GULP prevented Jedi-mediated engulfment, indicating that Jedi-1 interaction with GULP is essential for this process. NPXY motifs are known to play a role in the internalization of proteins from the plasma membrane through clathrin-coated vesicles (Bonazzi et al., 2011). Furthermore, clathrin and AP-2 directly bind to peptides containing an FXNPXY sequence (Boll et al., 2002; Kibbey et al., 1998), but increasing evidence suggests that this motif is actually recognized by PTB-containing proteins, such as Numb, ARH, Dab1, and Dab2 which work with AP-2 to promote clathrin recruitment and assembly (Traub, 2003); therefore GULP could function as such an adapter. Third, GULP itself was shown to bind to clathrin by yeast-2-hybrid and GST-pull down, and it colocalized with clathrin in SN56 cells (Martins-Silva et al., 2006). In addition, the *Drosophila* vitellogenin receptor Yolkless was recently demonstrated to associate with CED-6/GULP through a FXNPXA sequence in Yolkless, and internalization of the receptor required CED-6 expression (Jha et al., 2012). Similarly, we found that Jedi-1 internalization in response to addition of microspheres was GULP dependent (Figure 3.5). Furthermore, Jha et al (2012) found an interaction between dCED-6 and clathrin heavy chain (CHC) that depended on a C-terminal DLF/DPF bipartite sequence in dCED-6. We also demonstrated an interaction between mammalian GULP and CHC that depended on the C-terminal portion of GULP containing this bipartite motif (Figure 4.1). Importantly, in cells with GULP silenced, Jedi-1-mediated phagocytosis could be rescued by wild type GULP, but not a mutant lacking the CHC binding domain (Figure 4.1), indicating that GULP association with clathrin is necessary for Jedi-1-induced engulfment.

The requirement for clathrin in phagocytosis was rather surprising given the small size of a clathrin coated vesicle (typically <200nm) (McMahon & Boucrot, 2011) and the large size of an apoptotic cell or microsphere (2 microns) engulfed via a Jedi-1-dependent mechanism. The triskelion clathrin cage that forms around vesicles would be too small to accommodate a phagocytosed particle as large as the microspheres; nevertheless, clathrin was required for this process. To the best of our knowledge, this is the first

report to identify a direct role for mammalian clathrin in phagocytosis of apoptotic cells. However, clathrin was previously implicated in internalization of other large bodies such as bacteria (Veiga & Cossart, 2005), fungi (Moreno-Ruiz et al., 2009), and some viruses (Ehrlich et al., 2004; Rust et al., 2004). The Cossart group reported that pathogenic bacteria associate with their host cells and are internalized through a clathrin-dependent mechanism (Bonazzi et al., 2011; Veiga & Cossart, 2005). They demonstrated that bacterial adhesion stimulated localized formation of clathrin-coated pits, which served as docking sites for actin accumulation rather than forming conventional clathrin coated vesicles. The actin recruitment and bacterial internalization required phosphorylation of clathrin heavy chain (CHC) (Bonazzi et al., 2011). Exactly how phosphorylation facilitated the recruitment of actin is not clear; however it was necessary for the formation of a complex that included phospho-CHC and clathrin light chain as well as the actin binding protein Hip1R. We similarly found that CHC is phosphorylated in response to microsphere addition to cells expressing Jedi-1 and that the phospho-clathrin as well as actin accumulated around the engulfed spheres and colocalized with Jedi-1. Furthermore, silencing GULP inhibited CHC phosphorylation (Figure 4.3) and microsphere internalization, and the phosphorylation of CHC was required for engulfment mediated by Jedi-1 (Figure 4.6). It will be interesting to determine if a similar Hip1R complex is formed in response to Jedi-1 activation.

CHC has been reported to undergo tyrosine phosphorylation by Src family kinases during the internalization of a number of cell surface receptors such as the EGF (Wilde et al., 1999), NGF (Beattie, Howe, Wilde, Brodsky, & Mobley, 2000), T cell (Crotzer, Mabardy, Weiss, & Brodsky, 2004), and IL-7 (Jiang et al., 2004) receptors. In addition, upon binding to its receptor Gb3, the bacterial Shiga toxin was shown to undergo endocytosis by promoting an interaction between the tyrosine kinase Syk and clathrin, which results in a Syk-dependent phosphorylation of CHC (Lauvrak et al., 2006). Similarly, human rhinovirus binding to cells resulted in Syk recruitment to the cell surface and association with clathrin (Lau et al., 2008). Interestingly, we recently

demonstrated that during engulfment, Jedi-1 recruits and activates the tyrosine kinase Syk through its immunoreceptor tyrosine based activation motif (ITAM) domains (Scheib et al., 2012). Syk recruitment and activity was required for Jedi-1 mediated phagocytosis. Therefore, it is possible that binding of apoptotic bodies to Jedi-1 results in Syk activation and recruitment to the ITAM domain of Jedi-1, which then associates with CHC and phosphorylates it. The phosphorylation of CHC has been reported to promote its association with actin, which facilitated internalization (Bonazzi et al., 2011), and we demonstrated that phosphorylation of CHC was required for internalization of microspheres (Figure 4.6). Notably, we also observed actin accumulation around phagocytosed microspheres, particularly at early stages of internalization (Figure 4.5). Since Syk can also regulate actin polymerization through activation of the Rac exchange factor Vav (Cougoule, Hoshino, Dart, Lim, & Caron, 2006; Deckert, Tartare-Deckert, Couture, Mustelin, & Altman, 1996), Jedi-1 may utilize both GULP-mediated CHC recruitment and Syk activation of Rac to form actin structures that facilitate engulfment.

Together with our previous findings (Scheib et al., 2012; Wu et al., 2009), the findings presented here suggest a remarkable conservation of engulfment mechanisms from *C. elegans* to *D. melanogaster* to mammals. Like its homologs Draper and CED-1, Jedi-1 is expressed by “amateur” phagocytes and signals engulfment of apoptotic cells through recruitment of the adapter protein GULP/CED-6. It is notable that a very recent report demonstrated that in *C. elegans* CED-6 forms a complex with CHC and the adaptor AP2 during cell corpse engulfment (Chen et al., 2013).

CHAPTER V

JEDI -/- MICE DEVELOP SEVERE AUTOIMMUNITY

Introduction

Apoptosis is an essential part of mammalian development that occurs in various tissues throughout the lifespan of the organism. Approximately 200 billion cells undergo apoptosis every day in humans (Ravichandran, 2010). Apoptotic cells, unlike necrotic cells, are efficiently cleared to prevent unwanted release of their cellular contents and triggering an immune response. “Professional” phagocytes such as macrophages and dendritic cells usually carry out apoptotic cell removal. However, it is now known that “amateur” phagocytes can also engulf neighboring cells rapidly due to their close proximity. Apoptosis plays a major role in the developing mammalian nervous system, including establishment of correct connections between groups of neurons and their target tissues. Approximately 50% of the neurons generated during development undergo apoptosis (Burek and Oppenheim, 1996); the mechanisms by which these corpses are cleared to prevent an immune response are unclear in the peripheral nervous system (PNS). Microglia are known to be the professional phagocytes of the CNS (Ashwell, 1991; Ashwell, 1990; Ling et al., 1990); however, recent evidence suggests that astrocytes (Park et al., 2007; Magnus et al., 2002) and even neural progenitor cells (Lu et al., 2011) can participate in efferocytosis.

The Carter lab demonstrated that satellite glial cell (SGC) precursors are the principal phagocytes during development of the dorsal root ganglia (DRG) (Wu et al., 2009). Macrophage engulfment of apoptotic neurons in the DRG was a rare event. Even in neurotrophin 3 knockout mice, in which 70% of DRG neurons die, only 3% of the corpses were engulfed by macrophages while 75% were engulfed by SGC precursors (Wu et al.,

2009). These findings revealed a novel role for SGC precursor cells as amateur phagocytes in the PNS. Satellite glial cells are neural crest derived and share a common precursor with Schwann cells (Woodhoo and Sommer, 2008). SGCs are located around neuron soma and form tight junctions between the glial cells to establish a protective barrier around the neuron (Ohara et al., 2009). Although SGCs are the main phagocytes in the DRG during development, macrophages have a more prominent role in phagocytosis of neuronal debris after injury (Lu and Richardson, 1993).

Neuronal cell death can also occur due to injuries or neuropathologies such as Alzheimer's disease, stroke, and chemotherapeutic treatment. Insufficient removal of dead cells may result in autoimmune diseases or an inflammatory response (Nagata et al., 2010); however, many gaps exist in the knowledge of dead neuron clearance. In many cases, autoimmune conditions such as rheumatoid arthritis and lupus are associated with peripheral neuropathy (Spirin et al., 2007). There are also autoimmune neuropathies such as chronic inflammatory demyelinating polyneuropathy and Guillain-Barre Syndrome, which are believed to be due to an autoimmune attack specifically on peripheral nerves. How the immune system begins to recognize "self" in these pathologies is unknown, but it is possible that inefficient clearance of cell types such as neurons may contribute to an immune response over time. Therefore, determining the mechanisms by which apoptotic neurons are cleared will not only benefit our understanding of the development of the mammalian nervous system, but will possibly provide vital insights into the etiology of autoimmune disorders. The primary goal of my dissertation research was to determine the signaling mechanisms downstream of the newly identified engulfment receptor, Jedi-1, and to evaluate the role of Jedi-1 in clearing dead neurons in vivo.

Proper recognition and clearance of apoptotic cells is thought to be important for the prevention of autoimmune diease and inflammation. For example, mice lacking a phosphatidylserine binding protein known as MFG-E8 (Hanayama et al., 2004) develop

autoimmunity. Mice lacking the engulfment receptor c-Mer expressed by macrophages (Cohen et al., 2002) also develop autoimmune phenotypes. Interestingly, c-Mer is expressed not only by macrophages but also in retinal pigment epithelial cells, where it is required for the engulfment of distal ends of photoreceptors. Mice lacking c-Mer undergo a progressive loss of photoreceptors due to defective clearance. Mice which lack all three of the TAM family of receptors (Tyro-3, Axl, and MerTK) develop blindness due to defective engulfment of photoreceptor outer segments, and male TAM knockout mice are sterile due to defective phagocytosis of sperm cell progenitors by Sertoli cells in the testes (Lu et al., 1999). Mice which lack SCARF-1, another engulfment receptor, develop autoimmunity including increased serum autoantibodies, glomerulonephritis, and skin lesions (Ramirez-Ortiz et al., 2013). It has been hypothesized that the result of inefficient engulfment of the large number of neuronal corpses generated during development would be an autoimmune response; however, this has not previously been tested. It is known that a fraction of lupus patients develop peripheral neuropathy (Spirin et al., 2007) and some of the autoantibodies found in these patients target neuronal proteins such as NMDA receptors and neurofilament (Zandman-Goddard et al., 2006). These findings suggest that neuronal debris may have accumulated improperly leading to autoimmunity.

