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The role of complement anaphylatoxins in CNS pathology and glial cell function

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THE ROLE OF COMPLEMENT ANAPHYLATOXINS IN CNS PATHOLOGY AND GLIAL CELL FUNCTION

by

Sarah Ingersoll

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
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Thesis Supervisor: Senior Scientist Brian K. Martin

ABSTRACT

Demyelination in the CNS is known to involve several immune effector mechanisms, including complement proteins. For this dissertation project the central hypothesis that C3 and downstream effector complement proteins exacerbate demyelination through activation of glial cells was tested. To investigate the role of C3 and downstream complement proteins in demyelination and remyelination pathology *in vivo* the cuprizone model was utilized. Demyelination was induced in C3 knockout mice ($C3^{-/-}$), which are lacking the central C3 protein and subsequently all downstream complement effector proteins, and transgenic mice expressing C3a or C5a under the control of the astrocyte GFAP promoter. Interestingly, there were no changes in demyelination or remyelination pathology between $C3^{-/-}$ and control mice. However, C3a and C5a transgenic mice had exacerbated demyelination and delayed remyelination in the corpus callosum compared to WT mice. Transgenic mice had increased cellularity due to increased activation and/or migration of microglia. There was also evidence of T cells in the corpus callosum during demyelination in C5a transgenic mice, suggesting C5a may modulate BBB permeability. During early remyelination, oligodendrocytes migrated to the corpus callosum in higher numbers in C3a and C5a transgenic mice, thus enabling these mice to remyelinate as effectively as WT mice by the end of the ten week study.

To determine the effects of anaphylatoxins on individual glial subsets, murine recombinant C3a and C5a proteins were created. MAPK pathway proteins JNK1 and ERK1/2 were activated in glia upon stimulation with recombinant anaphylatoxin proteins. When microglia and mixed glial cultures were stimulated with C3a/C5a, an increase in the production of proinflammatory cytokines and chemokines was observed. In contrast, anaphylatoxin-treated primary astrocytes had suppressed cytokine and chemokine production compared to untreated astrocytes. In vitro, primary microglia and astrocytes did not significantly migrate in response to stimulation with C3a or C5a

proteins, suggesting migration may not be a primary anaphylatoxin-mediated function in the CNS. Overall, my findings show that anaphylatoxin production in the brain plays a negative proinflammatory role during demyelination and that anaphylatoxin proteins can activate individual subsets of glia, initiating the production of inflammatory mediators.

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Iowa City, Iowa

CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

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Demyelination in the CNS is known to involve several immune effector mechanisms, including complement proteins. For this dissertation project the central hypothesis that C3 and downstream effector complement proteins exacerbate demyelination through activation of glial cells was tested. To investigate the role of C3 and downstream complement proteins in demyelination and remyelination pathology *in vivo* the cuprizone model was utilized. Demyelination was induced in C3 knockout mice ($C3^{-/-}$), which are lacking the central C3 protein and subsequently all downstream complement effector proteins, and transgenic mice expressing C3a or C5a under the control of the astrocyte GFAP promoter. Interestingly, there were no changes in demyelination or remyelination pathology between $C3^{-/-}$ and control mice. However, C3a and C5a transgenic mice had exacerbated demyelination and delayed remyelination in the corpus callosum compared to WT mice. Transgenic mice had increased cellularity due to increased activation and/or migration of microglia. There was also evidence of T cells in the corpus callosum during demyelination in C5a transgenic mice, suggesting C5a may modulate BBB permeability. During early remyelination, oligodendrocytes migrated to the corpus callosum in higher numbers in C3a and C5a transgenic mice, thus enabling these mice to remyelinate as effectively as WT mice by the end of the ten week study.

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proteins, suggesting migration may not be a primary anaphylatoxin-mediated function in the CNS. Overall, my findings show that anaphylatoxin production in the brain plays a negative proinflammatory role during demyelination and that anaphylatoxin proteins can activate individual subsets of glia, initiating the production of inflammatory mediators.

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CHAPTER I
INTRODUCTION

Complement proteins have long been known for their role in mediating functions that protect the host from harmful pathogens; however excess complement activation can be detrimental to the host. Although the brain was once thought to be an immune privileged site, it has since been shown that complement production occurs at a local level within the brain and cells of the brain are able to express complement receptors and produce complement proteins. Thus, a compromised blood brain barrier (BBB) is not required for the presence of complement proteins in the brain. In recent years, it has been established that various complement proteins play a role in CNS disease pathology. Complement production in the brain has been shown to be protective or detrimental during disease depending on the disease model and local stimuli, making it difficult to determine the overall role of complement and to establish therapies for disease pathology. The goal of this project was to better understand the role of C3 and downstream complement proteins and their involvement during demyelination and remyelination in the brain and to dissect the role of complement signaling at a cellular level.

Overview of complement pathways

The complement system consists of more than 30 proteins found in the plasma and on cellular surfaces. These proteins act together to make up three well defined enzymatic cascades; including the classical, alternative and lectin pathways (1). Each of these pathways requires a specific initiation event to activate downstream complement proteins, however all three pathways converge at the level of the central C3 protein (Fig. 1). Briefly, the classical pathway becomes activated when C1q binds to antigen-antibody complexes (2). Whereas antigen-antibody complexes were previously thought to be the principal binding partner of C1q, it has since been shown that C1q can also bind certain myelin breakdown products (3), apoptotic cells and cell blebs (4), C-reactive protein (5)

and serum amyloid P protein (6, 7) to initiate the classical pathway. The lectin pathway is activated by the binding of mannose binding lectin, a C1q-like molecule, to repeating carbohydrate moieties found primarily on the surface of microbes (Fig. 1) (8).

The alternative pathway can be activated by more than one mechanism. First, contact with foreign organisms, such as lipopolysaccharide (LPS), zymosan, teichoic acid and various cell surfaces are able to activate the alternative pathway (9). Additionally, unlike the classical and lectin pathways, the alternative pathway is capable of autoactivation through the spontaneous "tickover" of C3 (Fig. 1) (10). C3 contains an unstable thioester bond and is subject to slow spontaneous hydrolysis to yield C3a and C3b. This allows for the binding of factor B to C3b and subsequent cleavage of factor B by factor D, leading to the formation of a C3 convertase. Formation of C3 convertase allows for the cleavage of more C3, resulting in additional C3b production. Additionally, fixed C3b that has been formed via activation of the lectin or classical pathways can bind factor B, resulting in the formation of even more C3 convertase (10). This process can result in the amplification of complement effector proteins, resulting in increased proinflammatory events and cell lysis.

In addition to the three complement pathways described above, recent studies have found evidence of another mechanism by which C3 and C5 proteins are cleaved. Previous studies showed that proteolytic enzymes involved in coagulation, such as thrombin, are able to cleave C3 and C5 in the absence of activation of any of the three complement pathways previously described (11, 12). This method of complement activation has been called the extrinsic protease pathway. Although each pathway is initiated in a different manner, the activation of each of these pathways results in the production of several complement effector proteins that are able to protect the host from invading pathogens.

C3 and downstream effector complement products

Regardless of which complement pathway is activated, the pathways converge at the level of C3 and C3 convertase (Fig. 1). At this stage, several effector complement proteins are formed upon the cleavage of C3 and downstream C5. First, the cleavage of C3 results in the formation of C3a and C3b. C3b and its degradation products are capable of binding to foreign surfaces including polysaccharides and glycoproteins such as LPS, gram positive techoic acids and viral membrane glycoproteins (13). This process is known as opsonization and it marks pathogens for phagocytosis by cells expressing receptors for C3b and iC3b fragments, including complement receptor 3 (CR3/CD11b) and CR4 (CD11c) (14). CR1 (CD35) also binds C4b, C3b and iC3b and has been implicated in engaging in the phagocytosis of targets opsonized with complement fragments (15). C3b also acts as a component of C3 and C5 convertases, thus supporting more cleavage of C3 and C5. Cleavage of C5 by C5 convertases result in the formation of C5a and C5b. C5b forms a complex with the terminal complement proteins of the complement cascade. C5b-C9 act in concert to form the membrane attack complex (MAC) (Fig. 1), which is capable of destroying potential pathogens by binding to and penetrating the membrane of foreign cells, causing cell lysis (16).

The cleavage products C3a, C4a and C5a are collectively known as anaphylatoxin proteins. These proteins bind to specific receptors on a wide variety of cell types to induce many effector functions. C5a is capable of regulating vasodilation, enhancing phagocytosis, mediating oxidative bursts and inducing proinflammatory cytokine production (9, 17). C5a and C3a are both able to induce vascular permeability, smooth muscle contraction and histamine release in basophils and mast cells (18-20). Additionally, both C3a and C5a have been described as being mediators of chemotaxis, or cellular migration (21). While several proinflammatory mechanisms have been assigned to C3a and C5a, it has also been shown that anaphylatoxins can promote growth or repair in certain environments. Therefore, previously established roles for C3a and/or

C5a are expanding and in the absence of foreign pathogens, anaphylatoxin production seems to aid in some normal tissue development and function (20).

Complement regulation

Complement activation is regulated at several steps along the cascade to prevent excess activation from occurring and to provide protection to the host. Without proper regulatory mechanisms in place, complement proteins can cause damage and even death to host cells. There are several regulatory proteins present throughout the body, both in the plasma and on cell membrane surfaces. Many membrane inhibitors are in place to inhibit excess formation of C3 convertase. Decay accelerating factor (DAF, CD55), inhibits the formation and accelerates decay of C3 convertases (22), thus regulating the production of anaphylatoxins and other downstream complement products. Membrane cofactor protein (MCP, CD46) acts as a cofactor for factor I-induced cleavage of C3b and C4b, preventing these proteins from forming C3 and C5 convertases (23). MCP is a common regulatory protein in humans, however, MCP has only been found in mouse testis (24). In addition to enhancing phagocytosis, CR1 acts as a decay accelerating factor for C3 and C5 convertases (25). Complement receptor-1 related gene/protein Y (Crry) is a regulatory protein that is unique to rodents. This protein acts as a C3 convertase inhibitor, exhibiting both decay accelerating and membrane cofactor functions (22).

Soluble complement regulatory proteins also play an important role in protecting host cells from harmful effects of activated complement proteins. C1 inhibitor regulates activation of the classical pathway by binding the serine proteases C1r and C1s, preventing the formation of the C1 complex (26). C1 inhibitor has also been shown to bind mannose-assoicated serine protease-1 (MASP-1) and MASP-2, leading to the inhibition of the lectin pathway (27). Factor I is able to regulate all three complement pathways by cleaving C3b or C4b with the aid of soluble and membrane bound

complement protein cofactors. Cofactors essential for Factor I mediated cleavage include soluble and membrane-bound CR1, MCP, Factor H and C4 binding protein (C4bp) (28). Factor H is a serum protein that is involved in dissociation of the alternative pathway C3 convertase by binding to C3b and also acts as a cofactor for factor I-mediated cleavage of C3b (29). C4bp binds C4b to aid in dissociation of the classical and lectin pathway C3 convertase. C4bp also acts as a cofactor for factor I-mediated cleavage of C4b (29).

Excess formation of C3a and C5a can induce harmful results in the host; therefore, in addition to membrane bound and soluble complement regulatory proteins, there are other mechanisms that inactivate these potent molecules. C3a and C5a are largely regulated by serum carboxypeptidases that rapidly cleave the carboxy-terminal arginine of C3a and C5a (20, 30), which is essential for mediating effector functions. The cleavage of arginine from C3a or C5a leads to the formation of C3adesArg and C5adesArg, respectively. Previous data have shown that C3adesArg loses the ability to bind to C3aR, thus inhibiting many proinflammatory functions that occur upon C3a-C3aR interaction (31). C5adesArg shows a 10 to 100-fold decrease in biological activity, but is still able to bind C5aR and mediate some functions, such as chemotaxis (30, 32).

Complement protein expression in the brain

The majority of complement proteins that are circulating throughout the body are produced in the liver by hepatocytes (16). However, over the years, several studies have found that complement proteins are produced by many different cell types in the body. Additionally, many cells have been found to express complement receptors on their cell surfaces, enabling them to respond to various complement proteins. The brain is protected from exposure to many molecules found in the plasma via an anatomical structure, the blood brain barrier (BBB). The BBB is formed by endothelial cells of microvessels, smooth muscle cells (also known as pericytes) and astrocytes (discussed below). The tight junctions of this structure provide strict limits for the types of

molecules and cells that can pass into the CNS (14). For this reason, the brain was thought to be an immune privileged site that was protected from exposure to complement and other proteins found in the circulatory system in the absence of BBB compromise. However, it has since been shown that many brain cells, or glia, are capable of producing nearly all of the complement proteins including complement regulatory proteins (33). In addition, local cells in the brain express complement receptors, suggesting glia are equipped to respond to complement proteins present within the CNS. Finally, there is some evidence to suggest that complement may be involved in normal CNS development and function in the absence of pathogens, BBB compromise or CNS damage (34, 35).

Microglia

Microglia are CNS-resident macrophages and make up 10-20% of cells in the brain (36). The origin of these cells has been a source of controversy for several years. Other glial cell types arise from neuroectodermal precursors, however microglial progenitors arise from peripheral mesodermal, or myeloid, tissue (37). Microglia express the myeloid transcription factor, PU.1, and share common surface markers with mononuclear phagocytes (38, 39). Several research groups have postulated that early in development, circulating peripheral monocyte precursors may cross the BBB to develop into immature microglia (39). However, this was a controversial topic in the past because other groups provided evidence that tissue-resident macrophages, including microglia, are derived from a lineage of myeloid cells that is separate from the monocyte lineage (37). A recent paper from Ginhoux et al. supports findings that microglia arise from primitive myeloid progenitor cells that arise early in embryonic development (40).

Resting microglia are characterized by long processes and low expression of surface antigens that are associated with macrophage activation (41). In the normal CNS it was found that the bodies of resting microglia remained relatively stationary; however, their long processes were highly motile (42). While resting microglia are not actively

involved in phagocytosis, they are constantly sampling the microenvironment around them, similar to resting dendritic cells (43). Several factors can cause resting microglia to become activated; including certain serum products that indicate to microglial cells that the BBB has been compromised. In addition, altered synaptic activity, impaired neural function and pathogens also activate microglia (39). Upon sensing danger signals, microglia quickly migrate to sights of damage or infection (41). Similar to peripheral macrophages, there are several cell surface markers that indicate microglial activation and the expression of these markers varies depending on the stimuli (44). Once activated, microglia are thought to be responsible for clearing cellular and myelin debris by phagocytosis (39, 45). The activation state of microglia can also be monitored by their ability to produce many immune modulating molecules including cytokines, chemokines, reactive oxygen species and complement proteins (39, 46).

Microglia are thought to be one of the main cell types, along with astrocytes, that are responsible for the production of complement in the brain. When primary human microglia were stimulated with various cytokines and amyloid β peptides, complement proteins, including C1q and C3, were shown to be upregulated (47). Additionally, activated microglia express complement receptors, including CR3 and CR4 which play an important role in phagocytosis (48). Finally, primary microglia and several monocyte cell lines have been shown to express C5aR and C3aR, the receptors for C5a and C3a, respectively (49, 50). When stimulated with various complement proteins, microglia exhibit many effector functions including upregulation of neurotrophins and increased migration capabilities (51, 52).

Astrocytes

Astrocytes are the most prevalent cell type in the brain and have a wide variety of roles that are critical for proper function of the normal CNS. Astrocytes are responsible for maintaining a suitable environment for nerve conduction, which includes providing

structural, metabolic and trophic support to surrounding nerves (53). Astrocytes (along with endothelial cells, pericytes and microglia) are also required for proper BBB structure. When the BBB becomes compromised, astrocytes can become reactive, a process known as astrogliosis. There are two main events that characterize astrogliosis. First, the processes on astrocytes hypertrophy and second, many intermediate filaments are upregulated, including glial fibrillary acidic protein (GFAP), vimentin, and nestin (54). Under inflammatory conditions, reactive astrocytes can upregulate production of proinflammatory cytokines and growth factors in response to certain stimuli (55). In some instances, astrocytes are capable of phagocytosis; however, they are not nearly as efficient in this role as microglia and it is thought phagocytosis by astrocytes may only occur as a second line of defense when microglia become overtaxed (56).

Astrocytes are capable of producing many complement proteins and expressing receptors for complement (33, 49). In studies examining complement production, astrocytes have been shown to produce all complement proteins involved with the alternative and classical complement pathways. Additionally, they express the complement receptors CR1, CR2, C5aR and C3aR (33, 57). Astrocytes upregulate complement protein production when they are stimulated with certain inflammatory factors, including LPS, interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (49, 58). In response to complement stimulation, astrocytes have been shown to migrate and upregulate mRNA expression of certain cytokines and growth factors (59-61).

Oligodendrocytes and neurons

Oligodendrocyte cells are responsible for producing myelin and maintaining myelin structure in the brain (62). Myelin surrounds nerves, which are comprised of neurons, and aids in conduction of electrochemical impulses. Like many cell types, the maturation status of oligodendrocytes can be determined by observing surface markers,

morphology and migratory ability (63). Oligodendrocyte progenitors are highly mobile and migrate throughout the CNS to areas that need to be replenished with mature oligodendrocytes (64). Once oligodendrocytes are able to produce myelin, they are referred to as mature cells. A single mature oligodendrocyte can be responsible for maintaining up to 40 internodes of myelin and because of the high metabolic demand placed on oligodendrocytes, these cells are thought to be particularly vulnerable to injury and subsequent apoptosis (65). Human oligodendrocytes isolated from patients that had undergone surgery for temporal lobe resection for epilepsy were positive for complement protein synthesis (66) and the expression of complement receptors on oligodendrocytes was previously demonstrated (33, 67). Interestingly, neurons have also been shown to express receptors for both C3a and C5a (68, 69), in addition to producing nearly all complement proteins of the classical and alternative pathways (33).

Overall, many studies have shown that glial cell subsets are capable of expressing complement proteins, receptors and regulatory proteins (33). The ability of glia to produce complement regulatory proteins suggests these cells are most likely protected from the harmful effects of downstream complement effectors. Most of the experiments used to determine if glia produce complement proteins were performed *in vitro* using various stimuli to treat glia or alternatively, complement production was found, *in vivo*, in infectious or disease settings. However, in the normal CNS complement mRNA expression was low or non-detectable in control patients when compared to patients with Alzheimer's disease (AD) (70). Thus, in response to local insult and stimuli, glial cells provide an endogenous source of complement components in the CNS.

The role of complement in diseases of the CNS

Since the discovery of complement receptor expression on glial cells and complement production by glia, several groups have worked to determine the role complement proteins play in disease, trauma and infections in the CNS. Many of these

studies have utilized animal models deficient in certain complement proteins or complement receptors and transgenic mice, in addition to human studies. From these studies, there is strong evidence to support the conclusion that complement does indeed play a role in diseases of the CNS. In some disease models it is difficult to assess the overall role of complement, because some proteins play a protective role, while others exacerbate disease pathology.

There have been several studies implicating complement involvement in neurodegenerative diseases. The role of complement in AD has received much attention in recent studies. In brain sections from AD patients, C1q, C3 and C4 proteins were found in senile plaques and mRNA levels of C1 and C9 were increased compared to controls (71). In corresponding studies using a murine model of AD, pathology was attenuated in the presence of a C5aR antagonist, suggesting C5a plays a harmful role in AD pathology (72). Interestingly, another study found that when a soluble form of Crry (sCrry) was expressed, plaque formation was increased and neurodegeneration was worsened (73), suggesting that some complement activation is beneficial to AD pathology and may be necessary for clearance of debris. Overall, complement plays opposing roles in AD, demonstrating the dual nature of complement in diseases of the CNS.

A role for complement has also been suggested for other neurodegenerative diseases. C3a and/or C5a proteins exacerbated disease severity in murine models of amyotrophic lateral sclerosis (ALS) (74) and CNS lupus (75, 76). Complement protein expression was also shown to be upregulated in patients suffering from Huntington's and Parkinson's disease (71), suggesting a potential role for complement in these diseases. Finally, several studies have suggested that complement plays a role in multiple sclerosis (MS), an autoimmune disease that affects over two million people worldwide and is a major cause of disability in young adults (77, 78).

Multiple sclerosis (MS)

MS is a chronic, inflammatory disease of the CNS that targets the myelin sheath that surrounds nerves and myelin-producing oligodendrocytes, resulting in faulty nerve conduction (79). MS pathology is characterized by multifocal lesions in the CNS, demyelination, oligodendrocyte death, disruption of the BBB, infiltration of microglia/macrophages and T cells, axonal damage and astrogliosis, which all contribute to neurological disability (80, 81). The initial event that causes MS has not been clearly identified and disease prognosis varies significantly from patient to patient. MS is a complex disease that involves several cell types and proinflammatory molecules that mediate damage, making it difficult to provide a therapy that addresses all of the mechanisms involved in disease.

MS lesion types and demyelination

Demyelination is a process that involves destruction of the myelin sheath which surrounds nerves. In MS, BBB breakdown corresponds with lesion formation, thus many cell types are able to pass into the CNS to exacerbate inflammation and myelin destruction (80). Several effector cells and molecules have been identified within demyelinating lesions of MS patients and the requirement for these effectors in the pathogenesis of lesions is fairly well understood. In acute lesions, it is thought that $CD4^+$ T cells play an important role in initiating pathology (82). Additionally, HLA class II molecules have been positively associated with genetic risk for MS (82). Previous studies have shown $CD8^+$ T cells also play a prominent role in perpetuating the inflammatory response in lesions (80). Macrophages, microglia, dendritic cells and B cells have also been shown to contribute to lesion pathology in MS (83, 84). Along with effector cells, several proinflammatory molecules have been implicated in disease; including several chemokines, cytokines, perforin, proteolytic enzymes, reactive oxygen species and complement proteins (84).

While the cells necessary for lesion formation are fairly well understood, the exact mechanisms involved in myelin damage remain elusive. Because of the heterogenous nature of lesions in patients suffering from MS, myelin damage is not mediated in exactly the same way in each person. Luccinetti et al. described four primary demyelinating lesion types in MS patients. One common characteristic between these lesion types was the predominance of infiltrating T cells and macrophages/microglia into the inflammatory lesions (85). Briefly, type I and II lesions were centered on veins and venules. Type I lesions lacked complement and antibodies, suggesting proinflammatory effector molecules produced by macrophages, such as TNF-α and IFN-γ, may be the cause of damage to the myelin sheaths (85, 86). Type II lesions were defined by the presence of antibody and complement, in addition to large numbers of macrophages and T cells. Damage to myelin in these lesions most likely occurred due to complement-mediated lysis of myelin sheaths that were bound by anti-myelin antibodies. Type III and IV lesions were characterized by oligodendrocyte apoptosis. These lesions lacked complement deposition and the presence of antibodies and they were also characterized by high numbers of T cells and macrophages. Type II and III were the most common lesions found in MS patients, while type IV was found to make up <1% of active lesions (85).

Remyelination

In early stages of MS, most patients are diagnosed with a relapsing/remitting form of MS, in which demyelinated axons are eventually remyelinated by oligodendrocytes, thus leading to a period of remission (80). As the disease progresses, remyelination occurs less efficiently or not at all. This form of the disease is known as secondary progressive disease and accounts for many of the irreversible debilitating symptoms observed in long-term MS patients (79). Similar to the different lesion subtypes found in patients, the ability to remyelinate differs widely from patient to patient depending on

how far the disease has progressed, the extent of damage to oligodendrocytes and axons, as well as other factors. Myelin protects axons and in its absence, damage to axons may occur through association with inflammatory cytokines and/or other harmful mediators (79, 87). Therefore, it is hypothesized that in the absence of remyelination, damage to axons ultimately leads to the irreversible disability that is associated with later stages of MS.

The factors that dictate remyelination are complex and are not completely understood. Remyelination involves migration of oligodendrocyte precursors to areas of demyelination, adhesion of oligodendrocyte processes to the axon and spiraling of the oligodendrocyte processes around bare axons (63). Remyelination results in a thinner myelin sheath than the original sheath, so remyelinated plaques are sometimes called "shadow plaques", due to the difference in myelin thickness when compared to normal myelinated regions (80). The effectiveness of remyelination depends on the extent of damage and/or death to preexisting oligodendrocytes and the ability of oligodendrocyte precursors to migrate to the active lesion. Several trophic factors and cytokines have been implicated in the process of maturing precursor cells into the mature myelin-producing oligodendrocytes necessary for remyelination (84). Other groups have postulated that inflammation is ultimately necessary for oligodendrocyte precursors to migrate and mature within active lesions to remyelinate damaged areas (88). In active lesions, myelin-producing oligodendrocytes can be visualized in proximity to T cells and macrophages ingesting myelin, suggesting that demyelination and remyelination occur simultaneously within some lesions (89). In later, chronic stages of MS, oligodendrocytes are present within the lesion, but are sometimes incapable of producing myelin for unknown reasons. Future studies are required to fully understand the mechanisms that are necessary for oligodendrocyte precursor migration, maturation and what factors are necessary to initiate the remyelination of demyelinated axons.

Animal models of MS

There are several animal experimental models that share aspects of pathology with the different lesion types found in MS patients. While these animal models all mimic some aspect of disease, there is not one perfect model that completely captures all the events that occur in human disease (Table 1). The most widely used animal model for studying events that occur in MS, has been the experimental autoimmune encephalomyelitis (EAE) model. EAE is induced in animals by injection with different myelin proteins, in addition to an adjuvant, or by passive transfer of myelin antigen-stimulated lymphocytes (90). These methods of treatment result in an immune-mediated attack on the myelin sheaths surrounding neurons and mature myelin-producing oligodendrocytes, resulting in demyelinating lesions. These lesions are characterized by infiltrating T cells and macrophages, sharing some similar characteristics to acute MS lesions (91).

In addition to the EAE model, there are several other animal experimental systems that are used to analyze different aspects of MS pathology (Table 1). Several research groups hypothesize that viral infection triggers the onset of MS. To model this hypothesis, various viruses are used to induce demyelination in several animal models, including Theiler's murine encephalomyelitis (TMEV), murine hepatitis virus (MHV) and Semliki Forest virus (SFV) (90). There are also several "toxic demyelination models", including; the lysolecithin, ethidium bromide and cuprizone models, in which a neurotoxic compound is given to induce demyelination (91). One reason many investigators utilize toxic demyelination models is the ability to investigate the biology of remyelination. Because the experiments for my dissertation utilized the cuprizone model, this specific model will be discussed in more depth in the Introduction of Chapter II.

Evidence of complement involvement in MS and animal models of demyelination

There has been some evidence to suggest that complement plays a role in human MS, however results from human patients are complex and further studies are needed to clarify the precise role of complement involvement in this disease. There have been several studies over the years that examined various complement components in the blood and cerebral spinal fluid (CSF) of some MS patients (78). Several groups have tried to determine if certain complement products could be used as a biological marker for disease, but these studies have been contradictory and inconclusive (78). Previous studies have shown that receptors for anaphylatoxins, C3aR and C5aR, are upregulated in areas of inflammation in the CNS of some MS patients (50, 92, 93). Additionally, complement deposition has been shown in certain lesion types (85). Finally, in a study analyzing newly formed MS lesions, macrophages were shown to contain the opsonization target, C3d, and C9neo in acute MS plaques (94), suggesting complement may play a role in clearance of myelin debris and apoptotic cells during demyelination. While the precise mechanisms that individual complement proteins play in MS remain unclear for human demyelination and remyelination pathology, there have been several studies showing complement involvement in animal models of demyelination.

The most widely used animal model to study complement involvement in demyelination has been the EAE model. Results from studies employing the EAE model showed that several complement proteins contribute to the pathogenesis of demyelination. An EAE study using cobra venom factor (CVF) to deplete complement activation showed that complement was necessary for the proliferation of MOG-specific $CD4^+$ T cells (95). When mice were transiently treated with CVF, disease pathology was attenuated and disease onset was delayed. C3 knockout mice were shown to have less disease severity and delayed onset (96). Finally, when sCrry was overexpressed, EAE severity was decreased compared to control mice (97). A separate study also showed that

DAF knockout mice had worsened disease severity than control mice (98). Collectively, these studies all suggest C3 and downstream complement proteins play a role in EAE severity and progression.

Studies that have analyzed individual complement proteins and/or complement receptors have also determined that certain complement proteins exacerbated disease severity. For instance, C3a and C3aR were shown to play a role in exacerbating disease severity (99); however C5a and C5aR did not play a role in EAE pathology (100, 101). Mice that were deficient in CR3 had attenuated disease compared to control mice, with less disease severity and later disease onset, suggesting this complement receptor is critical for EAE pathology (102). Overall, studies in which the EAE model was utilized would suggest that the complement proteins play an exacerbating role in demyelination severity and disease onset.

In addition to *in vivo* animal studies, there have been several *in vitro* studies demonstrating that complement may play a role in the pathobiology of demyelination. For instance, *in vitro* studies have shown that C1q is able to bind to myelin to initiate classical pathway activation (103). In studies using myelinated CNS explant cultures, anti-myelin antibodies and fresh serum induced severe demyelination (104). Additionally, using the same technique, it was shown that C5b-9 was necessary for demyelination *in vitro*, because in the presence of C8 depleted serum, demyelination failed to occur (105). Many of the *in vivo* and *in vitro* studies suggest that complement plays a proinflammatory role leading to worsened demyelination or inflammation. However, there are some studies that indicate some complement proteins may play a protective role in disease. For example, sublytic levels C5b-9 protected oligodendrocytes by inhibiting apoptosis, thus assigning a protective role to complement in demyelination pathology (106).

