NORTHWESTERN UNIVERSITY

The Modulation of MFG-E8 Mediated Microglial Phagocytosis

A DISSERTATION

SUBMITTED TO THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

for the degree

DOCTOR OF PHILOSOPHY

Field of Cell and Molecular Biology

By

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EVANSTON, ILLINOIS
December 2008

ABSTRACT

The Modulation of MFG-E8 Mediated Microglial Phagocytosis

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Phagocytosis is an essential mechanism for clearance of pathogens, dying cells, and other unwanted debris in order to maintain tissue health in the body. Macrophages execute this process in the peripheral immune system, but in the brain microglia act as resident macrophages to accomplish this function. In the peripheral immune system, macrophages secrete Milk Fat Globule Factor-E8 (MFG-E8) that recognizes phosphatidylserine expressed on the surface of apoptotic cells. MFG-E8 acts as a tether to join the apoptotic cell and the macrophage and trigger a signaling cascade that stimulates phagocyte development, allowing the macrophage to engulf the dying cell. When this process becomes disrupted, inflammation can result. MFG-E8 resides in the brain as well as in the periphery, and microglia express MFG-E8. However, the function of MFG-E8 in the brain has not been elucidated. We measured MFG-E8 production in the BV-2 microglial cell line and the role of this protein in the recognition and engulfment of apoptotic SY5Y neuroblastoma cells. BV-2 cells produced and released MFG-E8, which apoptotic SY5Y cells and the chemokine fractalkine further stimulated. Conversely, lipopolysaccharide (LPS) and proinflammatory cytokines reduced the production of MFG-E8 by BV-2 cells. Furthermore, MFG-E8 increased phagocytosis of apoptotic SY5Y cells, and a dominant negative form of MFG-E8 inhibited phagocytosis by BV-2 cells, while LPS reduced

the phagocytic activity of BV-2 cells. Finally, brain MFG-E8 levels were altered in a mouse model of the amyloid pathology found in Alzheimer's disease. We hypothesize that MFG-E8 acts in the brain via microglia to aid in clearance of apoptotic neurons. Our data provide a foundation for further exploration of the involvement of MFG-E8 in the proper clearance of apoptotic cells, debris, and pathogenic material by microglia and suggest that a dysregulation of this protein may be involved in neurodegenerative disease.

ACKNOWLEDGEMENTS

The past six years have been an amazing time of personal and scientific discovery. There are a few people that have made my time in graduate school a rewarding experience, and I would like to thank them for their support.

First, I want to thank my husband Shan for his unending patience, love, and positive energy. I am very lucky to have such an wonderful partner along our journey together.

I would like to thank my parents for their constant encouragement. They have instilled in me that I am capable of accomplishing anything I dream, and the confidence this gives me has gotten me to where I am today. Also, thanks to my brother Bryan for his loving support and for reminding me what the finish line might look like and my brother Brad for showing me the power of a competitive spirit.

I am extremely lucky to have made some incredible friends in Chicago. Thank you to James, Eric, Jen, and Yvonne for being there through tears, laughs, and cocktails. I would not have made it without you as my family.

I would like to give a shout out to the wine group: Derek and Ryan, Tom and Yvonne, James and Lauren, Kelly, and Shan. Good times, good wine, incredibly smart people. Here's to wine nights across the country for years to come.

I would like to thank my mentor Dr. Linda Van Eldik for generously allowing me the opportunity to work in her lab and for her encouragement for me to explore my own passions in science.

Thank you to my committee members, Dr. D. Martin Watterson, Dr. Lester (Skip) Binder, and Dr. Sarah Rice, for their positive support and encouragement throughout this process.

Finally, thank you to all of the people I have worked with in the Van Eldik lab over the years: Wendy Thompson, Jeff Craft, Cassie Stephens, Jen May, Sarah Medgyesi, Jeff Crawford, Lucia Almeida, and Zhong Xie. These brilliant people have provided daily support and friendship and have transformed my time here into a positive experience.

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Chapter 1

INTRODUCTION

1.1 Microglia in health and disease

Microglia are the resident immunocompetent and phagocytic cells in the central nervous system (CNS) (Kreutzberg 1996; Perry et al. 1985). Cajal first described these cells in 1913 as "the third element" of the brain, the first element being neurons and the second being astrocytes, and Rio-Hortega further detailed the definition of the third element to include both microglia and oligodendrocytes during the first formal studies of microglial cells (Cajal 1913; Rio-Hortega 1921). It is believed that during embryonic development monocytes from the bloodstream enter the brain where they differentiate into resident brain microglia. Microglia share many of the surface antigens expressed by macrophages (Barron 1995; Hickey and Kimura 1988; Ling et al. 1980; Perry et al. 1985).

In the last few decades, the role microglial cells play in the brain has been further elucidated, and we now understand the importance of these cells in maintaining brain health and homeostasis while in the "resting" state, as well as the importance of timely response to acute injury or chronic disease. In the healthy adult human brain, resting microglia are characterized by fine, ramified processes extending from a small cell body and minimal expression of surface antigens. Insult to the CNS initiates a swift activation of microglial cells, resulting in the transformation to phagocytes capable of releasing noxious oxygen radicals, proteases, and proinflammatory cytokines (Banati et al. 1993; Colton and Gilbert 1987; Hanisch et al. 2002). This activation of microglia implicates them in the pathogenesis of various neurological disorders including Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS), and acquired immune deficiency syndrome (AIDS) dementia complex (Bo et al. 1994; Dickson et al. 1991; Gelman 1993; McGeer et al. 1993; McGeer and McGeer 1995).

The term “microglial activation” does not describe only a proinflammatory response to injury. As the resident phagocytes in the brain, microglia can also be activated to engulf and safely remove dead or dying cells and debris in the CNS (Streit 2002). Furthermore, microglia are part of an intricate network of communication amongst cells in the brain as well as with the peripheral immune system. Microglia signal each other and neighboring astrocytes by secreting cytokines and prostaglandins that act to amplify and spread the signal throughout the brain (van Rossum and Hanisch 2004). Microglia activation also functions to recruit other microglia to the site of activation as well as to promote infiltration of peripheral immune cells. Microglia also handle the downregulation of the immune response by secreting anti-inflammatory cytokines and inducing apoptotis.

1.2 Signals for microglial activation

It is generally accepted that proteins and debris expelled from damaged cells in the brain as well as invading toxins and pathological proteins prompt microglial activation, thus initiating long-term modifications of gene expression and adaptation of the cellular phenotype (Hetier et al. 1988; Kreutzberg 1996; Rogers et al. 2002; Streit 2002). One of the first descriptions of microglial activation in the literature is of the response induced by the bacterial endotoxin lipopolysaccharide (Hetier et al. 1988). Microglial cells exhibit a strong response to LPS challenge characterized by the release of a plethora of cytokines, chemokines, nitric oxide (NO), and proteases. TLR4, a member of the toll-like receptor (TLR) family mediates this dramatic microglial response to LPS (Takeda et al. 2003). Microglia express most TLRs, allowing a response to nearly any type of bacterial or viral challenge, but the microglial activation through LPS/TLR4 is undoubtedly the most rigorously described response (Rivest 2003). Furthermore,

LPS triggers microglial proliferation, migration, NO release, and cytokine production, rendering it a popular choice for positive controls in *in vitro* and *in vivo* experiments (Garden and Moller 2006).

β-amyloid (Aβ), the protein responsible for the hallmark senile plaque formation found in AD, potently activates microglia as well (Rogers et al. 2002). Aβ treatment of microglial cells leads to microglial proliferation and cytokine release, but the microglial receptor responsible for these actions is still to be determined. While candidates have been suggested, such as scavenger receptors, formyl peptide receptor-like 1, and receptor for advanced glycation end products, the Aβ receptor is still elusive due in part to the fact that different forms of Aβ may interact with different receptors (Garden and Moller 2006; Husemann et al. 2002; Verdier et al. 2004). The controversy over what species of Aβ causes the pathology of AD is ongoing and is possibly mediated by multiple forms, but it is known that reactive microglia surround plaques in AD (Carpenter et al. 1993; McGeer et al. 1988; Perlmutter et al. 1992), and that in culture Aβ treated microglia increase production of IL-1β, IL-8, TNFα, and MIP-1α, to name a few (Kim and de Vellis 2005). Furthermore, evidence suggests that lowering this proinflammatory response is beneficial to the health of neurons and slows the progression of AD (McGeer and McGeer 1995).

1.3 Microglia as phagocytes

Microglia are the primary phagocytic cells in the CNS capable of phagocytosing microbes and presenting antigens to T cells trafficking in and out of the CNS to modulate the adaptive immune response. Microglia are also responsible for the clearance of pathological proteins such as Aβ (Rogers et al. 2002), apoptotic cells, and cellular debris. While it is not completely understood

how microglia recognize and engulf apoptotic cells, some of the key elements to the process have been described. Some examples are the expression of phosphatidylserine (PS) on the apoptotic cell surface, interactions of apoptotic cells with the microglia vitronectin receptor and the CD36 scavenger receptor, and the binding of TREM-2 (Stolzing and Grune 2004; Takahashi et al. 2005; Witting et al. 2000). Activation of TREM-2 promotes microglial phagocytosis and inhibits proinflammatory cytokine expression, and deficiency in this protein leads to decreased phagocytosis and the transformation of microglia into cytokine-producing cells. Interestingly, humans deficient in this protein develop a frontotemporal dementia in the third or fourth decade of life (Takahashi et al. 2005). This implies a need for microglia not only for phagocytosis but also to regulate the immune response associated with the clearance of apoptotic cells.

1.4 Antigen presentation by microglia

In order to create an adaptive immune response, antigen-presenting cells (APCs) must engulf microbes, infected cells, or other foreign material, process the antigen from these species in vacuoles, and present the processed antigen on the cell surface for T cells to recognize. In the peripheral immune system, dendritic cells are professional APCs and can be identified by the expression of major histocampatibility complex class II (MHC-II) and CD11c. When properly activated, a subset of brain microglia are capable of presenting antigen for recognition by T cells back and forth across the blood brain barrier (Aloisi et al. 2000). Both CNS infection and the experimental autoimmune encephalomyelitis murine model of multiple sclerosis (MS) stimulate the expression of cells capable of presenting antigen (Fischer and Reichmann 2001). This is also true in the cases of stroke, amyotrophic lateral sclerosis (ALS), and neoplasms (Henkel et al. 2004; Reichmann et al. 2002; Watters et al. 2005). Moreover, experiments in bone marrow

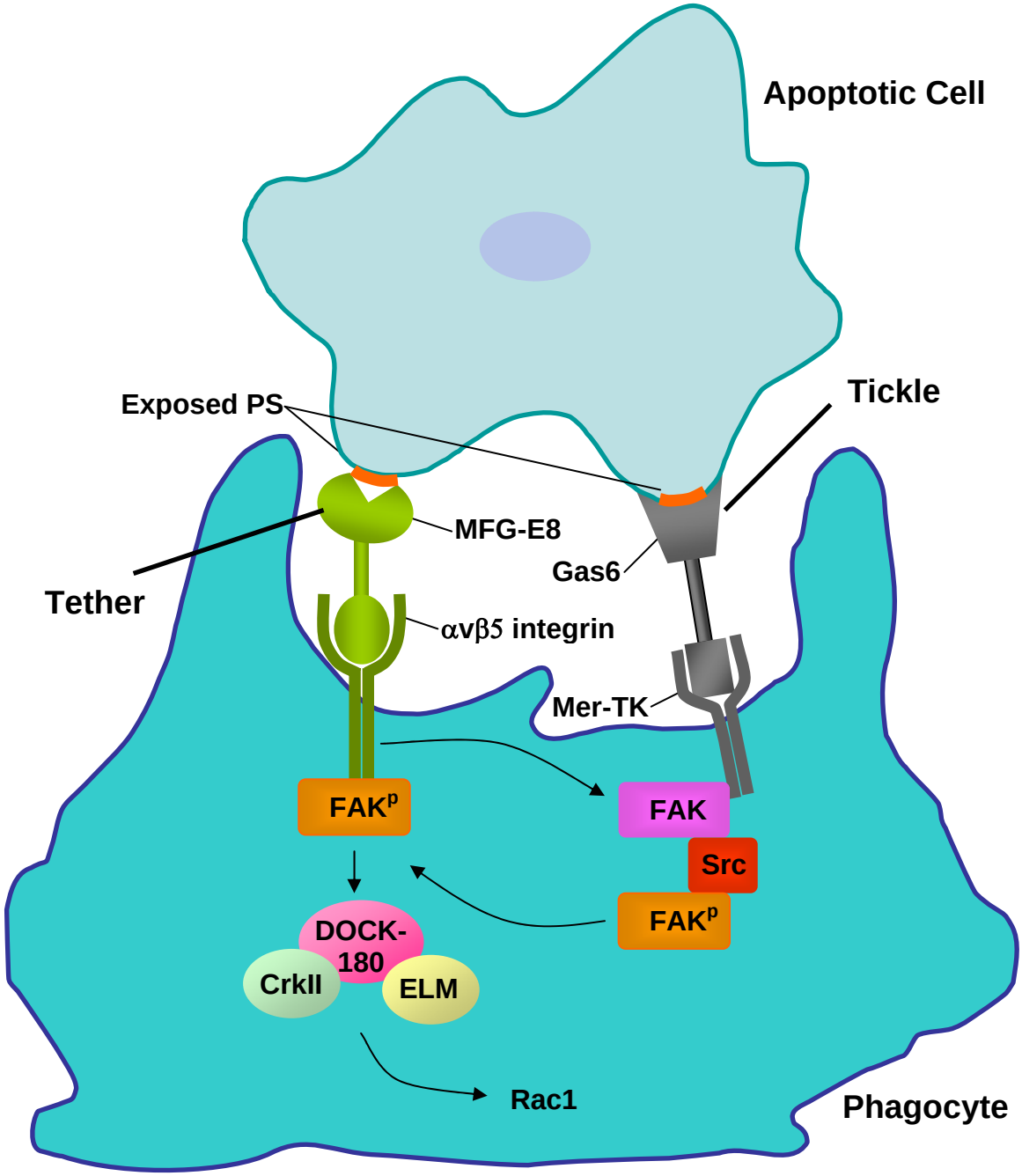
chimeric mice convincingly show that both peripheral blood and brain microglia function as APCs to regulate the neuroimmune response (Byram et al. 2004). These reports demonstrate that microglia are capable of behaving like APCs in a variety of neuropathological conditions.

1.5 Phagocytosis in the peripheral immune system

Apoptotic cells and potentially toxic materials are the result of diseases and normal aging processes (Mochizuki et al. 1996; Su et al. 1994; Thomas et al. 1995; Zhang et al. 1995), and the efficient removal of apoptotic material is of utmost importance for protecting the surrounding tissue from damage due to released proteins from dying cells (Savill and Fadok 2000). Therefore, it is critical that immune cells are capable of recognizing apoptotic cells so that they may be efficiently removed from the tissue. Phagocytes express specific receptors to recognize so called “eat me” signals on the surface of apoptotic cells. This is accomplished either directly by apoptotic cell-phagocyte interactions or indirectly through serum opsonizing proteins that link apoptotic cells to phagocytes for removal (Fadok et al. 2000; Henson et al. 2001). During apoptotic cell death, a redistribution of the plasma membrane occurs which exposes the normally inward facing phospholipid PS on the surface of the dying cell, which in turn acts as a cue for phagocyte recognition. However, PS is constitutively expressed on the surface of many cell types that are not undergoing apoptotic cell death. Therefore, a mechanism must exist for phagocytes to differentiate between PS on the surface of healthy and dying cells. One possibility is that fatty acyl oxidation of PS transpires prior to externalization and recognition as an apoptotic signal (Jiang et al. 2004; Tyurina et al. 2004). This model links apoptotic signaling and phagocytosis so that the proper recognition occurs.