In this chapter, I describe the analysis of our jedi-1 -/- mice for symptoms of autoimmunity. The jedi-1 -/- mice develop autoimmune disease including production of auto-antibodies, kidney dysfunction, and immune cell abnormalities. I also present data which suggest that loss of Jedi-1 results in defective clearance of apoptotic DRG neurons during development. Expression of Jedi-1 by other types of phagocytes suggests that it could play a role in professional phagocytes such as microglia and macrophages as well, although our current data suggests that Jedi-1 is not required for engulfment by macrophages. The jedi-1 -/- mice are a valuable tool for understanding how autoimmunity develops after defective clearance of apoptotic cells.

Experimental Procedures

Generation and verification of jedi-1 -/- mice

We generated the jedi-1 -/- mice using embryonic cells created by the Knock Out Mouse Project (KOMP). The consortium generated a null allele by insertion of a cassette containing the lacZ gene and Neo flanked by FRT sites with loxP sites positioned such that upon flipping out the FRT bounded region the lacZ and Neo will be removed leaving exons 4 (where the initiator methionine is) and flanked by loxP (Figure 5.1). The inserted cassette disrupts splicing, resulting in a nonsense RNA that is degraded; creating a functioning null allele in all tissues. Verification of successful deletion of Jedi-1 was verified by RT-PCR and immunoblotting (Figure 5.2). cDNA was generated using brain RNA from P5 KO or WT mouse pups and using primers for Jedi-1 as described in Wu et al., 2009. We determined that no Jedi-1 protein was produced using lysates from P5 KO and WT mouse brain. Briefly, Jedi-1 was immunoprecipitated using a polyclonal rabbit anti-Jedi antibody generated by our lab, and Jedi-1 was then detected by immunoblotting with a monoclonal mouse anti-Jedi antibody generated in collaboration with the Vanderbilt Antibody Core Facility.

TUNEL Staining

Timed pregnancies were used to obtain E17.5 WT and jedi-1 KO embryos. Embryos were fixed in 4% paraformaldehyde for 24 hours and cryoprotected in 30% sucrose. Embryos were embedded in OCT and cryosectioned to obtain 15 micrometer sagittal serial sections (taking every third section). Sections were stained with rabbit TuJ1 antibody (Covance) to label neurons and counterstained with TUNEL staining using the Millipore ApopTag Fluorescence Kit. Nuclei were counterstained with DAPI. Images were obtained using a fluorescence scope, and the number of TUNEL positive cells/section in the DRG were quantified in cervical, thoracic, and lumbar DRG.

Anti-dsDNA antibody ELISA

Blood was collected from the tails of mice using capillary tubes, and blood was spun for an hour at 4°C at 15,000 RPM. After spinning, the supernatant layer of serum was collected and stored at -80°C. Serum titers of anti-dsDNA antibodies were measured using the protocol developed by Shivakumar et al, 1989. Nunc MaxiSorp plates were blocked with mBSA in 1× PBS (0.1 mg/ml) at 37°C for 30 min. The plate was then washed with PBS and coated with 50 μg/ml dsDNA in PBS at 37°C for 30 min. The plate was washed twice with PBS and blocked overnight at 4°C with blocking buffer (3% BSA, 3 mM EDTA, and 0.1% gelatin in PBS). Plates were washed twice with PBS. Serum was diluted 1:1,000 in serum diluent (2% BSA, 3 mM EDTA, 0.05% Tween 20 in PBS) and added to the plate and incubated 2 h at room temperature (RT) on an orbital shaker. The plate was washed twice in PBS-Tween and twice in PBS. IgG-HRP (Promega, Madison, WI) was diluted 1:5,000 in secondary diluent (1% BSA and 0.05% Tween in PBS), added to the plate and incubated overnight at 4°C on an orbital shaker. The plate was washed twice with PBS-Tween and twice with PBS, and OptEIA TMB Substrate (BD Biosciences, San Diego, CA) was added to plate and allowed to incubate. Reaction was quenched with 1 M phosphoric acid, and the plate was promptly read at 450 nm.

Immunoglobulin staining of kidneys

Adult animals were anesthetized using isofluorane and perfused with cold PBS. Following perfusion, kidneys were dissected and fixed in 4% paraformaldehyde for 24 hours. The kidneys were cryoprotected in 30% sucrose and mounted in OCT. 15 micrometer cryosections of kidneys were obtained using a cryostat. Sections were stained with an Alexa-488 conjugated antibody recognizing mouse IgG heavy chain according to the protocol of Ramirez-Ortiz et al., 2013 and counterstained with To-Pro-3 to label nuclei. Images were obtained using an LSM-510 confocal microscope.

Hematoxylin and Eosin staining of kidneys

Kidneys were obtained using the same conditions as described in the methods for "Immunoglobulin staining of kidneys". Sections were submitted to the Vanderbilt Translational Pathology Core and stained with Hematoxylin and Eosin using an automated system.

Proteinurea measurements

Urine was obtained from mice by squeezing the lower abdomen over a petri dish. Urine was collected and frozen at -80°C until time of analysis. Urine was spotted onto colorimetric Siemens Uristix Reagent Strips, and the amount of protein in the urine was measured according to color change. The amount of protein in the urine was scored for each mouse on a numeric scale: 1=negative/trace, 2=30 mg/dL, 3=100 mg/dL.

Results

The targeting strategy used to generate the jedi-1 -/- mice was successful

We generated the jedi-1 -/- mice using embryonic cells created by the Knock Out Mouse Project (KOMP). The consortium generated a null allele by insertion of a cassette containing the lacZ gene and Neo flanked by FRT sites with loxP sites positioned such that upon flipping out the FRT bounded region the lacZ and Neo will be removed leaving exons 4 (where the initiator methionine is) flanked by loxP (Figure 5.1). The inserted cassette disrupts splicing, resulting in a nonsense RNA that is degraded; creating a functioning null allele in all tissues.

In order to validate the success of the deletion cassette we performed expression analysis in brain tissue of P5 wild type (WT) and jedi-1 KO mice. RNA was obtained from

whole brain and reverse transcribed to generate cDNA for PCR analysis using primers for Jedi-1 and GAPDH as a loading control as in Wu et al., 2009. We found that WT mice express Jedi-1 mRNA at high levels, and Jedi-1 was not detectable in *jedi-1* -/- brain (Figure 5.2). In order to verify loss of Jedi-1 protein expression, Jedi-1 was immunoprecipitated from P5 brain lysate and detected using a monoclonal Jedi-1 antibody. A robust Jedi-1 signal was seen by immunoblotting wild-type samples, but not in the *jedi-1* -/- mice (Figure 5.2). Based on the expression data, we conclude that the *jedi-1* targeting strategy used by KOMP was successful in disrupting Jedi-1 expression.

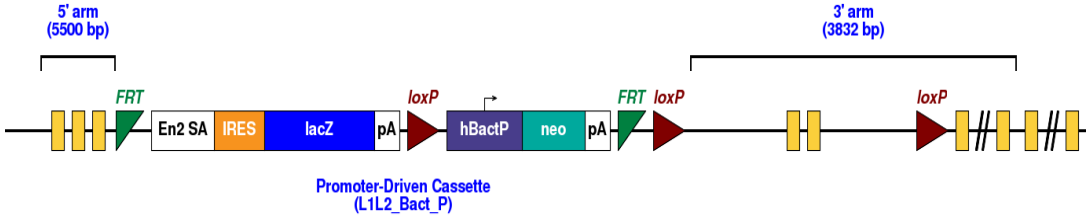


Figure 5.1. Targeting strategy for Jedi-1 knock-out. The depicted cassette, containing IRES-lacZ and Neo is inserted between exons 3 and 4 such that splicing is disrupted, creating a functional knock out. Upon deletion of the cassette by Flp recombinase, an allele containing exons 4 and 5 floxed remains.

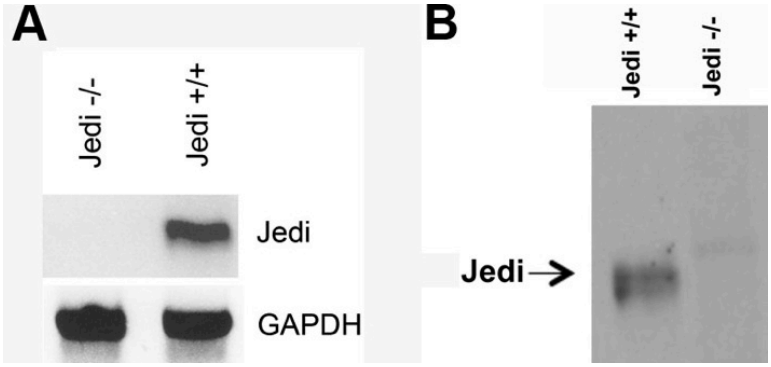


Figure 5.2. The jedi1 targeting allele produced a knock out. (A) RT-PCR for Jedi1 from brain of jedi1+/+ or -/- mice. GAPDH primers were used as a loading control for equal starting amounts of RNA. (B) Lack of Jedi1 protein expression confirmed by IP of Jedi1 from brain of jedi1+/+ or -/- mice followed by immunoblotting for Jedi1.

jedi-1 -/- mice have defective clearance of apoptotic cells in DRG

Due to the role of Jedi-1 in clearance of apoptotic neurons *in vitro* (Wu et al., 2009), we hypothesized that deletion of Jedi-1 would result in reduced clearance of apoptotic neurons in the developing DRG *in vivo*. Indeed, at E17.5, a time point after the normal period of sensory neuron apoptosis, we detected a significant increase in TUNEL positive cells in the lumber DRG of *jedi1*-/- mice, relative to wild type (1.83+0.11 TUNEL+/section in *jedi*-/- vs 0.83+0.07 in +/+, n=4 animals/genotype, 1 lumbar, thoracic and cervical DRG for each, every 3rd section counted, $p<0.01$) (Figure 5.3). The presence of excess apoptotic cells at other ages and in other tissues will be analyzed in future experiments. The increased number of apoptotic cells present in the DRG of *jedi-1* -/- animals supports our in vitro data that Jedi-1 is an important engulfment receptor for apoptotic neuron engulfment during development (Wu et al., 2009). We also evaluated the phagocytic capability of WT or *jedi-1* -/- satellite glial cells in vitro. In WT co-cultures, 56% of transfected glial cells were engulfing apoptotic neurons, while only 40.67% of *jedi-1* -/- glial cells had engulfed apoptotic fragments (Figure 5.4) (n=3, p=.017). These results are quite similar to what has been previously observed when Jedi-1 was knocked down in glial cells using siRNA (Wu et al., 2009).