Rationale

Since all glial cells are able to produce complement proteins, it is possible for localized activation of complement pathways to occur in the CNS without the BBB being breached (86). There have been several studies in human patients and in the EAE model that imply complement plays a significant role in disease. In MS and EAE the BBB is compromised and a wide variety of immune cells are able to cross into the CNS. While these studies have been important for understanding the role of complement in disease, it does not provide clear evidence for how complement proteins affect cells of the brain in the absence of BBB breakdown.

To determine the effect of complement on glial cells during demyelination I employed the murine cuprizone model, in which the BBB remains essentially intact, in order to assess the role of different complement factors on glial cells during demyelination and subsequent remyelination. Previous studies from our lab using the neurotoxicant cuprizone model have provided evidence that complement plays a role in demyelination pathology in this model. When sCrry was expressed on an astrocyte-specific promoter, mice were protected from demyelination and had reduced disease severity compared to wild-type mice (107). These data suggest that C3 and downstream proteins play a role in exacerbating demyelination in the cuprizone model. Therefore, for this research dissertation I proposed the following hypotheses to test to determine the roles of complement in CNS demyelination, remyelination and glial cell activation.

Hypothesis

There have been several studies suggesting complement plays a role in demyelination and remyelination events in the CNS, however the effect of complement proteins on glial cells in the absence of BBB breakdown remains uncertain. The goal of this thesis project was to determine the role of C3 and downstream C3a and C5a on demyelination and remyelination pathology in the murine cuprizone model.

Additionally, we wanted to establish the biological functions of C3a and/or C5a signaling on individual types of glia. The central hypothesis of this dissertation project was that C3 and downstream effector complement proteins would exacerbate demyelination through activation of glial cells. The central hypothesis was broken down into two specific research aims.

The first research aim tested the hypothesis that C3 and the production of downstream complement effector proteins, including C3a and C5a, would exacerbate demyelination pathology through the recruitment and/or activation of inflammatory cells. Additionally, it was hypothesized that an increased production of C3a and C5a proteins would lead to an enhanced and more rapid remyelination, through increased recruitment of oligodendrocyte precursor cells to sites of demyelination. The second research aim tested the hypothesis that C3a and/or C5a would activate signal transduction pathways and mediate effector functions, including chemotaxis and proinflammatory cytokine and chemokine production in glial cells. We also predicted that activation of signal transduction pathways would be differentially regulated in individual types of glia including, astrocytes and microglia.

Figure 1. An overview of the complement pathways

The classical, alternative and lectin complement pathways converge at C3 to form downstream effector proteins.

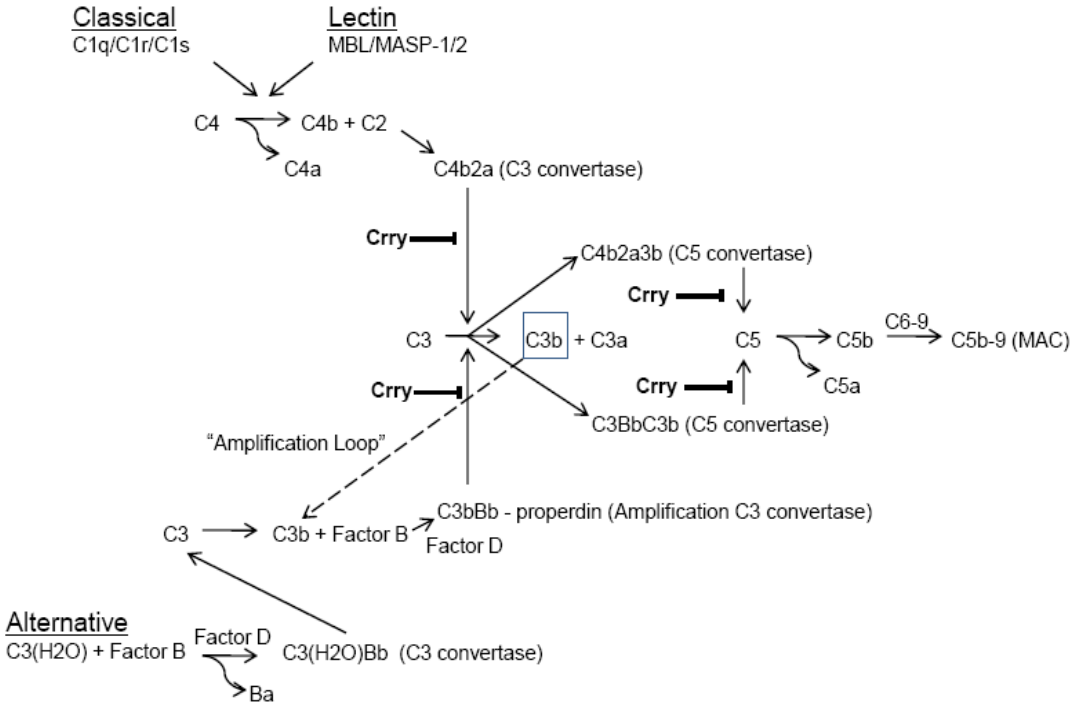

Classical
C1q/C1r/C1s
Lectin
MBL/MASP-1/2
C4
C4b + C2
C4a
C4b2a (C3 convertase)
Crry
C4b2a3b (C5 convertase)
Crry
C3
C3b + C3a
C5
C5b
C6-9
C5b-9 (MAC)
C5a
Crry
Crry
C3BbC3b (C5 convertase)
"Amplification Loop"
C3bBb - properdin (Amplification C3 convertase)
C3
C3b + Factor B
Factor D
Alternative
C3(H2O) + Factor B
Factor D
C3(H2O)Bb (C3 convertase)
Ba

Table 1. Advantages and disadvantages of murine demyelination models

	Advantages	Disadvantages
EAE	• Overall lesion cellularity similar to MS • Different EAE induction models mimic different stages of MS • Primary model used to test potential human drug targets • Immune response is myelin-specific • Symptoms mimic clinical manifestations in MS patients	• Successful EAE therapies do not always work in human patients • EAE lesions dependent on $CD4^+$ T cells, while $CD8^+$ T cells play a prominent role in MS lesion pathology
Virus-induced	• MS may have viral etiology • Used to study molecular mimicry and epitope spreading • Ability to study potential viral targets for induction of demyelination • Immune-mediated demyelination similar to MS	• Human to mouse virus correlation; some viruses used in these models do not cause disease in humans • More difficult to study remyelination • Certain viruses only induce demyelination in certain strains of mice
Cuprizone	• Reproducible kinetics • Same area of the brain affected upon treatment • Intact BBB • Reversible demyelination • Share some similarity to type III and IV lesions in MS	• Strain/species dependent • Less T cells and peripheral macrophages; does not mimic typical MS lesion cellularity • Demyelination is not immune-mediated like MS

CHAPTER II

THE ROLE OF C3 AND DOWNSTREAM COMPLEMENT PROTEINS IN DEMYELINATION AND REMYELINATION

Abstract

Demyelination in the CNS is known to involve several immune effector mechanisms, including complement proteins. To determine the effect of local complement production in the CNS on demyelination and remyelination pathology, I utilized C3 knockout mice ($C3^{-/-}$), which are lacking the central C3 protein. In addition to C3 deficiency, these mice lack activation of downstream complement effector proteins. To investigate the role of C3 and downstream complement in demyelination and remyelination pathology I utilized the cuprizone model. $C3^{-/-}$ and C3 heterozygous littermates ($C3^{+/-}$) were placed on the cuprizone diet to induce demyelination. Interestingly, no changes in demyelination severity were found between $C3^{-/-}$ and $C3^{+/-}$ mice. After cuprizone was removed from the diet, remyelination kinetics remained unchanged in $C3^{-/-}$ when compared to $C3^{+/-}$ mice. Overall cellularity, microglial infiltration and oligodendrocyte loss were not significantly altered from levels found in $C3^{+/-}$ mice. These results suggest that C3 is not relevant in the cuprizone model, which contradicts previous observations from our lab that production of sCrry in the CNS protects mice from demyelination during cuprizone exposure and previous results from studies utilizing the EAE model. Alternatively, C3 deficiency prevents both positive and negative effects of complement activation from occurring, thus negating any observable effects in this mouse strain. Further investigation into individual complement proteins will be needed to assess the role of complement on cuprizone demyelination and remyelination processes.

Introduction and rationale

It has previously been established that complement receptors are upregulated in some patients with MS (50, 93) and complement deposition is present within MS lesions (78, 85). In two separate studies utilizing the EAE model it was shown that when the central C3 complement protein was knocked out, disease was attenuated and onset was delayed (96, 108). These results suggest that C3 and downstream complement proteins may be involved in demyelination pathology in both MS and EAE. The EAE model is characterized by increased BBB permeability, so infiltrating immune cells and autoantibodies from outside the CNS are able to enter into areas of demyelination and exacerbate pathology (109, 110). Previous studies have shown that $CD4^+$ T cells play a crucial role in both EAE disease onset and severity (111-113). To better understand the localized effect of complement on glial cells, we utilized the neurotoxic cuprizone model of demyelination. One attractive aspect of the cuprizone model is the relative lack of BBB breakdown (114, 115), which makes it possible to analyze the local response of glial cells to different variables, including complement proteins.

Cuprizone is a copper chelating drug that when given at low doses in the diet of C57BL/6 mice, leads to apoptosis of oligodendrocytes, the myelin producing cells of the brain (Fig. 2) (116). The mechanism that makes oligodendrocytes specifically susceptible to cuprizone is unknown, but it is hypothesized that the high metabolic demand put upon oligodendrocytes to produce large amounts of myelin leaves them vulnerable to apoptosis in the absence of copper (117). It is also thought that copper may be necessary for mitochondrial function and energy metabolism in oligodendrocytes, since cytochrome oxidase, an important molecule for mitochondrial function, is decreased in cuprizone-treated mice (118). The death of mature oligodendrocytes results in demyelination in predictable, specific axonal tracts of the brain, including the corpus callosum, cerebellum and the superior cerebellar peduncle (117, 119). Demyelination in this model shares some characteristics of type III and IV lesions described in MS,

characterized by oligodendrocyte death, microglial infiltration and lack of antibodies present in areas of demyelination (85).

Another positive attribute of this model is that once cuprizone is removed from the diet, remyelination occurs in demyelinated areas with predictable kinetics (Fig. 2) (107, 117). The most common region of the brain studied during cuprizone treatment is the corpus callosum, a heavily myelinated tract in the brain. The primary effector cells in the demyelinating corpus callosum during treatment with cuprizone are reactive astrocytes and microglia, which migrate and/or proliferate in the corpus callosum throughout treatment with cuprizone (Fig. 2) (120). Several effector molecules have been shown to exacerbate demyelination in the cuprizone model; including lymphotoxin-α (121), neuronal nitric oxide synthase (nNOS) (122) and macrophage-inflammatory protein-1α (MIP-1α) (123).

Previous cuprizone studies have suggested that complement plays a role in demyelination. Arnett et al. showed that five complement genes were upregulated during peak demyelination, suggesting complement may be involved in pathogenesis that occurs during cuprizone treatment (124). When RNA samples from untreated mice and cuprizone-treated mice that were at peak demyelination were analyzed by gene chip analysis, *C1qA, C1qB, C1qC, C3a receptor* and *C4* were upregulated in the corpus callosum of cuprizone treated mice (124). Results from our lab also suggest that complement plays a role in the murine cuprizone model. When astrocyte-specific sCrry was expressed in the CNS, mice were protected from demyelination during cuprizone treatment, implicating a role for C3 and downstream complement proteins in this model (Fig. 3) (107). To determine the effect of C3 and downstream complement proteins on demyelination and remyelination pathogenesis, mice deficient in C3 and C3 sufficient mice were treated with cuprizone.

Hypothesis

Our lab has previously shown that if regulatory complement protein expression is upregulated during cuprizone treatment, mice do not undergo demyelination, implicating complement in demyelination pathology. Therefore, I hypothesized that C3 and downstream complement proteins would play a deleterious role during demyelination through harmful effects on oligodendrocytes/myelin proteins and increased inflammation in areas of demyelination. In addition, I predicted that complement would be necessary for effective remyelination to occur via increased clearance of apoptotic cells and myelin debris by microglial cells.

Materials and methods

Mice

Previously described $C3^{-/-}$ (125) and C57BL/6 mice were purchased from The Jackson Laboratories (Bar Harbor, ME). $C3^{-/-}$ mice were crossed more than 10 generations to the C57BL/6 background. Age-matched $C3^{+/-}$ littermates were used as the complement sufficient controls in cuprizone experiments. $C3^{-/-}$ and $C3^{+/-}$ were differentiated by RT-PCR using genomic DNA isolated from mouse tails. The Institutional Animal Care and Use Committee (IACUC) of the University of Iowa approved all mouse experiments.

Induction of demyelination and remyelination

To induce demyelination, 8–12 week old male mice were fed a diet of NIH-31 Modified 6% Mouse/Rat Sterilizable Diet-meal form (Harlan Laboratories, Madison, WI), containing 0.2% cuprizone (bis (cyclohexanone) oxaldihydrazone, Sigma-Aldrich, St. Louis, MO) for six weeks. Mice were returned to a normal diet after six weeks of cuprizone treatment to allow for remyelination (107, 117, 126).

Histological analysis

Tissue processing and microscopy were carried out as previously described (107, 127). Briefly, mice were anesthetized and transcardially perfused with PBS, followed by 4% paraformaldehyde. Brains were then removed and incubated in 4% paraformaldehyde for seven days and embedded in paraffin. Five micrometer coronal sections corresponding to Sidman sections 241-251 (128) were used for histological analyses. All demyelination and remyelination studies using Luxol Fast Blue-Periodic acid Schiff (LFB-PAS) were done at midline of the corpus callosum. A demyelination severity score of zero indicates no demyelination, while a score of three indicates complete demyelination. Each section was scored blindly by three different individuals and the scores averaged for each mouse.

Immunofluorescence

For immunohistochemistry, paraffin sections were rehydrated, heat-unmasked, and then blocked with a solution containing 5% normal goat serum and 0.1% Triton X-100 (Sigma-Aldrich) in PBS. Slides were incubated overnight with primary antibody diluted in blocking solution. Appropriate fluorochrome-labeled secondary antibodies (Invitrogen-Molecular Probes, Carlsbad, CA) were used for detection. The immunohistochemistry antibodies used were: Biotinylated *Ricinus communis* agglutinin-1 (RCA-1) (Vector Laboratories, Burlingame, CA; 1:100), a lectin used to detect microglia/macrophages; the secondary reagent used was streptavidin-Alexa Fluor-594; GFAP was detected using anti-GFAP antibody (Dako, Carpinteria, CA; 1:100); the secondary antibody was goat anti-rabbit Alexa Fluor-488; antibody to the Olig-2 transcription factor (129), a gift from C. D. Stiles (Harvard University), was used at 1:10,000 to detect total oligodendrocytes; the secondary reagent was goat anti-rabbit Alexa Fluor-488. For studies comparing different treatments, all sections were stained and digitally captured by microscopy at the same time. All of the brain sections were

analyzed at midline of the corpus callosum. All images were analyzed using ImageJ (http://rsbweb.nih.gov/ij/). Manipulations of the digital images after capture were performed equally on all pictures.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay

Paraffin-embedded sections were deparaffinized in Safeclear (Fisher Scientific, Pittsburgh, PA) and hydrated step-wise in ethanol. Sections were boiled in citrate buffer and allowed to cool. Sections were then washed in PBS-Tween-20 and pre-incubated in terminal deoxynucleotidyl transferase (Tdt) (Roche Diagnostics, Indianapolis, IN) reaction mix for 2 hours. Sections were washed in PBS-Tween-20 and incubated in PBS containing a 1:100 dilution of streptavidin conjugated to Alexa fluor-488. After washing, the sections were mounted in Vectashield containing diamidino-2-phenylindole (DAPI) (Vector Labs, Burlingame, CA). All sections were stained and analyzed at the same time. Any manipulations of the digital images after capture were performed equally on all captured pictures.

Statistics

Student's unpaired *t*-test (two-tailed distribution, homoscedastic, Microsoft Excel) was used to examine the probability that differences between treatment groups were statistically significant.

Results

The absence of C3 does not affect demyelination severity

Previous studies from our lab indicated that complement was active in the corpus callosum during cuprizone-induced demyelination (Fig. 3) (107). To verify these results demyelination was induced in $C3^{-/-}$ and $C3^{+/-}$ mice by feeding them a diet of 0.2% cuprizone for six weeks. To determine demyelination severity in the corpus callosum,

paraffin-embedded brain sections were stained with LFB to detect myelin. Interestingly, overall demyelination was not affected in the absence of C3 (Fig. 4). There was a slight delay in demyelination in the $C3^{-/-}$ mice after 3 weeks of cuprizone treatment, however these results were not significant ($p = 0.08$). Additionally, there were no significant changes in remyelination kinetics between $C3^{-/-}$ and $C3^{+/-}$ mice (Fig. 4). In contrast to previous results produced in our lab using transgenic sCrry mice, these results suggest that C3 and downstream complement proteins are not necessary for demyelination. An alternative hypothesis is that C3 deficiency prevents both positive and negative effects of complement activation from occurring during cuprizone treatment, thus negating any observable effect on demyelination severity.

Cellularity during cuprizone treatment is not dependent on C3 protein and downstream complement proteins

Although there were no significant changes in demyelination severity between $C3^{-/-}$ and $C3^{+/-}$ mice, I wanted to determine whether C3 was necessary for migration and/or activation of certain glial cells in the corpus callosum. First, DAPI nuclear stain was used to determine the total number of cells within the corpus callosum of the treated mice. It was predicted there would be more cells present in the corpus callosum of the $C3^{+/-}$ compared to the $C3^{-/-}$ mice, because certain downstream complement proteins, specifically C3a and C5a, are well known chemotactic agents. Upon quantification of cells in the corpus callosum, there were no significant changes in overall cellularity between $C3^{-/-}$ and $C3^{+/-}$ mice during demyelination or remyelination (Fig. 5A and 5B). These findings corroborate the demyelination severity data, in that there are no significant changes between mice that are able to produce C3 and mice that lack C3.

The presence of microglia in the corpus callosum during cuprizone treatment is not dependent upon C3

Microglial infiltration in the corpus callosum during demyelination is a well-characterized event that occurs during cuprizone treatment (117). Immunofluorescence was used to determine if C3 deficiency affects the presence of microglia in the corpus callosum of cuprizone treated mice. Microglia were detected in the corpus callosum using an RCA lectin stain. Similar to the demyelination severity and total cell numbers, there were no significant changes between $C3^{-/-}$ and $C3^{+/-}$ mice. The fluorescent intensity of the RCA increased throughout six weeks of cuprizone treatment in both groups (Fig. 6A), suggesting that C3 and downstream complement proteins are not necessary for the migration and/or proliferation of microglia during cuprizone treatment. At five weeks of cuprizone treatment, both groups had elevated microglial staining compared to the untreated control (Fig. 6A and 6B). After removal of cuprizone from the diet, RCA staining levels decreased in the corpus callosum of both groups (Fig. 6A).

Oligodendrocyte numbers and apoptosis levels are not altered in the presence of C3

It has previously been shown that cuprizone treatment leads to oligodendrocyte apoptosis in the corpus callosum beginning two weeks after initiation of cuprizone treatment (116). Oligodendrocyte progenitors begin to increase in the corpus callosum at four weeks, which is why some remyelination occurs before removal of cuprizone from the diet. However, mature oligodendrocytes do not return to levels found in untreated mice until eight to ten weeks of a typical cuprizone study (116). I wanted to determine the effect of complement on oligodendrocyte numbers in the corpus callosum throughout demyelination and remyelination. An antibody against an oligodendrocyte transcription factor, Olig-2, was used to detect both immature and mature oligodendrocytes. The number of oligodendrocytes in the corpus callosum were quantified and again, there were

no significant changes between $C3^{-/-}$ and $C3^{+/-}$ mice (Fig. 7). Both groups of mice had significant loss of oligodendrocytes beginning at week four of cuprizone treatment compared to the untreated control mice (Fig. 7A-C). Additionally, both groups of mice had increases in oligodendrocyte numbers once cuprizone was removed from the diet.

Previous data have shown that terminal complement proteins, C5b-9, are able to protect oligodendrocytes from apoptosis (130). To determine if there are fewer apoptotic cells in the presence of C3 and downstream complement proteins, a TUNEL detection assay was used to identify the number of apoptotic cells in the corpus callosum of cuprizone treated mice. TUNEL-positive cells were quantified and it was found that the presence of complement did not protect cells, most likely oligodendrocytes, from undergoing apoptosis (Fig. 8A and B). Overall, C3 was not involved in oligodendrocyte loss and/or apoptosis within the demyelinating corpus callosum.

Discussion

When $C3^{-/-}$ mice were put on the cuprizone diet I found no change in demyelination severity when compared to C3 sufficient mice. In addition, $C3^{-/-}$ mice showed remyelination kinetics that were similar to $C3^{+/-}$ mice, suggesting the lack of C3 had no effect on overall remyelination kinetics upon removal of cuprizone from the diet. Finally, I found no differences in the other disease parameters that I examined in the corpus callosum; including overall cellularity, microglia, oligodendrocyte death and apoptotic cells. These results suggest that C3 and downstream complement products do not play a role in demyelination or remyelination in the cuprizone model.

The results from these studies conflict with previous findings from our lab that provide data to suggest sCrry can prevent demyelination in the corpus callosum during cuprizone treatment. In addition to decreased demyelination severity, sCrry mice had decreased cellularity in the corpus callosum and decreased mature oligodendrocyte loss during early cuprizone treatment (107), suggesting complement plays a role in cell

migration/proliferation within the demyelinating corpus callosum. Previous studies using a different $C3^{-/-}$ strain (131) have produced conflicting results in EAE studies from two different labs. Two groups used these mice to determine the role of C3 in EAE disease severity and the outcomes of these studies were contradictory. Initially, a study by Nataf et al. showed that the genetic deletion of C3 lessened disease severity in the EAE model (96). Following this study, the results from a similar study were published by Calida et al. suggesting C3 was not required for the development of EAE or exacerbating disease severity (132). There were some differences in EAE induction methods between the two studies, however this has not been established as the reason for the disparity between the results. Since these two initial reports were published, several other studies have implicated specific complement proteins and receptors in the involvement of EAE disease progression and severity; including C3a, C3aR and CR3 (99, 102). Additionally, Szalai et al. duplicated results observed by Nataf et al. in 2000, demonstrating that disease was less severe in $C3^{-/-}$ mice compared to $C3^{+/-}$ and WT mice (108). Overall, these results suggest that certain complement proteins downstream of C3 activation are necessary for EAE pathology.

To determine if the lack of positive results was due to the specific $C3^{-/-}$ strain used in my studies, cuprizone experiments could be repeated in the $C3^{-/-}$ strain used in the above EAE studies. However, since the results found in EAE studies were contradictory, results obtained using this mouse strain may not be reliable. The results obtained from the two groups using the EAE model were vastly different, suggesting an unknown phenomenon could potentially be at work in this mouse strain. Personal communication with other labs utilizing the same $C3^{-/-}$ strain used in my studies suggests that results obtained with this strain have not always been consistent with other findings produced in these labs.

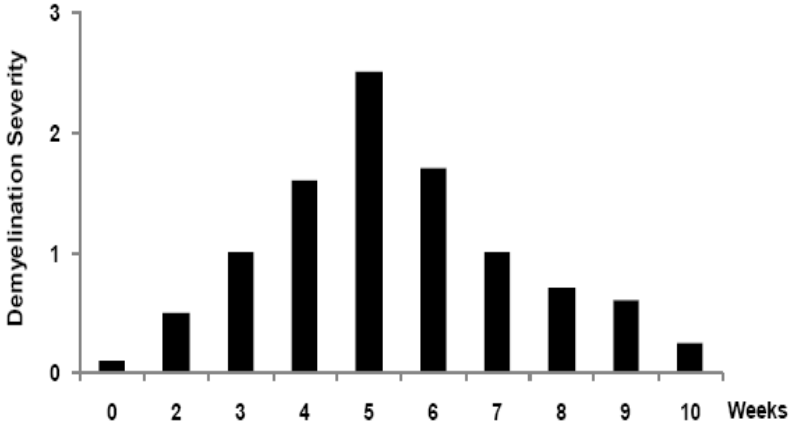
In these studies I used $C3^{+/-}$ mice as complement sufficient controls, however if these studies were to be repeated in the future, it would be beneficial to use C57BL/6 WT

($C3^{+/+}$) mice as controls instead of $C3^{+/-}$ mice. WT controls would theoretically be able to produce more complement upon cuprizone exposure and thus the differences between $C3^{-/-}$ and WT mice could potentially be greater. Szalai et al. found that WT mice had exacerbated EAE disease severity compared to $C3^{+/-}$ (108). Measuring the levels of C3 in the brains of $C3^{+/-}$ and WT mice will be important future studies to determine what levels of C3 are required for more severe demyelination.

An alternative theory for the lack of positive results in these studies may be that both harmful and protective complement mechanisms are being suppressed upon the genetic deletion of C3. There are several complement proteins that are formed once C3 has been activated and since none of these proteins have previously been studied in the cuprizone model, it is possible that knocking out all of these effector mechanisms could be negating any effect that could be seen with examining individual downstream complement proteins. Previous studies have shown a harmful role for C3a and CR3 in EAE (99, 102). On the other hand, low concentrations of terminal complement proteins, C5b-9, have been shown to be protective against oligodendrocyte apoptosis (106, 130). Because of the dual nature of complement in CNS pathology, future cuprizone studies will be required to determine the role of individual complement proteins and to better assess the effect of local complement on demyelination and remyelination pathology.

Figure 2. Kinetics of 0.2% cuprizone treatment in the corpus callsum of C57BL/6 mice

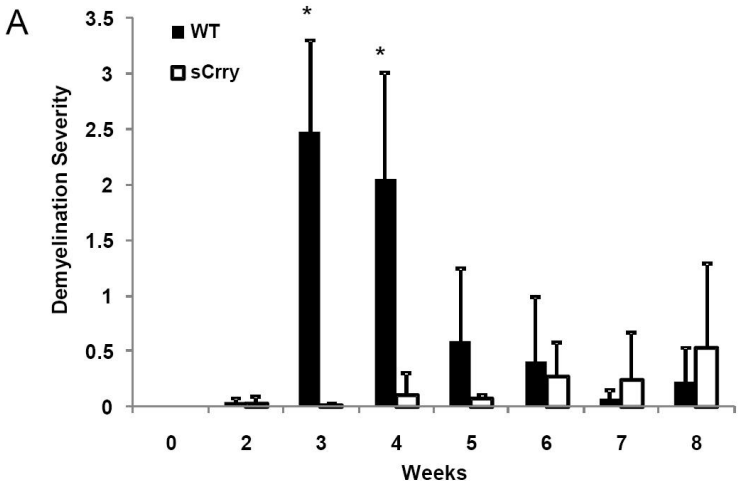
Cellularity and demyelination severity during six weeks of 0.2% cuprizone treatment are outlined below. Briefly, mature oligodendrocytes undergo apoptosis after 3-4 weeks of cuprizone treatment and myelin loss occurs. Oligodendrocyte precursors begin to make their way back into the corpus callosum around three weeks of cuprizone treatment. During demyelination, microglia and astrocytes increase within the corpus callosum. Once cuprizone is removed from the diet after six weeks, the numbers of inflammatory cells decrease in the corpus callosum, mature oligodendrocytes migrate or mature within the corpus callosum and full remyelination occurs.



	0	2	3	4	5	6	7	8	9	10
Mature oligodendrocyte apoptosis	-	+	+++	+++	-/+	-	-	-	-	-
Microglia/macrophages	-	-/+	+	++	+++	++	+	-/+	-/+	-/+
Astrocytes	-	-/+	-/+	++	+++	+++	++	+	+	+
Oligodendrocyte progenitors	-	-/+	-/+	+	+++	+++	++	++	++	++

Figure 3. CNS-specific expression of sCrry prevents demyelination during cuprizone treatment

(A) LFB-PAS staining of brain sections from C57BL/6 WT and sCrry-GFAP transgenic mice. Mice were treated with 0.2% cuprizone for six weeks and at the indicated times, the brains were harvested. Corpus callosum sections were stained using LFB-PAS and evaluated by microscopy. The stained sections were blindly scored by three individuals and averaged. Data are presented as mean demyelination severity scores for three to five mice from two different experiments per timepoint. Statistical significance was calculated using Student's *t*-test, * $p < 0.001$, where sCrry values were compared to WT values for each treatment timepoint. (B) Representative images of LFB staining at zero and four weeks of cuprizone treatment. This figure was created using data produced by Dustin Briggs (107).