The mechanics of PS-triggered phagocytosis have been studied in some detail in the peripheral immune system, where phagocytes recognize the exposed PS on apoptotic cells either directly through a PS receptor (Fadok et al. 2000) or through opsonized bridging proteins such as milk-fat globule EGF factor-8 (MFG-E8) (Akakura et al.; Hanayama et al. 2002) and Growth Arrest Specific Gene-6 (Gas-6) (Chen et al. 1997; Hall et al. 2005). MFG-E8 and Gas6 are the major players in a mechanism termed "tether and tickle" (Hoffmann et al. 2001). MFG-E8, shown to be released by macrophages, recognizes and binds PS on apoptotic cells and tethers the two cells by binding an integrin receptor ($\alpha_v\beta_3$ or $\alpha_v\beta_5$) on the macrophage (Hanayama et al. 2002). This triggers a conformational change in the integrin receptor that signals the recruitment of the CrkII-DOCK180-Rac1 complex and thus the activation of Rac1 (Akakura et al. 2004; Albert et al. 2000). Gas6 binds the receptor tyrosine kinase (RTK) Mer, and molecular crosstalk between the integrin receptor and the RTK creates a synergy between MFG-E8 and Gas6 by using post-translational modifications of the integrins and RTKs by phosphorylation to coordinate a complex intracellular response and the recruitment of signal proteins. The result is cytoskeletal reorganization in the macrophage and its transformation into a phagocyte capable of removing the apoptotic cell, as is diagrammed in Figure 1.1. This engulfment not only sequesters potentially harmful cellular products from surrounding tissue, it suppresses proinflammatory cytokines to create an anti-inflammatory environment to further protect the tissue (Fadok et al. 1998). However, when interference with this tethering occurs, such as insufficient production of MFG-E8, a proinflammatory state is created, evident in MFG-E8$^{-/-}$ mice that show the symptoms of an autoimmune disorder resembling systemic lupus erythematosus (SLE) (Hanayama et al. 2004).

Figure 1.1 Molecular crosstalk between RTK and integrin during PS-mediated phagocytosis. PS exposed on the surface of apoptotic cells is recognized by the opsonins MFG-E8 and Gas6. MFG-E8 then reacts with the $\alpha_v\beta_5$ or $\alpha_v\beta_3$ receptor and Gas6 binds the RTK Mer to trigger an intracellular signaling cascade. Activation of the integrin causes a phophorylation of FAK on Tyr397, which activates Mer. Once Mer is activated, a Src-dependent phosphorylation of FAK on Tyr861 occurs, causing FAK to associate with the β_5 integrin. The close association and crosstalk of this mechanism may be useful in the amplification of intracellular signals in the phagocyte to aid in efficient phagocytosis of apoptotic cells. Diagram is adapted from Wu and colleagues (Wu et al. 2006).


Apoptotic Cell
Exposed PS
Tickle
MFG-E8
Gas6
Tether
αvβ5 integrin
Mer-TK
FAK^p
FAK
Src
FAK^p
DOCK-180
CrkII
ELM
Rac1
Phagocyte

While a significant amount of work supports this mechanism in the peripheral immune system, no studies have been done to examine a role for MFG-E8 in the process of apoptotic cell phagocytosis in the brain. Microglia, which are similar to macrophages in the periphery of the body, act as the phagocytic cells in the brain to remove harmful material and dying cells (Garden and Moller 2006; Stolzing and Grune 2004; Witting et al. 2000), but little is known about MFG-E8 and microglia function. MFG-E8 is expressed in the brain (Boddaert et al. 2007; Hanayama et al. 2004), and one report indicates fractalkine (CX3CL1) treatment of cultured microglia increases MFG-E8 mRNA levels (Leonardi-Essmann et al. 2005). Fractalkine is a 95 kDa chemokine expressed by neurons throughout the brain in both membrane-bound and soluble forms (Harrison et al. 1998). The membrane-bound protein is cleaved to its soluble form following cell stress or injury, which then acts as a chemoattractant for T-cells, monocytes, and potentially microglia (Bazan et al. 1997). This is supported by the observation that microglia are the only cells in the brain that express CX3CR1, the fractalkine receptor (Harrison et al. 1998). Studies report abnormal levels of fractalkine in neurodegenerative diseases such as Alzheimer's disease (AD) and AIDS associated dementia (Erichsen et al. 2003; Kim et al. 2008), therefore suggesting a potential role for MFG-E8 regulation in the progress of these diseases. However, the function of MFG-E8 in the brain is currently unknown.

1.6 Hypothesis

In the present study, we tested the hypothesis that MFG-E8 is produced by microglia and regulates the ability of microglia to phagocytose apoptotic neurons. We report here that microglial production of MFG-E8 can be increased by fractalkine as well as apoptotic neurons. MFG-E8 production is also downregulated by LPS and proinflammatory cytokines. Second, we

studied the phagocytosis of apoptotic neurons by microglia in the presence of MFG-E8 or a dominant negative form of the protein and demonstrated that MFG-E8 regulates the phagocytosis in this system. Third, stimulation of BV-2 cells with LPS reduced the phagocytic ability of BV-2 cells. Finally, we report reduced levels of MFG-E8 protein in the brains of Tg2576 mice, a mouse model of Alzheimer's disease (AD) overexpressing $APP_{(swe)}$, as compared to wild-type littermates. Our findings suggest a role for MFG-E8-mediated clearance of apoptotic cells in the brain by microglia, and MFG-E8 production is downregulated by proinflammatory stimuli. This suggests that the downregulation in MFG-E8 production in an animal model of the Aβ pathology seen in AD is a result of the neuroinflammatory state seen in this disease and could perpetuate the neuroinflammatory cycle due to improper clearance of apoptotic material. Further studies could reveal dysregulation of MFG-E8 in other neurological diseases, and this protein is therefore a potential drug target or biomarker for such diseases.

Chapter 2

Materials and Methods

2.1 Materials

Recombinant mouse fractalkine, recombinant mouse MFG-E8, and recombinant mouse Gas6 (R&D Systems, Minneapolis, MN) were resuspended in sterile phosphate-buffered saline (PBS) at 10 μg/ml and used at the concentrations indicated. LPS from *Salmonella typhimurium* (Sigma, St. Louis, MO) was resuspended in

PBS at 100 μg/ml, and was used at a final concentration of 100 ng/ml. Recombinant mouse interferon-γ (IFN-γ) and recombinant mouse tumor necrosis factor-α (TNFα) were from R&D Systems, and were used at a final concentration of 25 ng/ml. Bovine serum albumin (BSA), fraction V (Sigma), was reconstituted at 1 mg/ml in sterile PBS and used at a final concentration of 4 μg/ml.

2.2 Cell culture

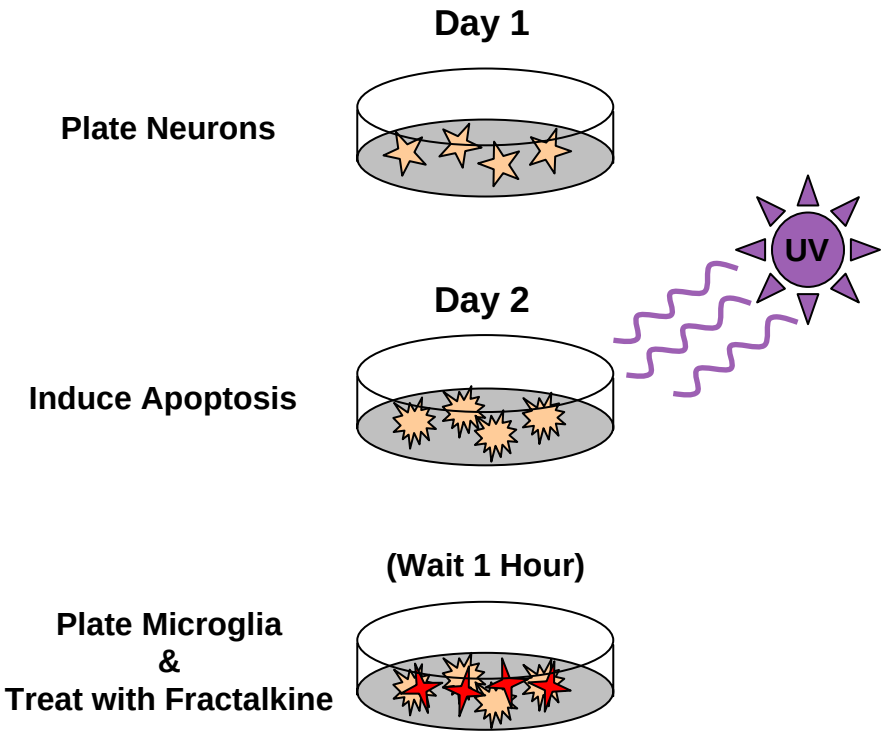
BV-2 cells, a murine microglial cell line, were originally derived by Dr. Elisabetta Blasi (Blasi et al. 1990) and cultured as previously described (Petrova et al. 1999). SH-SY5Y cells, a human neuroblastoma cell line, were obtained from the American Type Culture Collection (ATCC; Manassas, KS). Both cell lines were maintained in DMEM/F-12 media containing 10% fetal bovine serum (FBS; HyClone, Logan, UT), 2 mM glutamine, and antibiotics [100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA)] and incubated at 37˚C with 5% carbon dioxide.

2.3 Co-culture and cell treatments

For dose curve and time course experiments, cells were trypsinized (0.25% trypsin, 0.53 mM EDTA for 5 min at 37˚C) and seeded at a density of 2.5×10^5 cells/well (BV-2) or 1×10^5

cells/well (SY5Y) in 12-well tissue culture plates. For co-culture experiments, SY5Y cells were cultured for one day in advance of the experiment. After 24 hours, BV-2 cells were cultured with serum in the same wells as SY5Y cells so that the cells were able to share the same culture media and engage in direct cell-cell contact. Cells were treated with fractalkine or vehicle at the time of co-culture. In the case of UV treated cultures, SY5Y cells were exposed to 50 J/m^2 of UV light for 20 sec with a Stratalinker 1200 UV crosslinker 1 hour prior to co-culture. At the indicated time points, conditioned media was collected, centrifuged at 3,000xg for 3 minutes at 4˚C, and supernatants collected. Cells were washed one time with PBS then 100 μl of lysis buffer (20 mM Tris pH=8, 2 mM EDTA, 0.5% Triton X-100, 1 mM PMSF, 1 μg/ml leupeptin, 2 mM sodium orthovanadate) was added. Cell lysates were sonicated for 8 seconds on ice, centrifuged at 10,000xg for 10 minutes at 4˚C, and supernatants analyzed by Western blotting. See Figure 2.1 for a diagram of these experiments.

Figure 2.1 Diagram of glial-neuronal co-culture. On day 1, SY5Y cells are plated at a density of $2.5x10^5$ cells/ml in a 12 well tissue culture plate. After 24 hours, SY5Y cells are treated with 20 seconds of UV light to induce apoptosis and allowed to rest for 1 hour. BV-2 cells are then plated at a density of $1x10^5$ cells/ml directly on top of the SY5Y cells, and cell lysates and conditioned media are collected at the times indicated in the data.


Day 1
Plate Neurons
UV
Day 2
Induce Apoptosis
(Wait 1 Hour)
Plate Microglia
&
Treat with Fractalkine

2.4 Cell viability assays

The combination of two cell viability assays allowed the determination of a point when cells were dying but still maintained membrane integrity. Therefore, apoptotic cells would be expressing the appropriate cell surface cues (e.g., PS) for phagocytosis but not undergoing necrosis or cell lysis. The number of viable cells with and without UV radiation was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI). This assay, known as the MTS assay, utilizes the chemical reduction of a tetrazolium substrate by the NADPH or NADH present in living cells. Therefore, a higher number of living cells in a sample results in more formation of a color product at 490 nm. The assay was performed according to manufacturer instructions. To assess the effect of UV radiation on the membrane integrity of SY5Y cells, a LDH assay was performed on the conditioned media from control and UV exposed cells according to manufacturer instructions (Sigma). The assay measures released LDH by its ability to convert NAD to NADH, which in turn reacts with a tetrazolium dye substrate. The optical densities (OD) of the colored product were measured at 490 nm.

2.5 Western blotting

Western blotting was carried out as previously described (Petrova et al., 1999). Briefly, cell lysates (1:2) and media (1:3) were prepared in sodium dodecyl sulfate (SDS) containing sample buffer, and equal volumes of both cell lysates and media were separated by 10-14.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad, Hercules, CA). Proteins were transferred to Immobilon-P membranes (Millipore, Billerica, MA), and membranes were probed with rabbit polyclonal anti-mouse MFG-E8 primary antibody (0.2 μg/ml, R&D Systems) or mouse monoclonal anti-β-actin (1:50,000 dilution, Sigma) overnight at 4˚C. Membranes were

then probed with goat anti-rabbit (1:5000) or goat anti-mouse (1:2000) horseradish peroxidase conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Protein bands were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Images of blots were acquired for quantification using Kodak 440 digital imager and analyzed with Kodak Molecular Imaging software (Kodak, New Haven, CT). To quantify Western blot data, bands were normalized by dividing density values of each band by the corresponding density values of β-actin bands, and values were expressed as relative intensity. A background correction was performed by the software by subtracting the intensity of background one pixel width around each band.

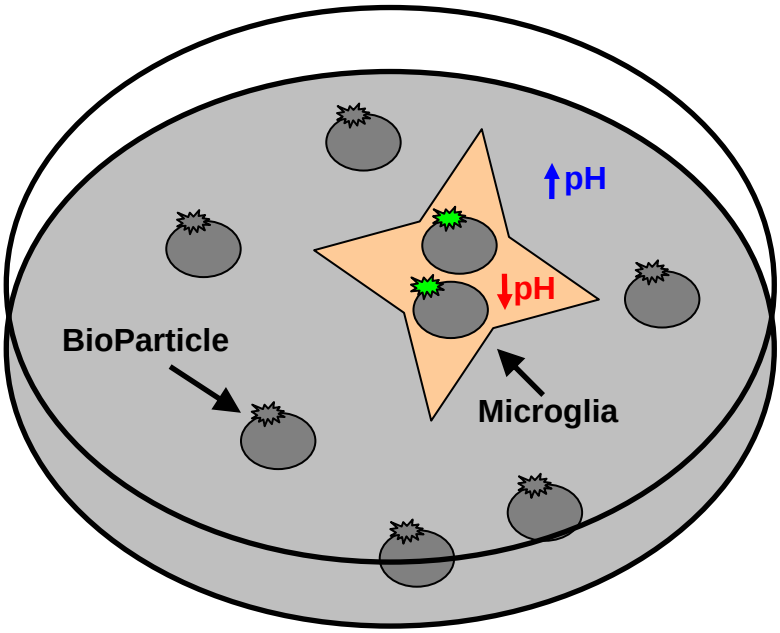
2.6 Transfection of D89E

The plasmid for the dominant negative form of MFG-E8 (D89E) was a generous gift from Dr. Shigekazu Nagata (Osaka University Medical School, Osaka, Japan), and the Flag-tagged D89E was expressed in human 293T cells using FuGene 6 transfection reagent (Roche Diagnostics Co, Indianapolis, IN). After 48 hours, conditioned media containing the secreted D89E was collected from the cells and purified by column chromatography using anti-Flag M2 affinity gel (Sigma) as recommended by the manufacturer. To determine the purity of the protein, samples were subjected to SDS-PAGE and Western blotting. For SDS-PAGE, proteins were separated by electrophoresis on a 10.5-14% polyacrylamide gradient gel and stained with Coomassie blue. For Western blotting, recombinant MFG-E8 and D89E proteins were separated by electrophoresis, transferred to PDVF membrane, and probed with anti-MFG-E8 antibody as described in section 2.5.

2.7 pHrodo phagocytosis assay

Phagocytosis by BV-2 cells was assayed using the pHrodo *E. coli* BioParticles Conjugate assay (Molecular Probes, Eugene, OR, cat# P35361). In this assay, dead *E. coli* labeled with a pH-sensitive dye, termed BioParticles, become fluorescent after they are engulfed by phagocytes, due to the acidic environment of endosome-like vacuoles formed during phagocytosis. The advantage of using this assay is the ability to distinguish engulfed (fluorescent) particles from those merely attached to the outer cell surface. This allows the certainty of accurate measurement of phagocytosis. The assay is conducted in a 96-well tissue culture plate, and the amount of fluorescence is quantified on a fluorescent plate reader set at 550 nm excitation and 600 nm emission. The pHrodo assay was conducted according to manufacturer instructions. Briefly, BV-2 cells were seeded at 5×10^5 cells/well in black 96-well tissue culture plates for 24 hours prior to the assay. To prep BioParticles, 2.5 ml of Hank's buffered saline solution with an additional 20 mM HEPES (Invitrogen, Carlsbad, CA) at a pH of 6.8 was used for reconstitution. BioParticles were sonicated in a bath sonicator at 30% intensity 3 times for 20 seconds each. BioParticles were examined by light microscopy on a hemocytometer to ensure dispersion. Recombinant mouse MFG-E8 (100 ng/ml), D89E (4 μg/ml), or BSA (negative control, 4 μg/ml) was added to BioParticles and vortexed briefly. Media was then removed from the cells and BioParticles were added along with treatments. Each condition was composed of five replicates, and a negative control of wells containing no cells was used to calculate background fluorescence and was subtracted from other wells. The positive control was BV-2 cells treated with BioParticles alone. Plates were read on a SpectraMax 250 (Molecular Dynamics,) spectrophotometer, excitation 550 nm and emission 600 nm. Data shown are representative of 4 separate experiments. See Figure 2.2 for a diagram of these experiments.