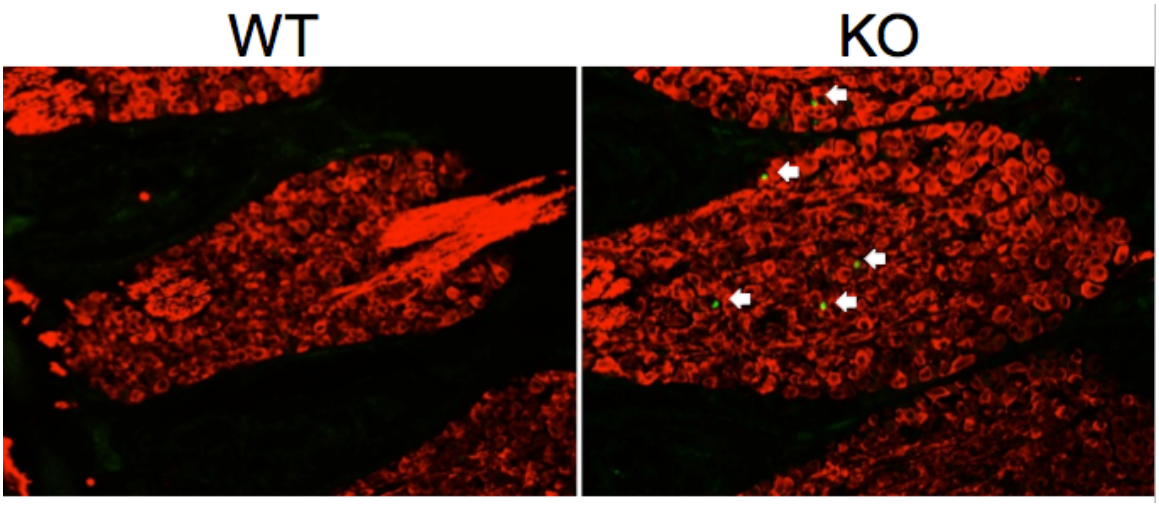


Figure 5.3. Apoptotic bodies accumulate in jedi1-/- DRG. Cryosections through E17.5 lumbar DRG from jedi1+/+ and -/- embryos were immunostained for TuJ1 (red) to mark neurons and TUNEL labeled (green) to label apoptotic cell corpses (indicated by arrowheads).

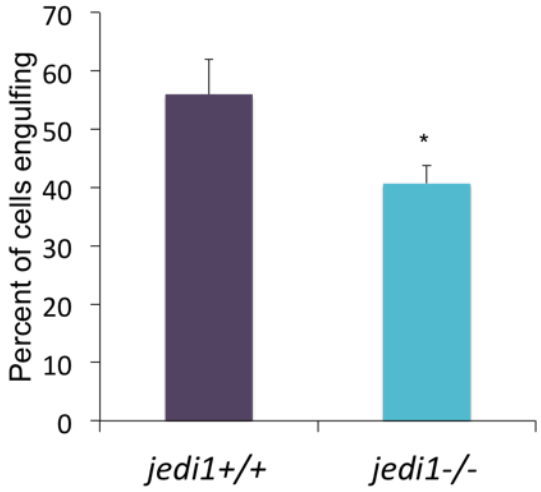


Figure 5.4. jedi-1 -/- SGCs have reduced engulfment capability in DRG co-cultures. DRGs from early postnatal mice were dissected and cultured with NGF. After 3 days, NGF was withdrawn to induce neuronal apoptosis. SGCs were transfected with GFP to identify the boundaries of cells, and the percentage of transfected SGCs which had engulfed one or more apoptotic nuclei was quantified using confocal microscopy (n=3, p=0.029).

Jedi-1 -/- mice have elevated dsDNA autoantibodies

Based on the delayed engulfment of apoptotic cells in *jedi-1* -/- KO mice, we hypothesized that autoimmunity would develop in the animals. Indeed, autoimmunity is reflected in our *jedi-1* -/- mice by the presence of autoantibodies in the serum; specifically, antibodies to ds-DNA were detected starting at 8 months, with a titer similar to the positive control, which are B6.SLE, a spontaneous model of Systemic lupus erythematosus (Morel et al., 1996) (Figure 5.5). Autoantibodies that recognize double stranded DNA are a hallmark of SLE pathology. In fact, approximately 95% of patients diagnosed with SLE have dsDNA antibodies in their serum (Reveille et al., 2000). These data suggest that *jedi-1* -/- mice suffer from autoimmune phenotypes similar to those of SLE patients.

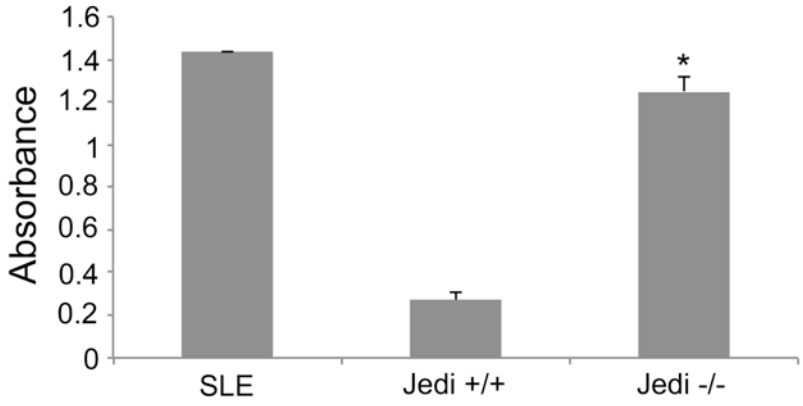


Figure 5.5. Jedi-1 -/- mice have elevated autoantibodies to dsDNA. Serum from *jedi-1* +/+ and -/- mice was collected and subjected to an ELISA for antibodies to ds-DNA. B6.SLE mice were used as a positive control (n=4-6 per genotype; *, $p<0.01$). ELISA analyses were completed by Jillian Rhoads.

Mice lacking Jedi-1 have glomerulonephritis and proteinurea

One consequence of antibodies that recognize self-antigens is deposition of immunoglobulin (IgG) in such tissues as the kidney, which can cause glomerulonephritis. In conditions of autoimmunity, IgG will accumulate in glomeruli (Muñoz et al., 2010). We assessed kidneys from wild-type and *jedi-1* -/- mice in 8 month old mice, and found that IgG accumulates in the glomeruli of *jedi-1* -/- mice (Figure 5.6). Another renal phenotype typically observed in SLE is hypercellularity of the glomeruli. We found that the glomeruli of *jedi-1* -/- mice are strikingly hypercellular in comparison to wild-type glomeruli (Figure 5.7). How the hypercellularity arises during SLE pathology is not completely clear, but one hypothesis is that accumulation of IgG causes infiltration of inflammatory cells (Couser, 1999). Another autoimmune phenotype associated with IgG accumulation in the glomeruli is increased protein in the urine. We collected urine from WT and *jedi-1* -/- mice for analysis using colorimetric strips. The *jedi-1* -/- animals had increased proteinuria that was consistent with the other renal phenotypes observed (Figure 5.8). Taken together, these data suggest that deletion of jedi-1 results in many of the hallmark pathologies of autoimmunity. This SLE-like pathology is likely due to deficient clearance of apoptotic cells that leak their contents due to secondary necrosis.

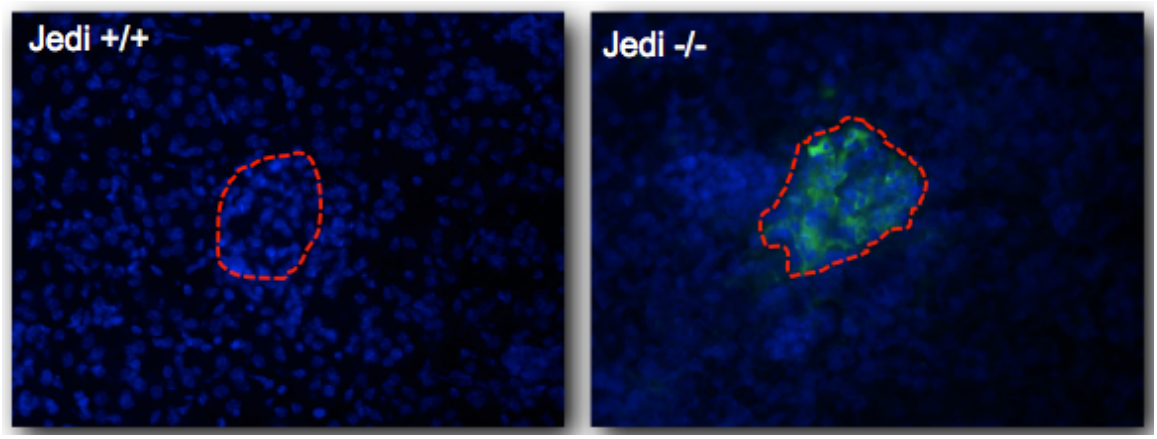


Figure 5.6. Jedi-1 -/- mice have elevated levels of immunoglobulin in their glomeruli. Cryosections of 8 month old jedi-1 +/+ and -/- mice were stained with an Alexa-488 conjugated anti-mouse secondary antibody. Glomeruli have been outlined using red dashed lines.