A
WT
sCrry
Demyelination Severity
3.5
3
2.5
2
1.5
1
0.5
0
*
*
0
2
3
4
5
6
7
8
Weeks

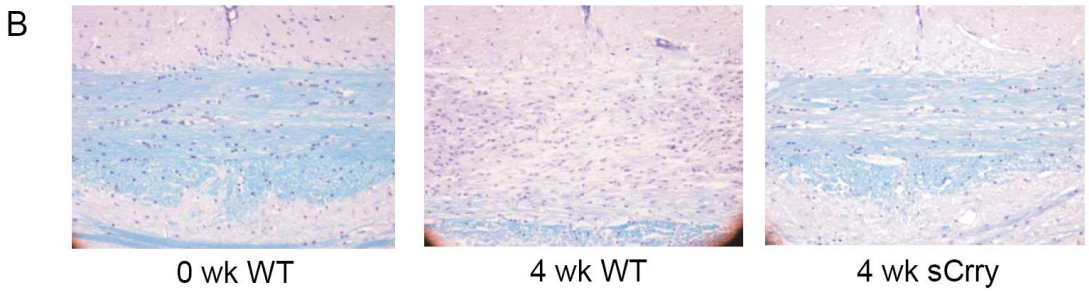

B
0 wk WT
4 wk WT
4 wk sCrry

Figure 4. Demyelination severity in the corpus callosum in C3 deficient and sufficient mice

Mice were treated with 0.2% cuprizone for six weeks and at the indicated times, the brains were harvested. Corpus callosum sections were stained using LFB-PAS and evaluated by microscopy. The stained sections were blindly scored by three individuals and averaged. At each timepoint, four to eight mice were analyzed for each experimental group. The error bars represent standard error of the mean (SEM). Statistical significance was calculated using Student's *t*-test, where $C3^{-/-}$ values were compared to $C3^{+/-}$ values for each treatment timepoint.

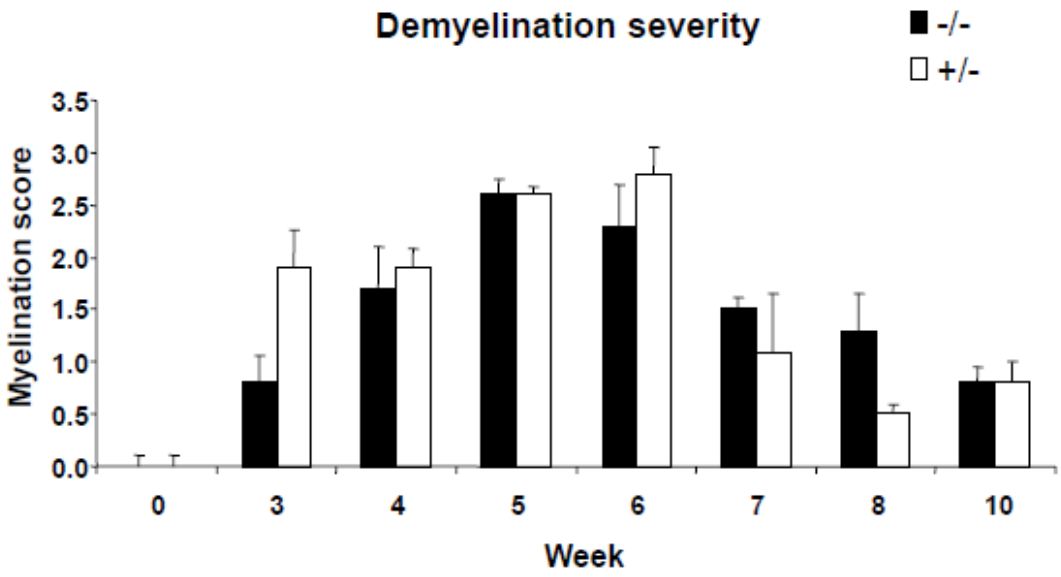
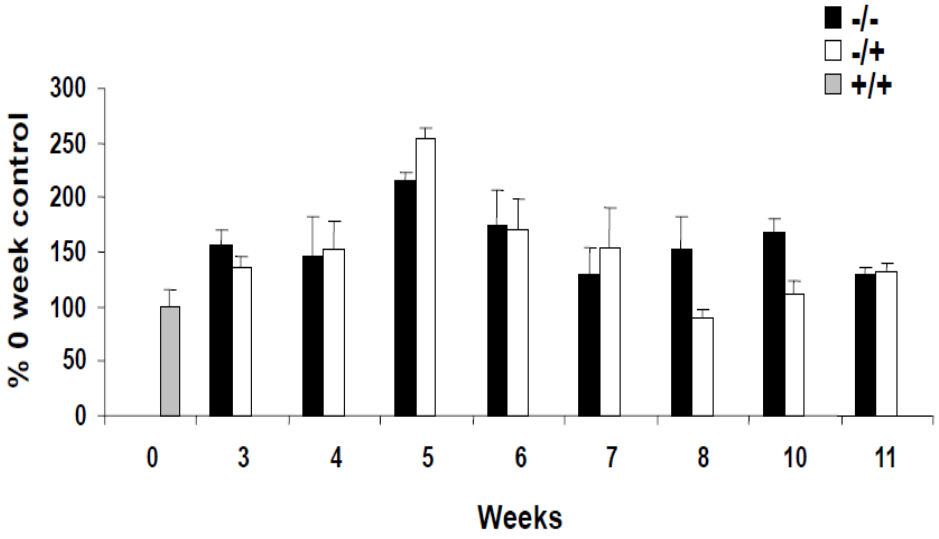

Demyelination severity
-/-
+/-
Myelination score
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
3
4
5
6
7
8
10
Week

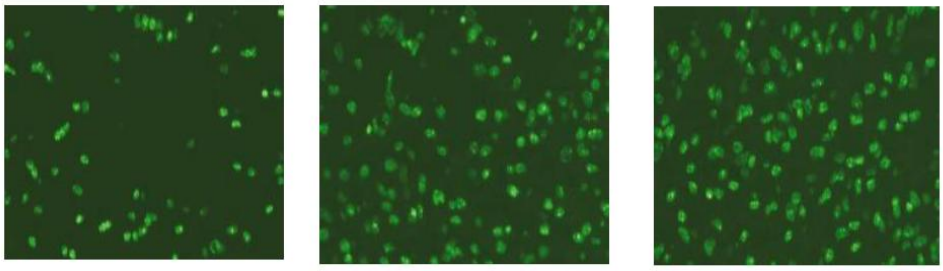
Figure 5. Cellularity is not dependent upon C3 and downstream complement proteins during cuprizone treatment

(A) Corpus callosum sections were stained with DAPI nuclear stain and the cell numbers were quantified using ImageJ. The results are presented as percent increase over untreated WT control. (B) Representative images of DAPI staining at zero and four weeks of cuprizone treatment. Statistical significance was calculated using unpaired Student's *t*-test, where $C3^{-/-}$ values were compared to $C3^{+/-}$ values for each treatment timepoint. Four to eight mice were used for each timepoint for each experimental group in the above experiments and the error bars represent SEM.

A



B



0 wk C57BL/6 4 wk C3 $^{-/+}$ 4 wk C3 $^{-/-}$

Figure 6. The presence of microglia in the corpus callosum during cuprizone treatment is not influenced by C3

(A) Brain sections were stained with RCA lectin and the mean fluorescence intensities measured in the corpus callosum below the midline of the brain. The results are shown as the percent increase over untreated WT control. (B) Representative images of RCA staining at zero and five weeks of cuprizone treatment. Statistical significance was calculated using unpaired Student's *t*-test, where $C3^{-/-}$ values were compared to $C3^{+/-}$ values for each treatment timepoint. Four to eight mice were used for each timepoint for each experimental group. The error bars represent SEM.

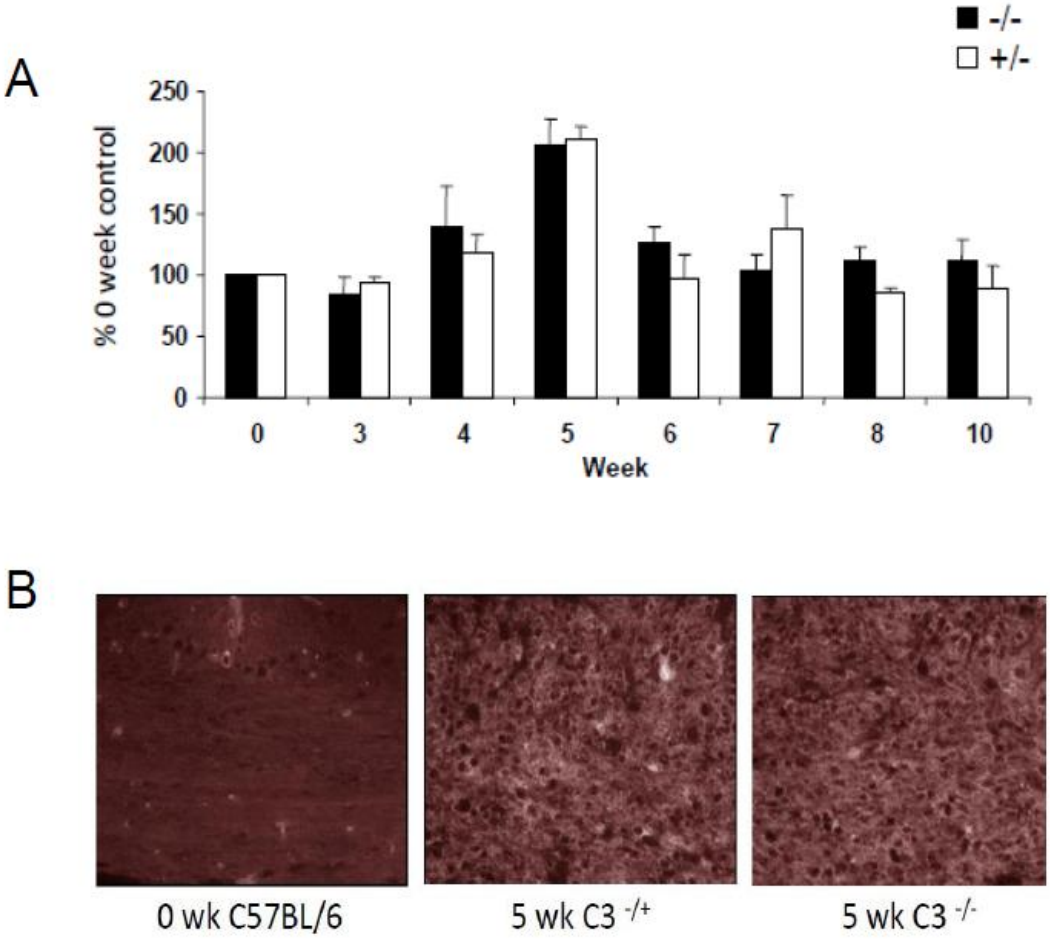

A
-/-
+/-
% 0 week control
250
200
150
100
50
0
0
3
4
5
6
7
8
10
Week
B
0 wk C57BL/6
5 wk C3 -/+
5 wk C3 -/-

Figure 7. Oligodendrocyte numbers are not affected by the absence of C3

(A) Sections were stained with Olig-2 antibody. The numbers of Olig-2^{+} cells were quantified in a predetermined area of the corpus callosum below the midline of the brain using ImageJ software. The results are presented as a percent change from WT untreated mice. (B) Representative images of Olig-2 staining at zero and six weeks of cuprizone treatment. (C) Olig-2^{+} cells as a percentage of total $DAPI^{+}$ cells. The same area of the corpus callosum was used for both DAPI and Olig-2 quantification. Statistical significance was calculated using unpaired Student's *t*-test, where $C3^{-/-}$ values were compared to $C3^{+/-}$ values for each treatment timepoint. Four to eight mice were used for each timepoint for each experimental group in the above experiments. The error bars represent SEM.

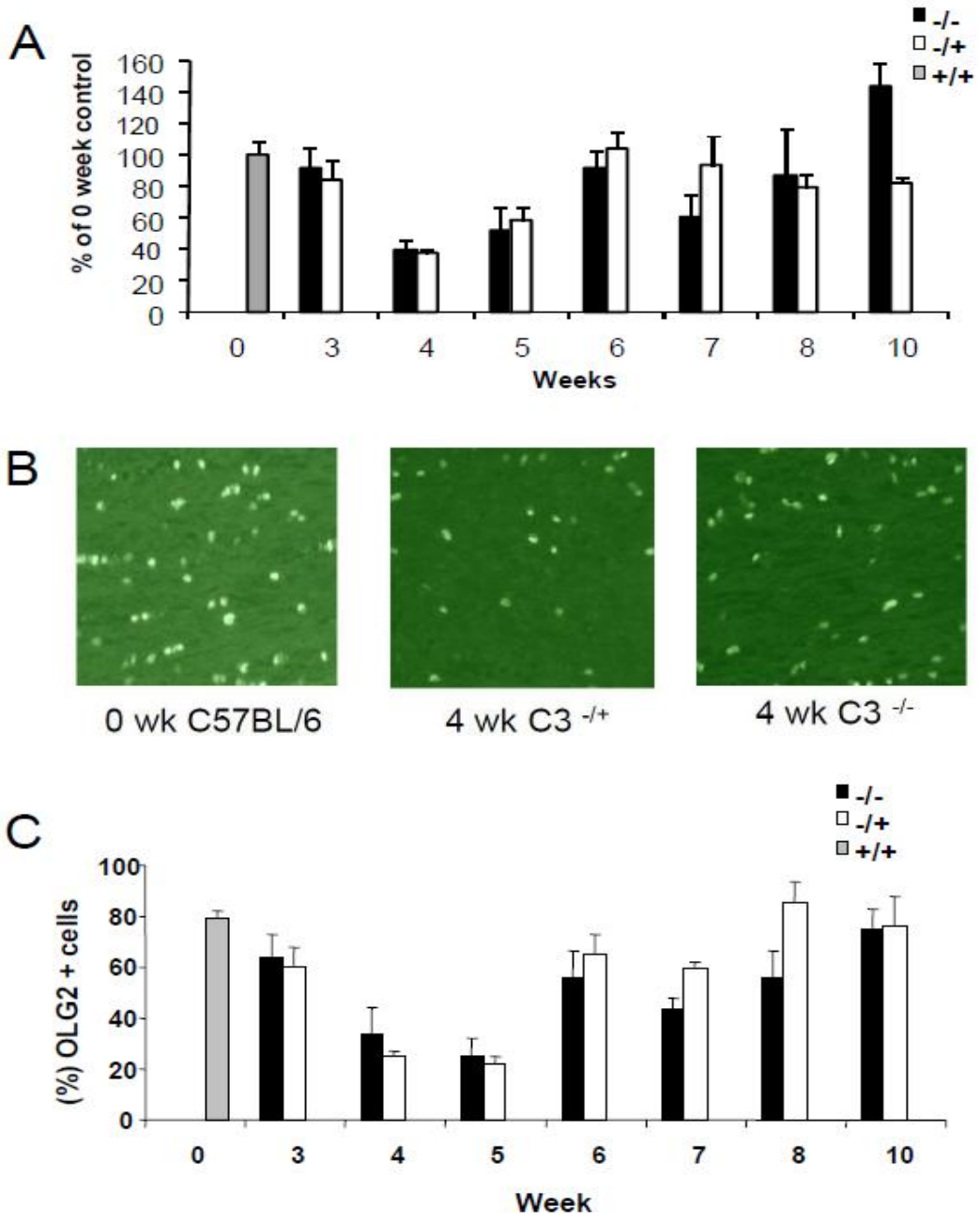
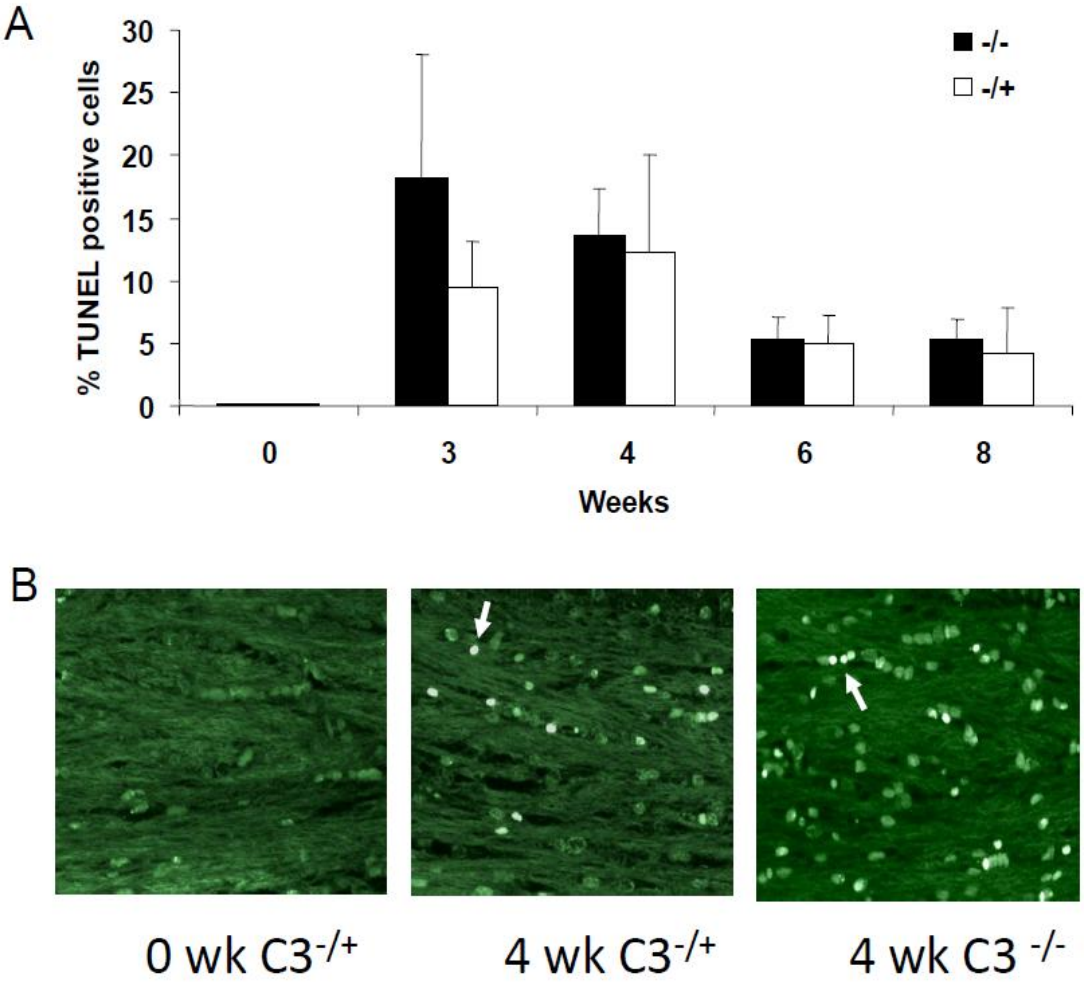

A
-/-
-/+
+/+
% of 0 week control
160
140
120
100
80
60
40
20
0
0
3
4
5
6
7
8
10
Weeks
B
0 wk C57BL/6
4 wk C3 -/+
4 wk C3 -/-
C
-/-
-/+
+/+
(%) OLG2 + cells
100
80
60
40
20
0
0
3
4
5
6
7
8
10
Week

Figure 8. C3 is not involved in apoptosis during cuprizone treatment

(A) Five μm sections were deparaffinized and incubated in Tdt reaction mixture for two hours. Sections were washed in PBS-Tween-20 and incubated in PBS containing a 1:100 dilution of streptavidin conjugated to Alexa fluor-488. The numbers of TUNEL-positive cells were quantified in a predetermined area of the corpus callosum below the midline of the brain using ImageJ software. The results are presented as a percent change from C57BL/6 untreated control mice. (B) Representative images of TUNEL staining at zero and four weeks of cuprizone treatment. Examples of the brightly staining TUNEL-positive cells are indicated by the arrows. Statistical significance was calculated using unpaired Student's *t*-test, where $C3^{-/-}$ values were compared to $C3^{+/-}$ values for each treatment timepoint. At each time point four to eight mice were analyzed for each experimental group and the error bars represent standard deviation.


A
% TUNEL positive cells
30
25
20
15
10
5
0
-/-
-/+
0
3
4
6
8
Weeks
B
0 wk C3-/+
4 wk C3-/+
4 wk C3 -/-

CHAPTER III

THE ROLE OF C3A AND C5A IN DEMYELINATION AND REMYELINATION

Abstract

C3a and C5a proteins mediate a wide range of proinflammatory functions; however, predicting the effect of C3a and C5a production in the CNS is complex, because these proteins can mediate both harmful and protective responses. The cuprizone model was utilized to investigate the roles of C3a and C5a in demyelination and remyelination pathology. Upon cuprizone treatment, transgenic mice expressing C3a or C5a under the control of the astrocyte-specific GFAP promoter had exacerbated demyelination and slightly delayed remyelination in the corpus callosum compared to WT mice. C3a and C5a transgenic mice had increased cellularity in the corpus callosum due to increased activation and/or migration of microglia. Additionally, there was evidence of T cells in the corpus callosum during demyelination in C5a and C3a transgenic mice, suggesting C3a and/or C5a may modulate BBB permeability in this system. Oligodendrocytes migrated to the corpus callosum in higher numbers during early remyelination events in C3a and C5a transgenic mice, thus enabling these mice to remyelinate as effectively as WT mice by the end of the ten week study. Overall, our findings show that anaphylatoxin production in the brain plays a negative role during demyelination; however, C3a and C5a stimulation enables effective remyelination.

Introduction and rationale

Activation of any of the complement pathways results in the downstream production of two small protein cleavage products, C3a and C5a, collectively known as anaphylatoxins. Anaphylatoxins are known to induce proinflammatory functions such as chemotaxis, upregulation of cytokine and chemokine production, and enhanced phagocytosis (31). C3a and C5a mediate their effects by binding to their receptors, C3aR

and C5aR (CD88), respectively, which are expressed on glial cells. Anaphylatoxins also bind C5a receptor-like 2 (C5L2), whose functions have not been clearly defined, but may act as a decoy or scavenger receptor (20). Previous studies have shown that astrocytes, microglia, neurons and oligodendrocytes express anaphylatoxin receptors (67, 68, 92, 133). Anaphylatoxin signaling in the CNS can induce a wide range of functions depending upon the cell type and local environmental stimuli. The overall effect of anaphylatoxin signaling on glial cell subsets and during CNS disease pathology can be complex, because anaphylatoxins have proven to be both protective and harmful. For instance, C5a has been shown to protect neurons against glutamate-mediated apoptosis both *in vitro* and *in vivo* (134, 135). Additionally, C3a has been implicated in neural stem cell regeneration and migration (34, 35). In contrast, anaphylatoxins have been shown to have exacerbating effects on CNS disease severity in mouse models of Alzheimer's (72), ALS (74) and lupus (75, 76) . Anaphylatoxin involvement has not been directly assessed in MS, however several studies have implicated complement involvement in this disease (78).

Glial cells are able to produce complement proteins, so it is possible for localized activation of complement pathways in the CNS to occur (86). C3aR and C5aR are expressed in areas of inflammation in the CNS of some MS patients (50, 92, 93) and complement deposition is present within some MS lesions (78, 85). Since complement deposition products have been found in MS lesions, it is likely that C3a and C5a are being produced in lesions as well. As discussed previously, C3a and C3aR were shown to play a role in exacerbating EAE disease severity. When C3a-GFAP and $C3aR^{-/-}$ strains were crossed, the worsened disease severity observed in the C3a-GFAP mice was attenuated, confirming that the elevated C3a production in the C3a-GFAP mice was the cause of exacerbated disease (99). Interestingly, it was shown that C5a and C5aR did not play a role in EAE pathology, even though experiments confirmed that C5a was expressed at increased levels in the CSF of C5a-GFAP EAE mice (100, 101). To

determine the localized role of C3a or C5a on glial cells during demyelination and remyelination processes, I utilized transgenic mice expressing C3a or C5a under the control of the astrocyte-specific GFAP promoter. Again, I utilized cuprizone to induce demyelination and removed cuprizone after six weeks to analyze different parameters of pathology during remyelination. In contrast to results observed in the EAE model, C3a and C5a both played a role in increased demyelination pathology in the cuprizone model.

Hypothesis

C3a and C5a are able to act as potent chemotactic factors; therefore, I hypothesized that an increase in the expression of C3a and C5a in transgenic mice would exacerbate demyelination severity by increasing the migration of inflammatory cells to the demyelinating corpus callosum during cuprizone treatment. There have been some reports that inflammation causes an increase in clearance of myelin and apoptotic debris by microglia, thus, I hypothesized that an increase in anaphylatoxin expression would aid in remyelination after the removal of cuprizone from the diet.

Materials and methods

Mice

Previously described C3a-GFAP (99) and C5a-GFAP (101) transgenic mice on the C57BL/6 background were provided by S. R. Barnum (University of Alabama). Five week old male C57BL/6 mice were purchased from The Jackson Laboratory.

Induction of demyelination and remyelination

To induce demyelination, 8–12 week old male mice were fed a diet of NIH-31 Modified 6% Mouse/Rat Sterilizable Diet-meal form (Harlan Laboratories, Madison, WI), containing 0.2% cuprizone (bis (cyclohexanone) oxaldihydrazone, Sigma-Aldrich) for six weeks. Mice were returned to a normal diet after six weeks of cuprizone treatment to allow for remyelination (107, 117, 126).

Histological analysis

Tissue processing and microscopy were carried out as previously described (107, 127). Briefly, mice were anesthetized and transcardially perfused with PBS, followed by 4% paraformaldehyde. Brains were then removed and incubated in 4% paraformaldehyde for seven days and embedded in paraffin. Five micrometer coronal sections corresponding to Sidman sections 241-251 (128) were used for histological analyses. All demyelination and remyelination studies using Luxol Fast Blue-Periodic acid Schiff (LFB-PAS) were done at midline of the corpus callosum. A demyelination severity score of zero indicates no demyelination, while a score of three indicates complete demyelination. Each section was scored blindly by three different individuals and the scores averaged for each mouse. Because of the large number of mice used in these studies, sections were stained in two separate groups. Untreated controls were stained along with each group to ensure that staining was consistent between experiments. WT, C3a-GFAP and C5a-GFAP mice from the same timepoint were stained together to guarantee an accurate comparison between WT and the transgenic mice.

Immunofluorescence

For immunofluorescence, paraffin sections were rehydrated, heat-unmasked, and then blocked with a solution containing 5% normal goat serum and 0.1% Triton X-100 (Sigma-Aldrich) in PBS. Slides were incubated overnight with primary antibody diluted in blocking solution. Appropriate fluorochrome-labeled secondary antibodies (Invitrogen-Molecular Probes) were used for detection. The immunohistochemistry antibodies used were: Biotinylated RCA-1 (Vector Laboratories, 1:100), a lectin used to detect microglia/macrophages; the secondary reagent used was streptavidin-Alexa Fluor-594; GFAP was detected using anti-GFAP antibody (Dako, 1:100); the secondary antibody was goat anti-rabbit Alexa Fluor-488; antibody to the Olig-2 transcription factor (129), a

gift from C. D. Stiles (Harvard University), was used at 1:10,000 to detect total oligodendrocytes; the secondary reagent was goat anti-rabbit Alexa Fluor-488. CD3 was detected using anti-CD3 antibody (Abcam, Cambridge, MA) at a dilution of 1:200, followed by goat anti-rabbit secondary antibody Alexa Fluor-488. Due to the large number of mice used in these studies, the sections were stained in two separate groups. Untreated controls were stained along with each group to ensure that staining was consistent between experiments. WT, C3a-GFAP and C5a-GFAP mice from the same timepoint were stained together to guarantee an accurate comparison between WT and the transgenic mice. For studies comparing different treatments, all sections were stained and digitally captured by microscopy at the same time. All of the brains were analyzed at midline of the corpus callosum. RCA and GFAP images were analyzed using ImageJ (http://rsbweb.nih.gov/ij/). Olig-2 and DAPI cells were quantified using NIS Elements 3.1 Imaging software (Nikon, Melville, NY). Manipulations of the digital images after capture were performed equally on all pictures.

Statistics

Student's unpaired *t*-test (two-tailed distribution, homoscedastic, Microsoft Excel) was used to examine the probability that differences between treatment groups were statistically significant. One-way ANOVA was also used to verify the significance in longitudinal studies between different treatment groups.

Results

Increased levels of C3a or C5a in the brain lead to exacerbated demyelination and delayed remyelination

To determine the role of C3a and C5a in demyelination and remyelination, transgenic mice expressing C3a or C5a under the control of the astrocyte-specific GFAP promoter were used. Results show that after four weeks on the cuprizone diet, both C3a

and C5a transgenic mice had worse demyelination severity compared to C57BL/6 wild type (WT) mice (Fig. 9A and 9B). Demyelination severity was increased through seven weeks in both transgenic groups (Fig. 9A). Although these mice showed worse demyelination severity at seven weeks, both transgenic groups were able to remyelinate completely by the end of the ten week study (Fig. 9A), suggesting increased C3a or C5a production does not affect the overall ability of these mice to undergo effective remyelination. Additionally, these results imply that C3a and C5a transgenic mice are able to remyelinate more quickly, since they had worse demyelination severity than WT mice.

Along with increased demyelination severity in the corpus callosum of transgenic mice, increased cellular infiltration and thickness of the inflammatory lesion within and surrounding the corpus callosum was observed. The increased thickness of the lesion is most likely a result of increased cell migration or cellular proliferation within the demyelinating corpus callosum. When the thickness of the lesion below the midline of the brain was measured, I determined that the corpus callosum and surrounding inflammation had almost doubled in width compared to the WT mice at five weeks (Fig. 9B and 9C). There were no differences in the thickness of the corpus callosum in untreated transgenic mice compared to untreated WT mice (Fig. 9C).