Figure 2.2 Diagram of pHrodo phagocytosis assay. To determine the effect of MFG-E8 and D89E on the ability of BV-2 cells carry out phagocytosis, BV-2 cells are first plated at a density of 10^6 cells/ml for 24 hours in a 96-well black tissue culture plate. The pHrodo BioParticles are prepared for use by sonicating for 30 seconds in HBSS, combined with MFG-E8 or D89E, and then added to the culture of BV-2 cells. As BV-2 cells take up the BioParticles, the fluorescein tag on the BioParticles is activated by the comparatively lower pH to become fluorescent, allowing only phagocytosed BioParticles to be measured 3 hours later on a fluorescent plate reader.


pH
pH
BioParticle
Microglia

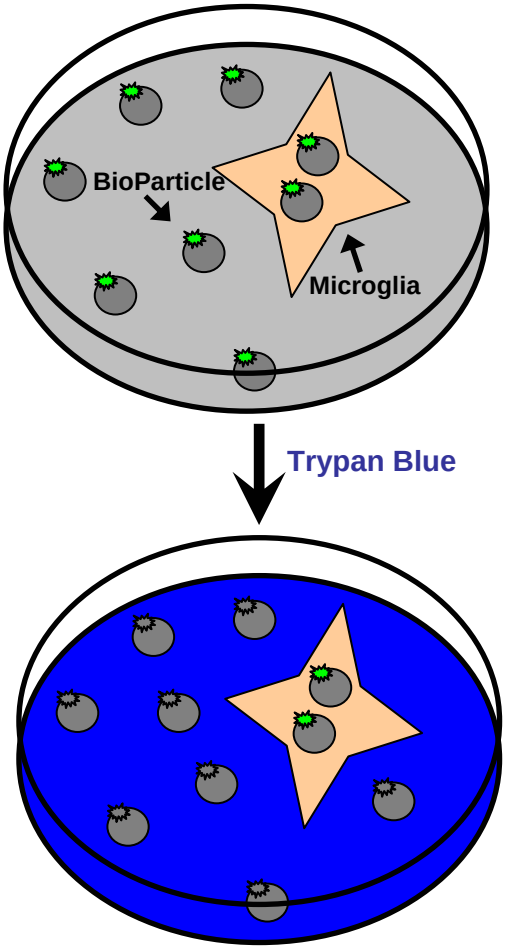
2.8 Vybrant phagocytosis assay

Where indicated, the Vybrant™ phagocytosis assay kit (Molecular Probes, cat# V6694) was used in place of the pHrodo assay, and the kit was executed according to manufacturer instructions. In this assay, fluorescein-labeled dead *E. coli*, or BioParticles, are incubated with phagocytic cells, and after phagocytosis occurs the assay is stopped with trypan blue, which quenches the fluorescein on BioParticles not engulfed by phagocytes. Fluorescence is quantified by reading the 96-well plate on a spectrophotometer, excitation 494 nm and emission 518 nm. BV-2 cells were seeded at 5×10^5 cells/well in black 96-well tissue culture plates for 24 hours, treated with LPS for 24 hours, and finally the assay was completed on day 3. To prep BioParticles, 2 ml of Hanks Buffered Sterile Saline (HBSS, provided in kit) was added to the lyophilized BioParticles and the solution was sonicated in a water-bath sonicator for 30 seconds. Media was removed from cultured cells and BioParticles were added to the cells along with LPS. After 2 hours, fluorescein labels were quenched with trypan blue for 60 seconds, and the plate was immediately read on a SpectraMax 250 spectrophotometer, excitation 494 nm and emission 518 nm. Data shown are representative of 4 separate experiments. See Figure 2.3 for a diagram of these experiments.

The use of Trypan blue to quench the fluorescein tag on BioParticles that have not been engulfed in this assay poses a few challenges to reproducibility and accuracy. First, the quench step is only 1 minute, and the plate takes 2-3 minutes to read. While the Trypan blue is removed from the assay, residual Trypan blue remains and can continue to act on cells. This also causes a second problem. Eventually, the Trypan blue will penetrate the cell and quench the engulfed particles as well, leading to inaccurate measurements. This can result in high readings at the

beginning of the plate and lower readings in the last wells. This assay was utilized in a few experiments before the pHrodo was made available by Molecular Probes. The data presented here with the Vybrant assay were rigorously controlled by working in small batches and including control lanes at the beginning and end of the assay plate. The Vybrant and pHrodo assays measure the same endpoint, so for time and cost considerations, we continued with the pHrodo assay.

Figure 2.3 Diagram of Vybrant phagocytosis assay. The Vybrant phagocytosis assay uses the same experimental design as the pHrodo assay, but the dynamics of the dye are notably different. In the Vybrant assay, the fluorescein labeled BioParticles are constitutively fluorescent, but after phagocytosis is allowed to occur for 3 hours, cells are treated with Trypan blue to quench any fluorescent BioParticles not phagocytosed by the BV-2 cells. Fluorescent BioParticles protected by the plasma membrane of the BV-2 cells are then quantified with a fluorescent plate reader.


BioParticle
Microglia
Trypan Blue

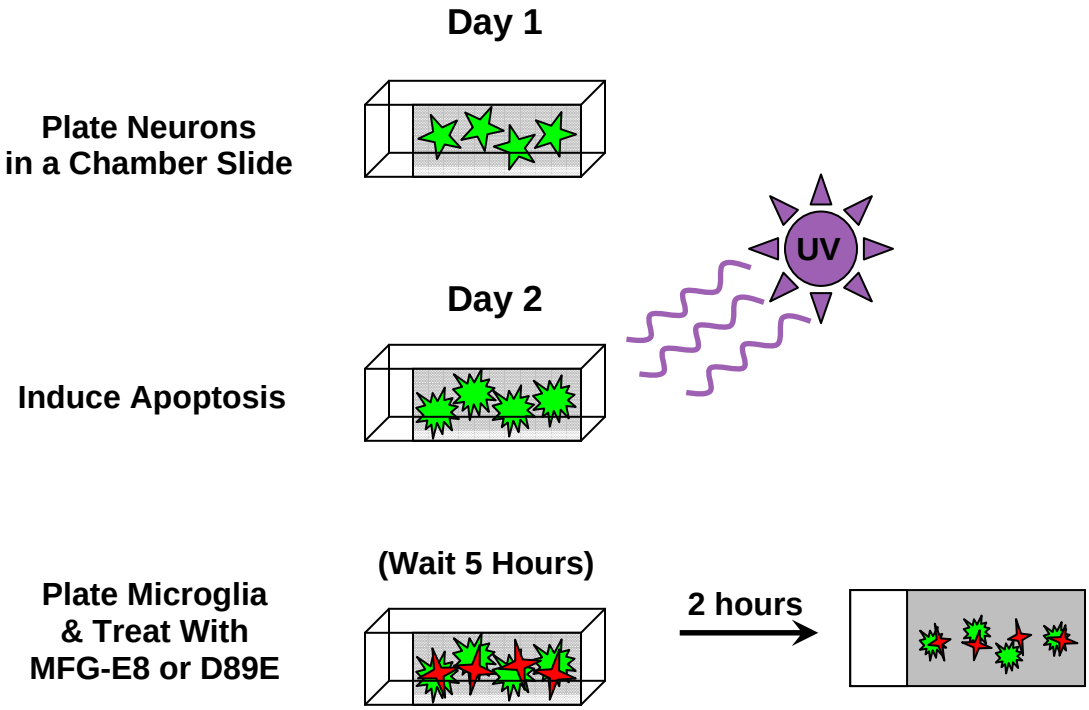
2.9 Imaging

SY5Y cells were subcultured for 3 days until cells were approximately 70-80% confluent. Media was then replaced with fresh complete media containing 7.5 μM CellTracker Green CMFDA (5-chloromethylfluorescein diacetate, Molecular Probes) made from a 10 mM stock in DMSO. After 30 minutes of incubation at 37˚C and 5% CO_2, media was exchanged for normal media for an additional 30 minutes. SY5Y cells were then plated in 8 well chamber slides (BD Transduction Labs) at a density of 5×10^4 cells/ml and allowed to incubate for 24 hours. Six hours prior to co-culture, SY5Y cells were exposed to 50 J/m^2 of UV light for 20 sec as described above when indicated and returned to incubation. One hour prior to co-culture, media on 70-80% confluent BV-2 cells was replaced with fresh complete media containing 4 μM CellTracker Red CMTPX (Molecular Probes) made from a 10 mM stock in DMSO for 30 minutes followed by normal fresh media for another 30 minutes. BV-2 cells were then seeded on top of SY5Y cells at a density of 2.5×10^4 cells/ml and treated with MFG-E8 or D89E where indicated. After 2 hours, cells were washed one time with sterile PBS, fixed using 4% paraformaldehyde in sterile PBS, washed once more with sterile PBS, and mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA). See Figure 2.4 for a diagram of these experiments.

To analyze the amount of phagocytosis that occurred in this co-culture, 4 images per condition were randomly captured and then analyzed in MetaMorph to determine the percent colocalization of red and green labels. This was repeated 3 times and data were pooled. Significance was measured with a Student's *t*-test. Briefly, the red and green channels of images were opened in MetaMorph, and inclusive thresholds of each image were set using the controls

in the software. This step allows for elimination of noise and for sharpening the measurement on the fluorescent cells. The program was then instructed to measure the colocalization area of the two fluorophores (red and green), and the area of overlap of the two images was presented as a percentage. Because the background was extremely low in these images and threshold levels were easily distinguishable, no background subtraction was made.

Figure 2.4 Diagram of glial-neuronal co-culture for imaging. To study the ability of BV-2 cells to phagocytose apoptotic SY5Y cells as well as the effect of MFG-E8 and D89E on this process, a co-culture system is utilized. To establish the co-culture, SY5Y cells are incubated with CellTracker Green, a dye that is taken up by viable cells, and then plated at a density of 5 x 10^4 cells/ml in an 8-well chamber slide for 24 hours. On day 2, SY5Y cells are treated with UV to induce apoptosis. Also, BV-2 cells are incubated with CellTracker Red and then plated on top of SY5Y cells at a density of 2.5 x 10^4 cells/ml. At the time of co-culture, cells are treated with MFG-E8 or D89E as indicated. After 2 hours, cells were washed one time with sterile PBS, fixed using 4% paraformaldehyde in sterile PBS, washed again with PBS, and mounted with Vectashield mounting medium containing DAPI. Cells are visualized on a fluorescent microscope.


Day 1
Plate Neurons
in a Chamber Slide
UV
Day 2
Induce Apoptosis
(Wait 5 Hours)
Plate Microglia
& Treat With
MFG-E8 or D89E
2 hours

2.10 Mouse brain tissue preparation

Hemibrain homogenates from Tg2576 mice (Hsiao et al. 1996) and age-matched wild-type littermates were a generous gift from Dr. Robert Vassar (Northwestern University, Chicago, IL), and samples were prepped as previously described (Oakley et al. 2006). Briefly, hemibrains were homogenized by douncing ten times in four volumes of PBS containing protease inhibitor cocktail (Calbiochem, La Jolla, CA) and then sonicated for ten seconds. Protein concentrations were determined using a BCA kit (Pierce), and 10–20 μg of protein analyzed by Western blot as outlined above.

2.11 Statistical analysis

Data were analyzed using a two-tailed Student's *t*-test for paired data or using one-way ANOVA followed by Tukey's post hoc tests of multiple comparisons to determine significant differences between groups. Statistical significance was established when $P<0.05$.

Chapter 3

MFG-E8 Production by Microglia

3.1 Overview

In the peripheral immune system, macrophages produce and secrete MFG-E8 which functions to remove apoptotic cells. Although data suggests MFG-E8 resides in the brain as well and that microglia express MFG-E8 mRNA, it is unknown if microglia secrete MFG-E8 or how MFG-E8 protein production is regulated in the brain. We report here that the BV-2 microglial cell line produced MFG-E8, allowing us to use this as a model system for the regulation of MFG-E8. We confirmed that fractalkine, shown previously to increase mRNA expression of MFG-E8 in primary microglia, upregulated the protein production and release of MFG-E8 in BV-2 cells. In addition, co-culturing an apoptotic SY5Y neuronal cell line with BV-2 cells also increased MFG-E8 levels. Conversely, treatment of BV-2 cells with the bacterial endotoxin LPS suppressed MFG-E8 production, as did tumor necrosis factor (TNF)-α and interferon (IFN)γ, proinflammatory cytokines known to stimulate microglia. These data suggest a similar regulatory mechanism for the production of MFG-E8 in the brain by microglia as is seen in the peripheral immune system by macrophages. Furthermore, the results suggest a neuroinflammatory environment could lead to lower than normal levels of MFG-E8 in the brain.

These results (Figures 3.1 and 3.3 in press in Fuller and Van Eldik, J. Neuroimmune Pharmacol. 2008) provided the first known data to suggest microglia secrete MFG-E8, and this production is mediated by fractalkine and apoptotic cells.

3.2 A microglial cell line produces MFG-E8 that is stimulated by fractalkine

In these studies, we utilized the BV-2 microglial cell line as a model system to study the production of MFG-E8 by microglia. Based on a previous report (Leonardi-Essmann et al. 2005) that MFG-E8 mRNA levels increased in microglial cells following treatment with the chemokine fractalkine, we investigated whether the levels of MFG-E8 protein in BV-2 cell lysates and conditioned media changed following treatment with 100 ng/ml fractalkine. As shown in Figure 3.1, Western blots of BV-2 cell lysates and media revealed MFG-E8 production and release by these cells. Furthermore, treatment of BV-2 cells with fractalkine led to an upregulation of the production and release of MFG-E8 above control levels, as is seen in both cell lysates and conditioned media after 24 and 48 hours (Fig. 3.1 A-C). Fractalkine also induced a concentration-dependent increase in MFG-E8 secretion by BV-2 cells (Fig. 3.2).

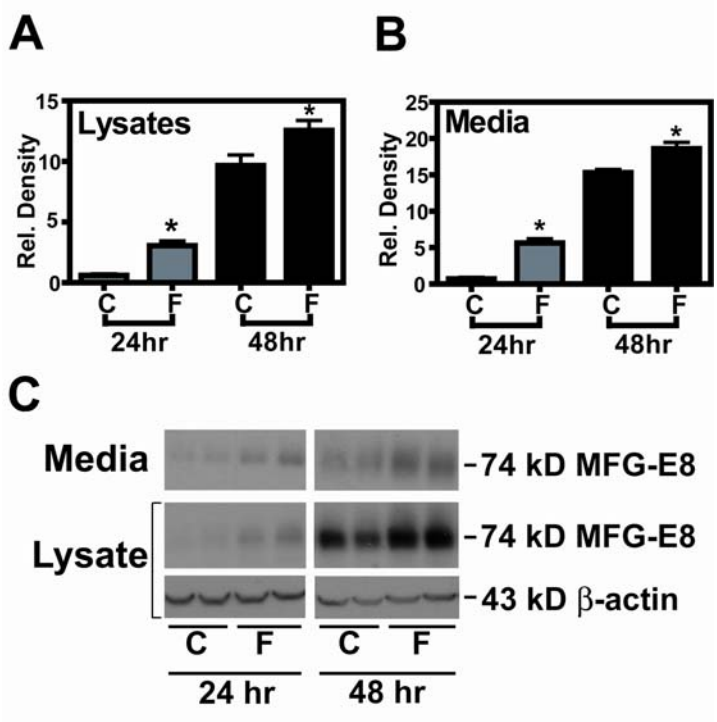


Figure 3.1 MFG-E8 production by microglia is upregulated by fractalkine. BV-2 microglia cells were treated with vehicle (C) or 100 ng/ml fractalkine (F) for 24 or 48 hours before lysates (A) and conditioned media (B) were collected and analyzed for the presence of MFG-E8 by Western blot (C). Western blots were quantified by densitometry and normalized to β-actin. The data are mean ± SEM of quadruplicate determinations and are representative of 3 independent experiments. Significant difference, as determined by Student's *t*-test, is from vehicle control, *$p<0.05$.