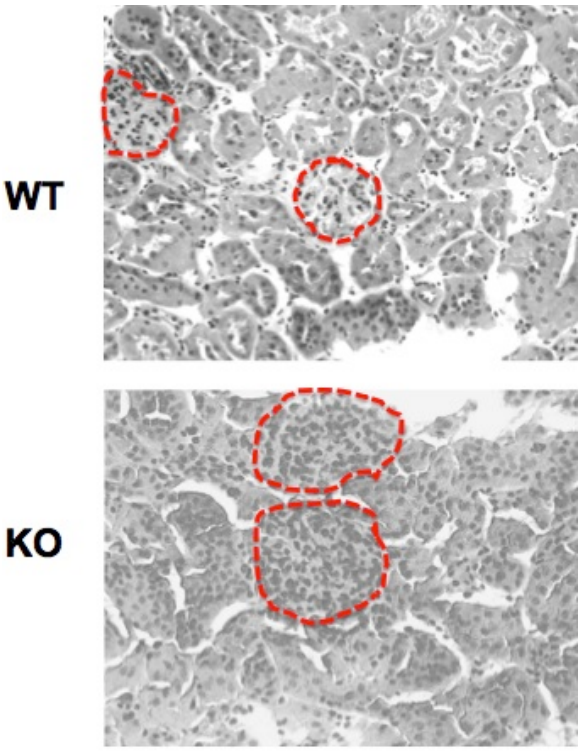


Figure 5.7. Glomeruli of jedi-1 -/- mice are hypercellular in comparision to wild-type glomeruli. Cryosections of kidney from WT and KO Jedi-1 mice were obtained and stained using hematoxylin and eosin by the Vanderbilt Translational Pathology Core. Glomeruli have been outlined using red dashed lines for size comparison.

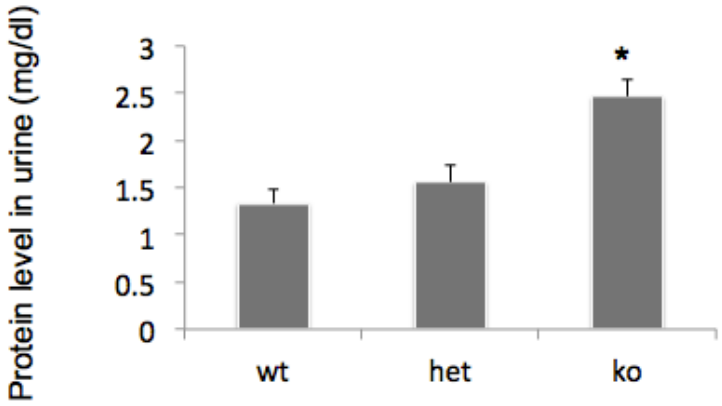


Figure 5.8. Mice lacking jedi-1 exhibit proteinurea. Urine was collected from jedi-1 +/+ (wt), +/- (het), and -/- (ko) mice and protein measured using Siemens Multistix urine reagent strips and scored as 1= 0/trace, 2= 30 mg /dL, 3= 100 mg/dL (n=9-12 per genotype; *,$p<0.01$).

Discussion

The timely and efficient removal of cell corpses is necessary to stave off progression to secondary necrosis and potential autoimmune responses. Mutation or genetic deletion of apoptotic cell engulfment receptors or bridging molecules have been linked to autoimmune pathologies in both humans and mice. We hypothesized that based on the role of Jedi-1 in engulfment of apoptotic neurons *in vitro*, jedi-1 -/- mice would have defective clearance of apoptotic cells and potential autoimmune phenotypes. After ablating Jedi-1 expression in all tissues, we detected several phenotypes indicative of SLE-like pathology.

The first place that we chose to assess apoptotic cell uptake *in vivo* was in the E17.5 dorsal root ganglia. This developmental stage lies outside of the normal time of programmed cell death of neurons, and we would not have expected to see many apoptotic cells in the DRG. In fact, we did see increased numbers of apoptotic cells in the DRG of jedi-1 -/- mice. This evidence indicates that Jedi-1 is an important receptor for apoptotic cell recognition and clearance *in vivo*. The phagocytic capability of the

satellite glial cells from jedi-1 -/- mice was also diminished in comparison to wild-type SGCs *in vitro*. These data suggest that deletion of Jedi-1 decreases the ability of SGCs to clear apoptotic cells and also suggests that apoptotic cells persist in the DRG past the normal time of engulfment. The consequences of this defective clearance are potentially contributing to the autoimmune phenotype observed in the Jedi-1 null mice. Defective clearance of apoptotic cells can result in accumulation of apoptotic cell remnants. The consequence of this improper clearance is the initiation of systemic autoimmunity in diseases such as SLE. If apoptotic debris accumulates in germinal centers, activates the complement system and promotes survival of B cells that have become autoreactive, autoimmunity could arise (Muñoz et al., 2010). The improper clearance of postapoptotic remnants and secondary necrotic cells results in production of autoantibodies against self antigens and autoinflammation. Complexes made up of autoantibodies, nucleic acids, and proteins can be mistaken by the immune system for opsonized viruses, which leads to production of type I interferons (a common hallmark of SLE)(Muñoz et al., 2010). The potential harmfulness of autoantibodies is thought to be much higher when autoantigens are accessible for immune complex formation, as in the case of defective apoptotic cell engulfment. Indeed, there are numerous examples of genetic mouse models in which key engulfment signaling receptors or adapter proteins have been deleted which exhibit SLE-like pathologies. For example, mice lacking the novel engulfment receptor SCARF-1 exhibit many SLE-like symptoms including autoantibodies, glomerulonephritis, and skin lesions (Ramirez-Ortiz et al., 2013).

After observing defective clearance of neurons in the DRG, we began to assess the mice for typical signs of autoimmunity. We observed elevated levels of autoantibodies that recognize ds-DNA, a hallmark of SLE pathology. Other autoantibodies such as those that recognize cardiolipin will also be assessed in future studies in collaboration with the Major lab at Vanderbilt. Another symptom of autoimmunity, accumulation of IgG in the glomeruli of kidneys, was observed very consistently in the jedi-1 -/- mice.

Autoantibodies can clog the capillary networks of the glomerulus, and this can result in damage and hypercellularity of the glomeruli. The IgG buildup in the glomeruli can promote infiltration of immune cells and damage to the glomerulus due to inflammation. We did observe IgG accumulation in the glomeruli of the Jedi-1 null mice at 8 months and 1 year of age. The resulting pathology of IgG deposition and immune cell infiltration of the glomeruli is increased protein in the urine, or proteinuria. Indeed, urine samples taken from Jedi-1 KO mice consistently showed increased levels of protein as assessed by colorimetric strips. These data suggest that an aberrant immune response is chronically occurring in the jedi-1 -/- mice, and we hypothesize that the cause of the renal phenotype is defective clearance of apoptotic cells.

We hypothesize that the autoimmunity exhibited by the Jedi-1 null mice is due to defective clearance of apoptotic cells, specifically by amateur phagocytes such as satellite glial cells. We have preliminary data that suggests that Jedi-1 is not expressed in dendritic cells, which are professional phagocytes. While Jedi-1 is expressed in peritoneal macrophages, preliminary data suggest that loss of Jedi-1 does not impair phagocytosis by these primary phagocytes. Jedi-1 expression has also been detected in retinal pigment epithelial cells, a known phagocytic cell type. Future studies will assess expression of Jedi-1 in microglia, another professional phagocyte, as well as other known amateur phagocytic cell types, such as astrocytes.

CHAPTER VI

JEDI-1 -/- MICE DEVELOP CHRONIC ITCH

Introduction

The extensive programmed cell death that occurs in the dorsal root ganglia during development results in a burden of apoptotic cell corpses that must be recognized and cleared. Many autoimmune conditions, such as lupus and rheumatoid arthritis can be associated with peripheral neuropathy (Hanly, 2007; Sofat et al., 2006). The mechanism by which the immune system begins to recognize "self" in these conditions is not well understood nor is it clear how neuropathy develops. One hypothesis is that failure to clear apoptotic cells, such as neurons, may lead to an immune response against neuronal antigens over time. Extensive evidence in mouse models suggests that delayed clearance of apoptotic cells can lead to autoimmunity. For example, mice lacking c-Mer, an engulfment receptor expressed on macrophages and retinal pigment epithelial cells, develop autoimmune phenotypes similar to lupus patients (Cohen et al., 2002). In addition to the hallmark phenotypes of autoimmunity observed in our *jedi-1* -/- mice (circulating auto-antibodies and renal dysfunction), we observed the striking phenotype of chronic itch, or pruritis.

Itch sensation, or pruritis, is a normal physiological response to an unpleasant sensation; however, chronic pruritis is a recurrent and often debilitating condition that affects a widespread population. Numerous pathologies can result in chronic itch, including various injuries (e.g. burn, viral infection, and kidney damage), autoimmune disease (e.g. eczema and autoimmune hepatitis), and a number of neurological conditions including psychiatric disturbances and sensory nerve damage (Paus et al., 2006; Ikoma 2013; Bowcock and Cookson, 2004; Yosipovitch and Samuel, 2008). The molecular and

cellular mechanisms that regulate pruritis are unclear, and it is unknown why some autoimmune disease patients develop pruritis. Sensory neuron C-fibers mediate pain and itch response; however, there is a specific subset of C-fibers that selectively transduce itch sensation. Pruriceptive (itch sensitive) neurons express the TrpV1 channel (Imamachi et al., 2009), although nociceptors also express this channel (Karai et al., 2004). Pruriceptive neurons can be divided into two subgroups: those that respond to non-histamine pruritogens and those sensitive to histamine (Paus et al., 2006; Ikoma, 2013). Recent studies showed that the G-protein coupled receptor MrgprA3 (Han et al., 2013) and natriuretic polypeptide b (Nppb) (Mishra and Hoon, 2013) are key signaling molecules required for itch sensation. Loss of Nppb (Mishra and Hoon, 2013) or mrgprA3 (Han et al., 2013) in mice eliminated both non-histamine and histamine pruritic responses, but nociception (pain sensation) and proprioception (the detection of limb position) were not altered. Several groups have reported that gastrin-releasing peptide (GRP)-expressing sensory neurons specifically transmit histamine independent itch sensation (Sun and Chen, 2007; Nattkemper et al., 2013; Liu et al., 2014; Mishra and Hoon, 2013). One study showed that GRP receptor (GRPR)-positive neurons within the spinothalamic tract of the spinal cord transmit itch to the somatosensory cortex because genetic deletion of GRPR neurons selectively decreases itch sensation but not nociception (Sun et al., 2010). Mast cells release histamine, a natural pruritigen; however, many more pruritogenic substances exist (Paus et al., 2006). Examples of other pruritogens include cytokines and ATP, and these substances can be released by satellite glial cells (SGCs) (Ohara et al., 2009). The capability of SGCs to release pruritogens suggests that glial cells may be able to modulate itch response. Even though the connection between SGCs and nociception has been well established, the possibility that SGCs regulate pruritis has not been investigated.