Previous results from our lab and others have shown that mice lose weight during cuprizone treatment and regain weight after cuprizone treatment is discontinued (120, 136). Throughout the ten week study all groups of mice lost 10-20% of their original weight while on the cuprizone diet. When mice were put back onto a normal diet after six weeks, the C3a transgenic mice did not regain as much weight as the WT and C5a transgenic mice (Fig. 10A). Interestingly, untreated C3a transgenic mice started the study weighing significantly more than untreated WT and C5a transgenic mice (Fig. 10B). These results may suggest that a currently unknown systemic effect is occurring in the C3a transgenic mice. The C3a degradation product, C3adesArg, also known as

acylation-stimulating protein (ASP), is involved in lipid biology and can influence body weight (137). Potentially, this protein could be playing a role in the weight of C3a transgenic mice throughout development; however it is unlikely that C3adesArg is able to cross the BBB in these mice to affect systemic levels of C3adesArg, since the molecular weight of C3adesArg is ~ 9kDa (69). Previous drug studies have shown that most molecules larger than 500 Da are unable to cross the intact BBB without some type of transporter (138). Further investigation of this phenomenon will be required to determine the mechanism for overall increased weight over WT mice and decreased weight gain after cuprizone removal in the C3a transgenic mice.

C3a or C5a expression leads to increased cellularity in the corpus callosum during cuprizone treatment

It was evident from the LFB-stained sections that there were greater numbers of cells in the demyelinated corpus callosum of the C3a and C5a transgenics compared to WT mice. A DAPI nuclear stain was used to quantify the number of cells in the corpus callosum. After five weeks of cuprizone treatment, both transgenic strains had increased numbers of cells compared to WT mice (Fig. 11A and 11B). The cell numbers in the corpus callosum remained elevated in C3a and C5a mice through eight weeks (Fig. 11A). There were no significant changes in the number of cells in the corpus callosum of untreated WT mice compared to untreated C3a and C5a transgenic mice (Fig.11A and 11B), suggesting there was no preexisting inflammation in the corpus callosum of transgenic mice prior to the cuprizone treatment.

Astrogliosis and microglial infiltration in the corpus callosum during demyelination are well characterized events that occur at predictable timepoints during cuprizone treatment (117). When GFAP antibody was used to detect astrocytes in the corpus callosum, it was determined that GFAP levels in the transgenic mice followed a similar trend compared to the WT mice throughout the ten week study. The increase in

GFAP expression in all groups of mice started at three weeks and persisted through eight weeks (Fig. 12A), which implies a rise in C3a and C5a transgene production in the transgenic mice. However, the increase observed in GFAP expression during cuprizone treatment may be posttranscriptional. In this case, C3a and C5a levels in the transgenic mice would not be affected, due to the absence of GFAP promoter activity. At nine weeks, GFAP levels started to decrease in WT and transgenic mice (Fig. 12A). The increased expression of GFAP at five weeks in the transgenic mice may suggest that C3a and C5a are inducing proliferation, activation or migration of astrocytes; however five weeks was the only timepoint where C3a and C5a transgenic mice showed a significant difference in kinetics from WT mice (Fig. 12A and 12B). These results suggest that C3a and C5a are not playing a significant role in activation and/or proliferation of astrocytes during the course of cuprizone treatment.

Microglial staining was greatly increased in C3a and C5a transgenic mice beginning at five weeks (Fig. 13A and 13B) and continued through seven weeks (Fig. 13B). At five weeks, there was a 2.2-fold and 2.6-fold increase in C5a and C3a transgenic RCA staining, respectively (Fig. 13B). Surprisingly, microglial staining in the C3a transgenic mice surpassed levels found in C5a transgenic mice at five weeks, although these results were not statistically significant ($p = 0.06$) (Fig. 13A and 13B). RCA staining in the WT mice was greatest at four weeks and decreased throughout the rest of the study (Fig. 13A). These findings were similar to what our lab has shown in previous cuprizone studies (107). Conversely, the peak of microglial staining occurred at five weeks in both C3a and C5a transgenic groups (Fig. 13A). Again, there were no differences between untreated mice from WT and transgenic groups, suggesting that there were not increased levels of GFAP or microglia in the corpus callosum of the transgenics prior to cuprizone treatment (Fig.12A-B, 13A-B). The highly elevated levels of RCA staining in the corpus callosum of C3a and C5a transgenic mice during demyelination,

suggests both C3a and C5a stimulate migration and/or proliferation of microglia during cuprizone treatment.

Oligodendrocyte numbers are increased during early remyelination in C3a and C5a transgenic mice

It has previously been shown that cuprizone treatment leads to oligodendrocyte apoptosis in the corpus callosum beginning two weeks after initiation of cuprizone treatment (116). To examine the effect of excess C3a or C5a on oligodendrocytes during demyelination and remyelination, we used an antibody against the transcription factor Olig-2. We found that the decrease in oligodendrocyte numbers during cuprizone treatment was similar in WT and both groups of transgenic mice (Fig. 14A). These results suggest that oligodendrocyte death was a function of the cuprizone toxicity and was not affected by increased C3a or C5a levels. Additionally, there was no change in the number of oligodendrocytes in the untreated control mice versus the untreated transgenic mice.

During early remyelination events at week six of cuprizone treatment, C3a and C5a transgenic mice had increased numbers of oligodendrocytes in the corpus callosum compared to WT mice (Fig. 14A and 14B). Oligodendrocyte numbers remained elevated in C3a and C5a transgenic mice through week eight of the study (Fig. 14A). Increased oligodendrocyte numbers may be required in the C3a and C5a transgenic mice for complete remyelination, due to the increased severity of demyelination in the corpus callosum. Interestingly, when I calculated the percentage of total cells that are Olig-2^+ cells, I found that there was a significantly higher percentage of Olig-2^+ cells in the WT mice at five weeks (Fig. 14C). These findings are most likely due to the increased number of microglia in the corpus callosum of C3a and C5a transgenic mice at five weeks, thus making the ratio of oligodendrocytes to total cells decrease in the transgenic

mice. The percentages of cells that are positive for Olig-2 began to increase at five weeks and were not significantly different between WT and transgenic mice between six and ten weeks (Fig. 14C).

Increased CD3 expression in C5a and C3a transgenic mice

In past cuprizone studies, it has been shown that very few T cells are able to cross the BBB, because the BBB remains relatively impermeable throughout treatment (114). Preliminary studies have suggested that C5a may induce disruption of the BBB (139, 140). To determine if T cells entered the corpus callosum during cuprizone treatment, anti-CD3 antibody was utilized in immunofluorescence experiments. CD3 staining occurred in the corpus callosum of all C5a transgenic mice that were treated for five weeks with cuprizone (Fig. 15). There was positive CD3 staining in some of the C3a transgenics; however some C3a transgenic mice had no CD3 staining at all. WT mice that received cuprizone treatment for five weeks did not have positive staining for CD3 in the corpus callosum (Fig. 15). Additionally, there were no CD3 positive cells detectable in the corpus callosum of untreated transgenic or WT mice. While there was some positive staining in the transgenic mice, these cells were not nearly as bright as the positive cells within the spleen section, which was used as a positive control for CD3 staining. Overall, these results suggest that the blood brain barrier in the C5a transgenic mice may be compromised upon cuprizone treatment and subsequent upregulation of C5a. Although $CD3^+$ T cells are detected in the corpus callosum of C5a transgenic mice, it is clear that microglia and astrocytes make up the majority of cells present at peak demyelination.

Discussion

In this research aim I showed that C3a and C5a anaphylatoxins exacerbate demyelination severity in the murine cuprizone model. Increased expression of either anaphylatoxin during peak demyelination amplified microglial staining and GFAP

expression in the corpus callosum. C3a transgenic mice tended to have greater demyelination severity and higher RCA levels in the corpus callosum compared to C5a transgenic mice, although these results were not statistically significant (Fig. 9 and Fig. 13). Previous *in vivo* data have suggested a role for C3a and C3aR in diseases affecting the CNS (75, 99); however it is not clear how C3a mediates its effects within the CNS. There have been no published reports indicating C3a involvement in glial cell migration, therefore other effector mechanisms may be at play. For instance, C3a may mediate production of other proinflammatory mediators, such as chemokines, by glial cells to induce migration to sites of demyelination. Future studies are required to determine if C3a induces chemokine production by glial cells.

C5a has been shown to mediate migration of microglia and astrocytes *in vitro* (52, 59, 141), which could explain the increased levels of RCA staining observed in the corpus callosum of C5a transgenic mice during demyelination (Fig. 13). Recent studies have also demonstrated that C5a may play a role in increased BBB permeability (139, 140). $CD3^+$ T cells were detected in the demyelinated corpus callosum of C5a transgenic mice, suggesting C5a may increase permeability of the BBB. $CD3^+$ T cells were observed in approximately 50% of the C3a transgenic mice, while the remaining C3a transgenics were CD3 negative. Therefore, in addition to increased microglia in the corpus callosum of C3a and C5a transgenic mice during demyelination, transgenic mice also had increased numbers of T cells within the corpus callosum. Additionally, the presence of T cells in the corpus callosum of cuprizone-treated transgenic mice may suggest that other immune cells can enter the parenchyma of these mice. Future studies will be required to determine if the BBB is more permeable in C5a transgenic mice and if immune cells are able to target areas of demyelination in these mice. A simple experiment to assess the permeability of the BBB would be to inject a tracer molecule into the circulatory system and then look for the presence of this tracer molecule in the

brain. Finally, further experimentation will be needed to determine if the detected $CD3^+$ cells are $CD4^+$ or $CD8^+$ T cells.

Interestingly, previous results showed that C5a and C5aR do not exacerbate disease severity in the EAE model (100, 101). The disparity between our results and those found in the EAE model most likely reflect the differences in the induction of demyelination and differing effector mechanisms between the two models. There are different lesion types found in MS (85) and both models address different aspects of lesion pathology. Overall, my data suggest both C3a and C5a induce a proinflammatory response during cuprizone treatment, exacerbating demyelination.

Anaphylatoxins exacerbate demyelination pathogenesis in the cuprizone model; however their role in remyelination is complex. Although transgenic mice had worse demyelination severity through seven weeks, C3a and C5a transgenic mice were able to remyelinate as effectively as WT mice. Additionally, the data suggest that remyelination occurred more quickly in the presence of C3a and C5a, since inflammation and demyelination severity was heightened in C3a and C5a transgenic mice. Interestingly, oligodendrocytes returned to the corpus callosum of transgenic mice in greater numbers than WT mice at six through eight weeks of the study. Previous studies have shown that C5a does not induce chemotaxis of oligodendrocytes progenitors *in vitro* (59) and there were no significant increases of oligodendrocytes in the corpus callosum until six weeks of the cuprizone study, suggesting chemotaxis to C3a or C5a is not the primary mechanism for the increase in oligodendrocytes in the corpus callosum.

One potential explanation for the increase in oligodendrocytes in C3a/C5a transgenic mice is the increased presence of microglia within the corpus callosum of these mice. Previous studies have suggested that some inflammation is necessary for microglia-mediated clearance of apoptotic cells and myelin debris and this is necessary for subsequent remyelination to occur (142). While microglia are thought to be the primary effector cell during demyelination in the cuprizone model, it has also been found

that cytokines and growth factors most likely produced by microglia are necessary for remyelination. For instance, IL-1β, leukemia inhibitory factor (LIF) and TNF-α, all thought to be produced by microglia and astrocytes, have been shown to enhance remyelination in the cuprizone model (127, 143, 144). Additionally, growth factors such as insulin growth factor (IGF), are necessary for remyelination by oligodendrocytes in the cuprizone model (145). Therefore, the increase in oligodendrocytes in C3a and C5a transgenic mice could be explained by the increase in proinflammatory microglia in the corpus callosum at five to seven weeks. Whether or not anaphylatoxins play a role in upregulation of growth factor and cytokine production by glial cells that are necessary for oligodendrocyte migration, maturation or proliferation has yet to be established.

Figure 9. Increased levels of C3a or C5a in the brain lead to exacerbated demyelination and delayed remyelination in the cuprizone model

(A) Mice were treated with cuprizone for the indicated times and the brains were harvested. Sections were stained using LFB-PAS and evaluated by microscopy. The stained sections were blindly scored by three individuals and averaged. At each time point, four to eight mice were analyzed for each experimental group. (B) Representative images of LFB-PAS staining at zero and five weeks of cuprizone treatment. (C) The extent of inflammation in and surrounding the corpus callosum at zero and five weeks was measured in μm using NIS Elements 3.1 Imaging software. In this figure, 100% is the mean width of the corpus callosum at midline of the untreated WT group. Statistical significance was calculated using unpaired Student's *t*-test and one-way ANOVA. For these calculations, the experimental groups were compared to WT values for each timepoint, where * $p < 0.05$. The error bars represent standard deviation.

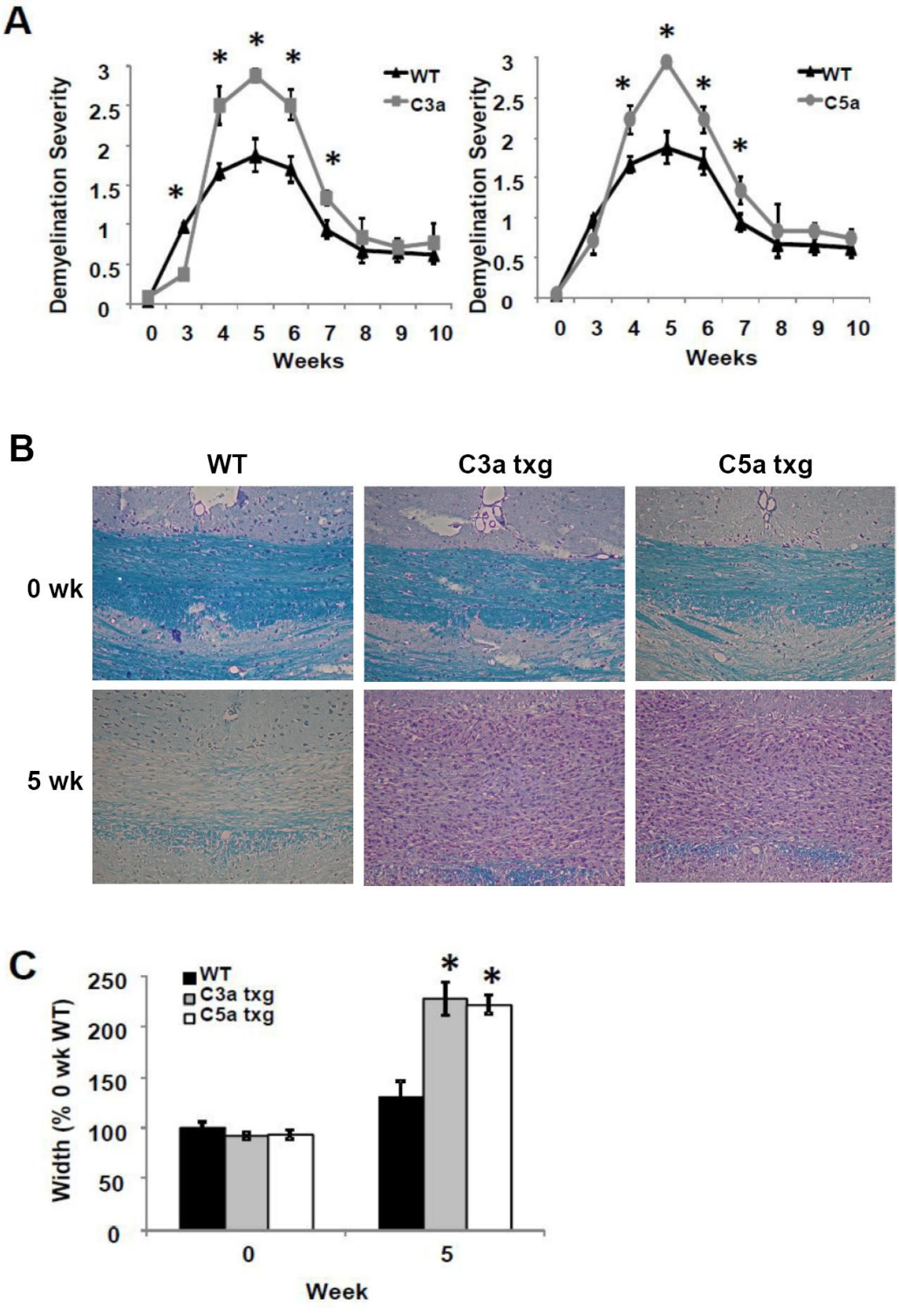
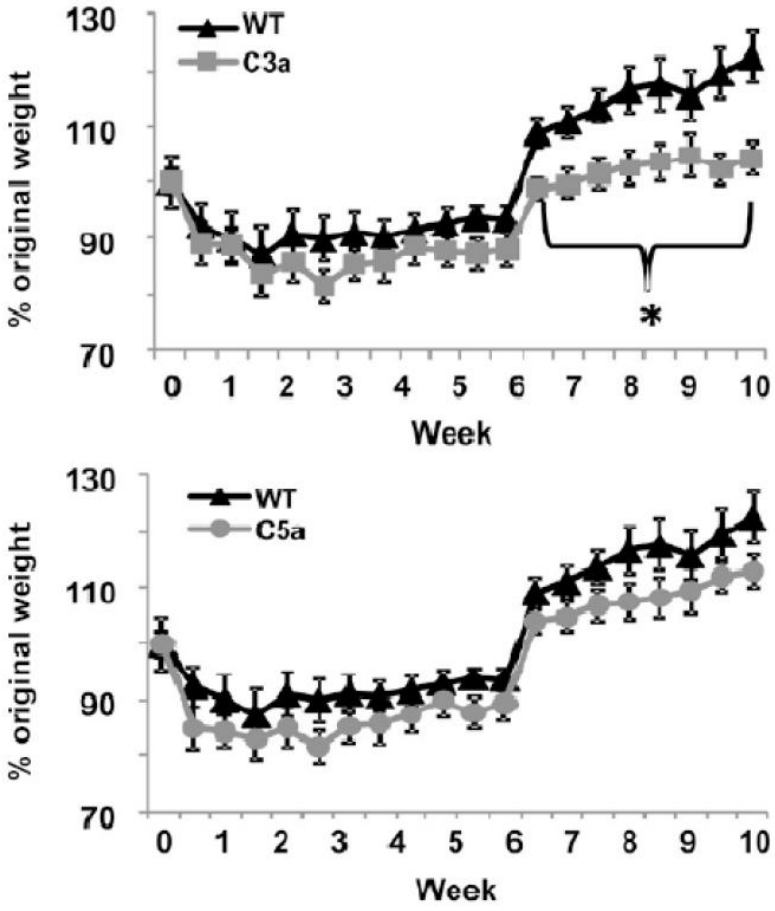

A
Demyelination Severity
Weeks
WT
C3a
C5a
B
WT
C3a txg
C5a txg
0 wk
5 wk
C
Width (% 0 wk WT)
WT
C3a txg
C5a txg
Week

Figure 10. Weight of WT and transgenic mice throughout cuprizone treatment
(A) Mice were weighed bi-weekly throughout the ten week study. (B) Initial weight of mice at zero weeks before cuprizone treatment was initiated. 7-16 mice were weighed bi-weekly per group. Statistical significance was calculated using unpaired Student's *t*-test and one-way ANOVA. For these calculations, the experimental groups were compared to WT values for each timepoint, where * $p < 0.05$ and ** $p < .001$. Error bars represent standard deviation.

A


WT
C3a
% original weight
130
110
90
70
0 1 2 3 4 5 6 7 8 9 10
Week
*
WT
C5a
% original weight
130
110
90
70
0 1 2 3 4 5 6 7 8 9 10
Week

B

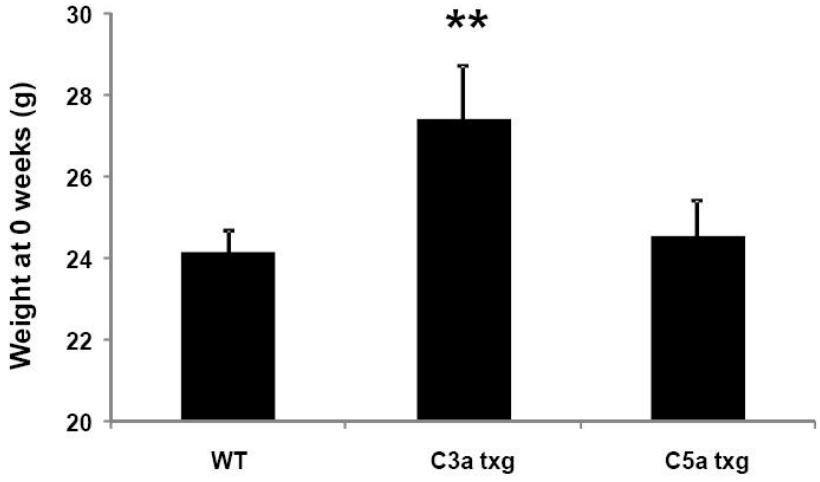
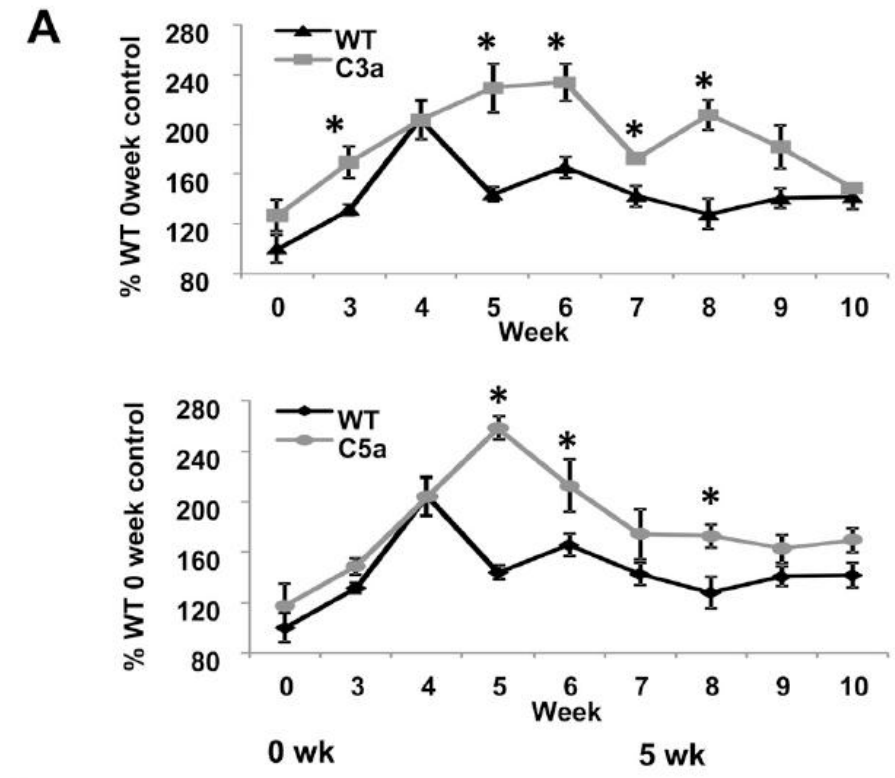

**
Weight at 0 weeks (g)
30
28
26
24
22
20
WT
C3a txg
C5a txg

Figure 11. C3a or C5a expression leads to increased cellularity in the corpus callosum during cuprizone treatment

(A) Sections were stained with DAPI nuclear stain and the cell number was quantified using NIS Elements 3.1 Imaging software. The results are presented as percent increase over untreated WT control. (B) Representative images of DAPI staining at zero and five weeks of cuprizone treatment. The scale bar equals 50μm. Statistical significance was calculated using unpaired Student's *t*-test and one-way ANOVA. For these calculations, the experimental groups were compared to WT values for each timepoint, where * $p < 0.05$. Four to eight mice were used for each time point for each experimental group in the above experiments and the error bars represent standard deviation.


A
WT
C3a
% WT 0week control
280
240
200
160
120
80
0
3
4
5
6
7
8
9
10
Week
WT
C5a
% WT 0 week control
Week
0 wk
5 wk

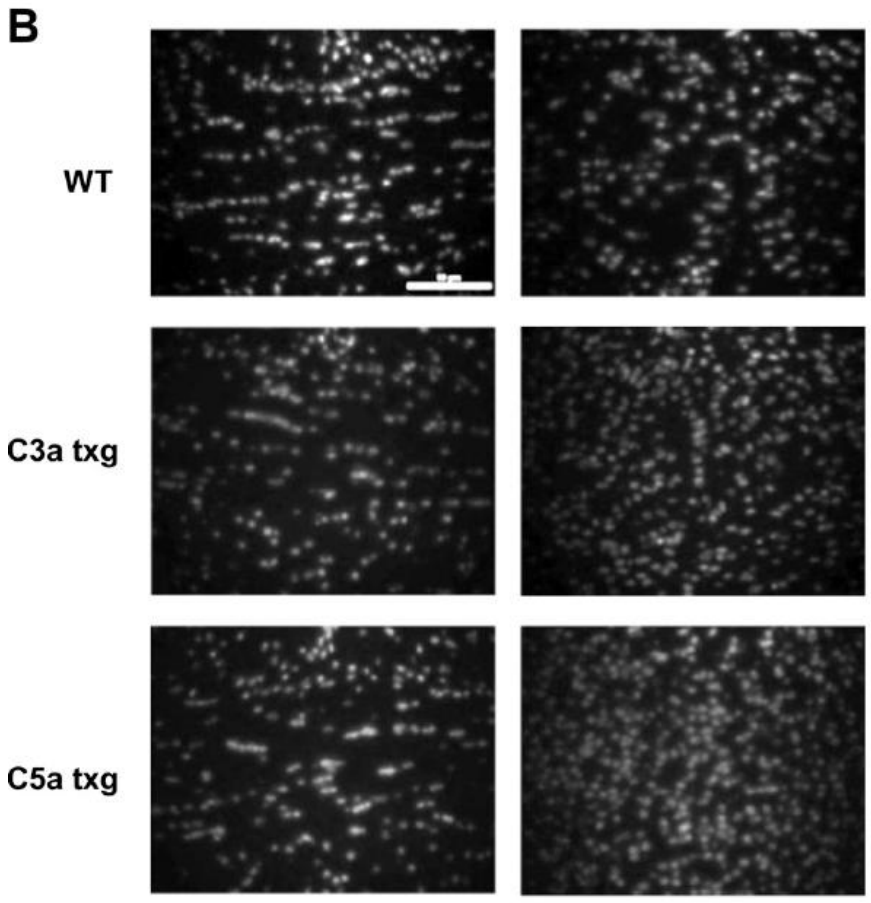
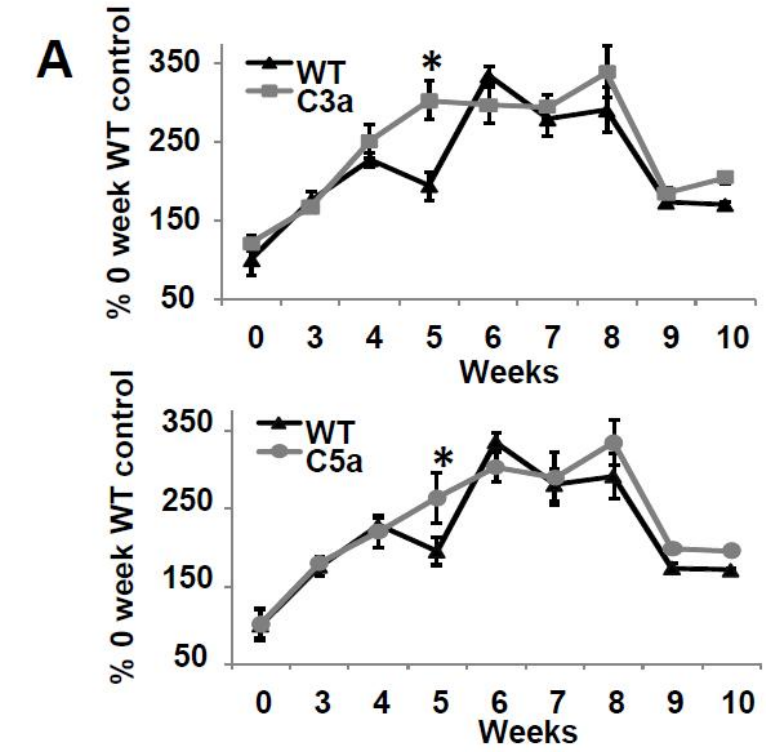

B
WT
C3a txg
C5a txg

Figure 12. Transgenic expression of C3a or C5a in the corpus callosum leads to increased GFAP expression at five weeks after cuprizone treatment

(A) Sections were stained with antibody against GFAP and the mean fluorescence intensity measured. The results are shown as a percent increase over untreated WT control. (B) Representative images of GFAP staining at zero and five weeks of cuprizone treatment. The scale bars equal 50μm. Statistical significance was calculated using unpaired Student's *t*-test and one-way ANOVA. For these calculations, the experimental groups were compared to WT values for each timepoint, where * $p < 0.05$. Four to eight mice were used for each time point and for each experimental group in the above experiments and the error bars represent standard deviation.