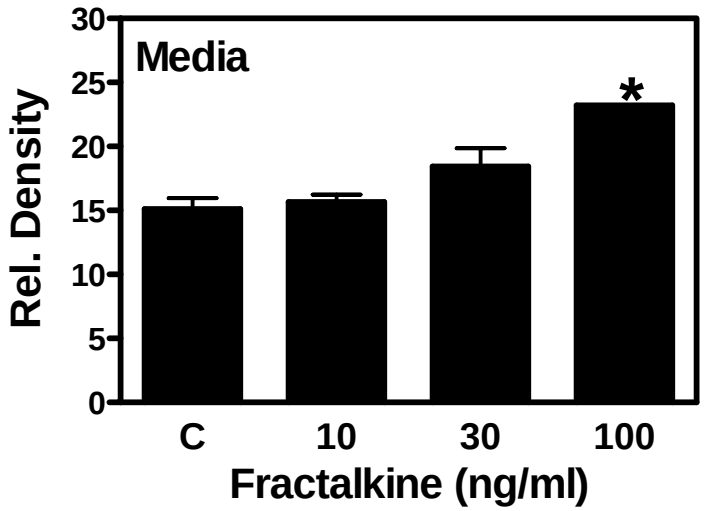


Figure 3.2 Fractalkine induces a concentration-dependent increase in MFG-E8 secretion by BV-2 cells. BV-2 cells were treated with different concentrations of fractalkine (10, 30, 100 ng/ml), and media was collected after 24 hours for Western blot analysis of MFG-E8. Western blots were quantified by densitometry and normalized to β-actin. The data are mean ± SEM of quadruplicate determinations and are representative of 3 independent experiments. Significant difference, as determined by one-way ANOVA, is from vehicle control, *$p<0.05$.

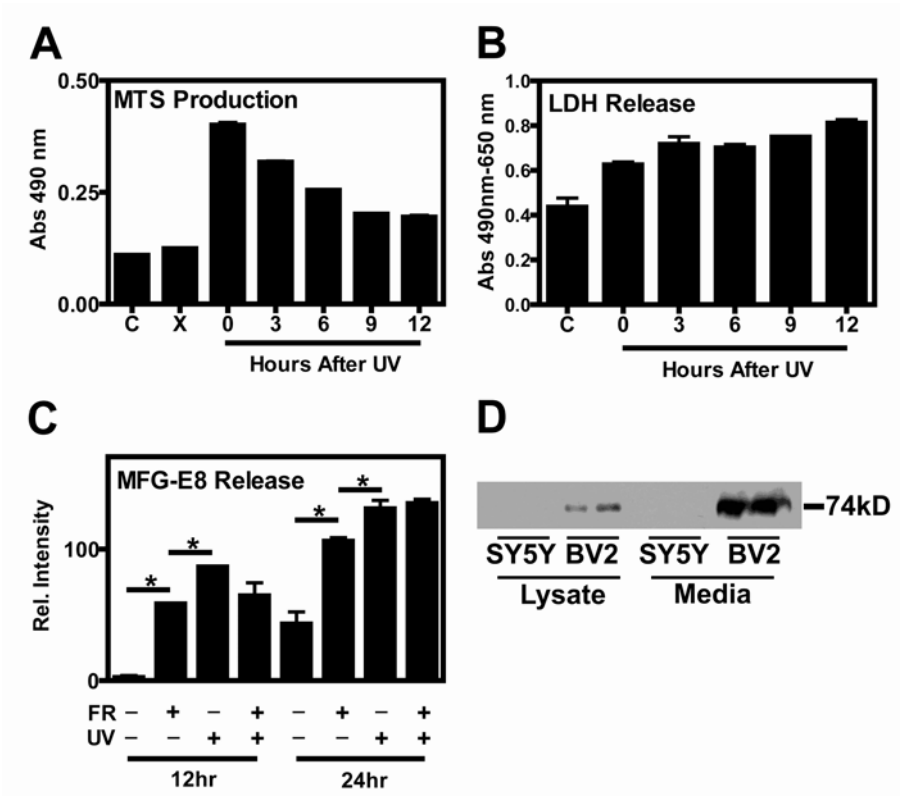
3.3 Dying SY5Y cells stimulate MFG-E8 production in co-culture with BV-2 cells

Because fractalkine is likely released in response to cellular stress and apoptosis (Harrison et al. 1998), we hypothesized that MFG-E8 aids in the clearance of apoptotic cells in the brain by enabling the recognition and engagement of microglia with dying cells, a similar mechanism to that observed in the peripheral immune system (Hanayama et al. 2002). Once we established MFG-E8 production and fractalkine-stimulated upregulation by BV-2 cells, we then explored the possibility that apoptotic cells also induce MFG-E8 release. To address this question, we measured MFG-E8 released from BV-2 cells when co-cultured with either healthy or dying SY5Y cells, a neuroblastoma cell line. We induced apoptosis in the SY5Y cells by exposing them to UV light, a proven method that eliminates the possibility of also killing the BV-2 cells with a chemical method (Chan and Yu 2000; Kulms and Schwarz 2000). We first verified that exposure of SY5Y cells to 20 seconds of UV light induced cell death as determined by the MTS and LDH assays (Fig. 3.3 A and B). In the MTS assay (Fig. 3.3 A), media containing Triton X-100 added to SY5Y cells served as a positive control, which yielded similar levels of MTS production as a negative control containing no cells. MTS production measured at 0, 3, 6, 9, and 12 hours after the 20 second UV treatment showed a reduction in MTS as soon as 3 hours, leveling off at 9-12 hours. LDH release measured at the same time points indicated membrane disruption beginning at 3 hours (Fig. 3.3 B).

To establish the co-culture (see Fig. 2.1 for diagram of experimental setup), we plated SY5Y cells for 24 hours prior to co-culture and exposed cells to 20 seconds of UV 1 hour prior to co-culture. After 12 and 24 hours of co-culture, 100 ng/ml fractalkine in the presence of healthy SY5Y cells induced a significant upregulation of MFG-E8 production by BV-2 cells (Fig. 3.3 C). However, untreated apoptotic SY5Y cells stimulated an even higher level of

production at both timepoints, indicating that apoptotic neurons elicit a more robust response from microglia than fractalkine alone. The combination of the two stimuli induced similar MFG-E8 levels as apoptotic cells alone, suggesting a maximal response at each timepoint. A Western blot of the individual cells types insured all MFG-E8 detected in these assays came from BV-2 cells and not SY5Y cells. In both media and lysate fractions, we detected MFG-E8 only in BV-2 cells (Fig. 3.3 D).

Figure 3.3 Dying SY5Y cells induce an increase in MFG-E8 release by BV-2 cells. SY5Y cells were exposed to UV light for 20 seconds to induce apoptosis, and the MTS assay (A) and LDH assay (B) were run to confirm UV-induced cell death. No cell wells were used as a negative control (C), Triton-X 100 treated cells were used as a positive control (X). Results were taken at 0, 3, 6, 9, and 12 hours after UV exposure. For co-culture experiments (C), SY5Y cells exposed to 20 seconds of UV 1 hour prior to co-culturing with BV-2 cells were treated with vehicle or 100 ng/ml fractalkine (FR) immediately following co-culture. Media was collected from cultures 12 or 24 hours after co-culture and analyzed for MFG-E8 by Western blot. Westerns were quantified by densitometry and normalized to β-actin (C). A representative Western blot is shown in D, demonstrating that MFG-E8 is detected only in BV-2 cells. The data are mean ± SEM of quadruplicate determinations and are representative of 3 independent experiments. Significant difference was determined by Student's *t*-test, *$p<0.05$.


A
MTS Production
Abs 490 nm
0.50
0.25
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C X 0 3 6 9 12
Hours After UV
B
LDH Release
Abs 490nm-650 nm
1.0
0.8
0.6
0.4
0.2
0.0
C 0 3 6 9 12
Hours After UV
C
MFG-E8 Release
Rel. Intensity
100
0
FR – + – + – + – +
UV – – + + – – + +
12hr
24hr
D
74kD
SY5Y BV2 SY5Y BV2
Lysate
Media

3.4 Pro-inflammatory stimuli suppress the production of MFG-E8 in BV-2 cells

In the peripheral immune system, macrophages treated with LPS, a bacterial endotoxin that induces inflammation, showed a reduction in MFG-E8 production, and MFG-E8$^{(-/-)}$ knockout mice exhibited an autoimmune disease resembling systemic lupus erythematosus (Hanayama et al. 2004). Furthermore, in an animal model of sepsis, animals produced less MFG-E8 than healthy animals, suggesting a role for infection in downregulating MFG-E8 secretion (Miksa et al. 2006). We hypothesized that microglia react in a similar manner and that treatment of BV-2 cells with pro-inflammatory stimuli would lead to a suppression of MFG-E8 protein. To test this, we treated BV-2 cells with LPS, a known activator of microglia, as well as with IFNγ, a cytokine often used as a co-stimulator when activating microglia. BV-2 cells were plated at a density of 1 x 10^5 cells/ml for 24 hours before treatment with vehicle, 100 ng/ml LPS, or LPS in combination with 25 ng/ml IFNγ, and cell lysates and conditioned media were collected at 5, 10, and 22 hours. Samples analyzed for changes in MFG-E8 levels by Western blot and normalized to β-actin levels (Fig. 3.4) showed that LPS initially stimulated intracellular MFG-E8 after 5 hours, but eventually LPS treatment led to a dramatic decrease in MFG-E8 levels in both the cell lysates (Fig. 3.4 A) and the conditioned media (Fig. 3.4 B). Co-stimulation with IFNγ prevented this initial stimulation and resulted in a more pronounced suppression of MFG-E8 production at 22 hours. The early stimulation by LPS could be due to LPS-induced cell death leading to apoptotic cell stimulation of MFG-E8 as was seen in figure 3.3, and decreased levels of β-actin support this proposition which are reflected in the normalization of the Western blot.

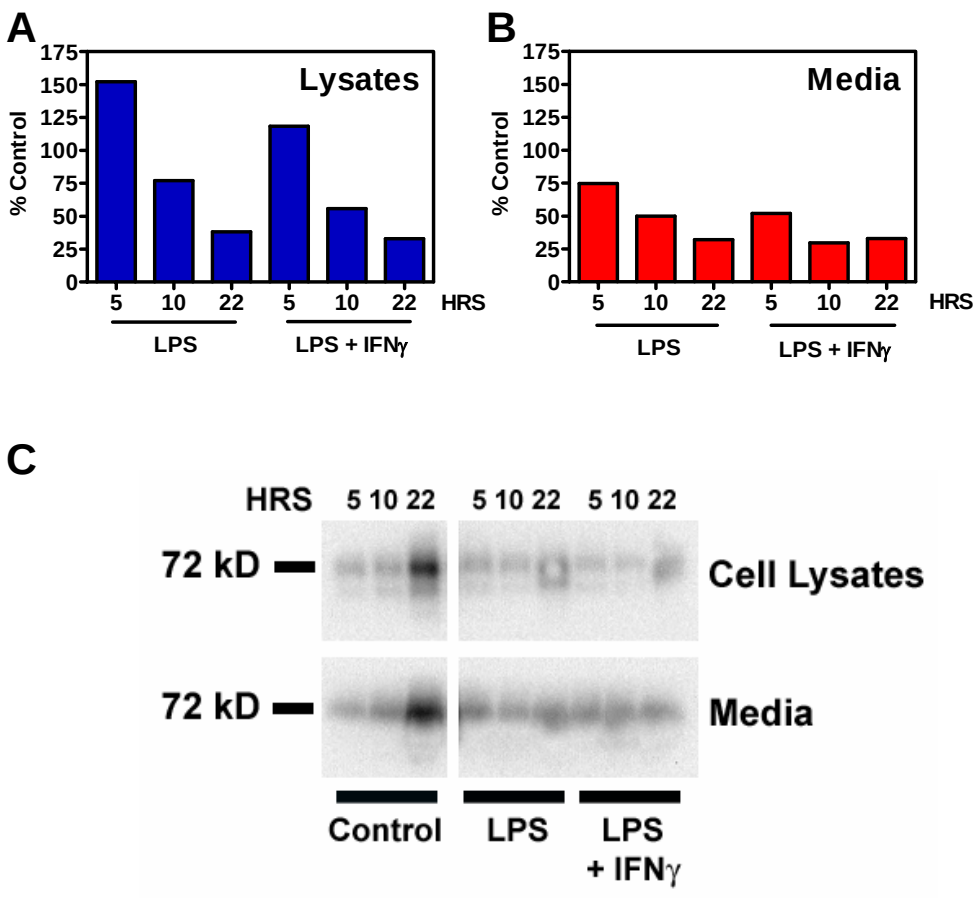


Figure 3.4 LPS and IFNγ suppress MFG-E8 production by BV-2 cells over time. BV-2 cells were treated with sterile PBS (Control), 100 ng/ml LPS, or LPS in addition to 25 ng/ml IFNγ before cell lysates (B) and conditioned media (C) were collected and analyzed for the presence of MFG-E8 by Western blot (A). Western blots were quantified by densitometry, normalized to β-actin, and expressed as percent of control. Because repeated experiments showed the same trend but differences in magnitude varied, the data are from one of three independent experiments.

To further probe the inhibitory effect of proinflammatory stimuli on the production of MFG-E8, we compared the levels of MFG-E8 in cell lysates and conditioned media following treatment with LPS, TNFα, IFNγ, or a combination of LPS or TNFα with IFNγ. TNFα is a proinflammatory cytokine that is stimulated by LPS as well as by β-amyloid (Aβ), a key protein in Alzheimer's disease (AD) pathology. TNFα is involved in the proinflammatory cytokine cycle that has been proposed to perpetuate neuroinflammation in disease such as AD (Fillit et al. 1991; Perry et al. 2001). In our experiments, cell lysates and conditioned media collected 24 hours after treatment with these stimuli were analyzed for MFG-E8 protein levels by Western blot and normalized to β-actin (Fig. 3.5). LPS and LPS in combination with IFNγ induced a significant inhibition of MFG-E8 in both cell lysates (Fig. 3.5 A) and conditioned media (Fig. 3.5 B) at the 24 hour timepoint. Interestingly, TNFα alone did not alter MFG-E8 levels in either lysate or media fractions. TNFα in conjunction with IFNγ yielded the same lowered MFG-E8 levels as was seen by IFNγ alone in cell lysates; however, this combination of TNFα and IFNγ decreased MFG-E8 amounts in the conditioned media where these cytokines yielded no effect independently. This indicates that the cytokine production seen in neuroinflammation associated with diseases like AD could be sufficient to suppress MFG-E8 production in the brain.