Satellite glial cells express a number of neurotransmitter transporters, including the glutamate transporter GLAST, and it has been suggested that SGCs buffer glutamate similar to astrocytes (Ohara et al., 2009; Berger and Hediger, 2000). Decreasing the

expression of GLAST in SGCs resulted in increased evoked pain (nociception), suggesting a role for glia in regulating sensory responsiveness (Ohara et al., 2009). Neuronal environment regulation by glial cells has an influence on neuronal excitability, and this is important in the response to nerve injury and chronic pain. After nerve insult, SGCs respond similarly to activation of astrocytes by up regulating GFAP and proliferating. The SGCs also release several cytokines (e.g. IL-1β and TNF) as well as ATP, which can induce hyperexcitability in the neuron (Ohara et al., 2009). Resident macrophages are also present in the adult DRG (Dijkstra and Damoiseaux, 1993), and after injury or immune system activation there is an increase in these cells within the DRG due to invasion of circulating macrophages (Hu and McLachlan, 2002). Like SGCs, macrophages can relase cytokines, many of which have been associated with pain sensation (Hu and McLachlan, 2002). Even though SGCs are the primary phagocytes during development of the DRG, macrophages have an important role in phagocytosis of neuronal debris following injury (Lu and Richardson, 1993). Owing to the similarities between macrophage and SGC function in the DRG, it is important to analyze the respective contribution of each cell type to sensory processing and immune cell activation.

Experimental Procedures

Skin analysis

Paraffin embedded sections of lesioned or un-lesioned skin were submitted to the Vanderbilt Pathology Core to be stained according to standard methods with hematoxylin and eosin. Skin sections were also submitted to the Vanderbilt Pathology Core to be stained with individual markers of immune cells in the skin: B220 (B cells), CD3 (T Cells), F480 (macrophages), and toluene blue (Mast cells). The percentage of each cell type in skin sections was quantified using ImageJ. Innervation of the skin was assessed using an anti-Pgp 9.5 antibody. The total Pgp 9.5 fluorescence in the epidermis was quantified and divided over the area of the epidermis.

Behavioral Analysis

Behavioral studies were conducted in the Vanderbilt Neurobehavioral Core Facility. Hot plate analysis was completed at 55 °C. Briefly, mice were placed on a hot plate and the latency to lift a paw or jump in response to heat was recorded. Touch sensation was determined using Von Frey monofilaments. Mice were placed into a holding device with a grid floor and allowed to acclimate to the new environment for 30 minutes. After acclimation, the back right paw of each mouse was stimulated with Von Frey monofilaments of increasing force. Each mouse was assessed three times, and the stiffness at which a response was generated was recorded and averaged for each mouse. Home cage behavior was analyzed using HomeCageScan software. Mice were placed into individual cages and placed into an incubator with controlled heat and humidity. The software was trained to recognize the mice as well as the dimensions of the cage, and an infrared camera was used to track the behavior of mice over a 24-hour period and record time spent engaging in various activities in an Excel file.

GFAP activation studies

DRG were dissected from paraformaldehyde perfused 8-month old WT and Jedi-1 KO mice. DRG were cryosectioned and stained with an anti-GFAP antibody and anti-TuJ1 (to label neurons). Sections were counterstained using DAPI. Sections were imaged using a Zeiss fluorescence microscope. GFAP activation was also assessed *in vitro* using co-cultures of sensory neurons and glial cells from *jedi-1* +/+ or -/- mice. Co-cultures of DRG neurons and glial cells were cultured in the presence of nerve growth factor (NGF) for 5 days or in NGF for 3 days followed by 2 days of NGF withdrawal as described in Wu et al., 2009. After 5 days, cells were fixed using 3.7% formaldehyde and stained with GFAP and To-Pro-3 (nuclear stain). Cells were imaged using a LSM 510 confocal microscope.

Results

Jedi-1 -/- mice develop skin lesions

One of the first phenotypes observed in the *jedi-1* -/- mice was a significantly increased tendency to aquire skin lesions such as hair loss and ulcerative wounds. When the incidence of skin lesions was recorded over time (between 6 months and 1 year), 31% of the knock-out mice developed skin lesions while only 5% of wild-type mice developed lesions (Figure 6.1). The *jedi-1* -/- mice are on the C57/BL6 background, which is prone to ulcerative dermatitis of the skin. C57/BL6 mice have a 4.1% overall incidence of developing dermatitis (Kastenmayer et al., 2006), but this cannot account for what we see in our *jedi-1* -/- mice.

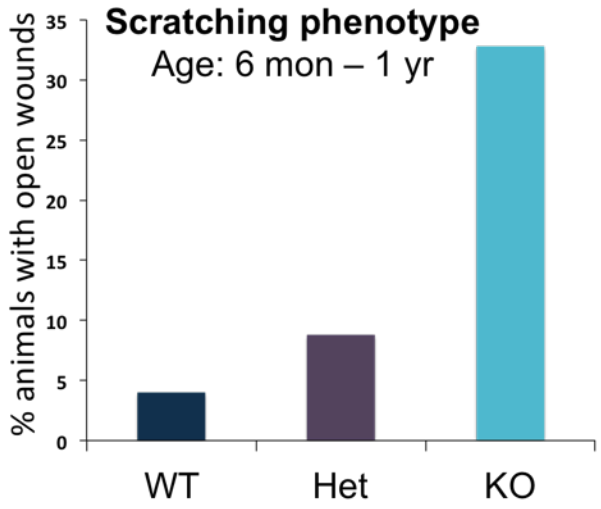


Figure 6.1. *Jedi-1* -/- mice have severe skin lesions compared to jedi-1 +/+ animals. The percentage of animals with open lesions by one year of age is shown. *Jedi-1* +/+ (WT; n=25), +/- (Het; n=45), -/- (KO; n=76).

Jedi-1 -/- mice have increased grooming/scratching behavior

Upon observation, we noted that the *jedi-1* -/- mice appeared to be scratching excessively, which seemed to be the cause of the skin lesions. To evaluate the unconstrained behaviors of the mice noninvasively, we used an automated video analysis system (HomeCageScan) available in the Vanderbilt Neurobehavioral Lab, which uses information about the entire body of the animal and its location within the cage to automatically recognize and quantify specific behaviors over a 24 hour period (maintaining 12 hour/12 hour light/dark schedule). We found that the *jedi-1* -/- mice spend 37% more time grooming/scratching than *jedi-1* +/+ mice (n=8 for each genotype; $p<0.05$). Interestingly, we did not detect any significant change in the Von Frey Hair threshold or hot plate latency (55°C), indicating that the touch and thermal sensation of *jedi-1* knock out mice was normal (data not shown). However, it should be noted that the animals used for this analysis were specificially selected based on not having any signs of scratching (balding or wounding); therefore, it is possible that these mice had not yet developed the pruritic phenotype or may have been among the population that fail to exhibit such a phenotype.

The skin of jedi-1 -/- mice has normal populations of immune cells

To determine the underlying cause of the itch, we first examined the skin. We hypothesized that there could be higher levels of immune cell infiltration in the skin of jedi-1 -/- mice, and perhaps these immune cells could be triggering an itch response (for example, mast cells releasing histamine). However, we found that this was not the case. The non-lesioned areas of both wild type and *jedi-1* -/- mouse skin were unremarkable when we stained sections with hematoxylin and eosin (Figure 6.2). While no gross morphological differences were seen in skin from non-lesioned areas in *jedi-1* +/+ or -/- mice, we wanted to assess specific populations of immune cells in the skin with specific stains. We stained sections of skin for B cells (B220), T cells (CD3), macrophages (F480),

and mast cells (toluene blue), and found no statistically significant differences in any of these cell populations in non-lesioned skin (Figure 6.3). The lack of differences in immune cell populations in the skin indicate that there is not an immune cell component in the skin, leading to the itch phenotype that we observe in *jedi-1* -/- mice.

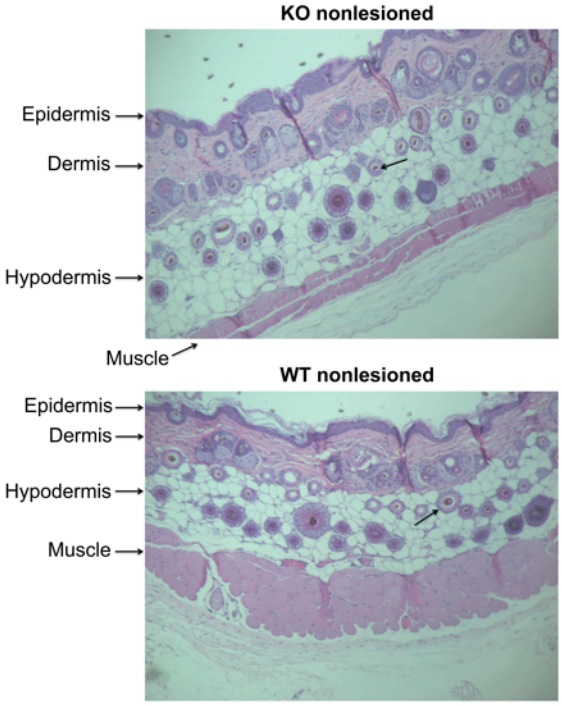


Figure 6.2. No gross morphological differences are observed in the skin of jedi-1 +/+ or -/- mice. Sections of skin were stained using H&E, and various layers of the skin have been labeled. The architecture of the skin seems grossly normal in both wild type (WT) and knock-out (KO) mice. An example of a hair follicle has been marked using an unlabeled arrow in each skin image.