A
% 0 week WT control
350
250
150
50
WT
C3a
*
0 3 4 5 6 7 8 9 10
Weeks
% 0 week WT control
350
250
150
50
WT
C5a
*
0 3 4 5 6 7 8 9 10
Weeks

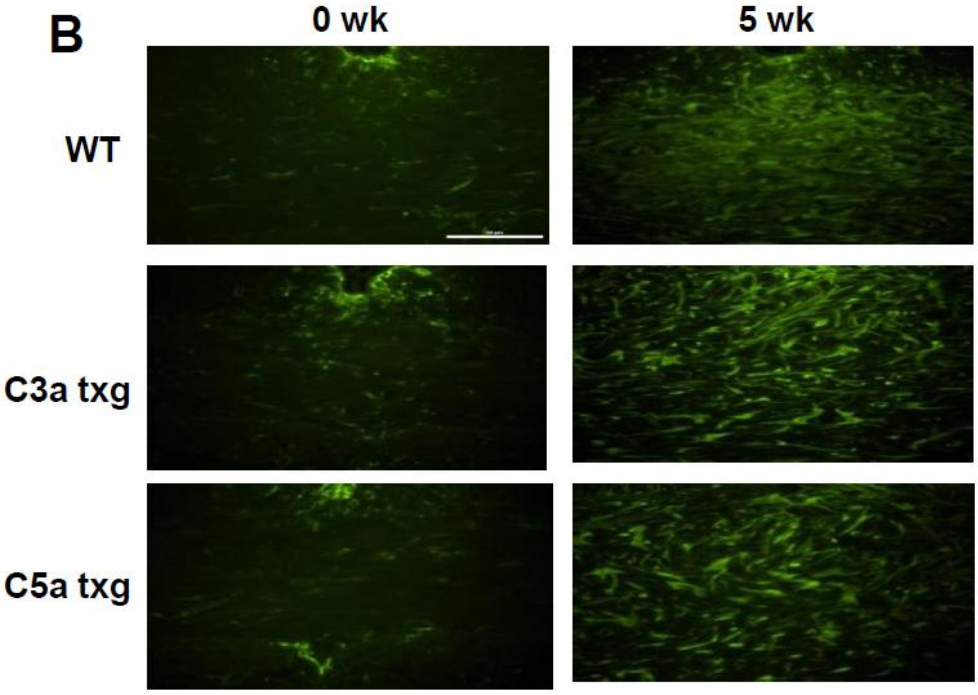

B
0 wk
5 wk
WT
C3a txg
C5a txg

Figure 13. Transgenic expression of C3a or C5a in the corpus callosum leads to increased microglial migration and/or proliferation

(A) 5μm sections were stained with RCA lectin and the mean fluorescence intensities measured. The results are shown as the percent increase over untreated WT control. (B) Representative images of RCA staining at zero and five weeks of cuprizone treatment. The scale bars equal 50μm. Statistical significance was calculated using unpaired Student's *t*-test and one-way ANOVA. For these calculations, the experimental groups were compared to WT values for each timepoint, where * $p < 0.05$. Four to eight mice were used for each time point and for each experimental group in the above experiments and the error bars represent standard deviation.

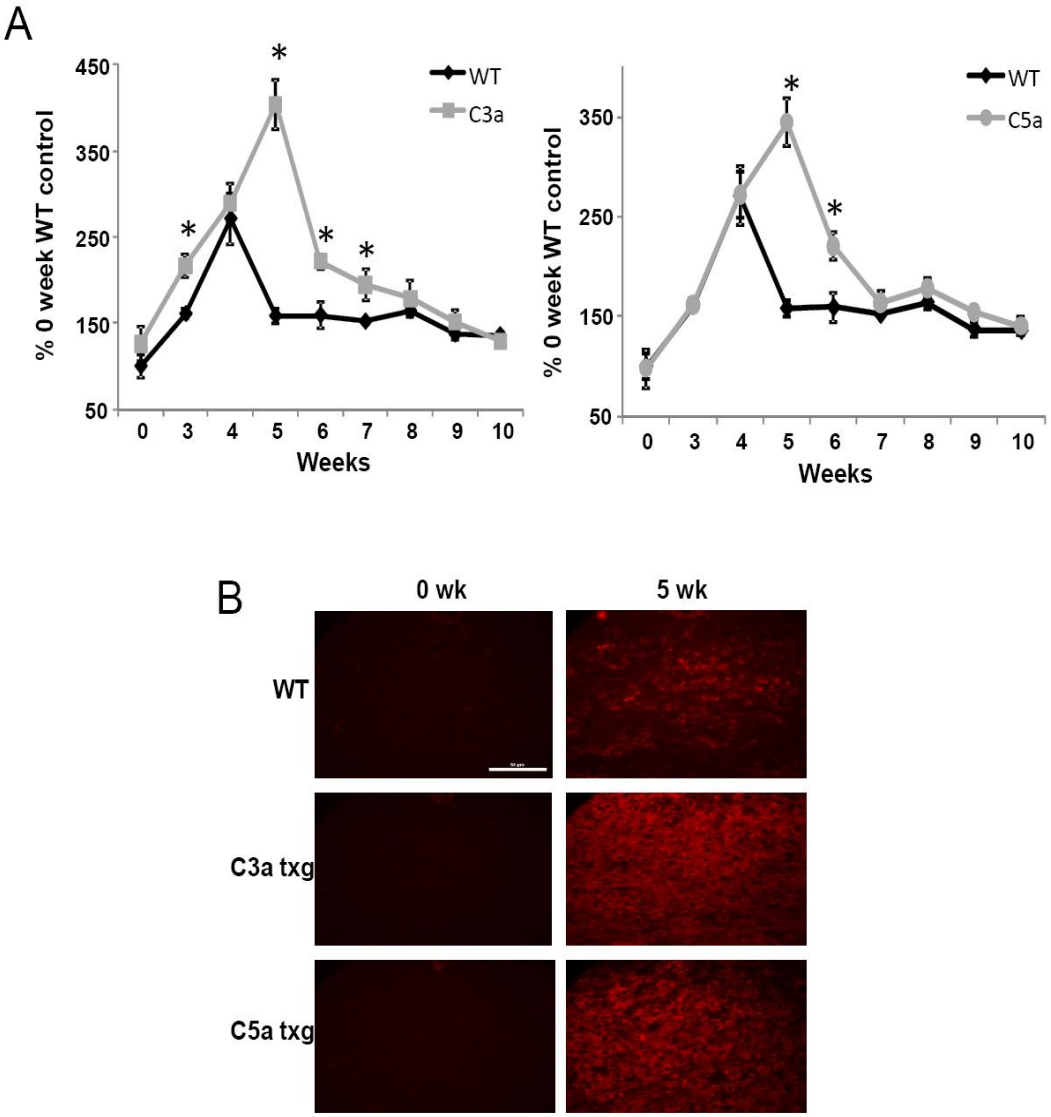

A
% 0 week WT control
450
350
250
150
50
0 3 4 5 6 7 8 9 10
Weeks
WT
C3a
*
% 0 week WT control
350
250
150
50
0 3 4 5 6 7 8 9 10
Weeks
WT
C5a
*
B
0 wk
5 wk
WT
C3a txg
C5a txg

Figure 14. Oligodendrocyte numbers are increased during early remyelination in C3a and C5a transgenic mice

(A) Sections were stained with Olig-2 antibody. The numbers of Olig-2^+ cells were quantified in a predetermined area of the corpus callosum below the midline of the brain using NIS Elements 3.1 Imaging software. The results are presented as a percent change from WT untreated mice. (B) Representative images of Olig-2 staining at zero and six weeks of cuprizone treatment. (C) Olig-2^+ cells as a percentage of total $DAPI^+$ cells. The same area of the corpus callosum was used for both DAPI and Olig-2 quantification. The scale bar equals 50μm. Statistical significance was calculated using unpaired Student's *t*-test and one-way ANOVA. For these calculations, the experimental groups were compared to WT values for each timepoint, where * $p < 0.05$. At each timepoint four to eight mice were analyzed for each experimental group and the error bars represent standard deviation.

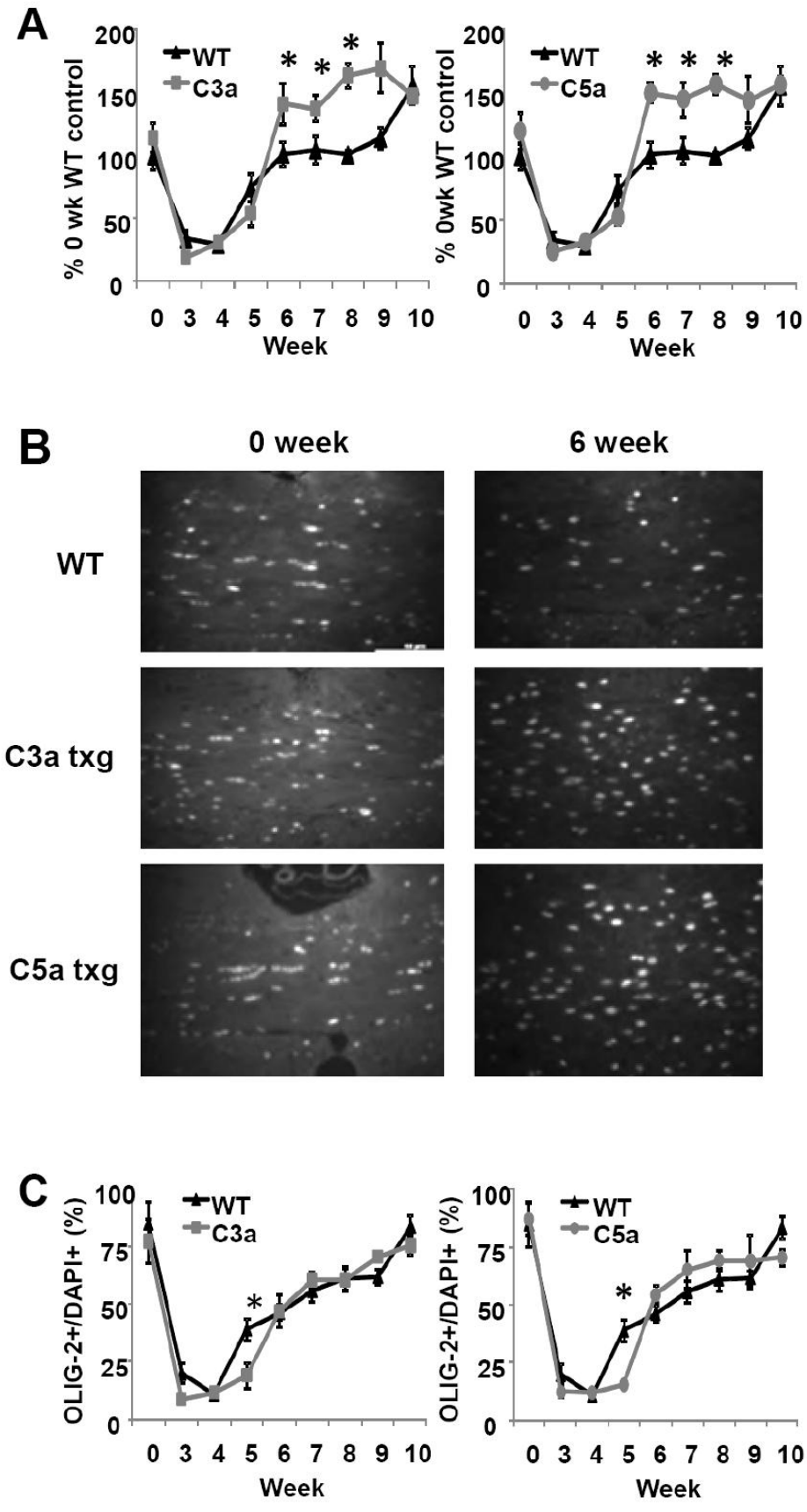
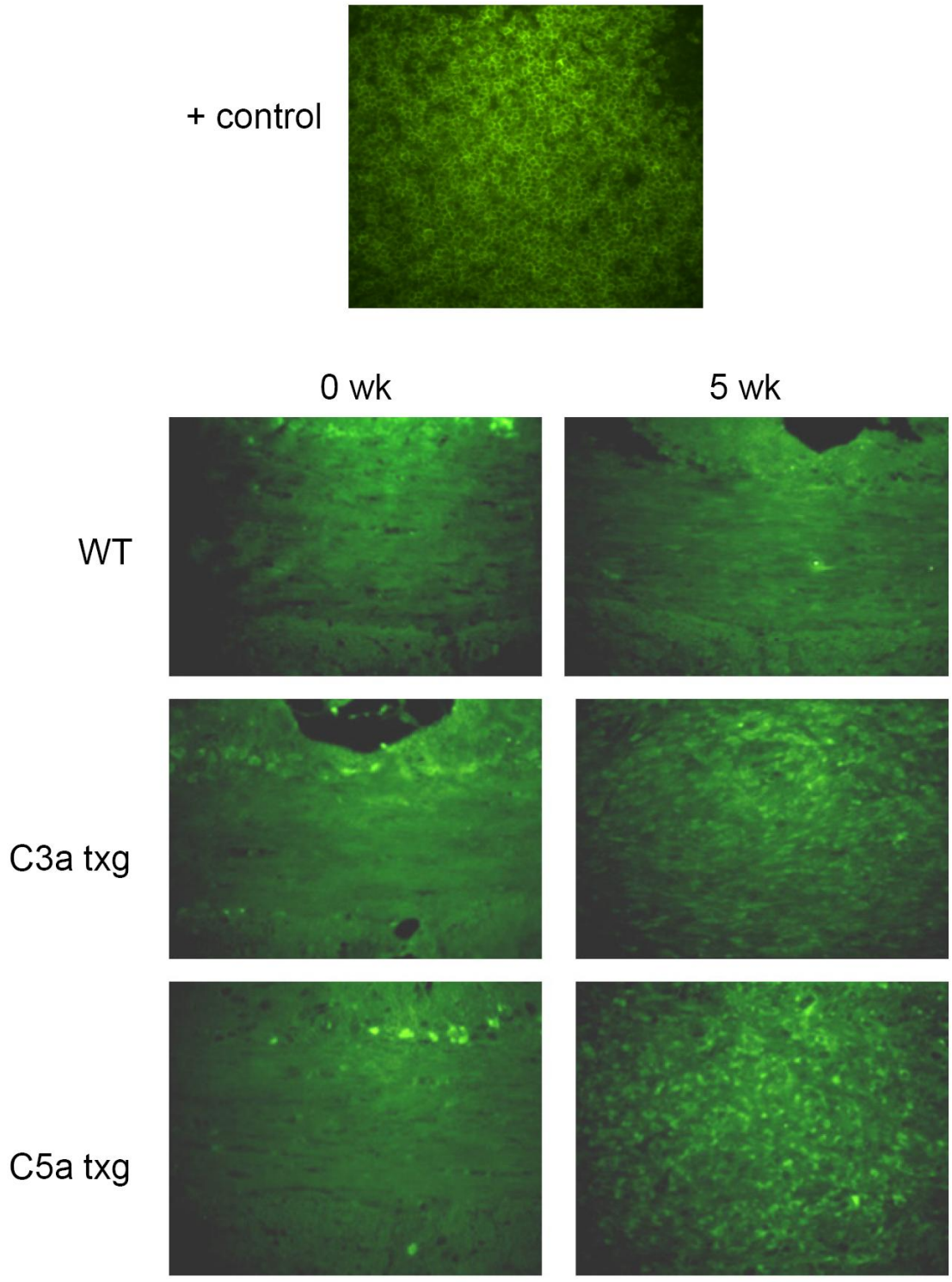

A
% 0 wk WT control
200
150
100
50
0
WT
C3a
* * *
0 3 4 5 6 7 8 9 10
Week
% 0wk WT control
WT
C5a
* * *
Week
B
0 week
6 week
WT
C3a txg
C5a txg
C
OLIG-2+/DAPI+ (%)
100
75
50
25
0
WT
C3a
*
Week
OLIG-2+/DAPI+ (%)
WT
C5a
*
Week

Figure 15. Increased CD3 expression in C3a and C5a transgenic mice

Sections were stained with an anti-CD3 antibody to detect any T cells that may be present in the corpus callosum at five weeks of cuprizone treatment. Four mice from each experimental group were used in this experiment. A C57BL/6 spleen section was used as positive control for the CD3 antibody.


+ control
0 wk
5 wk
WT
C3a txg
C5a txg

CHAPTER IV

C3A AND C5A SIGNALING AND DOWNSTREAM EFFECTOR FUNCTIONS IN GLIAL CELLS

Abstract

Previous studies have shown that glial cells produce complement proteins and express complement receptors; however the precise role of anaphylatoxin-mediated signaling in individual glial subsets remains unclear. To determine the effects of anaphylatoxins on glial subsets, murine recombinant C3a and C5a proteins were created. Mitogen-activated protein kinase (MAPK) pathway proteins c-Jun amino-terminal kinase 1 (JNK1) and extracellular signal-regulated kinases 1 and 2 (ERK1/2) were activated in glia upon stimulation with recombinant anaphylatoxin proteins. When microglial and mixed glial cultures were stimulated with C3a and/or C5a, the production of proinflammatory cytokines and chemokines was increased. In contrast, anaphylatoxin-treated primary astrocytes had suppressed cytokine and chemokine production compared to untreated astrocytes. In vitro, BV-2 microglia and primary astrocytes did not significantly migrate in response to stimulation with C3a or C5a proteins, suggesting migration may not be a primary anaphylatoxin-mediated function in the CNS or that other factors are required in addition to C3a and/or C5a to induce chemotaxis. These data provide evidence that anaphylatoxins activate cells of the CNS, as well as induce cytokine and chemokine production in glial cells. Understanding the downstream effects of anaphylatoxin signaling in specific glial cell types is important for developing future therapies for diseases of the CNS.

Introduction and rationale

The activation of the classical, alternative or lectin complement pathway results in the downstream cleavage of C3 and C5 proteins. C3 and C5 proteins both consist of two polypeptide chains, an α- and β-chain. Cleavage of the α-chain by C3 or C5 convertase

results in C3a and C5a proteins, respectively (31). Cleavage products C3a and C5a are small proteins, 77 and 74 amino acids, respectively, which bind to C3aR and C5aR. C3aR and C5aR receptors belong to the rhodopsin subfamily of G protein-coupled receptors (GPCRs) that contain seven transmembrane loops and are found on many cell types, including glial cells (67, 68, 92). Upon C3a or C5a binding, a conformational change of the intracellular receptor occurs, allowing certain heterotrimeric G proteins to interact with the receptor. C5aR has been shown to couple to pertussis toxin-sensitive $G_{i\alpha 2}$ (146, 147) or pertussis toxin-insensitive $G_{\alpha 16}$ (148), while C3aR may interact with $G_{\alpha 1}$, $G_{\alpha 12}$, $G_{\alpha 13}$ or $G_{\alpha 16}$ depending on the cell type (20). A GDP-GTP exchange leads to the release of the heterotrimeric G protein from the receptor and separation of the α-subunit from the βγ-subunit occurs. These subunits are then able to interact with distinct effector proteins, leading to the activation of various signal transduction pathways (149). After exposure of the receptor to agonist, the intracellular portion of the receptor becomes susceptible to phosphorylation, which promotes high affinity binding of arrestins. Binding of arrestins leads to rapid internalization of the anaphylatoxin receptors, by a clathrin/dynamin-mediated mechanism (31, 149, 150). This regulatory mechanism is termed homologous desensitization and can occur within minutes of C3a or C5a exposure (150).

C5L2, a seven-transmembrane receptor structurally similar to C5aR, binds C5a and the C5a degradation product, C5adesArg (151). There have been controversial reports of C3a and C3adesArg binding to C5L2, so the ability of C3a and C3adesArg to bind C5L2 remains uncertain (152, 153). Unlike C3aR and C5aR, previous data have suggested that C5L2 does not couple to intracellular G proteins (153). A recent report suggested that C5L2 regulates expression of C5aR on the cellular surface of neutrophils. C5aR and C5L2 were shown to interact with β-arrestin upon exposure to C5a (154). Interestingly, before agonist binding occurred, C5aR was most likely to be found on the cellular surface, while the majority of C5L2 expression was found within the cell (154).

In addition to modulating C5a signaling, previous studies have suggested that the binding of C3adesArg (ASP) to C5L2 may be involved in the regulation of triglyceride synthesis and other lipid biology functions (137, 155).

Nearly all cell types in the brain express C3aR and C5aR; and currently, most of what is known about the functions of anaphylatoxins in the CNS has come from murine disease models that utilize knockout or transgenic mice to study the role of these proteins. *In vivo* studies are important for understanding the overall involvement of C3a and C5a in disease pathology, but they have not addressed how C3a and C5a signaling affect individual glial subsets. In addition, many *in vitro* studies have used human reagents in murine systems, which may not fully recapitulate the activity of murine proteins. Previous *in vitro* studies have shown that anaphylatoxins are capable of activating signal transduction pathways in glial cells, including ERK proteins (52, 156). Other studies have shown that microglia and astrocytes migrate in response to anaphylatoxin signaling (52, 59, 141). Additionally, stimulation with C3a or C5a was shown to upregulate IL-8 and monocyte chemotactic protein-1 (MCP-1) mRNA expression in a human glioblastoma cell line (60). IL-6 mRNA expression was increased in human astrocyte cell lines after stimulation with C3a or C5a; however IL-6 protein levels were not altered upon treatment (157). Finally, C3a has been shown to induce neurotrophin and hormone production in glia (51, 158).

A major caveat in past studies has been the lack of available murine C3a and C5a recombinant proteins. To address this issue and to study additional anaphylatoxin signaling transduction pathways used to modulate glial function, our lab created viral constructs to express murine recombinant C3a and C5a proteins. Recombinant murine C3a and C5a were used to stimulate primary astrocytes, BV-2 microglia and mixed glial cultures *in vitro* to determine which signaling pathways are activated, in addition to determining the downstream effects of C3a and/or C5a including, migration and cytokine/chemokine production.

Hypothesis

Previous data have shown that anaphylatoxins are able to signal through their respective receptors in glial cells to activate downstream pathways and cellular events. The hypothesis of this aim is that C3a and C5a differentially activate signal transduction pathways in individual glial cell subsets. Once cell signaling is initiated, I hypothesize that C3a and/or C5a will mediate downstream proinflammatory effects, such as chemotaxis and proinflammatory cytokine production.

Materials and methods

Glial cell lines and enrichment of glial cultures

The BV-2 murine macrophage cell line was a gift from Jenny P.-Y. Ting (The University of North Carolina at Chapel Hill). BV-2 cells were cultured in DMEM (Invitrogen) with penicillin–streptomycin (Invitrogen) and 10% fetal bovine serum (Hyclone, Logan, UT). Primary astrocytes were obtained from one to three day-old neonatal mouse brains based on methods previously described (159, 160). Briefly, the brains of one to three day-old mice were dissected, meninges removed and cells dissociated in trypsin–EDTA. Mixed glial cells were cultured for 10-12 days at which time the microglia and oligodendrocytes precursors were shaken off at 225 rpm overnight. Following trypsin–EDTA treatment, astrocytes were cultured on new plates. Enriched astrocytes were >95% GFAP positive by FACS analysis (data not shown). Astrocytes were cultured in DMEM (Invitrogen) with penicillin–streptomycin (Invitrogen) and 10% fetal bovine serum (Hyclone). All animals were housed in the Laboratory of Animal Resources facilities at Iowa State University and all mouse protocols were approved by the Iowa State University Animal Care and Use Committee.

Characterization of anaphylatoxin receptors on glial cells

RNA was extracted from BV-2 microglia and astrocytes using Trizol reagent (Invitrogen). Amplification was performed using reagents from a Superscript one-step

PCR reaction kit (Invitrogen) and using the following primers from Integrated DNA Technologies (IDT) (Coralville, IA); GAPDH sense, GGC ATT GTG GAA GGG CTC AGT; GAPDH anti, TGG GAG TTG CTG TTG AAG TCG; C5aR sense, CCA TTA GTG CCG ACC GTT TCC; C5aR anti, ACG CAC AGG GAG TTC AGC TTC; C3aR sense, TCC TTG GCT CAC CTG ATT CTC; and C3aR anti, AGG AGA TGT TAG GAA TGG CTG. A 50 μL reaction was prepared for each sample and 10 μL aliquots were removed from the reaction at the indicated cycle number. Products were separated on an agarose gel containing ethidium bromide. The gel was exposed to ultraviolet light and the image was digitally captured. GAPDH was used as a housekeeping gene to ensure that equal amounts of RNA were utilized for each reaction.

To determine the protein level of anaphylatoxin receptors on glial cells, primary astrocytes and BV-2 microglial cells were analyzed by flow cytometry. Briefly, $1x10^6$ cells were placed in F_c block (BD Biosciences, San Jose, CA), washed and were then incubated with chicken anti-mouse C3aR or C5aR monoclonal antibody (1:100). The C3aR and C5aR antibodies were a gift from SR Barnum (University of Alabama). Cells incubated with chicken IgY (1:100) (VWR, Radnor, PA) were used as isotype controls. Next, cells were incubated in secondary antibody, anti-chicken IgY-FITC (1:50) (VWR). Cells were analyzed on a Beckman Coulter Epics Altra flow cytometer and the collected data were analyzed using FCS Express (De Novo Software, Los Angeles, CA).

Adenoviral infection and purification of C3a- and C5a-FLAG proteins

Sequences for murine C3a and C5a were cloned from mouse liver cDNA using reverse-transcription-PCR. A FLAG-tag sequence was cloned at the 5' end of both minigene constructs for purification and identification purposes. The Genbank accession numbers assigned to C3a and C5a sequences are HM105585 and HM105584, respectively. Since both C3a and C5a are internal sequences within the C3 and C5 genes,

respectively, a heterologous signal sequence from the murine IFN-γ gene was included at the 5' end. The adenoviral vector (pacAd5) was provided by the University of Iowa Gene Transfer Vector Core (GTVC). Each construct included an internal GFP cassette so that transduction efficiency could be monitored. The GTVC performed the adenoviral preparation. The recombinant Ad vector is based on the human adenovirus serotype 5, from which the E1a and E1b replication genes have been deleted in order to make virus replication defective (161). A549 human lung cells were transduced with the adenovirus at high MOI in serum-free Hybridoma SFM media (Invitrogen). After 48 hours, supernatants from transduced cells were collected, filtered and purified over an anti-FLAG-M2-agarose affinity column (Sigma-Aldrich). Proteins were eluted with low pH buffer and eluted proteins along with defined amounts of human C5a were run on a 15% SDS-PAGE gel and visualized by silver stain to quantify the amount of murine C3a or C5a.

Recombinant C3a and C5a binding

C3a- and C5a-FLAG proteins were dialyzed against PBS overnight to remove glycine. Next, C3a- and C5a-FLAG were conjugated with FITC using the FluoroTag FITC conjugation kit (Sigma). Briefly, C3a- and C5a-FLAG were incubated with FITC that had been resuspended in 0.1M sodium carbonate-bicarbonate buffer, pH 9.0, at a concentration of one mg/mL for two hours and dialyzed against PBS overnight to remove any unbound FITC. FITC-labeled anaphylatoxins were incubated with BV-2 microglia, J774 macrophages or primary astrocytes for five minutes. Cells were washed, fixed with 2.5% paraformaldehyde and immediately analyzed by flow cytometry.

Chemotaxis to anaphylatoxins

Chemotactic plates (Neuro Probe, Inc., Gaithersburg, MD) were used to determine if recombinant C3a and C5a induced migration of primary microglia, BV-2 microglia or primary astrocytes. The chemoattractants, various concentrations of C3a or

C5a, were placed in the bottom of a 96-well plate and a five or eight μm porous membrane was placed over the wells. 1 x 10^5 cells were pipetted on top of the membrane and the plate was incubated three to four hours at 37° C to allow for migration through the membrane towards the chemoattractant. Migrated cells were quantified using an ATPlite kit (Perkin Elmer, Waltham, MA), which determined the amount of ATP in each well using chemiluminescence. A serial dilution standard curve was set up for each plate where known numbers of cells were used to determine the corresponding luminescence value. The values obtained from the serial curve were then used to extrapolate cell numbers based on the luminescence value of the chemotaxis wells. Chemotaxis controls included a negative control where medium alone was placed in the bottom of the well and also a chemokinesis control where chemoattractant was placed in the bottom well and on top of the membrane. This control was used to account for the random migration of the cells across the membrane. J774 and RAW 264.7 murine macrophages were used as positive controls for chemotaxis to C5a.

Signaling assays

Primary astrocytes and BV-2 microglia were treated with 50nM C3a or C5a for 0-60 minutes or treated with varying concentrations of C3a or C5a (0-100nM) for five minutes. At the indicated times, cells were lysed with RIPA buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 1mM EDTA, 1% NP-40 and 1% sodium deoxycholate) containing protease inhibitor (Roche Diagnostics) and phosphatase inhibitor cocktails (Sigma-Aldrich). Protein lysates were quantified using Bio-Rad Protein Assay reagent (BioRad, Hercules, CA) and equal amounts of protein lysate (40-60 μg) were loaded and run on a 10% SDS-PAGE gel for 1.5-2 hours. Gels were then transferred onto 45μm PVDF membrane using a semi-dry transfer system. Blots were blocked in 5% fat-free dry milk (w/v) and then incubated overnight in primary antibody (pERK1/2, pJNK or p-p38 MAPK) (Cell Signaling Technology, Danvers, MA). Blots were developed on film using

LumiGLO chemiluminesence reagent (Cell Signaling Technology). Blots were then stripped using Restore Western blot stripping buffer (Thermo Fisher Scientific Inc., Rockford, IL), re-blocked and incubated with total ERK1/2, JNK or p38 MAPK antibodies (Cell Signaling Technology). Films were scanned and the densities of the bands were measured using ImageJ analysis software. Phosphorylated band values were normalized to their respective total band value to account for protein loading differences.