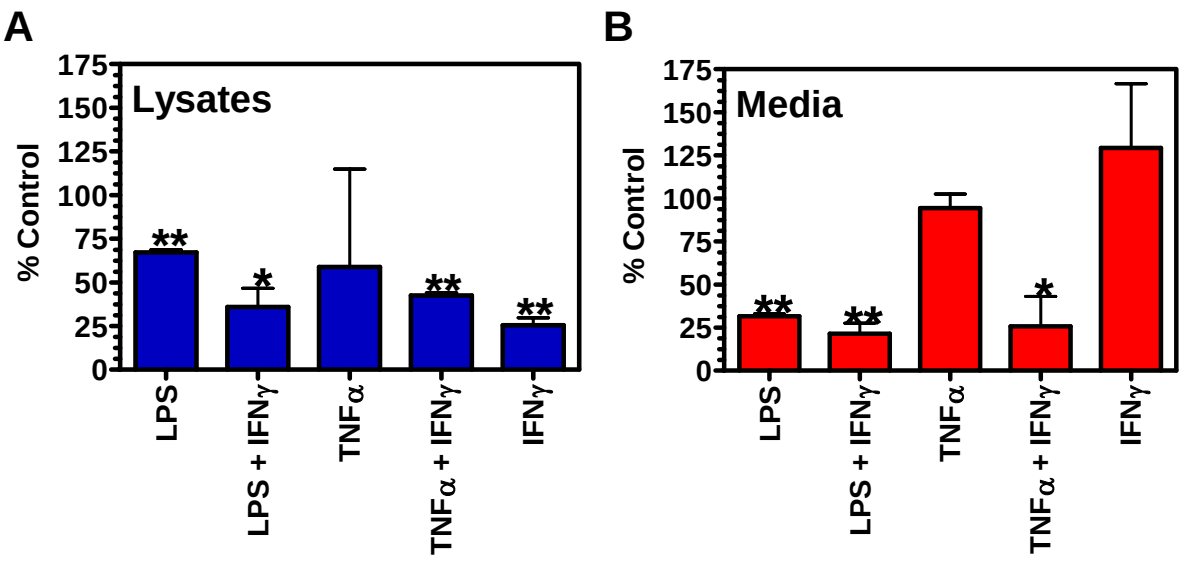


Figure 3.5 Common stimulators of proinflammatory responses in microglia suppress the production of MFG-E8 in BV-2 cells. BV-2 cells were treated with LPS (100 ng/ml), IFNγ (25 ng/ml), TNFα (25 ng/ml), LPS + IFNγ, or TNFα + IFNγ for 24 hours before lysates (A) and conditioned media (B) were collected and analyzed for the presence of MFG-E8 by Western blot. Western blots were quantified by densitometry and normalized to β-actin. The data are mean ± SEM of duplicate determinations and from one of three independent experiments. Significant difference, as determined by one-way ANOVA, is from vehicle control, *$p<0.05$, **$p<0.001$.

3.5 Summary

Treatment of microglia with the chemokine fractalkine was previously linked to MFG-E8 mRNA expression (Leonardi-Essmann et al. 2005), and we have extended these findings to MFG-E8 protein production and release by a microglial cell line. Fractalkine is known to be released from injured neurons, and the addition of fractalkine to microglia has been reported to induce chemotaxis resulting in a 3-fold increase in the number of glial cells, a response which is blocked by anti-fractalkine antibodies (Harrison et al. 1998). This recruitment of microglia to a site of neuronal injury coupled with the induction of MFG-E8 by fractalkine led us to hypothesize that fractalkine stimulation of MFG-E8 played a role in enabling microglial-mediated phagocytosis of apoptotic neurons. The robust increase in MFG-E8 induced by co-culturing an apoptotic neuronal cell line with a microglial cell line supports this theory and suggests that this protein is involved in the recognition of apoptotic cells in the brain in a similar way as in the periphery.

We have also shown for the first time that the bacterial endotoxin LPS and proinflammatory cytokines TNFα and IFNγ lower the levels of MFG-E8 produced by BV-2 cells. This suggests that a proinflammatory environment downregulates the phagocytic action of microglial cells, much like treatment with LPS leads to lower levels of MFG-E8 secretion by macrophages and dendritic cells in peripheral tissues (Miksa et al. 2006; Miyasaka et al. 2004). This implies that a state of neuroinflammation like that seen in AD and other neurodegenerative disorders may induce a decrease in MFG-E8 levels, which would in turn prevent proper phagocytosis of apoptotic cells. Because the clearance of apoptotic cells is a vital mechanism for maintaining homeostatis and preventing further inflammation, this lack of MFG-E8 could perpetuate the neuroinflammatory cycle in these diseases.

Chapter 4

MFG-E8 Mediates Phagocytosis by Microglia

4.1 Overview

MFG-E8 acts as a linker protein between apoptotic cells and macrophages or immature dendritic cells in the peripheral immune system to enable the phagocytosis of the dying cell. Blocking the action of MFG-E8 with a dominant negative form of the protein prevents phagocytosis by these cells, and this inhibition leads to a proinflammatory state resembling autoimmune disease. In addition to showing that the BV-2 microglial cell line produces MFG-E8, we show for the first time that MFG-E8 enables phagocytosis by this cell type, and likewise a dominant negative form of MFG-E8 (D89E) blocks phagocytosis by BV-2 cells. In addition, we demonstrate the ability of BV-2 cells to phagocytose an apoptotic neuroblastoma cell line, and this phagocytic activity is increased with MFG-E8 and decreased with D89E. Furthermore, we provide evidence that the "tether and tickle" mechanism described in the peripheral immune system involving MFG-E8 in concert with Gas6 is also at work in the brain. Finally, we further explore the impact of a proinflammatory environment on the phagocytic function of microglia by showing that BV-2 cells stimulated by LPS have a reduced capacity for phagocytosis. These data indicate an important role for MFG-E8-mediated phagocytosis by microglia in the brain and suggest a crucial modulation of this function during a state of neuroinflammation.

These results (Figures 4.1- 4.3 in press in Fuller and Van Eldik, J. Neuroimmune Pharmacol. 2008) provide the first direct evidence that MFG-E8 aids in phagocytosis by a microglial cell line.

4.2 Purification of D89E

MFG-E8 is composed of two epidermal growth factor (EGF) domains E1 and E2, a proline/threonine (PT)-rich domain, and two factor-VIII-homologous domains (C1 and C2; Fig. 4.1 A). A point mutation in the RGD (arginine-glycine-aspartic acid) integrin-binding motif to RGE (aspartic acid to glutamic acid) of one EGF domain at residue 89 results in a dominant negative form of MFG-E8, called D89E, which does not bind to the integrin on the phagocyte (Hanayama et al. 2002), effectively blocking the function of the protein. We utilize this dominant negative form of MFG-E8 in the following experiments to verify the involvement of MFG-E8 in phagocytosis by BV-2 cells. D89E constructs were created by Hanayama and colleagues as described (Hanayama et al. 2002). D89E expressed in HEK293T cells and purified from the conditioned media reacted with an antibody to MFG-E8 and ran at the correct molecular weight (Fig. 4.1B).

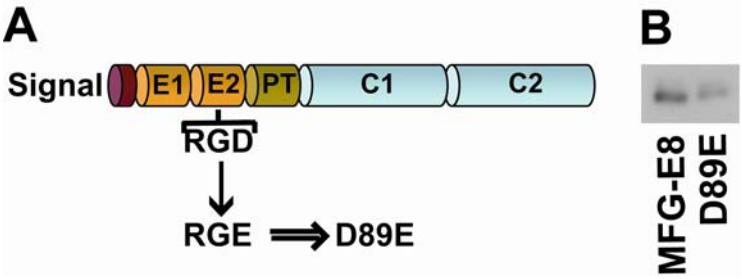


Figure 4.1 Diagram and purification of D89E. The construct for the D89E dominant negative form of MFG-E8 was obtained from Dr. Nagata (University of Osaka, Japan). Panel A demonstrates the point mutation of the D residue at position 89 in the second E2 repeat of the protein to an E that prevents the protein from binding to an integrin receptor while allowing normal PS binding. This mutation functionally inhibits the linking of macrophages to PS-expressing apoptotic cells, thus acting as a dominant negative protein. Transfection of the purified, FLAG-tagged D89E construct into human embryonic kidney 293T (HEK 293T) cells led to the secretion of D89E protein into conditioned media. Affinity column purification of the conditioned media using a FLAG resin yielded pure D89E to use in subsequent phagocytosis assays. Shown in panel B, Western blotting of the sample compared to a sample of recombinant mouse MFG-E8 probed with an antibody to MFG-E8 showed that the antibody recognized both the recombinant MFG-E8 and dominant negative protein which ran at the same molecular weight.

4.3 MFG-E8 increases and D89E decreases phagocytosis by BV-2 cells in the pHrodo Assay

After we confirmed MFG-E8 production by a microglial cell line and regulation by both fractalkine and co-culture with an apoptotic neuronal cell line, we next investigated the ability of MFG-E8 to increase the phagocytic capacity of microglia (Fig. 4.2). We hypothesized that adding recombinant MFG-E8 to a phagocytosis assay would result in more connections formed between BV-2 cells and the material intended to be phagocytosed, thereby enabling the engulfment of more particles. To first address this question, we employed the pHrodo assay from Molecular Probes, which measures the uptake of BioParticles, dead *E. coli* particles tagged with a pH sensitive dye which fluoresces once it enters the intracellular environment (See Figure 2.2). BV-2 cells were untreated (positive control) or treated with 100 ng/ml recombinant mouse MFG-E8, 4 μg/ml dominant negative MFG-E8 (D89E), or 4 μg/ml BSA as an unrelated protein control.

The addition of MFG-E8 to the pHrodo assay resulted in a 27.3 ± 6.8% increase in phagocytosis (Fig. 4.2). In the peripheral immune system, macrophages co-cultured with apoptotic cells show an increase of about 40% in the phagocytosis of PS expressing cells when MFG-E8 is added that is similar in scale to the roughly 27% increase in phagocytosis that we report here (Hanayama et al. 2002). Conversely, the addition of D89E suppressed phagocytosis in this assay by 65.8 ± 18.4%. A higher concentration of D89E (4 μg/ml) as compared to recombinant MFG-E8 (100 ng/ml) was needed in order to see an inhibition in phagocytosis, possibly due to the ability of the endogenous MFG-E8 to potentially bind multiple PS residues and thus tether multiple apoptotic cells, or BioParticles in this experiment. Therefore, an excess of the dominant negative form of

MFG-E8 was necessary to flood the PS targets and block phagocytosis, and reductions below control levels were seen, likely due to the blocking of endogenous MFG-E8. To control for the possibility that adding a protein at 4 μg/ml non-specifically inhibited phagocytosis, BSA added at an equivalent concentration showed no significant decrease in BioParticle engulfment.

Interestingly, an excess of MFG-E8 added to microglia in the Vybrant phagocytosis assay leads to an inhibition of phagocytosis (Figure 4.3). A similar effect is seen in a selection of patients with systemic lupus erythematosus (SLE) where overproduction of MFG-E8 leads to a proinflammatory response and autoimmune disease (Yamaguchi et al. 2008). One possible explanation for this is that excess MFG-E8 blocks other proteins involved in the proper clearance of apoptotic cells, such as Gas6.

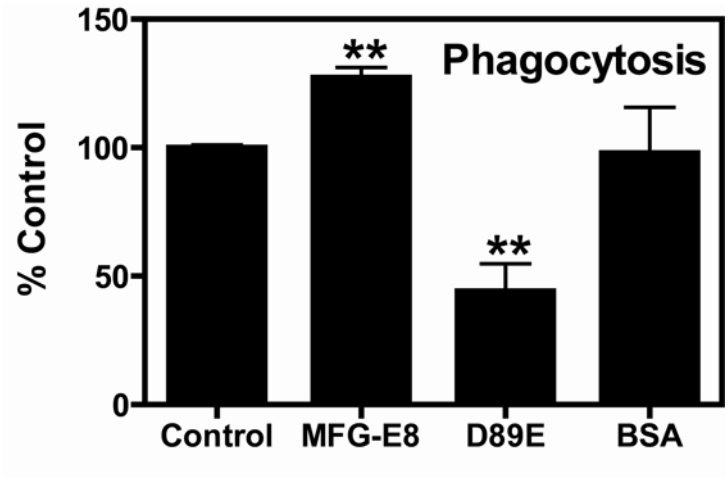


Figure 4.2 MFG-E8 enhances and D89E blocks phagocytosis by BV-2 cells in the pHrodo phagocytosis assay. BioParticles in the pHrodo assay from Molecular Probes were prepared in Hank's buffered saline solution, pH 6.8 and sonicated in a bath sonicator for 20 seconds 3 times. BioParticles were added to cultures of BV-2 cells after 24 hours in culture, and the BV-2 cells phagocytosed BioParticles after 2 hours in culture. This phagocytosis was increased with the addition of 100 ng/ml MFG-E8 and inhibited by treatment with 4 μg/ml dominant negative D89E added at the same time as BioParticles. Cells treated with a 4 μg/ml BSA control showed no difference from the untreated control. The data are the mean ± SEM from 5 replicates per experiment of 3 separate experiments, and are expressed as percent of untreated control wells. Significant differences, as determined by one-way ANOVA, are from control, **$p<0.001$.

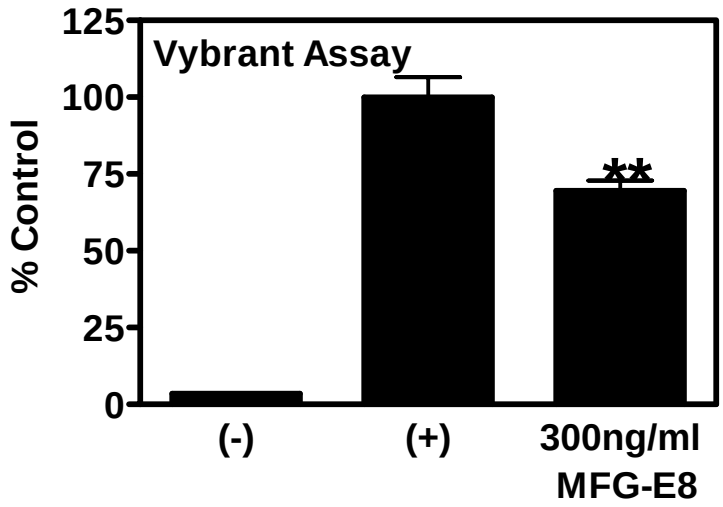
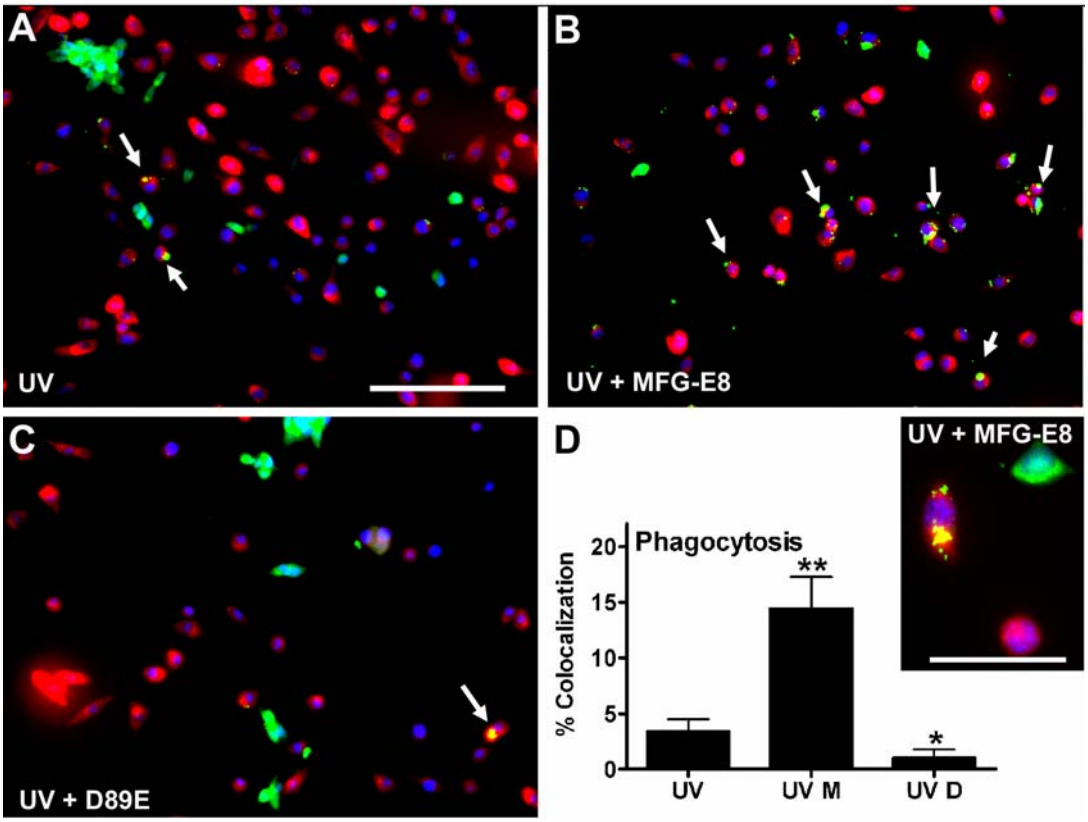


Figure 4.3 Excess MFG-E8 inhibits phagocytosis by BV-2 cells in the Vybrant phagocytosis assay. The negative control (-) is a no cell assay control. The BV-2 cells phagocytosed BioParticles in the Vybrant assay from Molecular Probes after 2 hours in culture, which is the positive control (+). This phagocytosis was decreased with the addition of 300 ng/ml MFG-E8. The data are the mean ± SEM from 5 replicates per experiment of 3 separate experiments, and are expressed as percent of untreated control wells. Significant difference, as determined by Student's *t*-test, is from the control unless otherwise indicated, **$p<0.01$.