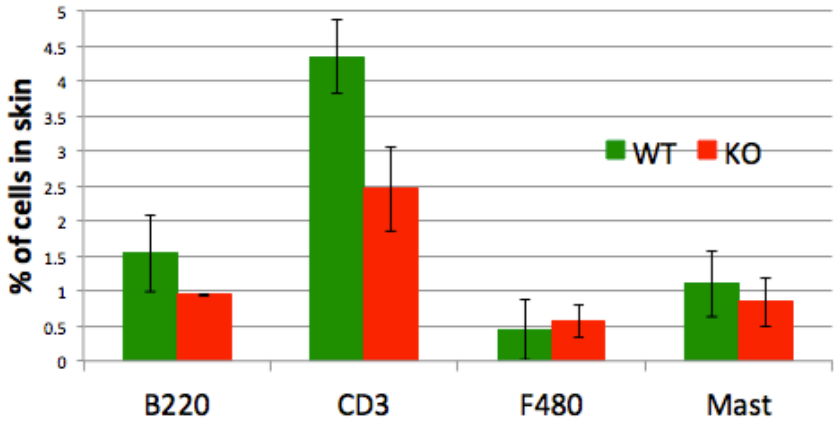


Figure 6.3. Immune cell populations are normal in jedi-1 +/+ and -/- skin. Skin sections from areas that were not lesioned in *jedi-1* +/+ (WT) or -/- (KO) mice were sectioned and stained in collaboration with the Vanderbilt Animal Pathology core facility. B cells were labeled with B220, T-cells with CD3, macrophages with F4/80, and mast cells with toluene blue (looking for characteristic granules). No differences in the percentage of each cell population in the skin were observed. Alexandra Trevisan performed quantification of the populations of cells in the skin.

Apoptotic cells do not accumulate in jedi-1 -/- mouse skin

We considered the possibility that chronic pruritis could result from excessive cell corpses in the skin either during development or the normal cycle of hair follicle growth, which could trigger an inflammatory response and the activation of auto-reactive T cells in the skin. However, preliminary analysis of the skin by Alexandra Trevisan did not reveal a statistically significant increase in apoptotic cells even in lesioned areas (data not shown). This finding, in combination with the lack of differences in immune cell populations in the skin, led us to explore other hypotheses as to why the *jedi-1* -/- mice develop skin lesions and increased scratching/grooming behavior.

Jedi-1 -/- mice have increased activation of SGCs *in vivo* and *in vitro*

We hypothesized that pruritis could develop due to cell corpses lingering in the DRG, which could activate satellite glia, causing them to release factors (e.g. ATP or Nppb)

that activate pruriceptive neurons. Glial fibrillary acidic protein (GFAP) expression is often up regulated in conditions of nerve injury by astrocytes in the CNS and by SGCs in the PNS (Aldskogius and Kozlova, 1998). Interestingly, preliminary data suggests that the SGCs in the DRG of adult *jedi-1* -/- mice in early stages of chronic itch, indicated by balding areas due to scratching, were activated (Figure 6.4) as shown by increased GFAP staining. Similarly, in cultures of DRG neurons and SGCs from *jedi-1* +/+ and -/- mice GFAP was strongly expressed in *jedi-1* -/- SGCs, suggesting that the SGCs are more activated in *jedi-1* -/- mice even under conditions in which little neuronal death should occur (Figure 6.5). Following NGF withdrawal for 2 days, to induce neuronal apoptosis, GFAP was up regulated in wild type glia, supporting the hypothesis that the presence of apoptotic neurons activates SGCs. However, the expression of GFAP in *jedi-1*-/- SGCs was even more intense, indicative of greater activation. The *in vivo* and *in vitro* results suggest that the SGCs of jedi-1 -/- mice have increased activation in comparison to jedi-1 +/+ SGCs.

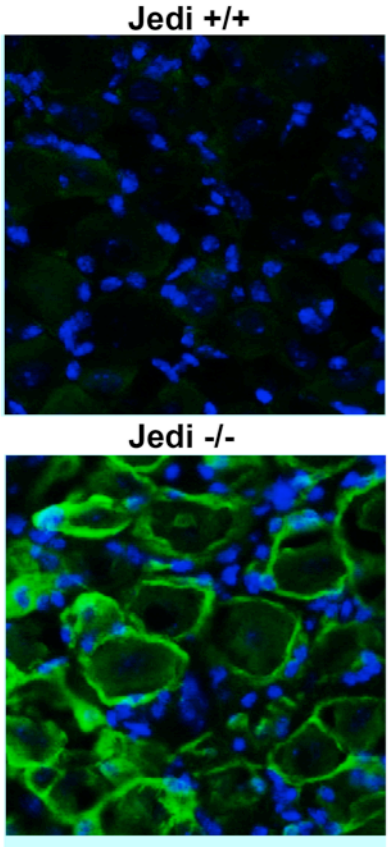


Figure 6.4. SGCs are activated in *jedi-1* -/- mice in vivo. DRG from 8 month-old *jedi-1* +/+ or -/- mice were dissected from paraformaldehyde perfused mice and cryosectioned to obtain 15 micrometer sections. DRG sections were stained for GFAP (green) and counterstained with the nuclear stain, DAPI. Images were taken using a Zeiss fluorescence microscope.

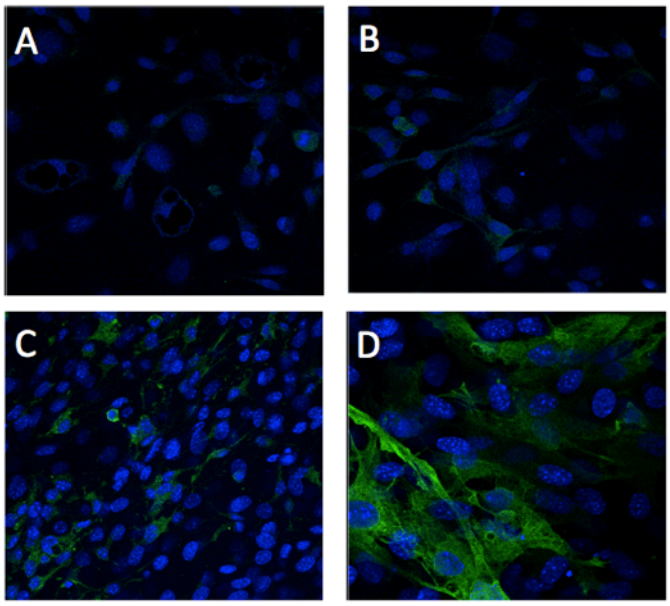


Figure 6.5. SGCs of *jedi-1* -/- mice are excessively activated, as shown by increased GFAP expression. Wild type (A) or *jedi-1* -/- co-cultures (C) were cultured in the presence of NGF for 5 days. Alternatively, wild-type (B) or *jedi-1* -/- (D) co-cultures were cultured in NGF for 3 days followed by 2 days of NGF withdrawal. Cells were stained for GFAP (green) and counterstained with To-Pro-3 to label nuclei. Images were taken using an LSM 510 confocal microscope (n=2).

Mice lacking Jedi-1 have increased skin innervation

Persistant increases in neuronal activity often leads to fiber sprouting. Therefore, we assessed the levels of innervation in the skin of *jedi-1* -/- and +/+ mice (note that this analysis was completed in nonsymptomatic skin with no balding or ulcers) by immunostaining with an antibody to the neuronal marker PGP9.5 (Figure 6.6). Fibers with bare endings in the epidermis are pruriceptive or nociceptive afferents, thus, the knockout mice may be hypersensitive to itch due to increased innervation. We detected a significant increase in PGP9.5 staining in the epidermis of the *jedi-1-/-* mice relative to the wild type, indicating increased skin innervation, which suggests that the neurons may have increased activity. As discussed in the previous chapter, there was no change in Von Frey filament threshold or hot plate latency was observed, suggesting that there is not hyperinnervation of all C fibers. Studies suggest that nerve sprouting occurs due

to an increase in NGF, which is elevated in lesioned skin and serum of patients with atopic dermatitis (Dou et al., 2006; Rukwied et al., 2010; Nockher and Renz, 2006). It is known that application of NGF blocking antibodies in a mouse model of dermatitis improved the symptoms and reduced skin hyperinnervation (Takano et al., 2005). We hypothesize that NGF could be released either by the SGCs or by local immune cells, thereby promoting sprouting. Alternatively, the SGCs could become activated by the persistence of neuronal corpses and secrete factors that increase activation of pruriceptive neurons, similar to the ability of SGCs to enhance nociception (Ohara et al., 2009). On the other hand, infiltration of macrophages or T cells into the DRG could also increase the sensitivity of pruriceptive neurons. The role of satellite glia in the observed pruritis of jedi-1 -/- mice requires further investigation.

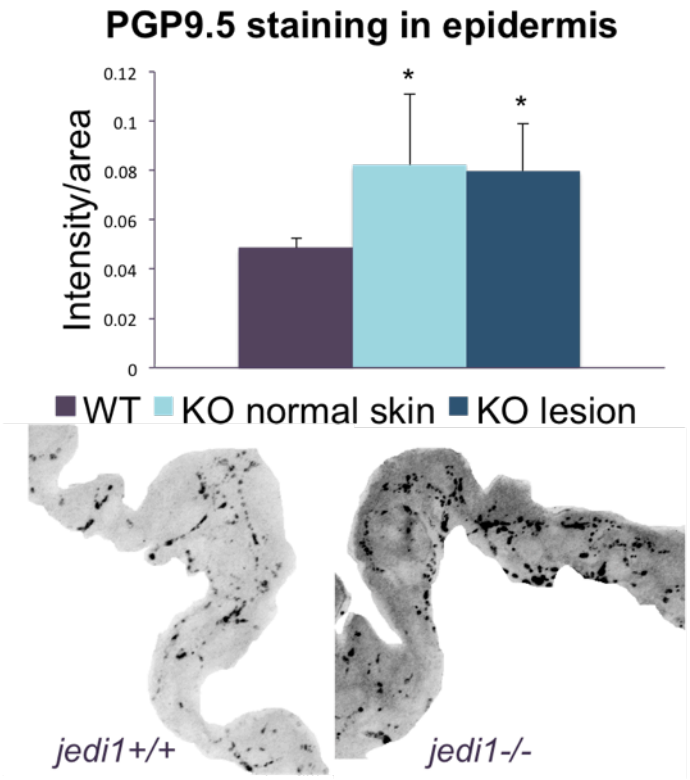


Figure 6.6. Increased innervation of jedi-1 -/- skin. Immunofluorescence staining for pan neuronal marker PGP9.5 was quantified in the epidermis of jedi-1 +/+ and -/- mice by fluorescence intensity. 3 animals analyzed per genotype, 3 non-consecutive sections per animal. p=3x10-4 and p=2.6x10-5 respectively. Innervation analysis and quantification was performed by Alexandra Trevisan.