BioPlex and enzyme-linked immunosorbent assays (ELISAs)

BV-2 microglia, primary astrocytes and mixed glial cells were plated in six-well tissue culture treated dishes. Primary mixed glial cultures were allowed to mature for 10-12 days before treatment. Cells were treated with 50nM C3a, 50nM C5a or a combination of 50nM of both anaphylatoxins for the indicated times. Supernatants were collected from each treatment group and were run in duplicate using a 23-multiplex assay (BioRad). Supernatant IL-6 levels were verified using an ELISA (BioLegend, San Diego, CA). CCL4 levels in the supernatants were also verified by ELISA (R&D Systems Inc., Minneapolis, MN). Untreated cell supernatants were used as controls for each time point.

Statistics

Student's unpaired *t*-test (two-tailed distribution, homoscedastic, Microsoft Excel) was used to examine the probability that differences between treatment groups were statistically significant.

Results

Glial cells express anaphylatoxin receptors and recombinant C3a and C5a bind these cells

Previous reports have shown that several types of glia express receptors for anaphylatoxins on their cellular surfaces (67, 68, 92, 133). To verify these results, I looked for mRNA and protein expression of C3aR and C5aR on primary astrocytes and BV-2 microglia. Both cell types expressed anaphylatoxin receptors at the mRNA and protein levels (Fig. 16A-C), confirming previous data. Primary astrocytes seemed to express less C5aR compared to BV-2 microglia, both at the mRNA and protein level. Also, C3aR expression in primary astrocytes appears to be higher at the mRNA and protein levels when compared to C5aR expression (Fig. 16A and C). C3aR and C5aR mRNA and protein were expressed at similar levels on BV-2 microglial cells (Fig. 16A and B). These results show that both BV-2 microglia and primary astrocytes express C3aR and C5aR at the mRNA and protein levels and should be able to respond to C3a and C5a proteins, assuming these cells also express functional G proteins.

Anaphylatoxins bind to anaphylatoxin receptors on glia

In order to determine the function of anaphylatoxins on glial cells, recombinant murine anaphylatoxins were created. C3a and C5a recombinant proteins were labeled with FITC to determine if they were able to bind to receptors on BV-2 microglia and astrocytes. These experiments were to ensure that the recombinant proteins were functionally able to bind to anaphylatoxin receptors and to rule out the possibility that the recombinant proteins were unable to bind to receptors due to the addition of the FLAG epitope. J774 macrophages were used as a positive control in these experiments, since previous data from our lab demonstrated that J774 cells migrate to our recombinant C5a.

Results demonstrated that both FITC-labeled C3a and C5a bound to BV-2 microglia. FITC mean fluorescence intensities were 12- and 15-fold higher for C5a and

C3a, respectively, when compared to negative control values (Fig. 16D and E). The mean fluorescence intensities were much lower when primary astrocytes were treated with FITC-labeled C3a or C5a. Only a two-fold increase of C3a or C5a binding was observed in astrocytes when compared to the negative control (Fig. 16D and E).

Anaphylatoxins activate MAPK signaling pathways in primary astrocytes and BV-2 microglia

It has been shown that human recombinant C3a and C5a activate MAPK proteins ERK1/2 in a human astrocyte cell line and human recombinant C5a stimulation can activate ERK1 in primary murine astrocytes (156, 162). To determine how anaphylatoxins differentially activate MAPK pathways in glial cell subsets, recombinant anaphylatoxin proteins were used to stimulate primary astrocytes and BV-2 microglia. When BV-2 cells were stimulated with 50 nM C5a, ERK2 was phosphorylated as early as one minute after treatment (Fig. 17A and 17B). Both ERK1/2 were significantly activated after five minutes of stimulation with C5a. Activation of ERK1/2 was short lived and by 15 minutes the phosphorylation levels of ERK1/2 were equal to levels found in untreated cells (Fig. 17A and 17B). When BV-2 cells were treated with 50 nM C3a, there was not an increase in phosphorylated ERK1/2 at any of the time points tested (Fig. 17D), suggesting 50 nM C3a does not activate the ERK-MAPK pathway in BV-2 microglia. Cells were treated for five minutes with C3a concentrations ranging from 0-100nM and still there was not an increase in phosphorylation of ERK1/2 (Fig. 17C). Additionally, p38 and JNK MAPK pathways were not activated in BV-2 cells when stimulated with 50 nM C3a or C5a (data not shown).

When primary astrocytes were treated with 50nM C3a or 50nM C5a, ERK proteins were activated. In contrast to BV-2 cells treated with C3a, significant phosphorylation of ERK2 occurred at 15 minutes (Fig. 18C and 18D). By 60 minutes, pERK1/2 levels were decreased to levels seen in untreated control cells. When astrocytes

were treated with 50nM C5a, levels of pERK1 were higher than untreated control cells starting at 15 minutes (Fig. 18A and 18B). Levels of pERK1/2 were not significantly higher than untreated control cells at 30 minutes ($p = 0.08$ for both pERK1/2), but both ERK1/2 were significantly activated at 60 minutes. The basal levels of ERK1/2 phosphorylation were higher in untreated primary astrocytes compared to BV-2 cells, thus making the overall fold-increase less in primary astrocytes. I also looked for activation of p38 and JNK proteins in primary astrocytes. In C3a or C5a treated cells, p38 MAPK was not phosphorylated (data not shown). Interestingly, JNK1 was phosphorylated in primary astrocytes treated with C3a or C5a (Fig. 19A and 19B). Similar to ERK1/2 activation, significant activation of JNK1 occurred between 15 and 30 minutes. At 60 minutes after C3a or C5a treatment, JNK1 phosphorylation was not significantly higher than untreated cells. JNK2/3 were not activated in C3a or C5a stimulated primary astrocytes.

C3a and C5a do not induce chemotaxis of primary astrocytes or microglia.

Previous findings have shown that C5a can induce downstream functions in glial cells, such as chemotaxis and upregulation of cytokine expression (52, 59, 60, 141). To determine if anaphylatoxins induce migration of glia, I utilized a chemotaxis assay. First, J774 murine macrophages were used as a positive control for migration to C5a. Approximately 30% of J774 cells migrated in response to 10 nM C5a (Fig. 20A). There was also significant migration to 5, 25 and 50 nM concentrations of C5a. However, when C3a was used as the chemoattractant, there was not a significant migration of J774 cells (Fig. 20A).

Next, the ability of primary astrocytes and microglia to migrate to C3a or C5a was tested. Primary microglia did not significantly migrate to either anaphylatoxin at the concentrations tested (Fig. 20B). Because of the small number of microglia that can be

isolated from mixed glial cultures, I could only look at a small concentration curve per experiment. Additionally, results for microglia had to be calculated in relative light units (RLU), because there were not enough cells per experiment to utilize for a serial dilution curve for calculating the number of cells. Primary microglia did not migrate to higher concentrations of either anaphylatoxin (data not shown). Also, primary astrocytes failed to significantly migrate towards C3a or C5a (Fig. 20C). Overall, the data suggest that C3a and C5a are not acting as strong chemoattractants at the concentrations tested for primary astrocytes or microglia.

Anaphylatoxins induce cytokine and chemokine production in mixed glia and BV-2 microglia

To establish if cytokines and/or chemokines are upregulated by glial cells in response to anaphylatoxin treatment mixed glial cultures, BV-2 microglia and primary astrocytes were treated with media alone, 50nM C3a, 50nM C5a or a combination of 50nM C3a and C5a. Mixed glia, which contain astrocytes, microglia and oligodendrocyte progenitors, were utilized to gain insight on how cellular interaction may play a role in regulating the cytokine response to anaphylatoxin stimulation. Using a multiplex assay, it was observed that glial cells were capable of upregulating proinflammatory chemokines and cytokines. In mixed glial cultures IL-6, CCL5 (RANTES) and IL-13 were upregulated by 2-fold or greater. Mixed glial cultures produced the proinflammatory cytokine IL-6 in response to C3a or C5a one day after treatment (Fig. 21A and Table 2). These data were verified using an IL-6 ELISA (Fig. 21B). A significant additive effect on IL-6 production was observed when cells were treated with C3a and C5a in combination. In a previous cuprizone experiment, it was shown that CCL4, or macrophage inflammatory protein-1β (MIP-1β), mRNA levels were upregulated upon cuprizone treatment (123). To determine if CCL4 was upregulated by mixed glia in response to anaphylatoxin stimulation, we looked for CCL4 production by

ELISA. While the multiplex results did not show a 2-fold increase of CCL4 after one day of anaphylatoxin treatment, CCL4 was increased by 1.7-fold in C5a treated cell supernatants compared to untreated cells (Table 2). ELISA experiments confirmed that mixed glial cultures produced significantly more CCL4 in response to C5a treatment (Fig. 21B). Interestingly, when cells were treated with C3a there was less CCL4 present in the supernatant after one day compared to untreated cells (Fig. 21B).

The multiplex experiment with BV-2 cell supernatants demonstrated increased levels of CCL4, CCL5 and CCL11 (Eotaxin) upon treatment with anaphylatoxins. ELISA results confirmed that BV-2 microglial cultures treated with 50nM C5a, 50 nM C3a or a combination of both anaphylatoxins had increased expression of CCL4 in cell supernatants compared to untreated control three days after treatment (Fig. 22A-B and Table 3). Significant additive effects on CCL4 production were not detected when BV-2 cells were treated with both C3a and C5a compared to when BV-2 cells were treated with either C3a or C5a alone (Fig. 22B). Multiplex data showed an increase in CCL4 production after one day of C5a stimulation in microglia, but these results could not be reproduced in ELISA experiments. In both mixed glia and BV-2 stimulated cells some chemokine and cytokine levels decreased after one day. The decrease in levels of IL-6 and CCL4 between day one and day three in mixed glia and BV-2 supernatants, respectively, in the multiplex analysis most likely reflects protein degradation.

When primary astrocytes were treated with anaphylatoxins, an interesting trend in the multiplex and ELISA results occurred. Interestingly, none of the cytokines tested for were increased upon treatment with anaphylatoxins; however, the presence of C3a and/or C5a decreased cytokine production in astrocyte cultures. After one day of anaphylatoxin treatment, IL-13 was decreased in astrocyte cultures treated with C3a, C5a, or both anaphylatoxins (Fig. 23A and Table 4). On day six, IL-13, IL-9 and CCL4 were decreased in the cell supernatant (Fig. 23A-B and Table 4). The CCL4 multiplex data

were verified by ELISA. Similar to the multiplex results, six days after anaphylatoxin treatment CCL4 levels were decreased in astrocyte cultures (Fig. 23B).

Discussion

These studies have shown that both microglia and primary astrocytes express C3aR and C5aR at the mRNA and protein levels (Fig. 16). JNK and ERK proteins were phosphorylated in primary astrocytes in response to C3a or C5a stimulation (Fig. 18 and 19). These results differed from what was observed in microglia, where ERK1/2 proteins, but not JNK, were phosphorylated in response to C5a only (Fig. 17). These results suggest that signal transduction pathways are differentially regulated in these two glial cell types. It has previously been shown that ERK1/2 are phosphorylated in response to C3a and C5a human recombinant proteins in a human glioblastoma cell line (156) and recombinant human C5a in primary murine astrocytes (162); however JNK activation in response to C3a or C5a stimulation has never been shown in primary astrocytes or astrocyte cell lines. While there have not been any previous reports on C3a and C5a induction of JNK activation, previous studies have shown that JNK activation in astrocytes can result in upregulation of cytokines (163, 164), growth factors (165) and apoptosis (166), depending on the stimuli. Future studies will be required to determine the downstream effects of anaphylatoxin-mediated JNK and ERK1/2 activation in primary astrocytes. In addition, the role of C5L2 remains elusive and future signaling studies must take into account the potential involvement of C5L2 in glial cells and its effect on C5a signaling.

C3a has been implicated in EAE pathology and in our own cuprizone studies, yet the exact mechanisms by which C3a mediates its exacerbating effects *in vivo* have yet to be established. There have been no published reports indicating C3a involvement in glial cell migration and results from our lab suggest that recombinant murine C3a does not induce migration of microglia, primary astrocytes or J774 macrophages (Fig. 20);

suggesting chemotaxis may not be the primary effector function of C3a in the CNS. It was previously shown that human recombinant C5a mediates migration of microglia and astrocytes *in vitro* (52, 59, 141); however in our chemotaxis experiments there was not significant migration of either of these cell types in response to recombinant murine C5a. These results may suggest a difference in experimental design or differences between human and murine reagents.

Previous studies have shown that when astrocytes (61) and microglia (51) were stimulated with C3a, production of growth factors were upregulated, suggesting C3a may have the potential to induce cytokine and chemokine production in glia. In my studies, C3a and/or C5a treated BV-2 microglia produced more CCL4, CCL5 and CCL11 than untreated control cells (Fig. 22 and Table 3). Also, when mixed glia were treated with C3a or C5a, IL-6 was upregulated after one day of treatment. Interestingly, when cells were treated with C3a, CCL4 was decreased compared to untreated control cells, suggesting C3a has the ability to induce certain cytokines while downmodulating others. Unlike C3a, C5a was able to upregulate CCL4 in mixed glial cultures after one day (Fig. 21 and Table 2). The ability of C3a and C5a to induce production of chemokines in glial cells provides an alternative mechanism for the induction of chemotaxis in the CNS, since CCL4 and CCL5 are known to be potent chemotactic factors (167).

In contrast to the proinflammatory cytokine and chemokine production in anaphylatoxin stimulated microglia and mixed glia, astrocytes treated with anaphylatoxins exhibited consistent regulatory characteristics. Untreated astrocyte supernatants had far greater levels of IL-9, IL-13 and CCL4 compared to astrocytes that were treated with C3a or C5a (Fig. 23 and Table 4), suggesting anaphylatoxin stimulation results in downmodulation of cytokine production in these cells. Previous studies showed that when astrocytes were stimulated with proinflammatory mediators, astrocytes acted in a regulatory manner through production of regulatory cytokines (168) or induction of regulatory signaling pathways (169, 170). Additionally, it has been shown

that when pituitary cell cultures are treated with C3a, the production of hormones involved in the regulation of inflammation are increased (158). When primary astrocytes were treated with C3a or C5a they did not upregulate any cytokines considered to be anti-inflammatory, including the regulatory cytokine IL-10 (Table 4). Although primary astrocytes reacted to anaphylatoxin stimulation in a regulatory or suppressive manner, previous studies have shown that astrocytes were able to upregulate proinflammatory cytokine production in response to specific proinflammatory stimuli, including TNF-α and IFN-γ (53).

Even though primary astrocytes downregulated cytokine production, the ability of mixed glial cultures to produce proinflammatory cytokines and chemokines in response to anaphylatoxin treatment *in vitro* suggests that the proinflammatory response, which is most likely due to microglial activation, overrides the regulatory response of astrocytes, the most prevalent cell type in mixed glial cultures. Alternatively, astrocytes may require cellular interaction with other types of glia to upregulate cytokine and chemokine production in response to anaphylatoxin stimulation. Further investigation into anaphylatoxin-mediated activation of alternative signaling pathways are needed to determine which signal transduction pathways may be required for suppressing cytokine production by astrocytes. Additionally, determining which type of cell is producing IL-6 or CCL4 in the mixed glial cultures would be of interest for future studies. These studies would help determine if cellular interactions are required for astrocytes to produce proinflammatory cytokines.

Figure 16. Primary astrocytes and BV-2 microglia express anaphylatoxin receptors (A) RT-PCR was used to detect mRNA levels of C3aR and C5aR on primary astrocytes and BV-2 microglia. 10 μL aliquots were taken out of the total PCR reaction every three cycles beginning at 18 cycles for GAPDH and 24 cycles for C3aR and C5aR. GAPDH was used as a housekeeping gene to ensure that equal amounts of RNA were used in the experiment. (B) C3aR and C5aR protein expression was detected on BV-2 microglia or (C) primary astrocytes by FACS analysis. Receptors on the cellular surfaces were detected using chicken anti-mouse C3aR or chicken anti-mouse C5aR. IgY was used as an isotype control. (D) Recombinant C3a- and (E) C5a-FLAG were labeled with FITC. The labeled protein was then used to detect C3a and C5a binding on J774 macrophages, BV-2 microglia and primary astrocytes. For each cell type, cells were stained with FITC alone to detect background staining, thus acting as a negative control. Results are presented as fold-increase in mean fluorescence intensity over the corresponding negative control. All of the experiments were repeated at least three times.

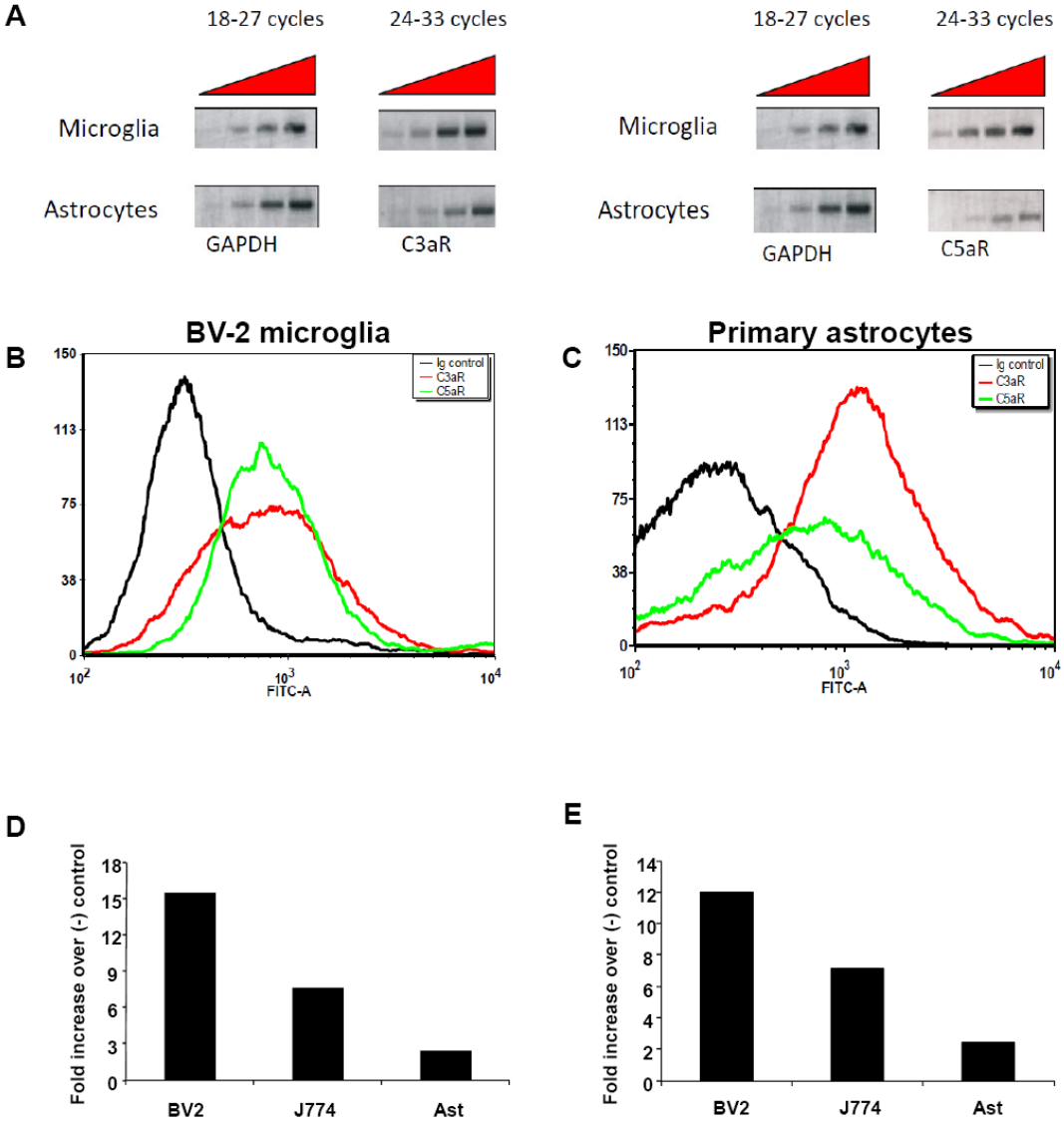
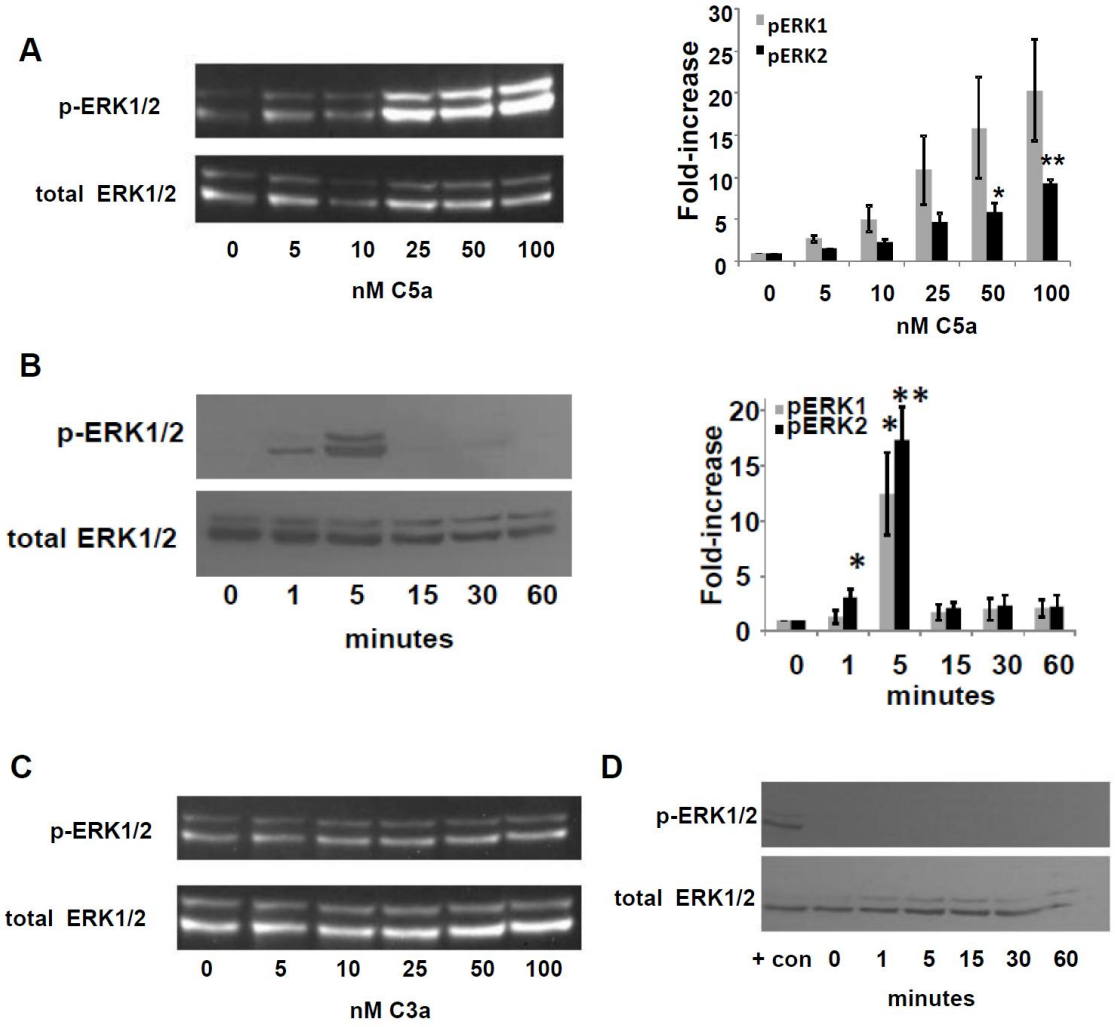

A
18-27 cycles
24-33 cycles
Microglia
Astrocytes
GAPDH
C3aR
18-27 cycles
24-33 cycles
Microglia
Astrocytes
GAPDH
C5aR
B
BV-2 microglia
Ig control
C3aR
C5aR
150
113
75
38
0
10^2
10^3
10^4
FITC-A
C
Primary astrocytes
Ig control
C3aR
C5aR
150
113
75
38
0
10^2
10^3
10^4
FITC-A
D
Fold increase over (-) control
18
15
12
9
6
3
0
BV2
J774
Ast
E
Fold increase over (-) control
14
12
10
8
6
4
2
0
BV2
J774
Ast

Figure 17. C5a activates MAPK signaling pathways in BV-2 microglia
(A) BV-2 cells were stimulated with 0-100 nM C5a for five minutes and whole cell lysates were used to analyze ERK1/2 activation by immunoblotting. After pERK1/2 were detected, all blots were stripped and re-probed for total ERK1/2. (B) BV-2 cells were stimulated with 50nM C5a for 0-60 minutes and whole cell lysates were used to analyze ERK1/2 activation by immunoblotting. After pERK1/2 were detected, all blots were stripped and re-probed for total ERK1/2. The density of each band was measured using ImageJ software. Phosphorylated values were normalized to total values to account for any discrepancies in protein loading. (C) BV-2 cells were stimulated with 0-100 nM C3a for five minutes and whole cell lysates were used to analyze ERK1/2 activation by immunoblotting. After pERK1/2 were detected, all blots were stripped and re-probed for total ERK1/2. (D) BV-2 cells were stimulated with 50nM C3a for 0-60 minutes and whole cell lysates were used to analyze ERK1/2 activation by immunoblotting. After pERK1/2 were detected, all blots were stripped and re-probed for total ERK1/2. Statistical significance was calculated using unpaired Student's *t*-test. For these calculations, values from each experimental timepoint were compared to untreated cells, where * $p < 0.05$ and ** $p < 0.001$. Three blots from three different experiments were used for measuring ERK1/2 activation. Error bars represent standard deviation.


A
p-ERK1/2
total ERK1/2
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nM C5a
pERK1
pERK2
Fold-increase
0 5 10 25 50 100
nM C5a
B
p-ERK1/2
total ERK1/2
0 1 5 15 30 60
minutes
pERK1
pERK2
Fold-increase
0 1 5 15 30 60
minutes
C
p-ERK1/2
total ERK1/2
0 5 10 25 50 100
nM C3a
D
p-ERK1/2
total ERK1/2
+ con 0 1 5 15 30 60
minutes

Figure 18. Anaphylatoxins activate ERK1/2 in primary astrocytes

(A) Primary astrocytes were stimulated with 50nM C5a for 0-60 minutes and whole cell lysates were used to analyze ERK1/2 activation by immunoblotting. After pERK1/2 were detected, all blots were stripped and re-probed for total ERK1/2. (B) The density of each band was measured using ImageJ software. Phosphorylated values were normalized to total values to account for any discrepancies in protein loading. (C) Primary astrocytes were stimulated with 50nM C3a for 0-60 minutes and whole cell lysates were used to analyze ERK1/2 activation by immunoblotting. After pERK1/2 were detected, all blots were stripped and re-probed for total ERK1/2. (D) The density of each band was measured using ImageJ software. Phosphorylated values were normalized to total values to account for any discrepancies in protein loading. Three blots from three different experiments were used for measuring ERK1/2 activation. Statistical significance was calculated using unpaired Student's *t*-test. For these calculations, values from each experimental timepoint were compared to untreated cells, where * $p < 0.05$. Error bars represent standard deviation.