4.4 MFG-E8 increases phagocytosis of an apoptotic neuronal cell line by BV-2 cells.

Given that BV-2 cells showed phagocytic activity in the pHrodo assay and that this activity increased in the presence of MFG-E8 and decreased with D89E, we next investigated the potential for BV-2 cells to engulf dying neurons and the effect of MFG-E8 in this system (Fig 4.3). SY5Y cells and BV-2 cells were labeled by CellTracker Green and CellTracker Red dyes, respectively, as described in Methods. SY5Y cells cultured for 24 hours prior to a 20 second UV exposure and then co-cultured with BV-2 cells for 6 hours after UV exposure were treated with MFG-E8 or D89E for 1 hour and then cells were analyzed by fluorescent microscopy. Colocalization of red BV-2 cells and green SY5Y cells indicated phagocytosis. We observed very little phagocytosis under control conditions where SY5Y cells were not undergoing apoptosis. However, phagocytosis increased with apoptotic SY5Y cells (Fig 4.3 A), and treatment with 100 ng/ml MFG-E8 further enabled the engulfment of SY5Y cells (Fig 4.3 B and D). As in the pHrodo assay, the presence of 4 μg/ml D89E inhibited phagocytosis (Fig 4.3 C). In many cases in the MFG-E8 treated cells, green SY5Y cells appeared fragmented inside red BV-2 cells (Fig. 4.3 D). Quantification of the data (Fig. 4.3 E) showed that MFG-E8 treatment resulted in a significant increase in co-localization compared to UV treatment alone, and treatment with the D89E dominant negative protein resulted in a significant decrease in co-localization. These results support the idea that microglial phagocytosis is mediated through an MFG-E8 dependent mechanism.

Figure 4.4 MFG-E8 increases and D89E decreases the phagocytosis of apoptotic neurons by microglia. SY5Y cells labeled with CellTracker green were exposed to 20 seconds of UV light, and five hours later, CellTracker red-labeled BV-2 cells were co-cultured with SY5Y cells for 1 hour. Slides were then coverslipped with VectaShield containing DAPI for imaging. At the time of co-culture, cells were treated with vehicle (A), 100 ng/ml MFG-E8 (B and inset in D), or 4 μg/ml D89E (C). Arrows point to cells with colocalization of red and green, therefore indicating the engulfment of SY5Y cells by BV-2 cells. Images were analyzed for co-localization of red and green dye with ImageQuant and results are expressed as percent co-localization, which is defined as the overlap area of red and green staining as compared to total area analyzed. The data (D) are the mean ± SEM from 3 independent experiments consisting of 6 randomly selected fields analyzed per experiment. The scale bar is equal to 100 μm for A-C and 50 μm for the inset in D. Significant differences, as determined by Student's *t*-test, are from vehicle-treated cells, $^{**}p<0.001$; $^{*}p<0.05$.


A
UV
B
UV + MFG-E8
C
UV + D89E
D
Phagocytosis
% Colocalization
20
15
10
5
0
**
*
UV
UV M
UV D
UV + MFG-E8

4.5 Gas6 increases phagocytosis by BV-2 cells in the Vybrant phagocytosis assay and combines with MFG-E8 to further increase phagocytosis.

Although we show an increase in the uptake of apoptotic cells by a microglial cell line in the presence of recombinant MFG-E8, this is not the only protein in the "tether and tickle" mechanism of phagocytosis. The studies of this mechanism using macrophages indicate that Gas6 also plays a very important role in the proper clearance of apoptotic material (Wu et al. 2006). Grommes and colleagues established a role for Gas6 in microglial phagocytosis; however, they were only able to show increased apoptotic cell attachment to microglia following Gas6 treatment (Grommes et al. 2008). This inability to demonstrate enhanced engulfment with the addition of Gas6 could indicate the dependence on MFG-E8 for proper clearance of apoptotic cells. In order to begin to address this question, we investigated the potential for the addition of both Gas6 and MFG-E8 to enhance the phagocytic capabilities of microglia (Fig. 4.4). Utilizing the Vybrant phagocytosis assay from Molecular Probes, we demonstrated that 100 ng/ml of either MFG-E8 or Gas6 in isolation modestly augments phagocytosis by BV-2 cells by approximately 30%. However, treatment with both recombinant proteins together, each at a lower concentration of 10 ng/ml, incited a more robust 50% increase in engulfment. Furthermore, a 90% increase resulted when 100 ng/ml of each protein was added to the assay. This possibly synergistic effect suggests a cooperative mechanism for these proteins in the brain that is similar to that seen in the tether and tickle hypothesis. Future experiments should further investigate this proposition by looking at the engulfment of apoptotic neurons by microglia in the presence of both MFG-E8 and Gas6. Furthermore, the effect of Gas6 treatment in combination with D89E would be interesting because it would further indicate whether Gas6 and MFG-E8 are mechanistically linked.

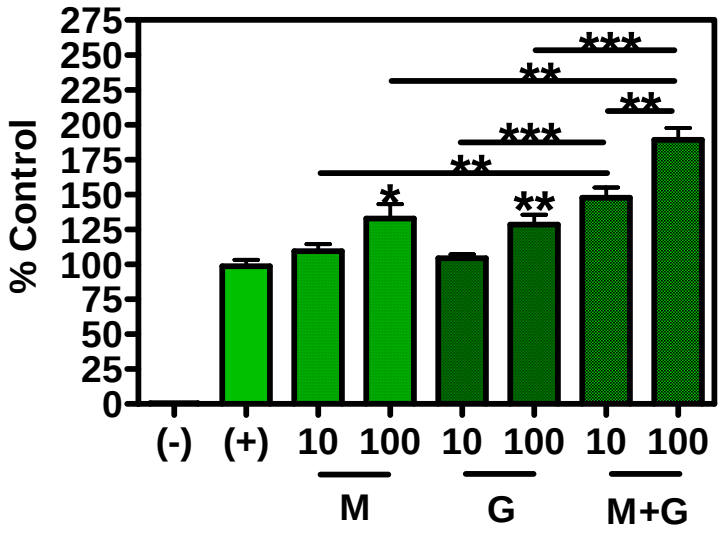


Figure 4.5 Gas6 and MFG-E8 in combination stimulate an increase in phagocytosis by BV-2 cells in the Vybrant phagocytosis assay. The negative control (-) is a no cell assay control. The BV-2 cells phagocytosed BioParticles in the Vybrant assay from Molecular Probes after 2 hours in culture, which is the positive control (+). This phagocytosis was increased with the addition of 100 ng/ml MFG-E8 as well as with 100 ng/ml Gas6. Cells treated with a combination of 10 ng/ml of both MFG-E8 and Gas6 stimulated more phagocytosis than with either MFG-E8 or Gas6 alone. Treatment with 100 ng/ml MFG-E8 and Gas6 together resulted in a significant increase over the 10 ng/ml level. The data are the mean ± SEM from 5 replicates per experiment of 3 separate experiments, and are expressed as percent of untreated control wells. Significant differences, as determined by one-way ANOVA, are from the control unless otherwise indicated, *$p<0.05$; **$p<0.01$; ***$p<0.001$.

4.6 The proinflammatory stimulus of LPS decreases phagocytosis by BV-2 cells in the Vybrant phagocytosis assay.

LPS is a frequently used stimulus of the proinflammatory response in microglia, and it is used often in experiments with BV-2 cells to model the neuroinflammatory response launched following a neural insult such as disease or infection. In addition, treatment of bone marrow derived immature dendritic cells with LPS induces maturation to antigen presenting cells, which then trigger an immune response by activating B and T cells in the peripheral immune system. During this maturation process, dendritic cells produce less MFG-E8 and also have dramatically reduced phagocytic capability (Miyasaka et al. 2004). Therefore, we investigated the effect of LPS treatment of BV-2 cells on phagocytosis in the Vybrant assay. Treatment of BV-2 cells for 24 hours with 100 ng/ml of LPS, a concentration that is typically used to show a robust increase in proinflammatory cytokines in these cells, resulted in a 70% decrease in phagocytosis below control levels (Fig. 4.5). These data, combined with our previous data that LPS and proinflammatory cytokines reduce MFG-E8 production (Figs. 3.4 and 3.5), suggest that when microglial cells are in a proinflammatory state, they are less capable of phagocytosing apoptotic cells due to a reduced level of MFG-E8 secretion.

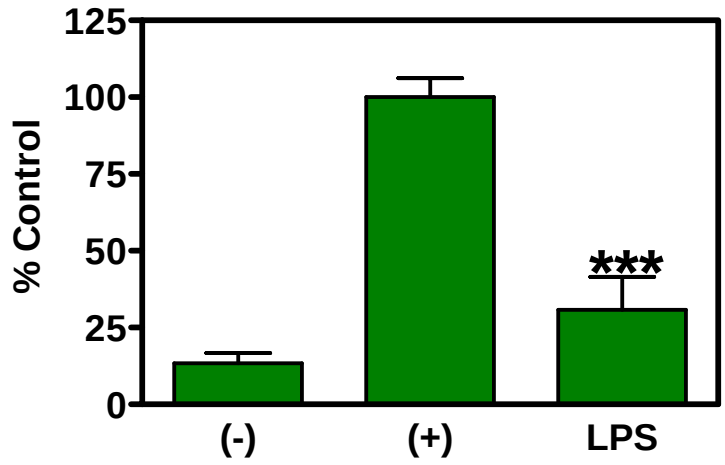


Figure 4.6 LPS reduces phagocytosis by BV-2 cells in the Vybrant assay. The negative control (-) is a no cell assay control. In the positive control (+), the BV-2 cells phagocytosed BioParticles in the Vybrant assay from Molecular Probes after 2 hours in culture. This phagocytic ability was decreased in BV-2 cells pre-treated with 100 ng/ml LPS for 24 hours. The data are the mean ± SEM from 5 replicates per experiment of 3 separate experiments, and are expressed as percent of untreated control wells. Significant difference, as determined by Student's *t*-test, is from the control, ***$p<0.001$.

4.7 Summary

The “tether and tickle” mechanism has proven to be an efficient mechanism for the clearance of apoptotic cells by the peripheral immune system (Hoffmann et al. 2001). The first step in this mechanism is defined by the tethering of apoptotic cells at an exposed PS to the integrin receptor on phagocytic cells by MFG-E8 (Hanayama et al. 2006; Hanayama et al. 2002). After the cells are linked, the tickle is carried out by the binding of the Mer receptor by Gas6, which triggers a signaling cascade in the phagocyte to internalize the apoptotic cell (Scott et al. 2001; Todt et al. 2004). Proper clearance of apoptotic cells by this process is important for preventing secondary necrosis and the seeping of noxious material into the surrounding tissue (Mitchell et al. 2002). Blocking either step of this process leads to lowered efficiency in apoptotic cell engulfment (Asano et al. 2004; Scott et al. 2001; Todt et al. 2004). Research on the tickle arm of this mechanism by microglia indicates that Gas6 is involved in the microglial phagocytosis process (Grommes et al. 2008). However, although addition of Gas6 to a culture of apoptotic cells and microglia increased attachment of the two cells types, Gas6 was not sufficient for the engulfment of the apoptotic cells. This indicates that the tether step is required for proper phagocytosis.

Here we provide evidence through studies with the BV-2 microglial cell line suggesting that MFG-E8 is also involved in phagocytosis by microglia. We show that MFG-E8 increases phagocytosis in a BV-2 assay and that this activity is inhibited by a dominant negative form of MFG-E8. Significantly, we have demonstrated a synergy between MFG-E8 and Gas6 that leads to a robust increase in phagocytosis. This is the first evidence suggesting that phagocytosis of apoptotic cells by microglia involves a two step process that includes recognition and binding of the apoptotic cell by MFG-E8 and signaling for engulfment of the cell by Gas6.

We have also provided data to imply that an environment of neuroinflammation may lead to impaired clearance of apoptotic cells by showing that LPS decreases phagocytosis by BV-2 cells. This may lead to further inflammation due to the presence of detrimental cellular debris in the extracellular space. This could have dire implications in neurodegenerative diseases where neuroinflammation is involved and apoptotic cell death occurs.

Chapter 5

MFG-E8 in the Brain

5.1 Overview

Data generated in the BV-2 cell culture system suggest that MFG-E8 plays a similar role in the clearance of apoptotic neurons in the brain as it does in the clearance of apoptotic cells in the peripheral immune system. Defects in this mechanism have been linked to several autoimmune and inflammatory disorders in the peripheral immune system (Asano et al. 2004; Hanayama et al. 2006; Hanayama et al. 2004; Miksa et al. 2006; Yamaguchi et al. 2008), so a reasonable hypothesis would be that MFG-E8 may play a role in neurological conditions as well. To begin to examine this idea, we screened the Tg2576 mouse model of Alzheimer's disease (AD) pathology for changes in brain MFG-E8 production compared to age matched wild-type animals. Tg2576 mice exhibit biochemical, pathological, and behavioral characteristics of AD, making it a popular model for studying the effects of Aβ on the brain during AD. Behaviorally, normal learning and memory in spatial reference and alternation tests is observed in these mice at 3 months of age, but impairment is detected at 9-10 months. Likewise, at 9-10 months of age $A\beta_{(1-40)}$ levels are increased by 5-fold and $A\beta_{(1-42)}$ levels are increased by 14-fold. Congo red positive plaques are detected in the cortical and limbic structures of mice showing increased Aβ levels (Hsiao et al. 1996). Furthermore, cognitive deficits in these mice correlate with soluble Aβ increases at 9-10 months (Lindner et al. 2006). Aged Tg2576 mice also display increased levels of the cytokines IL-1β, TNFα, and MCP-1 as well as increased complement proteins C1qA and C3 (Sly et al. 2001). Interestingly, fractalkine levels have also been shown to be lower in Tg2576 mice when compared to age-matched controls (Duan et al. 2008).

The following data show for the first time a decrease in MFG-E8 in the brains of Tg2576 mice as compared to age-matched controls (Figure 5.1 in press in Fuller and Van Eldik, J. Neuroimmune Pharmacol. 2008).

5.2 Tg2576 mice produce lower levels of brain MFG-E8

Whole brain homogenates from 2, 6, 12, and 18 month mice (Tg2576 and wild-type littermates) were compared by Western blot analysis. These ages allowed us to examine the levels of MFG-E8 in pathologically and behaviorally normal young animals as compared to older animals displaying Aβ deposition, proinflammatory cytokine upregulation, and behavioral deficits. When all protein levels were normalized to β-actin, wild-type animals displayed an increase in MFG-E8 levels with age that became significant and remained steady at 12 months. In contrast, Tg2576 animals showed much lower levels of MFG-E8 that increased a small but significant amount at 18 months, yet was still below wild-type levels.