Discussion

During development of the mouse dorsal root ganglia, approximately 50% of the neurons generated undergo apoptosis and must be recognized and cleared by phagocytes. Many autoimmune conditions, such as lupus and rheumatoid arthritis are associated with peripheral neuropathy (Hanly, 2007; Sofat et al., 2006). The mechanism by which the immune system begins to recognize "self" in these conditions is not well understood nor is it clear how neuropathy develops. In addition to the hallmark phenotypes of autoimmunity observed in our *jedi-1* -/- mice, we observed the prominent phenotype of chronic itch, or pruritis. We wanted to understand whether

the pruritis phenotype we observed was due to delayed apoptotic cell clearance in the skin, immune cell infiltration, or perhaps changes in glial cell activation and innervation of the skin.

Many autoimmune diseases have a skin lesion component. For example, lupus patients often develop a skin abnormality known as a "butterfly rash". These skin lesions are typically the result of immune cells being recruited to the skin, resulting in local inflammation (Werth, 2005). Several of the genetic mouse models lacking engulfment adapter proteins or receptors also exhibit skin lesions. For example, mice lacking MFG-E8, an adapter protein that links PS on dead cells to receptors on macrophages, develop a SLE-like phenotype. The mice exhibit a dermatitis phenotype due to enhanced CD8+ T cell responsiveness, and it was suggested that apoptotic cells in the null mice are not efficiently degraded in the lysosome but are instead presented as self-antigens in MHC I complexes to activate T cells in the skin (Hanayama et al., 2008). The autoimmune symptoms we detected (discussed in the previous chapter) suggested to us that the skin phenotype we observed in the *jedi-1* -/- mice may be due to an immune response in the skin. However, we did not detect any significant increase in B-cells, T-cells, macrophages or mast cells. In addition, we did not detect any increase in the number of apoptotic cells in the skin, indicating that the cell corpses that accumulate in the skin due to the normal anagen/catagen cycle are cleared normally. Together, these results suggest that there is no abnormal immune response in the skin of the *jedi-1*-/- mice that could account for the chronic itch.

Despite the many questions and controversy surrounding itch signaling, chronic pruritus or pain lead to physiologic changes that sensitize neurons to nociception or pruriception. Increasing evidence suggests that glial cells modulate synaptic plasticity during chronic pain (Watkins and Maier, 2003). In response to extensive stimulation of nociceptors, glia will become activated, resulting in changes in ion concentrations, morphology, proliferation, and gene expression (Ji et al., 2013). These activated glia can

also release factors that influence synaptic transmission by neurons and result in pain hypersensitivity (Scholz and Woolf, 2007).

While many treatments of chronic pain have attempted to target neurons, the newly studied influence of glia on nociception has identified them as a target for clinical intervention against hyperalgesia (Watkins and Maier, 2003). For example, each neuron soma in the DRG is enveloped by a group of interconnected SGCs which form a highly controlled microenvironment that is known to influence neuron firing (Hanani, 2005). Indeed, activated SGCs are known to play a role in gliopathic pain by regulating extracellular potassium levels through the Kir4.1 channel, releasing cytokines such as IL-1β, and regulating glutamate through the glutamate transporters GLAST and GLT-1 (Vit et al., 2008; Ohara et al., 2009; Takeda et al., 2007; and Takeda et al., 2008). While glial cells are known to contribute to chronic pain, the similarities in itch and pain circuitry suggest that perhaps hyperactive glia may sensitize neurons to itch and modulate pruritis.

We began to assess our mice for changes in glial cell activation both in *vivo* and *in vitro*. Neuronal environment regulation by glial cells has an influence on neuronal excitability, and this is important in the response to nerve injury. After nerve insult, SGCs respond similarly to activation of astrocytes by up regulating GFAP and proliferating. The SGCs also release several cytokines (e.g. IL-1β and TNF) as well as ATP, which can induce hyperexcitability in the neuron (Ohara et al., 2009). Interestingly, we saw increased expression of GFAP in SGCs of jedi-1 -/- adult animals in the early stages of skin lesion (balding) in comparison to a wild-type control. Under normal physiological conditions, SGCs do not express GFAP at robust levels in adult animals. The increased expression of GFAP in the jedi-1 -/- SGCs suggests that the glia were in a state of activation. Future studies should assess jedi-1 -/- animals with no apparent skin lesions as well as wild-type mice with lesions. To examine the activation of SGCs in vitro, we obtained data from co-cultures with or without NGF withdrawal. The GFAP level of jedi-1 -/- SGCs was higher than in jedi-1 +/+ co-cultures even in the absence of NGF withdrawal. This

suggests that even in conditions where little cell death occurs, the SGCs of jedi-1 -/- animals are primed for activation. We hypothesize that the activated SGCs could be stimulating activity of pruriceptive neurons and inducing itch.

The SGCs could become activated by the persistence of neuronal corpses and secrete factors that increase activation of pruriceptive neurons, similar to the ability of SGCs to enhance nociception (Ohara et al., 2009). On the other hand, infiltration of macrophages or T cells into the DRG could also increase the sensitivity of pruriceptive neurons. It is also possible that the itch phenotype of *jedi-1* -/- mice is due to a sensitization of itch related interneurons (GRP receptor positive neurons) in the CNS. Jedi-1 is expressed in microglia, and it is possible that defective engulfment in the CNS could induce microglial activation. This alternative hypothesis is interesting because microglia are known to influence synaptic transmission similarly to SGCs (Miyamato et al., 2013). The role of satellite glia in the observed pruritis of jedi-1 -/- mice requires further investigation, and our data suggests that the pruritis phenotype is rooted in a problem with glial activation and sensory innervation of the skin.

CHAPTER VII

CONCLUSIONS AND FUTURE DIRECTIONS

Recognition and engulfment of apoptotic cells during development is important to prevent progression to secondary necrosis and exposure of intracellular contents that can be immunogenic or toxic. Even though the consequences of defective removal of apoptotic cell corpses in the nervous system have not been studied in great detail, there is evidence that globally suppressing clearance of apoptotic cells leads to autoimmunity (Elliot and Ravichandran, 2010). In fact, macrophages with defects in phagocytosis have been linked to autoimmune disease in humans (Ren et al., 2003). One interesting facet of many autoimmune disorders is neurological symptoms; for example, lupus and rheumatoid arthritis are associated with peripheral neuropathy (Hanly, 2007; Sofat et al., 2006). The neurological symptoms associated with these autoimmune disorders suggest that, at least in some instances, the failure to clear apoptotic neurons or neuronal debris may contribute to the disease etiology.

The mechanisms of clearance of apoptotic cells in the peripheral nervous system are not well understood. The Carter lab recently showed that SGC precursors are the main phagocyte of apoptotic neurons during development in the DRG (Wu et al., 2009). Although the initial hypothesis was that macrophages would be the principal phagocyte of apoptotic neurons in the DRG, very few macrophages were engulfing apoptotic neurons during the time of developmental cell death. Further studies verified that SGC precursors act as amateur phagocytes for apoptotic neurons. The contribution of amateur phagocytes to engulfment in the nervous system has gained increased interest in recent years. Schwann cells in the PNS play a role in synapse pruning during development (Bishop et al., 2004), and they also promote neuronal debris clearance along with macrophages after injury (Fernandez-Valle et al., 1995). Although microglia

have been well-characterized as the professional phagocytes of the CNS, astrocytes can also act as amateur phagocytes to engulf damaged neurons (Magnus et al., 2002; Park et al., 2007). In addition to their role in the clearance of apoptotic neurons, astrocytes are also known to participate in synapse pruning in the mammalian CNS (Chung et al., 2013). The most novel amateur phagocytes in the CNS, neural precursor cells, engulf other neural precursor cells and regulate neurogenesis (Lu et al., 2012). Glia are the primary cell type responsible for engulfing neuronal cell corpses during development in *Drosophila* (Awasaki and Ito; 2004; Watts et al., 2004) and after injury (Aldskogius and Kozlova, 1998).

Jedi-1 and MEGF10 were identified as engulfment receptors required for engulfment of apoptotic cells by SGCs (Wu et al., 2009). The bulk of my dissertation research was trying to understand how Jedi-1 and MEGF10 signal to promote engulfment. The intracellular domains of Jedi-1 and MEGF10 share signaling motifs with the engulfment receptors CED-1 and Draper. I determined that SFKs phosphorylate the ITAM motifs of Jedi-1 and MEGF10, and this event is essential for engulfment mediated by Jedi-1 and MEGF10. This phosphorylation of ITAM motifs by SFKs promoted binding of Syk, a protein known to interact with proteins that are involved in phagocytosis (Scheib et al., 2012). Syk interacts with ITAM motifs on many phagocytic receptors (Mocsai et al., 2010) and signals to promote phagocytosis and actin rearrangement through activation of Vav (a Rac guanine nucleotide exchange factor) and phospholipase C (Mocsai et al., 2010; Tohyama and Yamamura, 2009). Although we have not explored signaling downstream of Syk in our system, it is possible that Syk interaction with Jedi-1 and MEGF10 promotes activation of Vav and PLC to influence actin rearrangement during engulfment.