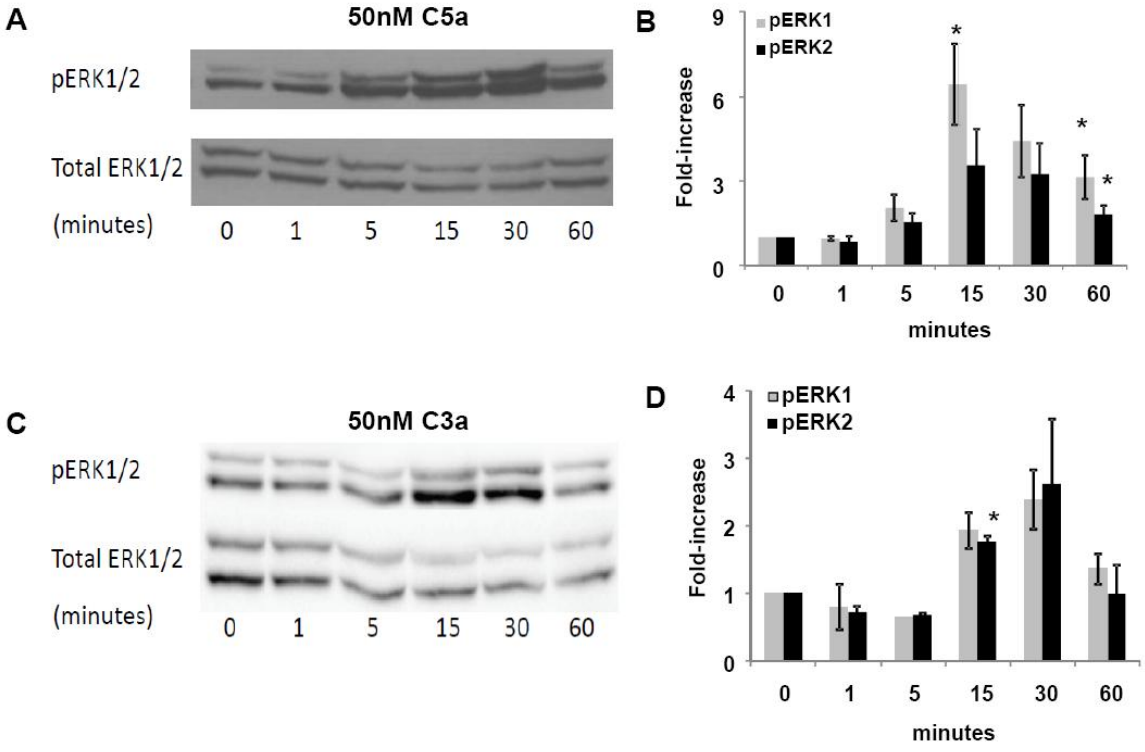

A
50nM C5a
pERK1/2
Total ERK1/2
(minutes)
0 1 5 15 30 60
B
pERK1
pERK2
Fold-increase
minutes
C
50nM C3a
pERK1/2
Total ERK1/2
(minutes)
0 1 5 15 30 60
D
pERK1
pERK2
Fold-increase
minutes

Figure 19. Anaphylatoxins activate JNK in primary astrocytes

(A) Primary astrocytes were stimulated with 50nM C5a for 0-60 minutes and whole cell lysates were used to analyze JNK activation by immunoblotting. After pJNK was detected, all blots were stripped and re-probed for total JNK. The density of each band was measured using ImageJ software. Phosphorylated values were normalized to total values to account for any discrepancies in protein loading. (B) Primary astrocytes were stimulated with 50nM C3a for 0-60 minutes and whole cell lysates were used to analyze JNK activation by immunoblotting. After pJNK was detected, all blots were stripped and re-probed for total JNK. The density of each band was measured using ImageJ software. Phosphorylated values were normalized to total values to account for any discrepancies in protein loading. Statistical significance was calculated using unpaired Student's *t*-test. For these calculations, values from each experimental timepoint were compared to untreated cells, where * $p < 0.05$ and ** $p < 0.001$. Three or more blots were used for measuring JNK activation in primary astrocytes. Error bars represent standard deviation.

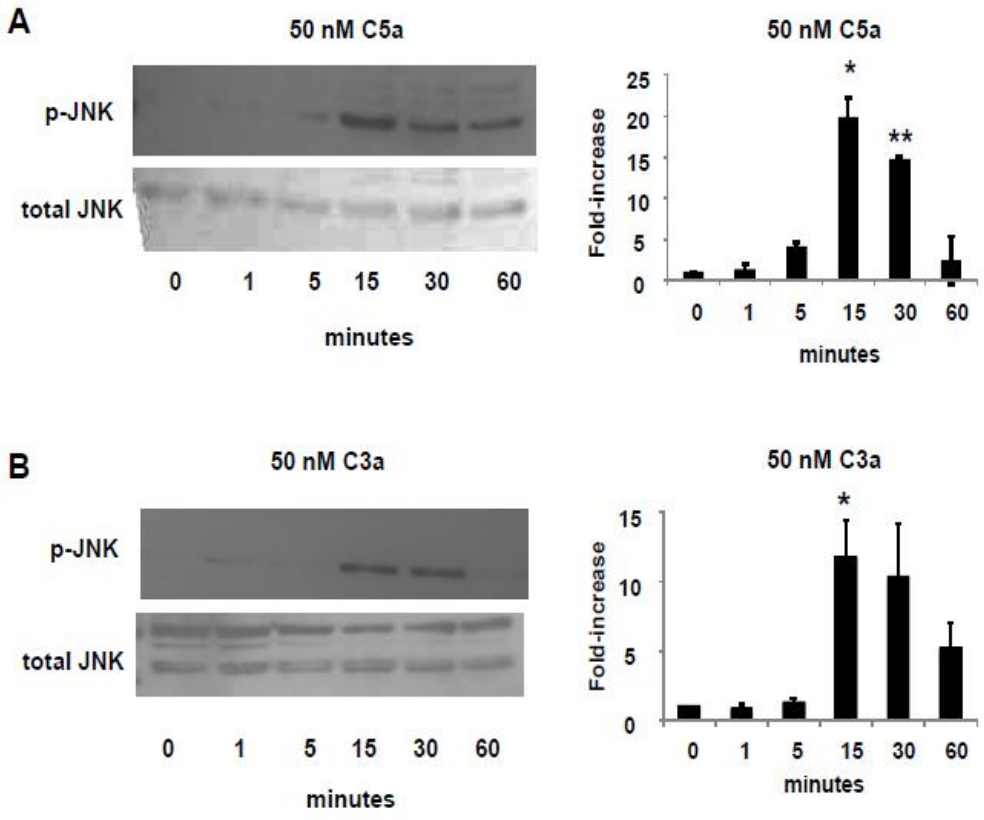

A
50 nM C5a
p-JNK
total JNK
0 1 5 15 30 60
minutes
50 nM C5a
Fold-increase
25
20
15
10
5
0
*
**
0 1 5 15 30 60
minutes
B
50 nM C3a
p-JNK
total JNK
0 1 5 15 30 60
minutes
50 nM C3a
Fold-increase
15
10
5
0
*
0 1 5 15 30 60
minutes

Figure 20. C3a and C5a do not induce chemotaxis of primary astrocytes or microglia (A) The indicated concentrations of C3a or C5a were placed in a 96-well chemotactic plate and a membrane with 5 um pores was placed over these wells. 1×10^5 J774 macrophages were placed on top of the membrane and the corresponding well containing either C3a, C5a or medium alone. The cells were allowed to incubate for three hours at 37º C. Cells were quantified using an ATP luminescence assay. Relative light units (RLU) from the samples were compared to a standard curve RLUs of known quantities of J774 cells. The error bars represent standard deviation. (B) Indicated amounts of C3a or C5a were placed in a 96-well chemotactic plate and a membrane with 5 um pores was placed over these wells. 1×10^5 primary microglia were placed on top of the membrane and the corresponding well containing either C3a, C5a or medium alone as a negative control. The cells were allowed to incubate for four hours at 37º C. Cells were quantified using an ATP luminescence assay. The values represent relative light units (RLU) and the error bars represent standard deviation. (C) Indicated amounts of C3a or C5a were placed in a 96-well chemotactic plate and a membrane with 8 um pores was placed over these wells. 1×10^5 primary astrocytes were placed on top of the membrane and the corresponding well containing either C3a, C5a or medium alone. The cells were allowed to incubate for four hours at 37º C. Cells were quantified using an ATP luminescence assay. Relative light units (RLU) from the samples were compared to a standard curve RLUs of known quantities of primary astrocyte cells. The error bars represent standard deviation. All of the above experiments were repeated at least three times. Statistical significance was calculated using unpaired Student's *t*-test. For these calculations, the experimental anaphylatoxin concentrations were compared to negative control values, where * $p < 0.05$.

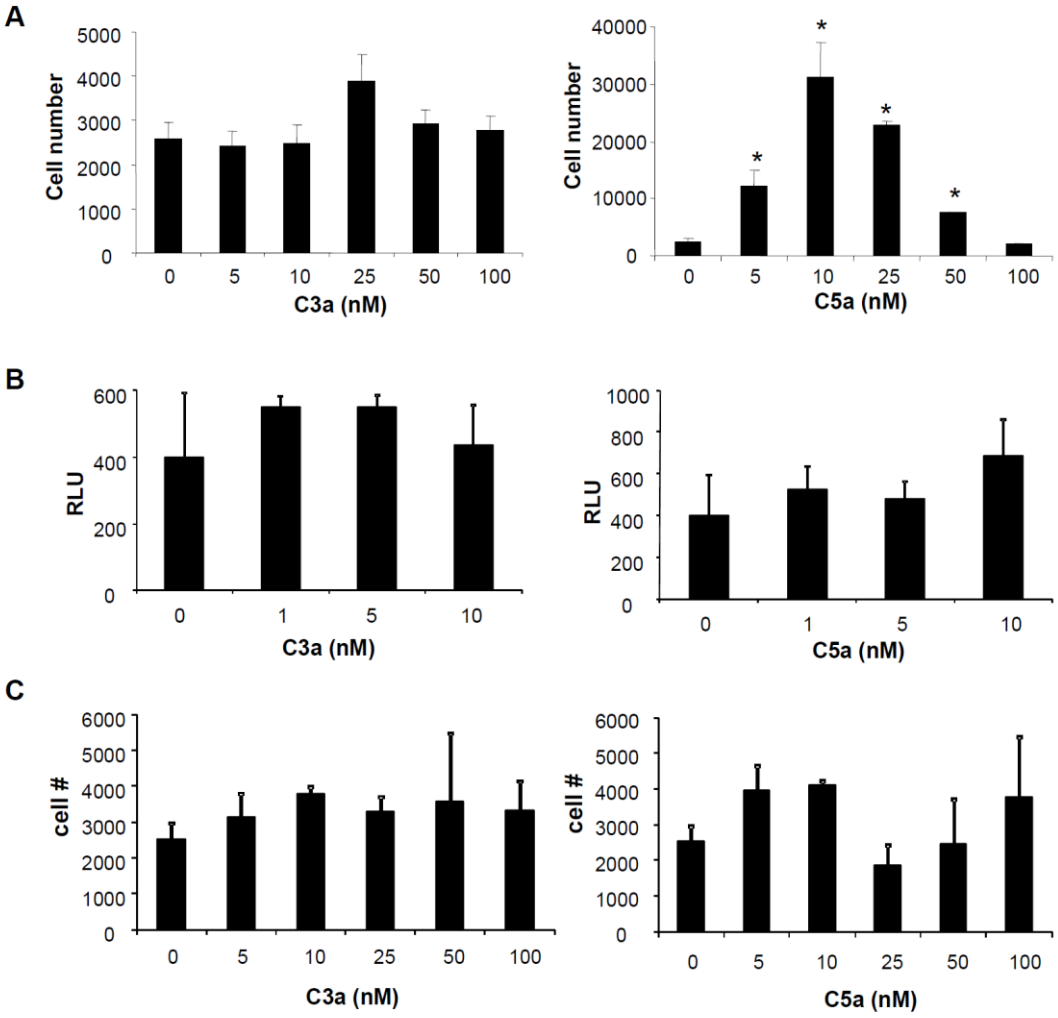

A
Cell number
5000
4000
3000
2000
1000
0
0 5 10 25 50 100
C3a (nM)
Cell number
40000
30000
20000
10000
0
*
*
*
0 5 10 25 50 100
C5a (nM)
B
RLU
600
400
200
0
0 1 5 10
C3a (nM)
RLU
1000
800
600
400
200
0
0 1 5 10
C5a (nM)
C
cell #
6000
5000
4000
3000
2000
1000
0
0 5 10 25 50 100
C3a (nM)
cell #
6000
5000
4000
3000
2000
1000
0
0 5 10 25 50 100
C5a (nM)

Table 2. C3a and/or C5a induce cytokine and chemokine production in mixed glia

	1 day treatment				3 day treatment				6 day treatment			
Analyte	None	C3a	C5a	Both	None	C3a	C5a	Both	None	C3a	C5a	Both
IL-1α	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-1β	126	83	176	134	15	9	45	15	20	49	30	25
IL-2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-3	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-4	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-5	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-6	109	265	203	334	22	58	65	95	8	19	20	30
IL-9	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-10	2	3	4	3	5	3	11	4	5	16	9	5
IL-12 (p40)	225	266	356	299	185	187	280	270	177	343	309	252
IL-12 (p70)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-13	45	41	72	41	nd	nd	10	nd	nd	41	24	21
IL-17	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
CCL11	232	240	407	301	49	52	480	100	176	208	471	126
G-CSF	248	207	372	298	224	162	386	257	206	217	308	246
GM-CSF	21	26	33	31	nd	10	24	16	14	48	38	23
IFN-γ	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
CXCL1	2993	3024	3787	3163	2877	2323	2365	3130	3266	2934	3537	3285
CCL2	25981	25706	43278	33024	16567	13779	20672	19919	13604	13713	18086	14459
CCL3	2845	1665	3695	3114	74	46	132	64	88	110	91	64
CCL4	2097	1390	3589	2426	222	51	143	112	267	97	131	106
CCL5	831	657	1342	976	205	119	442	240	327	137	337	230
TNF-α	10	9	18	16	7	5	17	7	9	11	17	13

Note: Mixed glia were left untreated or stimulated with 50nM C3a, 50nM C5a or a combination of 50nM C3a and C5a. After one, three or six days of treatment, cell supernatants were collected from the cell cultures and analyzed using a multiplex assay. These data are from a single multiplex experiment and all samples were run in duplicate. The numbers reported are pg/mL concentrations. nd, analyte was non-detectable in the cell supernatant. The shaded boxes represent samples in which a two-fold change in analyte levels between the experimental treatment and the untreated cells occurred.

Figure 21. Anaphylatoxins induce cytokine and chemokine production in mixed glial cultures

(A) Mixed glia were stimulated with 50nM C3a, 50nM C5a or a combination of 50nM C3a and C5a. After one, three or six days, cell supernatant was collected from cell cultures and analyzed using a multiplex assay. These data are from a single multiplex experiment and all samples were run in duplicate. (B) Mixed glia were treated with 50nM C3a, 50nM C5a or a combination of 50nM C3a and C5a for one day. After one day of treatment, supernatants were collected and used in ELISAs specific for IL-6 or CCL4. Samples from three experiments were used for ELISAs. Statistical significance was calculated using unpaired Student's *t*-test. For these calculations, values from C3a/C5a treated cells were compared to cells that were left untreated for one day, where * $p < 0.05$. Error bars represent standard deviation.

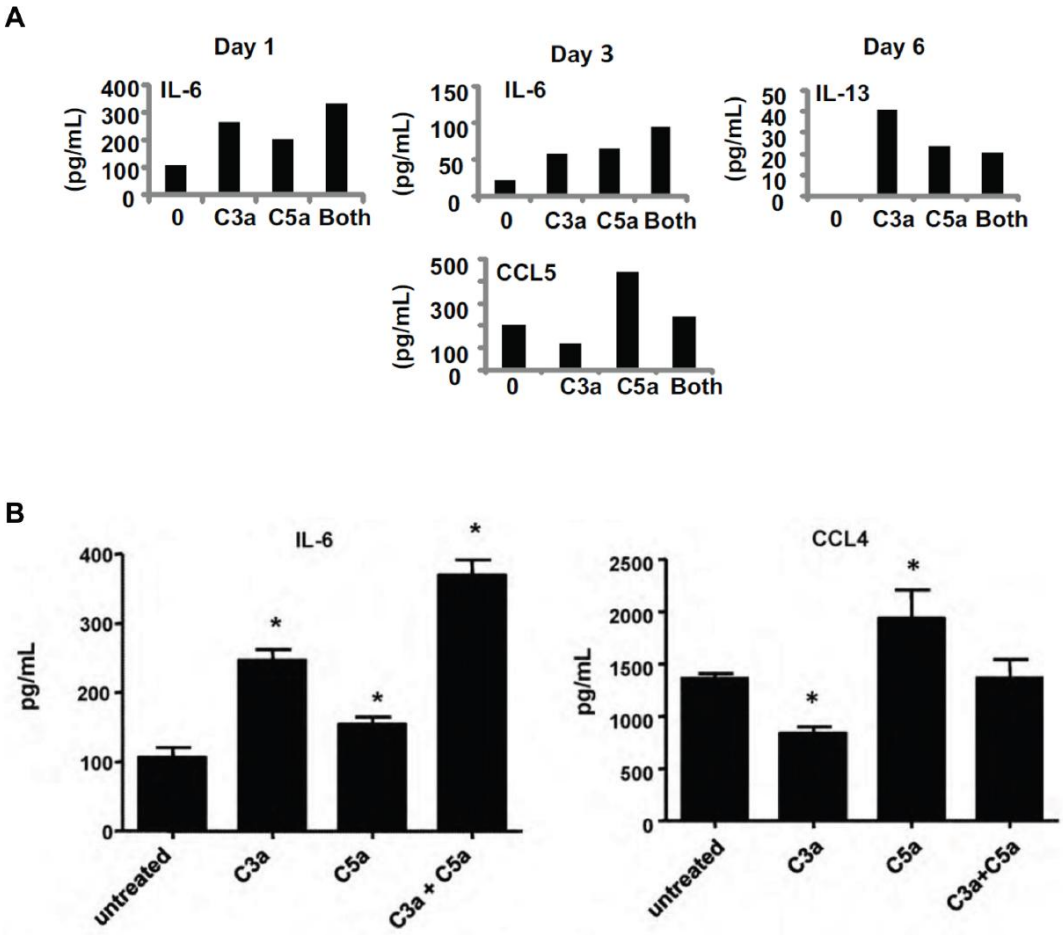

A
Day 1
IL-6
(pg/mL)
400
300
200
100
0
0 C3a C5a Both
Day 3
IL-6
(pg/mL)
150
100
50
0
0 C3a C5a Both
Day 6
IL-13
(pg/mL)
50
40
30
20
10
0
0 C3a C5a Both
CCL5
(pg/mL)
500
300
100
0
0 C3a C5a Both
B
IL-6
pg/mL
400
300
200
100
0
*
*
*
untreated
C3a
C5a
C3a + C5a
CCL4
pg/mL
2500
2000
1500
1000
500
0
*
*
untreated
C3a
C5a
C3a+C5a

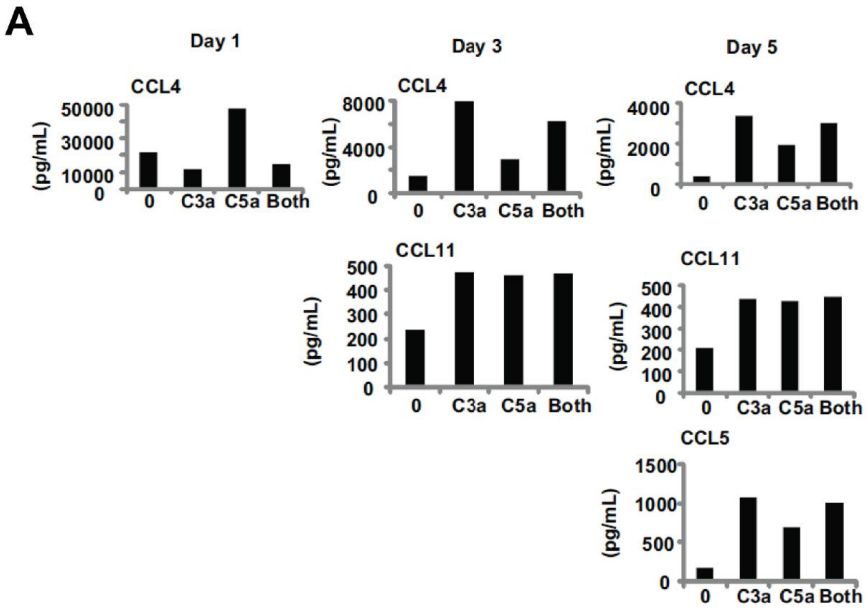
Table 3. C3a and/or C5a induce cytokine and chemokine production in BV-2 microglia

	1 day treatment				3 day treatment				5 day treatment			
Analyte	None	C3a	C5a	Both	None	C3a	C5a	Both	None	C3a	C5a	Both
IL-1α	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-1β	315	286	341	298	288	339	328	351	248	344	335	324
IL-2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-3	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-4	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-5	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-6	37	35	38	36	21	18	37	23	14	5	17	8
IL-9	100	60	90	73	73	118	83	102	60	81	77	73
IL-10	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-12 (p40)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-12 (p70)	34	40	43	38	23	14	33	21	25	15	35	33
IL-13	108	105	131	100	71	114	80	103	59	105	83	71
IL-17	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
CCL11	381	402	494	363	233	474	463	468	209	438	428	448
G-CSF	191	128	233	168	182	106	219	154	211	78	204	113
GM-CSF	44	38	41	31	30	41	38	42	31	37	38	34
IFN-γ	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
CXCL1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
CCL2	12213	10776	14948	13062	15392	16817	19883	19521	19943	14439	19322	15481
CCL3	5425	5282	5772	5445	5612	5970	5757	5833	5393	5819	5846	5762
CCL4	21665	11590	47916	14436	1514	7957	2963	6184	403	3370	1927	3016
CCL5	1244	1142	1231	1159	1129	1264	1219	1305	171	1067	691	1000
TNF-α	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

Note: BV-2 microglia were left untreated or stimulated with 50nM C3a, 50nM C5a or a combination of 50nM C3a and C5a. After one, three or five days of treatment, cell supernatants were collected from the cell cultures and analyzed using a multiplex assay. These data are from a single multiplex experiment and all samples were run in duplicate. The numbers reported represent pg/mL concentrations. nd, analyte was non-detectable in the cell supernatant. The shaded boxes represent samples in which a two-fold change in analyte levels between the experimental treatment and the untreated cells occurred.

Figure 22. Anaphylatoxins induce CCL4 production in BV-2 microglia

(A) BV-2 microglia were stimulated with 50nM C3a, 50nM C5a or a combination of 50nM C3a and C5a. After one, three or five days cell supernatant was collected from the cell cultures and analyzed using a multiplex assay. These data are from a single multiplex experiment and all samples were run in duplicate. (B) BV-2 microglia were treated with 50nM C3a, 50nM C5a or a combination of 50nM C3a and C5a for three days. After three days of treatment, supernatants were collected and used in an ELISA specific for CCL4. Samples from three experiments were used for ELISAs. Statistical significance was calculated using unpaired student's *t*-test. For these calculations, values from C3a/C5a treated cells were compared to cells that were left untreated for three days, where * $p < 0.05$. Error bars represent standard deviation.


A
Day 1
Day 3
Day 5
CCL4
(pg/mL)
50000
30000
10000
0
8000
4000
4000
2000
0 C3a C5a Both
CCL11
500
400
300
200
100
CCL5
1500
1000
500

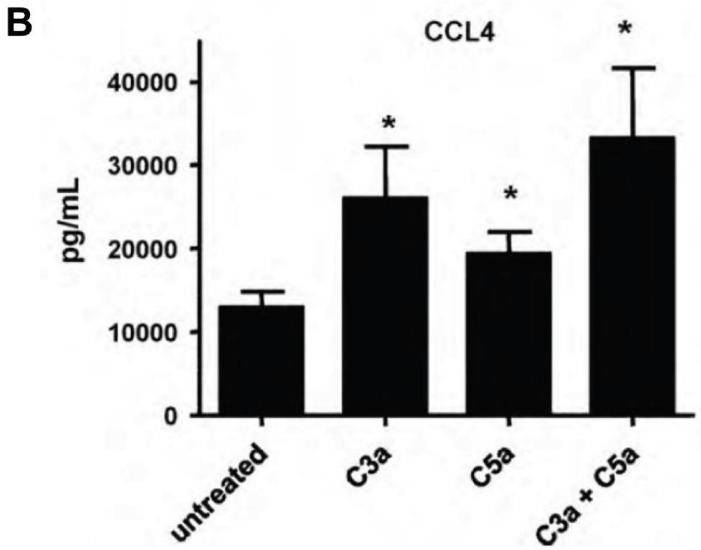

B
CCL4
pg/mL
40000
30000
20000
10000
0
*
untreated
C3a
C5a
C3a + C5a

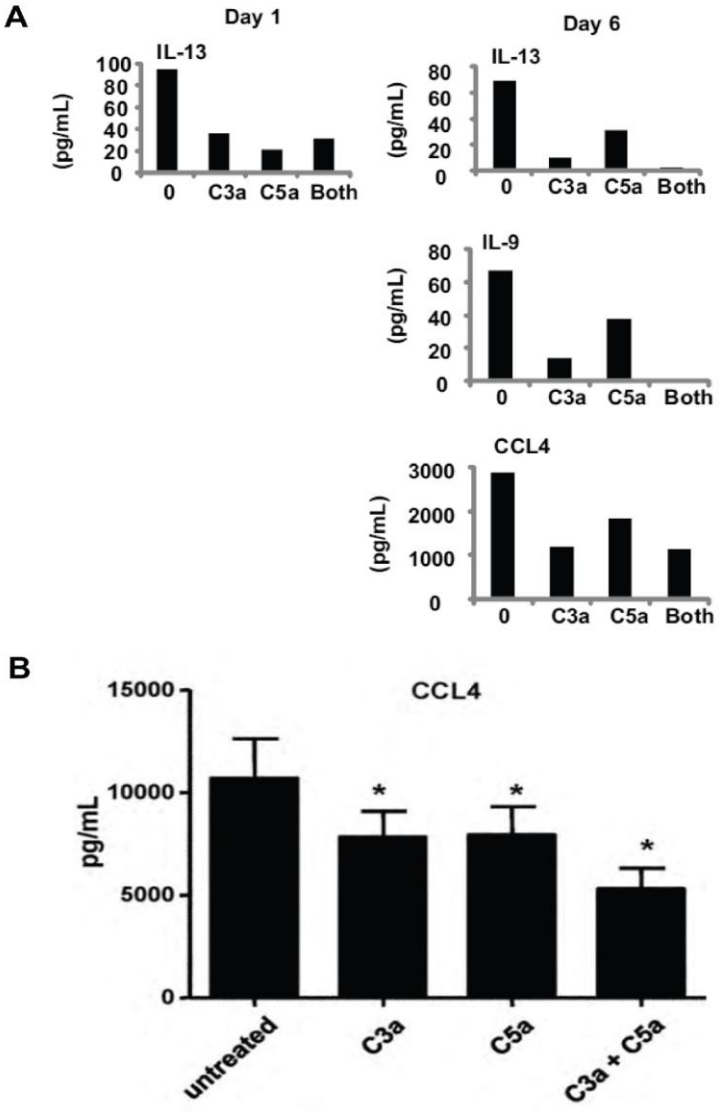
Table 4. C3a and/or C5a induce cytokine and chemokine production in primary astrocytes

	1 day treatment				3 day treatment				6 day treatment			
Analyte	None	C3a	C5a	Both	None	C3a	C5a	Both	None	C3a	C5a	Both
IL-1α	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-1β	151	126	139	118	185	144	152	132	182	98	141	92
IL-2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-3	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-4	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-5	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-6	205	229	187	242	121	164	122	155	61	69	63	88
IL-9	41	31	31	55	64	38	48	22	67	13	38	nd
IL-10	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-12 (p40)	121	97	104	100	107	89	93	88	96	65	83	80
IL-12 (p70)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-13	95	36	21	31	60	45	34	21	69	nd	31	nd
IL-17	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
CCL11	380	402	494	363	233	474	463	468	210	438	427	448
G-CSF	169	105	134	112	186	116	143	118	199	102	155	129
GM-CSF	29	26	25	28	35	35	29	24	26	23	19	18
IFN-γ	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
CXCL1	2083	1640	1748	1684	2354	1989	1962	1911	2854	2215	2470	2323
CCL2	14597	12692	13493	13343	19044	15895	15171	13869	17497	14045	15650	15121
CCL3	3420	2903	3216	3020	3858	3446	3517	3205	3953	2455	3273	2224
CCL4	2578	1948	2203	1864	3689	2544	2363	1982	2868	1206	1832	1133
CCL5	1188	974	1066	1056	1190	973	1083	985	1327	849	1127	901
TNF-α	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

Note: Primary astrocytes were left untreated or stimulated with 50nM C3a, 50nM C5a or a combination of 50nM C3a and C5a. After one, three or six days of treatment, cell supernatants were collected from the cell cultures and analyzed using a multiplex assay. These data are from a single multiplex experiment and all samples were run in duplicate. The numbers reported are pg/mL concentrations. nd, analyte was non-detectable in the cell supernatant. The shaded boxes represent samples in which a two-fold change in analyte levels between the experimental treatment and the untreated cells occurred.

Figure 23. C3a/C5a decrease cytokine production in primary astrocytes

(A) Primary astrocytes were stimulated with 50nM C3a, 50nM C5a or a combination of 50nM C3a and C5a. After one, three or six days, cell supernatant was collected from the cell cultures and analyzed using a multiplex assay. These data are from a single multiplex experiment and all samples were run in duplicate. (B) Primary astrocytes were stimulated with 50nM C3a, 50nM C5a or a combination of 50nM C3a and C5a. After six days, cell supernatants were collected from the cell cultures and analyzed using an ELISA specific for CCL4. Samples from three experiments were used for ELISAs. Statistical significance was calculated using unpaired student's *t*-test. For these calculations, values from C3a/C5a treated cells were compared to cells that were left untreated for six days, where * $p < 0.05$. Error bars represent standard deviation.