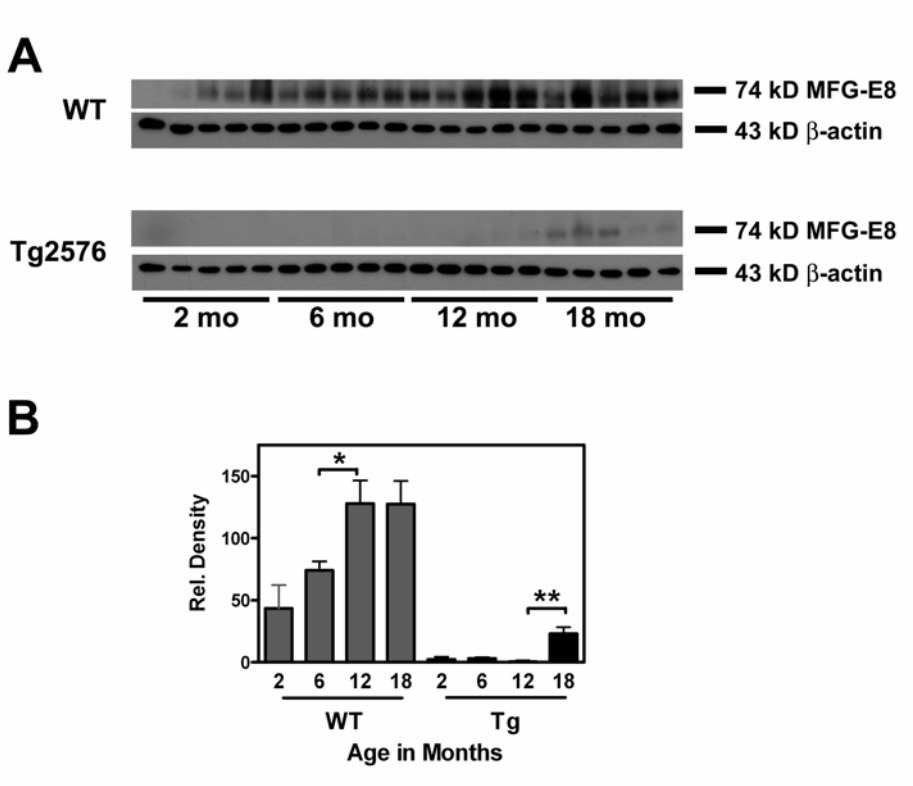


Figure 5.1 Tg2576 mice produce lower levels of brain MFG-E8 than wild-type mice. Whole brain homogenates from Tg2576 mice of various ages (2, 6, 12, 18 months) and from wild-type age-matched littermates were probed for MFG-E8 levels by Western blot (A). Blots were quantified by densitometry and normalized to β-actin (B). Significant differences, as determined by one-way ANOVA, are shown, **$p<0.001$; *$p<0.05$.

5.3 Summary

This is the first report of abnormal MFG-E8 levels in a mouse model of a neurodegenerative disease. In Chapter 3 we show the ability of the proinflammatory cytokines TNFα and IFNγ to reduce the levels of MFG-E8 produced by a microglial cell line. Similarly, we show in this chapter a reduction in MFG-E8 production in the brains of Tg2576 mice, which produce high levels of proinflammatory cytokines. However, MFG-E8 levels are very low as compared to age matched controls even at 2 months. This is earlier than reported increases in TNFα at 6 months and decreases in fractalkine at 9 months. Nonetheless, Tg2576 mice do produce large amounts of soluble Aβ even at a very early age (Lindner et al. 2006). One possibility for the low levels of MFG-E8 in the Tg2576 mice is that this soluble Aβ is acting to stimulate a proinflammatory response and inhibit the production of MFG-E8, much like LPS does to microglia (Chapter 3) as well as to peripheral macrophages (Miksa et al. 2007; Miyasaka et al. 2004). Further studies to test the ability of soluble Aβ to modulate MFG-E8 production by microglia are needed to properly address this proposition.

The results from the Tg2576 animals also validate the studies completed in the BV-2 cell line. The treatment of BV-2 cells with LPS and TNF-α predicts that microglia downregulate MFG-E8 production in the presence of proinflammatory stimuli. Likewise, we see decreased MFG-E8 levels in whole brain homogenates of animals known to have an active inflammatory response. Therefore, the use of BV-2 cells as a model for MFG-E8-mediated phagocytosis is a feasible approach for future studies as well.

Chapter 6

DISCUSSION

6.1 Summary

In this study, we report several key findings. First we establish that the BV-2 microglial cell line produces MFG-E8 and can therefore be used as a model for MFG-E8 production by microglia. In a cell culture model system of glial-neuronal interactions, apoptotic SY5Y neuroblastoma cells induce an increase in MFG-E8 production by the BV-2 microglial cell line. We also show that treatment of BV-2 cells with the chemokine fractalkine (CX3CL1) increases MFG-E8 protein production by these cells, suggesting a potential mechanism that drives the increase of phagocytosis of apoptotic SY5Y cells by BV-2 cells. This finding also builds on a report that fractalkine increases gene expression of MFG-E8 in primary rat microglia (Leonardi-Essmann et al. 2005). In contrast, LPS and proinflammatory cytokines TNFα and IFNγ reduce MFG-E8 from BV-2 cells, indicating that neuroinflammation could downregulate MFG-E8 production in the brain.

After exploring the pattern and modulation of MFG-E8 production by BV-2 cells, next we explored the ability of MFG-E8 to facilitate phagocytosis in these cells. The data demonstrate that MFG-E8 stimulates microglial phagocytosis of both BioParticles and apoptotic neurons, and a dominant negative form of MFG-E8, which acts to mask the PS expressed on the apoptotic cell, blocks this phagocytic activity. Furthermore, we report that a proinflammatory environment induced by LPS that was shown to downregulate MFG-E8 production also reduces the phagocytic activity of BV-2 cells. These data are further supported by the finding that MFG-E8 levels are reduced in an animal model of the Aβ pathology seen in AD. The data found in the animal model are a proof-of-concept that MFG-E8 regulation plays a role in the mechanism of glial phagocytosis in neurodegenerative diseases.

6.2 MFG-E8 production in BV-2 cells

Northern blots show that MFG-E8 is expressed in the spleen, lymph nodes, mammary glands, and brain of 10 week old mice (Hanayama et al. 2004). The MFG-E8 protein is secreted from dendritic cells, macrophages, and epithelial cells as part of exosomes, and mRNA for MFG-E8 is found in primary murine microglia (Asano et al. 2004; Hanayama et al. 2004; Leonardi-Essmann et al. 2005). However, secretion of MFG-E8 by microglia has not yet been shown.

The BV-2 cell line is an immortalized microglial cell line generated by infecting primary murine microglial cultures with the *v-raf/v-myc* oncogene carrying retrovirus J2 (Blasi et al. 1990). These cells exhibit many of the behaviors of primary microglia; for example, stimulation with LPS and poly[I]-poly[C] yields increased production of IL-1 and TNFα, respectively. BV-2 cells can phagocytose opsinized latex beads and opsonized inactivated *Candida albicans* (Blasi et al. 1990; Bocchini et al. 1992). In Chapter 3 we show that BV-2 cells also are capable of producing MFG-E8. Taken together, all of these factors make for an appropriate cell line for us to use as a model for the production and regulation of MFG-E8 in these studies.

With an established system with which to monitor MFG-E8 production, we treated BV-2 cells with fractalkine and monitored intracellular and released protein levels of MFG-E8. Previous data suggest an amplification of MFG-E8 gene expression in primary rat microglia following treatment with fractalkine (Leonardi-Essmann et al. 2005), and fractalkine also stimulates MFG-E8 in peritoneal macrophages (Miksa et al. 2007). We were able to corroborate this finding by showing a fractalkine-induced increase in MFG-E8 protein production by BV-2 cells. This is significant because fractalkine is known to be released from injured neurons (Harrison et al.

1998) and therefore alludes to the ability of apoptotic cells to signal microglia to increase MFG-E8 production. The treatment of microglia with fractalkine has been reported to induce chemotaxis resulting in a 3-fold increase in the number of glial cells, a response which is blocked by anti-fractalkine antibodies (Harrison et al. 1998). This recruitment of microglia to a site of neuronal injury coupled with the induction of MFG-E8 by fractalkine led us to hypothesize that fractalkine stimulation of MFG-E8 played a role in enabling microglial-mediated phagocytosis of apoptotic neurons.

In order to address this hypothesis, we established a co-culture system composed of SY5Y neuroblastoma cells and BV-2 cells. The SY5Y cells, exposed to UV light to induce apoptosis, generated a robust increase in MFG-E8 production by the BV-2 cells. The discovery that an apoptotic neuronal cell line significantly enhances MFG-E8 protein levels in BV-2 cells supports the theory that MFG-E8 could play a significant role in microglial phagocytosis of apoptotic neurons.

6.3 MFG-E8 mediates phagocytosis in BV-2 cells with Gas6

Microglia play a crucial role in maintaining homeostasis in the central nervous system as the resident macrophages in the brain. In the event of infection, trauma, neurodegeneration, or normal development, microglia transform into phagocytes in order to properly clear the infection or dying cells. Proper clearance of cellular debris and apoptotic cells prevents further neuronal toxicity and staves an inflammatory response. However, how microglia recognize and remove apoptotic cells in the brain has not been completely determined. During apoptosis, disruption of the plasma membrane exposes PS on the cell surface, which is known to signal the apoptotic

state of the cell. In the peripheral immune system, this signal is recognized by the opsonin MFG-E8, which bridges the apoptotic cell and a macrophage, enabling engulfment of the dying cell. In this study, we test the hypothesis that MFG-E8 is produced by microglia and facilitates the phagocytosis of apoptotic neurons by microglia. We also propose that the disruption of this process is involved in neurodegenerative diseases and discuss the potential for therapeutic strategies associated with MFG-E8.

Although the mechanism by which microglia recognize apoptotic cells in the brain has not been completely resolved, several important signals have been identified, such as the importance of PS expression (Witting et al. 2000), the CD36 scavenger receptor (Stolzing and Grune 2004), TREM-2 (Takahashi et al. 2005), and most recently Gas6 (Grommes et al. 2008). During apoptosis, a redistribution of the plasma membrane occurs which flips PS and closely associated annexinV from the inner leaflet of the cell membrane to the outer cell surface. This is one of the best characterized events to signal a cell is undergoing apoptosis, and phagocytes recognize this "eat me" signal in a variety of ways. The phagocyte can link to it directly via the PS receptor, or the phagocyte can also secrete other proteins with the capacity to recognize and bind the exposed PS.

MFG-E8 has two C1 lipid binding domains that allow it to bind apoptotic cells via interaction with PS. MFG-E8 then binds through an RGD domain to either $\alpha_v\beta_5$ or $\alpha_v\beta_3$ integrin on the macrophage to link the two cells together. Like MFG-E8, Gas6 binds to PS (Ishimoto et al. 2000) and links apoptotic cells to phagocytes; in this case Gas6 binds Mer receptor tyrosine kinase on the surface of the phagocyte. In the periphery, a link between Gas6 and MFG-E8 has

been identified, referred to as the “tether and tickle” (Hoffmann et al. 2001). The “tether,” recognition and binding of apoptotic cells to phagocytes, is often mediated by integrins, such as in MFG-E8 binding, and is not sufficient for engulfment. The “tickle” signals an internalization of bound particles and induction of downstream signaling cascades and functions through Mer (Scott et al. 2001; Todt et al. 2004). Taken together, strong adhesion through tethering with molecules like MFG-E8 in combination with the conversion of this binding to a signaling event through tickling molecules such as Gas6 create a multiple step model required for proper clearance of apoptotic cells. Likewise, Gas6 was shown to function in microglia to aid in phagocytosis via Rac activation and suppress inflammation as it does in the peripheral immune system (Grommes et al. 2008). However, while association of apoptotic cells and microglia was enhanced by the addition of Gas6 in the Grommes et al study, phagocytosis was not enhanced, indicating the Gas6/Mer interaction is not sufficient for engulfment. Based on our findings in the current study that co-treatment with MFG-E8 and Gas6 results in a synergistic effect on the increase in phagocytosis, we suggest that Gas6 and MFG-E8 are fulfilling their roles in the brain of the tether/tickle mechanism modeled in the periphery. A model of this proposed scenario is diagramed in Figure 6.1.

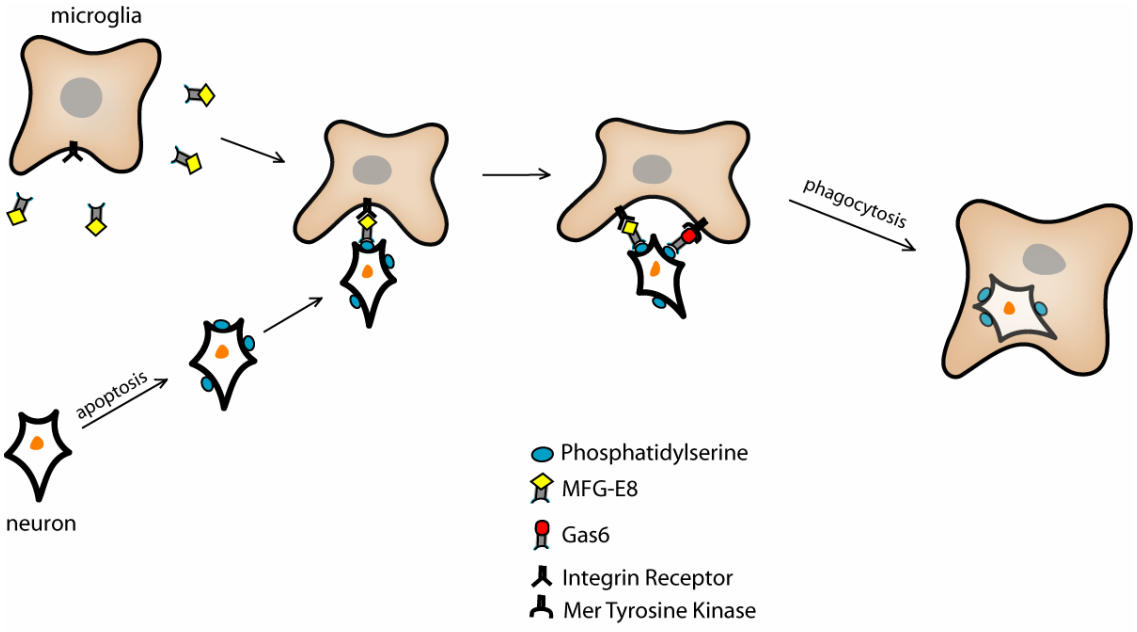


Figure 6.1 Proposed model for MFG-E8 and Gas6 mediated phagocytosis of apoptotic neurons by microglia. Cell stress from normal aging, disease, or injury induces apoptosis in neurons, resulting in a redistribution of the plasma membrane and the exposure of PS on the cell surface. Microglia produce and release MFG-E8 to scan the environment for apoptotic cells. Once PS is detected, MFG-E8 binds to it, allowing it to bind back to an integrin receptor on the microglia thus acting as a tether. This association of the cells allows the microglia to develop into a phagocyte and engulf the apoptotic neuron, ultimately protecting the surrounding tissue from potentially harmful cellular debris. Figure is adapted from Zullig and colleagues (Zullig and Hengartner 2004).

Our data showing that MFG-E8 added to a BioParticle phagocytosis assay with BV-2 increases phagocytosis also support our hypothesis that MFG-E8 is mediating microglial phagocytosis. Furthermore, MFG-E8 increases the phagocytosis of apoptotic SY5Y cells by BV-2 cells, and the dominant negative form of MFG-E8 inhibits phagocytosis in both of these assays. In the peripheral immune system, macrophages co-cultured with apoptotic cells show an increase of about 40% in the phagocytosis of PS expressing cells when MFG-E8 is added (Hanayama et al. 2002) similar in scale to the roughly 27% increase in phagocytosis that we report here. The involvement of endogenous MFG-E8 produced by BV-2 cells may account for the high basal levels of phagocytosis that we see in this assay, and therefore the addition of recombinant MFG-E8 yields a modest stimulation of phagocytic activity. In addition, masking the PS with D89E, the dominant negative form of the protein, inhibits peripheral phagocytosis by about 60% (Hanayama et al. 2002), the same magnitude of inhibition that we found in microglia. The addition of Gas6 to the Vybrant phagocytosis assay also increased phagocytosis by BV-2 cells, which reinforces the findings published by Grommes and colleagues. Interestingly, the combinatorial effect of Gas6 and MFG-E8 is an appropriate explanation as to why Grommes and colleagues were only able to show an increase in the uptake of beads and not of apoptotic cells. Our finding suggests both MFG-E8 and Gas6 are required for the efficient clearance of apoptotic material in the brain. Altogether, these data support the hypothesis that MFG-E8 binds apoptotic neurons and enables their clearance by microglia. Furthermore, a second step involving Gas6 assists in the engulfment by microglia. To provide further support for this hypothesis, future studies with microglia and apoptotic neurons in the presence of MFG-E8 and Gas6 will need to be conducted to confirm that the combination of these proteins elicits a greater amount of phagocytosis than each individually. Studies of the signaling process associated with the binding

of both MFG-E8 and Gas6 will indicate whether this combination is synergistic or additive. Also, because our results implicate fractalkine in stimulating MFG-E8 in microglia, the ability of fractalkine to stimulate phagocytosis of apoptotic neurons by microglia needs further examination.