Jedi-1 and MEGF10 each contain an NPXY motif, a common binding site for phosphotyrosine binding domain (PTB) containing proteins. Ced-1 and Draper also contain conserved NPXY motifs, and studies have shown that the NPXY motif is essential

for engulfment signaling by both receptors and is the site of interaction for the adapter protein Ced-6/GULP. GULP has been identified as an important player in engulfment downstream of multiple receptors, but the mechanism by which GULP promotes phagocytosis has been unclear. MEGF10 is known to interact with GULP (Hamon et al., 2006; Suzuki and Nakayama, 2007), and MEGF10 also interacts with the clathrin assembly protein complex 2 medium chain and beta actin (Suzuki and Nakayama, 2007). I have shown that GULP binds to the NPXY motif of Jedi-1 to promote recruitment of phosphorylated clathrin heavy chain and actin polymerization. This is the first instance of clathrin being implicated in engulfment of apoptotic cells in mammals. While my studies focused on the more novel engulfment receptor, Jedi-1, it would be interesting to see whether MEGF10 also signals through a clathrin-dependent pathway. It has also been recently shown that GULP regulates activation of Arf6, a GTPase known to regulate phagocytosis and membrane trafficking (Beemiller et al., 2006; Niedergang et al., 2003; Zhang et al., 1998). Arf6 is also known to promote recruitment of the clathrin adapter protein AP2 (Ma et al., 2007). Whether Arf6 is involved in engulfment downstream of Jedi-1 or MEGF10 is unknown at this time.

The focus of my work was on the signaling of Jedi-1, but the ligands this receptor recognizes on apoptotic neurons are unknown. Because CED-1 has been suggested to recognize the well known "eat me" signal phosphatidylserine (PS) (Venegas and Zhou, 2007), we hypothesize that Jedi-1 may recognize PS as well. Our findings show that Jedi-1 and MEGF10 promote binding of apoptotic CHO cells (Wu et al., 2009) as well as engulfment of carboxylated microspheres, which are thought to mimic the negative charge of PS. Unlike Ced-1, Draper does not depend on PS as a ligand (Manaka et al., 2004), but instead recognizes Pretaporter, a protein typically localized within the endoplasmic reticulum (Kuraishi et al., 2009). Future studies will attempt to identify the ligands of Jedi-1 that label apoptotic neurons.

Jedi-1 and MEGF10 mRNA has been detected in both neurons and glia (Wu et al., 2009), but at this time the function of Jedi-1 and MEGF10 in neurons is unclear. Recently, homotypic interactions of MEGF10 were found to be necessary for the patterning and spacing of subtypes of retinal interneurons (Kay et al., 2012). In this context, MEGF10 was acting as both a receptor and ligand to form a repulsion signal to prevent interneurons from being spaced too close together. The ECD of MEGF10 is large and contains many signaling motifs (EMI and multiple EGF-like repeats), thus it makes sense that it could act across extracellular space. Within the DRG, several SGCs form a tight envelope around each sensory neuron body, and it would be interesting to see whether Jedi-1 or MEGF10 play a role in this envelope formation. Possible homotypic interactions of Jedi-1 and MEGF10 may also be important for "zippering" the engulfment synapse between apoptotic cells and SGC precursors or creating the close extracellular space between SGCs and healthy neurons (Hanai, 2005).

Although we have made progress in understanding how Jedi-1 and MEGF10 promote engulfment of apoptotic neurons during development, we do not know whether these receptors play a role in clearance in adult animals. SGCs will proliferate and become stellate after sciatic nerve injury in rats when neurons undergo death (Fenzi et al., 2001; Gehrmann et al., 1991), but their engulfment capability has not been studied in injury conditions. It would be interesting to study the role of Jedi-1 and MEGF10 under injury conditions, and we could carry these studies out in our jedi-1 -/- mice. Expression of Draper is highly upregulated after injury in flies, so it would be reasonable to hypothesize that Jedi-1 or MEGF10 expression could also be regulated during injury response (MacDonald, 2013). Our studies have centered on SGC precursors in the DRG, but we have preliminary evidence that Jedi-1 and MEGF10 are expressed by Schwann cells. Schwann cells are known to engulf debris after nerve injury, so it would be interesting to study the role of Jedi-1 and MEGF10 in this clearance process.

Even though our studies have been centered in the PNS, both Jedi-1 and MEGF10 are expressed in whole brain and spinal cord (Hamon et al., 2006; Krivtsov et al., 2007; Wu et al., 2009). We also have data that Jedi-1 and MEGF10 are expressed in cultures of primary microglia from mice as well as in peritoneal macrophages. The fact that microglia, the primary phagocytes of the CNS, express Jedi-1 and MEGF10 suggest that they may be important for engulfment in the CNS as well. Future studies will assess whether microglia from jedi-1 -/- or megf10 -/- mice have diminished phagocytic capabilities and also examine whether there are clearance defects *in vivo* in the CNS. Another phagocytic cell type in the CNS, astrocytes, express MEGF10 (Cahoy et al., 2008), and neural precursor cells express both engulfment receptors. It remains to be seen whether Jedi-1 and MEGF10 are involved in engulfment of apoptotic cells during CNS development, as well as during adult brain neurogenesis, where neural precursor cells are responsible for engulfing apoptotic neurons (Lu et al., 2011).

Whether MEGF10 and Jedi-1 are involved in engulfment by professional phagocytes is unknown. The fly engulfment receptor, Draper, not only plays a role in amateur phagocytosis by glia but also promotes engulfment by macrophages (Awasaki et al., 2006; Manaka et al., 2004). Preliminary data from our lab shows that peritoneal macrophages express both Jedi-1 and MEGF10 transcripts. Interestingly, microsphere engulfment assays using peritoneal macrophages from jedi-1 +/+ or -/- mice showed no change in engulfment. This could be due to the fact that macrophages express a variety of engulfment receptors that can compensate for the lack of Jedi-1, or it is possible that Jedi-1 is not required for professional phagocyte engulfment. Future studies should examine whether macrophages from jedi-1 -/- mice are defective in engulfment of actual apoptotic cells. Dendritic cells, another well known phagocyte type, do not express Jedi-1 or MEGF10 mRNA. Future studies may shown a role for Jedi-1 and MEGF10 in engulfment by professional phagocytes, and it would also be interesting to study whether Jedi-1 and MEGF10 signal in combination with other well known engulfment receptors. For example, in astrocyte engulfment of synapses in the CNS,

MEGF10 cooperates with another engulfment receptor, c-Mer (Chung et al., 2013). Whether this type of cooperation with other engulfment receptors occurs in other cell types is unknown.

As detailed in Chapter I, there are a multitude of engulfment receptors involved in the recognition and internalization of apoptotic cells. Further studies should address the question of whether professional and amateur phagocytosis are mediated by different receptors or whether there is crossover between the two. Although new engulfment receptors have consistently been discovered throughout the years, the question of why so many exist despite their seemingly redundant functions remains a mystery. One hypothesis is that the process of engulfment is highly complex and requires each receptor to regulate some specific function in the process. As an example, some receptors are involved in tethering the apoptotic cell to the phagocyte. Others recognize eat-me signals or trigger signaling cascades to promote cytoskeletal and membrane rearrangement. We have noticed morphological differences in SGCs transfected with Jedi-1 or MEGF10 that indicate that the two receptors may regulate different parts of the engulfment process. SGCs overexpressing Jedi-1 exhibit an elongated morphology, which suggests that Jedi-1 activation may regulate cytoskeletal changes necessary for engulfment. MEGF10 transfected SGCs exhibit a noticeable increase in vacuoles, suggesting that MEGF10 may promote endocytosis of targets (Wu et al., 2009. Our results suggest that MEGF10 and Jedi-1 converge on a signaling pathway involving Syk, but it is possible that each receptor also activates other signaling cascades during engulfment.

The study of engulfment receptors is of great interest to the field of autoimmune disease research, which is hypothesized to occur when cell corpse clearance is defective. A large part of my dissertation research has been devoted to evaluating the jedi-1 -/- mice for signs of autoimmunity. Our findings suggest that the jedi-1 -/- mice exhibit many of the hallmark phenotypes of autoimmunity: elevated auto-antibodies,

glomerulonephritis, and skin lesions. We have also obtained MEGF10 mice which will be evaluated for autoimmune phenotypes in the future. Due to the possible redundant function of the two receptors, it would be interesting to generate double-knock out mice to study the phenotypes of defective engulfment.

One of the most studied autoimmune disorders, systemic lupus erythematosus (SLE), affects numerous parts of the body, including the peripheral nervous system in a subset of patients (Honczarenko et al., 2008). Interestingly, the jedi-1 -/- mice do exhibit skin phenotypes due to excessive scratching behavior (as detailed in Chapter VI). After assessing the skin for abnormal levels of immune cells or apoptotic cells, we determined that there seems to be no immunological cause behind the scratch phenotype at the level of the epidermis. Our preliminary data suggests that SGCs are abberantly activated as shown by increased GFAP expression *in vivo* and *in vitro*. While the link between glial cell activation and nociception has been well studied, the possible link between glial cell activation and pruritis has not been assessed. The jedi-1 -/- mice may be a new model of pruritus that could shed light on the connection between glial cell activation and chronic pruritis.

The etiology of SLE is very complex, and genetics play at least a partial role in development of the disease. Many genetic screens have attempted to identify SLE susceptibility genes, and several chromosomal regions have been identified, including chromosome 1q22-24 (Olson et al., 2002; Shai et al., 1999). The Jedi-1 gene is located within this identified region at 1q23.1, but it should be noted that the genes for FcγRs are also at 1q23. While several polymorphisms in the FcγRs have been definitively linked to SLE susceptibility (Pradhan et al., 2008), it would be interesting to screen the Jedi-1 gene for polymorphisms to determine whether it is an SLE susceptibility gene.

Although many questions remain about how Jedi-1 and MEGF10 signal to promote engulfment, my research has identified several signaling partners of both receptors. I

determined that SFKs promote engulfment by phosphorylating the ITAM motifs of Jedi-1 and MEGF10, and this step is required for binding of the kinase Syk and engulfment signaling (Wu et al., 2009). I also determined that the NPXY motif of Jedi-1 and the adapter protein GULP are required for engulfment downstream of Jedi-1, and this process involved a non-canonical role for clathrin as a scaffold for actin rearrangement. In addition to the characterization of Jedi-1 signaling, I also have made progress in defining the phenotype of the jedi-1 -/- mice. This may be a useful model of autoimmune diseases such as lupus, and the link between lack of jedi-1 and chronic pruritus could also be of great interest. It is my hope that my work can be built upon to provide a better understanding of engulfment and the consequences of defective engulfment *in vivo*.

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