6.4 Dysregulation of MFG-E8

Evidence suggests that abnormal levels of MFG-E8 play a role in the pathological process of several diseases. The first indication that deficiencies in MFG-E8 may lead to disease came from studies of a MFG-E8$^{(-/-)}$ knockout mouse (Hanayama et al. 2004). The tingible body macrophages of MFG-E8$^{-/-}$ mice are incapable of removing apoptotic cells in the germinal centers of secondary lymphoid tissues, which leads to an autoimmune syndrome that resembles systemic lupus erythematosus (SLE). Because humans with SLE also have impaired phagocytosis by tingible body macrophages, the MFG-E8$^{-/-}$ strain was considered to be a suitable mouse model of the disease. In addition, it was expected that serum levels of MFG-E8 in SLE patients would be lower than control subjects, but blood samples from SLE patients surprisingly had much higher serum MFG-E8 levels (Yamaguchi et al. 2008). Further experiments indicated that MFG-E8 increased phagocytosis in a concentration-dependent manner to a certain level, but at high concentrations MFG-E8 inhibited phagocytosis. We observe similar results in the pHrodo assay with BV-2 cells, where 100 ng/ml of recombinant MFG-E8 stimulates and 300 ng/ml inhibits phagocytosis. When considered in with our Gas6/MFG-E8 data in Chapter 4, these data may suggest that overproduction leads to the saturation of exposed PS on the surface of apoptotic cells. If this occurs, Gas6 would be inhibited from binding the apoptotic cell, and efficient removal of the apoptotic cell would be hindered. Figure 6.2 shows this idea.

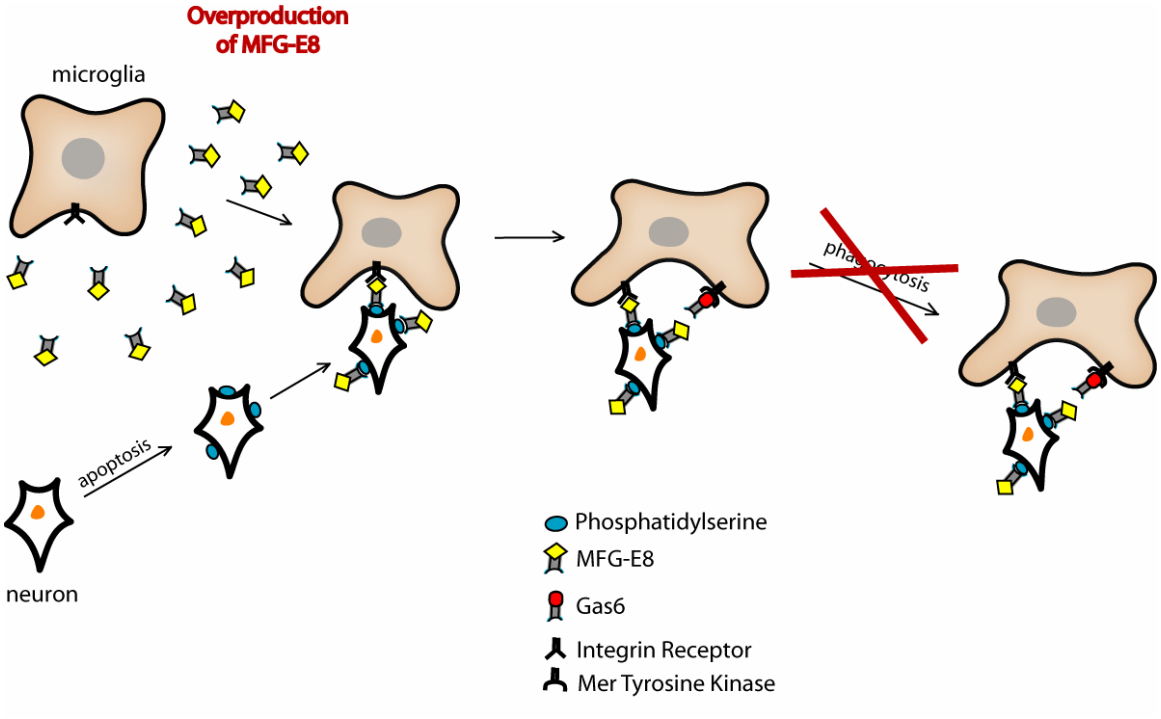


Figure 6.2 Modified model of MFG-E8/Gas6 mediated phagocytosis indicating the effect of overproduction of MFG-E8. Overproduction of MFG-E8, as is seen in some SLE patients, saturates the exposed PS on the surface of apoptotic neurons. This inhibits Gas6 from binding the apoptotic cell and thus inhibits efficient phagocytosis of the cell.

Another example of MFG-E8 dysregulation contributing to sickness is seen in sepsis (Miksa et al. 2006). Sepsis is characterized by a whole-body inflammatory response due to systemic infection, and apoptotic cell death in many cell types results. Cecal ligation and punction (CLP), a common animal model used to study sepsis, induced a 48% decrease in MFG-E8 production in the spleen and a 70% decrease in the liver. In addition, the endotoxin LPS which induces a sepsis-like endotoxemia decreased MFG-E8 production by RAW 267.4 macrophages by 38%. Intriguingly, the adoptive transfer of exosomes containing MFG-E8 induced a 2.8 fold increase in engulfment of apoptotic cells in the CLP model in rats. In addition, lower levels of TNF and IL-6 were reported, and survival increased from 44% to 81% (Miksa et al. 2006). Not only do these data show that a downregulation of MFG-E8 production can be initiated by a proinflammatory stimulus, but they also indicate that the resulting inflammatory response can be reversed by supplementing MFG-E8, a potential therapy that may be employed in other diseases as well.

In the peripheral immune system as well as in microglia (Chapter 3), data show that LPS, a toll-like receptor (TLR) agonist, inhibits MFG-E8 production by macrophages and dendritic cells (DCs) (Miyasaka et al. 2004). Dendritic cells are professional antigen-presenting cells (APCs) that stimulate B and T lymphocytes to modulate the immune response (Ardavin 2003; Banchereau and Steinman 1998; Shortman and Liu 2002). Undifferentiated progenitor cells proliferate in the blood but stop proliferating and become immature DCs once they enter peripheral tissues. Immature DCs recognize and engulf apoptotic cells, and they process and display foreign and self antigens as MHC-peptide complexes. At this point, immature DCs mature into APCs by upregulating coreceptors like CD11c, CD40, and CD86 and can then

activate B and T lymphocytes. Treatment of immature DCs with LPS stimulates their conversion to APCs, and concurrently MFG-E8 is downregulated resulting in a reduced ability to phagocytose apoptotic cells (Miyasaka et al. 2004). Because LPS also significantly reduces MFG-E8 production by BV-2 cells, it is likely that a similar method of MFG-E8 modulation occurs in microglia, and therefore proinflammatory stimuli could downregulate MFG-E8 production in the brain.

Our data showing that LPS inhibits MFG-E8 production in microglia cells support this idea. We report in Chapter 3 an LPS-induced decrease in MFG-E8 production by a microglial cell line, and in Chapter 4 we show LPS also reduces phagocytosis by BV-2 cells. It is therefore possible that downregulation of MFG-E8 could occur in diseases with associated neuroinflammation. A recent report shows MFG-E8 mRNA expression was decreased in AD brains as compared to age-matched controls, and less MFG-E8 was found in areas of abundant plaques. In addition, the study suggested an MFG-E8-dependent mechanism for phagocytosis of $A\beta_{1\text{-}42}$ (Boddaert et al. 2007). Similarly, we found lower levels of MFG-E8 expression in the Tg2576 mouse model of AD, a model which overexpresses the $APP_{(swe)}$ mutation (Chapter 5).

The finding of low levels of MFG-E8 protein in the Tg2576 mouse model of AD suggests that dysregulation of the protein is somehow involved in the pathological process of this disease. Previous research in this model shows that fractalkine is also inhibited in these mice at 9 months and returns to wild type levels at 11 months (Duan et al. 2008). Likewise, we have shown in Chapter 3 that fractalkine is capable of regulating MFG-E8 production in microglial cells.

However, the low levels of MFG-E8 protein detected in young Tg2576 mice suggest that something in this model is acting to suppress the protein product.

In AD, Aβ deposition stimulates a neuroinflammatory response by microglia causing them to become macrophage-like (Abbas et al. 2002; Dudal et al. 2004; Giovannini et al. 2002). In this state, microglia are characterized by CD11b presence and immunohistochemistry indicative of attempted phagocytosis of Aβ (Giovannini et al. 2002; Koenigsknecht and Landreth 2004; Monsonego et al. 2003; Sheng et al. 1997). However, the attack on Aβ plaques mounted by microglia is not sufficient for clearance of plaques and instead contributes to chronic progression of AD (Fiala et al. 2007; Streit 2002; Streit 2006). Chronically activated microglia in AD release neurotoxic cytokines, especially IL-1β and TNFα, leading to further neuronal deterioration (Akiyama et al. 2000; Griffin 2006; Wyss-Coray 2006). Recently, studies have shown that modulating the microglial response in AD from macrophage-like to a more dendritic phenotype so that they become APCs stimulates the adaptive immune response. The presence of MHC-II and CD11c indicates the transition to the adaptive immune response and coincides with a decrease in proinflammatory cytokines. Furthermore, these antigen presenting microglia are capable of phagocytosing Aβ and can prevent the formation and eliminate the presence of Aβ deposits in mice (Simard et al. 2006). The ability of newly differentiated microglia to downregulate neuroinflammation, present surface antigens, and become functionally phagocytic indicates that something in the AD brain is blocking this function in resident microglia. Intriguingly, the inability of microglia to clear Aβ resembles the effect of low levels of MFG-E8, which interacts with $A\beta_{1-42}$ in vitro. Reduced amounts of MFG-E8 have been shown near Aβ plaques in AD brains (Boddaert et al. 2007), which indicates a possible downregulation due

either directly by soluble Aβ or by proinflammatory cytokines such as TNFα stimulated by Aβ. This may also explain the proinflammatory state of surrounding microglia that are unable to phagocytose the plaque and suggests that the lack of inhibition of proinflammatory responses following MFG-E8 clearance of Aβ would lead to increased levels of IL-1β and TNFα.

Young Tg2576 mice have high levels of soluble Aβ, and cognitive deficits are seen in these mice before plaque deposition occurs (Lindner et al. 2006). It may be possible that soluble Aβ species, which have been shown to activate microglia, inhibit MFG-E8 production by a similar mechanism as LPS. As soluble Aβ accumulates into insoluble plaques in this animal model, a corresponding rise in MFG-E8 production is seen, suggesting a shift back toward normal MFG-E8 production or stimulation by apoptotic cells in the brain tissue. In order to address this directly, future experiments exploring the ability of different species of Aβ to inhibit MFG-E8 production by microglial cells and also microglial-mediated phagocytosis should be conducted.

Because we have shown that TNFα is capable of decreasing MFG-E8 production in BV-2 cells, we propose that blocking the production of TNFα in the AD brain using a small molecule inhibitor could produce higher levels of MFG-E8. Blocking neuroinflammation could release the inhibition of MFG-E8 and allow for the clearance of Aβ and apoptotic cells, essentially breaking the neuroinflammatory cycle seen in AD. Correspondingly, adaptive transfer of exosomes containing MFG-E8 could provide a similar result, based on the precedent in sepsis using a similar approach (Miksa et al. 2006).

6.5 Concluding remarks

In conclusion, the data reported in this dissertation have implicated MFG-E8 in the clearance of apoptotic material in the brain by microglia. In addition, our findings taken with current findings in the field suggest that MFG-E8 works in tandem with the Mer receptor tyrosine kinase agonist Gas6 to efficiently engulf apoptotic cells by microglia in the brain. Fractalkine, which is produced following neural insult, is a modulator of MFG-E8 production in a microglial cell line. This not only implicates dying neurons as a potential target for MFG-E8 action, but it also points to possible involvement of this protein in neurodegenerative diseases where fractalkine levels are altered, such as AD and HIV-1 associated dementia.

The finding that LPS, TNFα, and IFNγ significantly reduce levels of MFG-E8 production in BV-2 cells suggests a "maturation" of microglial cells that leads to a downregulation of phagocytosis and an upregulation of neuroinflammation. Our discovery of a reduction in MFG-E8 in the Tg2576 animal model of AD generates a new hypothesis that Aβ stimulation of microglia in AD leads to a inhibition of MFG-E8 production. It also raises the possibility of MFG-E8 as a potential drug discovery target. Further studies on the effect of inhibiting Aβ-induced neuroinflammation and TNFα specifically could show that a small molecule anti-neuroinflammatory drug could restore normal levels of MFG-E8 in the brain. Alternatively, MFG-E8 could be augmented in the brain by adoptive transfer of exosomes containing MFG-E8.

In the peripheral immune system, MFG-E8 aids in the phagocytosis of apoptotic T cells in order to downregulate the immune response. Microglia also phagocytose apoptotic T cells which downregulates the secretion of IL-12, IFNγ, and TNFα and also reduces microglial stimulation

of T cell expansion (Magnus et al. 2001). IFNβ is an agent used in multiple sclerosis (MS) and enhances the ability of microglia to phagocytose apoptotic T cells, resulting in a suppression of the immune response (Chan et al. 2003). Now that it is known that microglia also secrete MFG-E8, the mechanism described in this work and dysregulated in AD should also be investigated in MS.

The experiments described in this document represent the earliest studies of the function of MFG-E8 in the brain. The data successfully establish a role for MFG-E8 in the clearance of apoptotic cells in the brain, but the work here also effectively raises many more questions as to the extent that MFG-E8 works in the brain. Our data allude to the potential involvement of MFG-E8 in diseases involving a neuroinflammatory aspect, and our findings taken in light of other work in the field raise the possibility of MFG-E8 as a potential target for the development of therapeutics for these neurodegenerative diseases.

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NIH Ruth L. Kirschstein National Research Service Award Predoctoral Fellow. 2005-2008

PUBLICATIONS

AD Fuller and LJ Van Eldik. "MFG-E8 regulates microglial phagocytosis of apoptotic neurons." *Journal of Neuroimmune Pharmacology*. In press (2008).

AD Fuller and LJ Van Eldik. "The role of MFG-E8 in glial-neuronal interactions and neuroinflammation." *FASEB Journal*. 21(2007):785. Meeting Abstract

BL Moss, **AD Fuller**, CL Sahley, BD Burrell. "Serotonin modulates axo-axonal coupling between neurons critical for learning in the leech." *Journal of Neurophysiology*. 94(2005): 2575-2589.

PRESENTATIONS

November 2004	"The role of neuroinflammation in tau nitration," Poster Session, Northwestern University Drug Discovery Conference, Chicago, IL.
June 2005	"The role of glial-induced tau nitration in Alzheimer's disease," Cell and Molecular Biology Departmental Seminar, Northwestern University, Chicago, IL.
November 2005	"The role of glial-induced tau nitration in Alzheimer's disease," Poster Session, Northwestern University Center for Drug Discovery and Chemical Biology Symposium, Chicago, IL.
November 2006	"The role of MFG-E8 in glial-neuronal interactions," Poster Session, Northwestern University Center for Drug Discovery and Chemical Biology Symposium, Chicago, IL.
May 2007	"The microglial response to neuronal apoptosis and a role for MFG-E8 in the brain," Poster Session, Experimental Biology Annual Meeting, Washington, DC.
July 2008	"The microglial response to neuronal apoptosis and a role for MFG-E8 in the brain," Poster Session, International Conference on Alzheimer's Disease, Chicago, IL.

NATIONAL SCIENTIFIC MEETINGS

Society for Neuroscience Convention, San Diego, CA, 2004.
International BIO Conference, Chicago, IL, 2006.
Experimental Biology Annual Meeting, Washington, DC, 2007
International Conference on Alzheimer's Disease, Chicago, IL 2008

OTHER SKILLS AND ACHIEVEMENTS

InNUvation (Entrepreneurship and Innovation at Northwestern), www.InNUvation.org
Member of the founding Board of Directors, 2003-2005

- Helped to establish an interdisciplinary entrepreneurship and innovation forum for students of different professional backgrounds to network with the goal of developing new businesses.
- Organized seminars by professionals in the drug and device development industry.