A
Day 1
IL-13
100
80
60
40
20
0
(pg/mL)
0
C3a
C5a
Both
Day 6
IL-13
80
60
40
20
0
(pg/mL)
0
C3a
C5a
Both
IL-9
80
60
40
20
0
(pg/mL)
0
C3a
C5a
Both
CCL4
3000
2000
1000
0
(pg/mL)
0
C3a
C5a
Both
B
CCL4
15000
10000
5000
0
pg/mL
*
*
*
untreated
C3a
C5a
C3a + C5a

CHAPTER V

DISCUSSION

Summary

The goal of these experiments was to test the central hypothesis that the production of C3 and downstream effector complement proteins would exacerbate demyelination pathology through activation of glial cells, leading to upregulation of inflammatory mediators. From these studies, I have determined that complement proteins C3a and C5a play a detrimental role in demyelination in the murine cuprizone model. When C3a or C5a were overexpressed specifically in the CNS, increased demyelination severity was observed. Demyelination in transgenic mice was characterized by increased cellularity within the demyelinating corpus callosum. Upon further experimentation, I found that RCA staining intensity was increased in the corpus callosum of C3a and C5a transgenic mice, indicating an increased presence of microglia. Cells staining positive for CD3 were also detected in C5a transgenic mice, indicating an increase of T cells in the corpus callosum. Even though demyelination was exacerbated in C3a and C5a transgenic mice, these mice were still able to remyelinate effectively by the end of the ten week study. There was evidence that increased C3a and C5a expression in the brain led to increased oligodendrocytes in the corpus callosum upon the removal of cuprizone. Interestingly, various parameters of demyelination and remyelination were not affected in $C3^{-/-}$ mice when compared to control mice.

When individual glial subsets were analyzed, microglia and astrocytes were found to express receptors for C3a and C5a at mRNA and protein levels. *In vitro* studies provided evidence for cellular activation of different glial cell subsets when these cells were treated with C3a or C5a. C5a stimulated BV-2 microglia showed upregulation of activated ERK1/2 proteins, while C3a and C5a stimulated astrocytes showed upregulation of activated ERK1/2 and JNK proteins. Finally, upon stimulation with

anaphylatoxins, mixed glia and BV-2 microglia produced inflammatory cytokines and chemokines; including IL-6 and CCL4. In contrast to previous data, microglia and astrocytes did not significantly migrate to C3a or C5a in *in vitro* migration assays.

Discussion

Results of $C3^{-/-}$ studies

Initially, $C3^{-/-}$ mice were utilized to establish an overall role for complement in demyelination and remyelination pathology in cuprizone treated mice. These mice were tested based on previous results from sCrry transgenic mice, suggesting complement plays a crucial role in exacerbating disease pathology in the cuprizone model (107). The results obtained from the $C3^{-/-}$ studies imply that C3 and downstream complement proteins are inconsequential for pathology in the cuprizone system. However, deleting so many potential effector mechanisms from these mice could have eliminated both harmful and protective responses, negating any observable effects in these mice. For these reasons, I moved on to testing individual complement proteins and their role in cuprizone-mediated demyelination.

Past studies utilizing a different strain of $C3^{-/-}$ mice provided conflicting data. When EAE was induced in these mice, one group found that C3 exacerbated disease severity (96, 108) while another group, similar to our results, found no changes when compared to control mice (132). These results may suggest that this specific strain is an unreliable model for testing complement function in the CNS; however it would be interesting to know if cuprizone had the same effect on this strain of $C3^{-/-}$ mice, or if the results I obtained were a strain-specific issue. Also, if these studies were to be repeated, it may be beneficial to use C57BL/6 WT mice as controls instead of $C3^{+/-}$ mice, as I did. WT mice would theoretically be able to produce more complement upon cuprizone exposure and thus the differences between $C3^{-/-}$ and WT mice could potentially be greater.

C3a involvement in demyelination and glial cell activation

While results from $C3^{-/-}$ mice showed no change compared to control mice that were treated with cuprizone, CNS-specific expression of C3a or C5a was shown to exacerbate demyelination severity upon cuprizone treatment. Previous *in vivo* data from EAE studies have also suggested a role for C3a and C3aR in demyelination severity (99); however *in vivo* data have not provided a clear picture of how C3a mediates its specific effects. Our *in vitro* studies suggest that recombinant murine C3a does not induce significant migration of microglia or primary astrocytes, suggesting C3a may not be able to induce chemotaxis of glial cells on its own. However, it is possible that other growth factors, chemokines or cytokines are needed in conjunction with C3a to induce significant migration of astrocytes and microglia. Additionally, because individual cells were isolated, they may lack the necessary interactions with other types of glia to induce cellular migration. Future studies should address whether mixed glial cells can migrate to C3a/C5a to determine if cell interactions are needed for this process. Therefore, while there was not significant migration *in vitro*, it is possible that C3a encourages cellular migration in cooperation with other trophic or inflammatory molecules in an inflammatory *in vivo* setting, such as cuprizone treatment.

Previous studies have shown that when astrocytes or microglia are stimulated with C3a, they have the capability to upregulate neural growth factor (NGF) and cytokine mRNA expression (51, 60, 61, 157); however the ability of C3a to upregulate cytokine and chemokine protein levels has not been previously tested. I found that when BV-2 microglia were treated with C3a, CCL4, CCL5 and CCL11 were increased when cell supernatants were analyzed in a multiplex experiment. In previous experiments, microglia have been shown to express CCR3 and CCR5, receptors for CCL4 and CCL5 (171), while astrocytes express CCR3, in addition to CCR1, another receptor for CCL5 (172, 173). Additionally, previous cuprizone studies have shown that CCL4 and CCL5 mRNA transcripts are upregulated in the brain during cuprizone treatment (123, 174).

The expression of chemokine receptors on glia in conjunction with upregulated chemokine production by C3a stimulated microglia *in vitro* may be a potential mechanism by which C3a indirectly induces migration of reactive microglia and astrocytes to the demyelinating corpus callosum during cuprizone treatment.

IL-6 was also upregulated in mixed glial cultures stimulated with C3a/C5a. Because of the many functions IL-6 is known to regulate, it is difficult to determine what role IL-6 might be playing in the presence of C3a/C5a stimulation. Overexpression of IL-6 can play a detrimental role in the CNS, leading to BBB breakdown, neurodegeneration, microglial/macrophage activation and gliosis (175, 176). Despite the many known proinflammatory effects of IL-6, there has been evidence to suggest this cytokine may also have neurotrophic functions. For instance, IL-6 protects neurons from *N*-methyl-*D*-aspartate (NMDA) toxicity *in vitro* (177). Also, previous findings have shown that IL-6 promotes neuronal survival, neural stem cell differentiation and neurite growth (178, 179). Finally, IL-6 mediates the release of many neurotrophins; including, glial cell-derived neurotrophic factor (GDNF), neurotrophin-3 (NT-3) and NGF (180, 181). These molecules promote neuronal survival and interactions between neurons and immune cells. Future studies will be needed to determine the overall effect of IL-6 signaling in demyelination and remyelination models, including the cuprizone model.

Overall, our *in vivo* data suggest overexpression of C3a induces a proinflammatory response during cuprizone treatment, similar to results found in the EAE model (99). In contrast, previous data have shown that C3a is able attenuate inflammation in the CNS. For example, C3a-GFAP mice are protected from LPS-induced shock (182), suggesting that C3a expressed in the CNS is capable of inducing a neuroprotective response, as well as an exacerbating response. Additionally, when mixed glia were treated with C3a, there was a significant decrease in the amount of CCL4 present in the supernatant compared to untreated cells and cells that were treated with C5a alone or a combination of C3a/C5a. In contrast, C3a upregulated IL-6 production in

mixed glial cells. Therefore, depending on the stimuli in the CNS, C3a can induce detrimental or protective outcomes. Overall, these data suggest that the outcome of C3a signaling most likely depends on other inflammatory or regulatory factors present in the local environment.

C5a involvement in demyelination and glial cell activation

Upon treatment with cuprizone, C5a-GFAP mice had increased RCA staining compared to control mice during demyelination, indicating an increased presence of microglia. Despite C5a being a well known chemoattractant for many cell types, I found that microglia and astrocytes did not significantly migrate to this molecule in vitro, similar to results observed in C3a stimulated cells. J774 macrophages significantly migrated to C5a, indicating that our purified C5a recombinant protein was able to function as a chemoattractant. Similar to cells treated with C3a, glia produced upregulated levels of IL-6, CCL4, CCL5 and CCL11 in cell cultures treated with C5a. The ability of glia to produce chemokines in response to C5a presents an alternative mechanism for the observed increase in cellularity in the corpus callosum during demyelination in our *in vivo* studies. Future studies will be required to determine if any of these chemokines are necessary for demyelination events in the cuprizone model.

In addition to increased RCA staining, our studies demonstrate positive CD3 staining at five weeks of cuprizone treatment in C3a and C5a transgenic mice. Although this staining only occurred in approximately 50% of the C3a transgenic mice, staining was evident in all of the C5a transgenic mice that had received cuprizone treatment for five weeks. Since an increase in CD3 staining in untreated C5a-GFAP mice or cuprizone treated WT mice was not detected, it is assumed that the BBB was intact in transgenic mice before cuprizone treatment and in WT mice after five weeks of cuprizone treatment. Cuprizone studies in C5a transgenic mice suggest that during cuprizone treatment, the BBB may be more permeable compared to WT mice. A recent study has shown evidence

of $CXCR2^+$ neutrophils crossing into the brain during cuprizone treatment. Interestingly, neutrophil infiltration into the corpus callosum occurred at five days after the initiation of cuprizone treatment (183). These results may indicate that important inflammatory events occur before three weeks of cuprizone treatment. My data and the neutrophil study provide evidence that the BBB may be more permissive during cuprizone treatment than originally thought.

Past studies have provided some preliminary evidence that C5a alters BBB permeability *in vivo*. For instance, in a murine model of CNS lupus, when mice were treated with a C5aR antagonist, inter-cellular adhesion molecule-1 (ICAM-1/CD54) mRNA expression and the number of neutrophils were decreased in the brain (76). Additionally, when endothelial cells were treated with serum from lupus mice in the presence of a C5aR antagonist, endothelial cell permeability was decreased (140). Using an anti-C5a antibody in an experimental model of septic encephalopathy resulted in decreased BBB permeability (139). Finally, previous studies demonstrated that C5a stimulation upregulated adhesion molecule expression on various cell types (184). These data may suggest that upon exposure to C5a, cells of the BBB, such as endothelial cells and astrocytes are capable of upregulating adhesion molecules. If chemokines, such as CCL4 and CCL5, are upregulated within the parenchyma in response to C5a and adhesion molecules are upregulated on the endothelial or astrocyte cells of the BBB, this would create an ideal environment for inflammatory cell traffic across the BBB, which could explain the increase in $CD3^+$ T cells observed in C5a-GFAP mice. Previous studies have also shown that CCL4 increases the adherence of T-cells to endothelial cells, in *in vitro* experiments (185). Future studies should be directed at determining the role of anaphylatoxins on BBB permeability, and whether or not anaphylatoxins contribute to cellular traffic into the CNS.

The role of anaphylatoxins in remyelination

Using antagonists to the C3aR and/or C5aR may decrease inflammation in lesions; however the importance of C3a and C5a production during remyelination remains uncertain. One of the discouraging problems in some MS patients is the lack of remyelination by oligodendrocytes within areas of demyelination. This lack of remyelination has been associated with either an absence of migrating oligodendrocyte precursor cells into the demyelinating lesion, oligodendrocyte precursors that do not mature properly or do not receive the proper signals to begin remyelinating within the lesion (186).

My results suggest that C3a and C5a could enhance the recruitment of oligodendrocytes into demyelinated areas (Fig. 24). Consequently, blocking C3aR and C5aR activity could potentially be detrimental to remyelination processes. On the other hand, the increase in damage to the myelin sheaths and microglial presence in the corpus callosum could be responsible for the recruitment or proliferation of oligodendrocytes, in contrast to the actual C3a and/or C5a proteins themselves (Fig. 24). Previous studies have shown that an increase in inflammation could be necessary for recruitment of oligodendrocyte precursors into demyelinated lesions (142); however the mechanism that mediates enhanced remyelination in the presence of inflammatory mediators is unknown. This phenomenon could explain why remyelination is more likely to occur in acute inflammatory lesions, in contrast to chronic demyelinated lesions. Regardless of the mechanism at play, C3a and C5a transgenic mice were able to remyelinate as effectively as WT mice even though inflammation was more severe during peak demyelination.

Implications for human disease and proposed experimental model

It was previously established that complement deposition was present within type II lesions in MS patients (85) and C3a and C5a receptors were upregulated in some

patients with MS (50, 93). These results suggest that C3a and/or C5a may play a role in MS demyelination and lesion pathology. Results from my *in vitro* and *in vivo* experiments led me to propose the following model to detail how C3a and C5a affect demyelination and remyelination pathogenesis (Fig. 24). In MS, it is likely that the classical complement pathway is activated by C1q binding anti-myelin Ab-Ag complexes to initiate production of C3a and C5a, along with other downstream complement effector molecules, including C3b and MAC (Fig. 24). In lesions where antibody is absent, such as lesions induced by cuprizone, C1q could bind myelin breakdown products to initiate the classical pathway, which has previously been shown to occur in *in vitro* studies (3).

Upon activation of the classical pathway, several effector products are formed upon cleavage of C3 and downstream C5; including C3a and C5a proteins (Fig. 24). These complement effector proteins may all play some role in damage to the myelin sheaths. Finally, complement production may be further amplified by activation of the alternative pathway. In addition to increased migration of inflammatory cells to C3a and/or C5a, these molecules could be acting on several cell types to induce increased chemokine production, which also mediates cell migration, further stimulating increased cellularity within inflammatory lesions (Fig. 24).

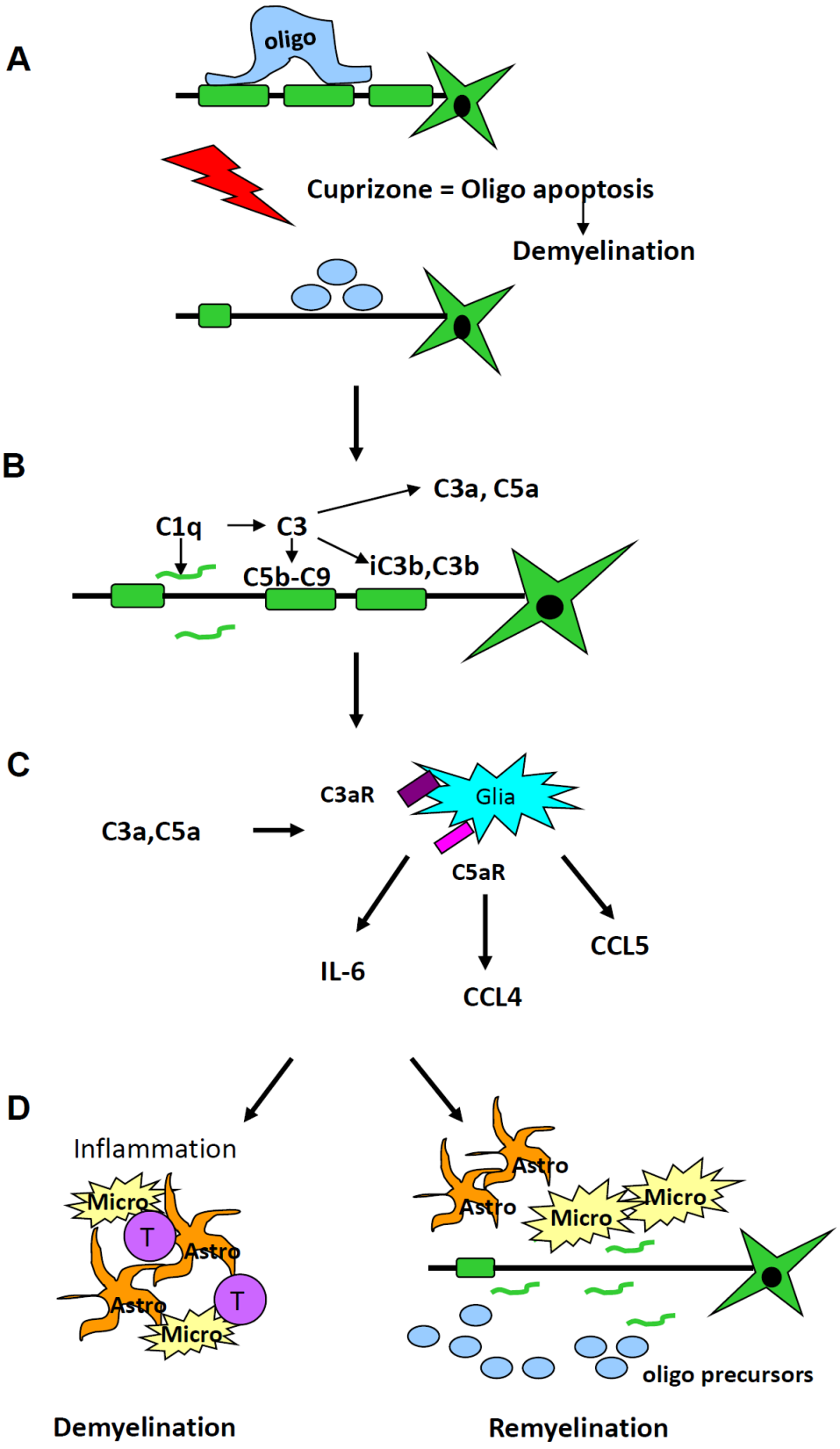
In MS, certain adhesion molecules are upregulated on vessels and inflammatory cells within MS lesions. ICAM-1 and ICAM-2 are upregulated on vessels in MS lesions and the ICAM-1/2 ligand, lymphocyte function antigen-1 (LFA-1) was found on the majority of infiltrating lymphocytes and monocytes (187). These data suggest that transendothelial migration by inflammatory cells could be mediated by the upregulation of cell adhesion molecules. Increased levels of soluble platelet/endothelial cell adhesion molecule-1 (PECAM-1) have also been found in serum samples from MS patients (187, 188). C5a has been shown to mediate upregulation of adhesion molecules on certain cell types (76, 189, 190), however it is unknown if C3a or C5a plays a role in the upregulation of adhesion molecules to affect BBB permeability in MS patients.

In addition to upregulation of adhesion molecules on brain endothelial cells, production of certain inflammatory molecules can affect BBB permeability. Matrix metalloproteinases (MMP) can damage parts of the basement membrane, which may also increase cellular traffic into the brain (191). Previous studies have shown that C5a is able to induce MMP-1 and MMP-9 in cultured human macrophages (192) and MMP-9 in eosinophils and neutrophils (193), aiding in the extravasation process; however, it is unknown if C5a-mediated production of MMPs affects BBB permeability in MS. The exact mechanism by which C5a affects BBB permeability has not been established; however, C5a is able to upregulate several mediators that are known to affect the ability of cells to enter the brain, so it is plausible that C5a plays a role in BBB permeability in MS and/or in animal models of demyelination.

Chemokines were upregulated in response to C3a and/or C5a treatment *in vitro* (Fig. 24). Several human studies have implicated certain chemokines and cytokines in MS lesion pathology, due to their upregulated expression in active lesions in MS patients. CCR1, CCR3 and CCR5, receptors for CCL4 and CCL5, were shown to be upregulated on microglia and macrophages within demyelinating lesions of MS patients (194, 195). Additionally, CCL4 and CCL5 were detected in active MS lesions and CCL5 was upregulated in CSF samples taken from relapsing MS patients (194). Chemokine gradients play a key role in cellular traffic crossing the BBB (187), but it is unknown if anaphylatoxin production is responsible for upregulation of any of these key chemokines in MS. It is certainly plausible that C3a and/or C5a have the capability, as shown in our *in vitro* studies, to induce chemokine upregulation by certain cells that may aid in the overall inflammatory response in active lesions (Fig. 24).

Figure 24. Proposed model.

(A) Insult to myelin and myelin-producing oligodendrocytes occurs in MS and during cuprizone treatment. (B) C1q can bind to myelin proteins or Ag-Ab complexes to initiate the classical pathway. Amplification of complement production may occur via the alternative pathway. Cleavage of C3 and downstream C5 results in several complement effector proteins which may cause direct damage to myelin through opsonization and subsequent phagocytosis and/or lysis via MAC. (C) Production of C3a and/or C5a has the ability to activate glial cell transduction pathways and subsequent effector functions. C3a and/or C5a mediate the production of inflammatory cytokines and chemokines including, IL-6, CCL4 and CCL5. (D) During demyelination, the expression of C3a and C5a leads to exacerbated demyelination, as well as increased microglial infiltration/activation in the corpus callosum. Upon removal of cuprizone, oligodendrocytes may migrate/proliferate in response to C3a or C5a. Alternatively, oligodendrocyte cells may be responding in higher numbers, because of the increased inflammation found in C3a and C5a transgenic mice.


A
oligo
Cuprizone = Oligo apoptosis
Demyelination
B
C1q
C3
C3a, C5a
C5b-C9
iC3b,C3b
C
C3a,C5a
C3aR
Glia
C5aR
IL-6
CCL4
CCL5
D
Inflammation
Micro
T
Astro
Astro
T
Micro
Astro
Micro
Micro
oligo precursors
Demyelination
Remyelination

CHAPTER VI

FUTURE DIRECTIONS

Anaphylatoxin receptors

While I established that increased expression of C3a or C5a leads to exacerbated demyelination, the role of anaphylatoxin receptors C3aR, C5aR and C5L2 in the brain during cuprizone treatment has not yet established. To confirm findings from studies utilizing C3a and C5a transgenic mice, C3aR KO and C5aR KO mice should also be utilized in cuprizone studies. It is assumed that in anaphylatoxin receptor KO mice, demyelination severity would be attenuated due to a lack of interaction between C3a, C5a and their respective receptors. It would also be of interest to determine the role of C5L2 in cuprizone-induced demyelination and remyelination. Some groups have postulated that C5L2 expression has the ability to dowmodulate C5aR expression on the cellular surface, which may regulate inflammation by C5a (154). Previous studies have utilized C5L2 KO to determine the overall role of this receptor during disease in murine models. Complementary experiments would include isolating glia from receptor knockout mice to establish the role of C5aR and C5L2 modulation on signal transduction pathways, as well as downstream effector functions. If C5L2 regulates C5aR cell surface expression and signaling, it is possible that upon C5a stimulation there would be more cytokine/chemokine production in the absence of C5L2. Future cuprizone and *in vitro* tissue culture studies should establish a role for all three receptors in demyelination/remyelination pathology and glial cell function.

Additionally, the levels of C3a and C5a produced in C3a- and C5a-GFAP mice during cuprizone treatment should be assessed to determine the level of complement activation throughout cuprizone treatment in transgenic mice compared to WT mice. Previous studies have confirmed that C5a is upregulated in the CSF of C5a-GFAP mice (101) and when C3aR KO and C3a-GFAP mice were crossed, severity of disease was

diminished, indicating that the production of C3a was responsible for worsened disease in the EAE model (182). However, determining the exact concentration of C3a and C5a present in the brains of transgenic mice during cuprizone treatment would be important for assessing the physiological relevance of these studies.

Effect of C3a and/or C5a on neurons

Previous results have shown that C3aR and C5aR are expressed on rodent neural precursor cells and mature neurons (34, 68, 196). Despite this fact, there have been few studies using C3a/C5a to stimulate neurons or examine the effect of C3a/C5a on neurons in neurodegenerative disease models. The *in vitro* studies performed thus far have suggested that signaling through C3aR or C5aR results in protective effects. For instance, C3a (but not C5a) was shown to play a role in neural precursor cell regeneration and migration (34, 35, 197) and C5a was shown to protect neurons from glutamate induced apoptosis (134, 135). Additionally, when human microglia were stimulated with C3a, NGF, a molecule associated with neuronal growth and survival, was shown to be upregulated (51). These data suggest that C3a and C5a are involved in neurogenesis, as well as the protection of mature neurons from harmful substances.

Future studies should determine the role(s) of C3a and C5a on neurons in progressive forms of MS and chronic demyelination. The lack of the protective myelin sheath around demyelinated axons is thought to encourage axon damage and subsequent disability (87). Thus, the role of C3a and C5a as therapeutic molecules for neurogenesis should be examined. To analyze chronic demyelination, previous studies have utilized the chronic form of the cuprizone model. In this model, cuprizone is not removed from the diet and areas of demyelination persist with little remyelination occurring (117). One study showed that after 12 weeks of cuprizone treatment, there was significant damage to axons within the corpus callosum (198). Thus, using C3a or C5a transgenic mice in chronic demyelination studies could provide useful information about whether or not

C3a/C5a provide protection to neurons experiencing chronic insult or stimulate neurogenesis within the damaged corpus callosum. There are methods for detecting damaged axons using immunofluorescence or immunohistochemistry (198, 199) and antibodies to detect neuronal (anti-NeuN, anti-microtubule associated protein-2 and anti-neuron specific beta III tubulin) and neural precursor cells (anti-CD133) are commercially available. These studies may provide information on whether or not excess C3a/C5a is harmful to neurons, or if these molecules are protective and stimulate increased neurogenesis and migration of neural stem cell precursors to areas of chronic demyelination.

Signal transduction pathways

C3a or C5a were able to activate certain proteins in MAPK signal transduction pathways. Future studies should focus on determining which pathways are necessary for downstream functions, including chemokine and cytokine production. The necessity of certain pathways can be tested using specific inhibitors to various proteins in signal transduction pathways. It is also interesting that C5a was able to activate ERK1/2 in BV-2 microglia, but C3a was not. These data suggest C3a and C5a may mediate activation via different signal transduction pathways in these cells. Previous results in anaphylatoxin-stimulated endothelial cells showed that C3a and C5a signal through different transduction pathways in these cells (200). A similar phenomenon could be occurring in BV-2 microglia, but further testing will be necessary to identify which signal transduction pathways are utilized by C3a. Activation of ERK1/2, JNK and p38 MAPK pathways did not occur in C3a-stimulated BV-2 microglia. C3a was able to mediate upregulation of CCL4 in BV-2 microglia when compared to untreated control cells, which would suggest that some type of signal transduction pathway is being activated by C3a in these cells.

Also, determining which specific signal transduction pathway is responsible for the decreased expression of chemokines and cytokines in astrocytes that were stimulated with C3a and/or C5a will be important future studies. Previous studies have shown that SOCS-1 and SOCS-3 activation are involved in downregulation of cytokine production in astrocytes (170), therefore future studies should involve determining if these proteins play a role in the down-modulation of cytokines observed in C3a/C5a stimulated primary astrocytes. Levels of SOCS-1 and SOCS-3 via anaphylatoxin stimulation in astrocytes could be analyzed by Western blot, using antibodies against SOCS-1/SOCS-3. Additionally, astrocytes from SOCS-1 and/or SOCS-3 knockout mice could be used to determine if cytokine production would be upregulated in response to C3a/C5a stimulation in the absence of SOCS proteins.

The effect of anaphylatoxins on the BBB function

As discussed previously, there was a consistent presence of $CD3^+$ cells in the corpus callosum of cuprizone-treated C5a-GFAP mice. This suggests that there is some disruption of the BBB making it leakier in the presence of increased C5a. Alternatively, C5a may be able to upregulate certain molecules, such as adhesion molecules and/or chemokines, to allow for $CD3^+$ T cells to extravasate into the brain parenchyma. Other groups have found some evidence to suggest that C5a may play a role in BBB permeability (76, 139), but more studies are needed to confirm these preliminary findings and to determine the precise mechanism for the disturbance in BBB function. Previous *in vitro* studies demonstrated that inflammatory cytokines and LPS induced increased expression of adhesion molecules, such as vascular cell adhesion molecule (VCAM), E-selectin and ICAM-1 on cerebral or brain microvascular endothelial cells (201-204). Similar studies could be performed using C5a to stimulate brain endothelial cells and then observing changes in adhesion molecule expression. It is also important to

determine the level of adhesion molecule expression on primary astrocytes, as they play an important functional role in BBB structure.

The presence of T cells in the corpus callosum of cuprizone-treated C5a transgenic mice also poses the question of whether or not other types of cells in the circulatory system are able to extravasate into the brain. If $CD3^+$ cells are able to cross the BBB in these mice, then it is plausible that other immune cell types are able to cross the BBB as well. Simple BBB permeability tests could be performed in C5a-GFAP transgenic mice to determine if these mice have disrupted BBB compared to control mice. To determine the permeability of the BBB, several past experiments utilized tracer molecules. Tracer molecules were injected into the circulatory system and the CNS was examined to observe the presence or absence of the tracer molecule (114, 139). Finally, utilizing cell specific antibodies could lead to the detection of other immune cell types entering the brain in C5a-GFAP mice using immunohistochemistry or immunofluorescence techniques.

Anaphylatoxins and stimulation of remyelination

As previously described, many MS patients suffer from demyelinating lesions in which proper remyelination fails to occur. There are several steps at which failure by oligodendrocyte precursors can occur. First, some demyelinated lesions do not contain any oligodendrocyte precursors, suggesting these cells fail to migrate to sites of damage (205). Second, some lesions have precursor cells at the outer edge of the demyelinating lesion, but do not infiltrate the lesion to remyelinate the damaged area completely (205). Some areas of demyelination have oligodendrocytes present, but the cells are quiescent and do not produce any myelin to repair damaged sites (206). Finally, some chronic lesions have been shown to contain premyelinating oligodendrocytes that contain myelin proteins in their processes and demyelinated axons, but remyelinated axons are not

present in the lesion (207). Therefore, there are several issues to address when looking for potential therapeutic targets to enhance remyelination in MS patients.

In our cuprizone studies, there were increased numbers of oligodendrocytes present in the corpus callosum of C3a and C5a transgenic mice once cuprizone was removed from the diet. Future studies should determine the reason for the increased presence of oligodendrocytes and whether oligodendrocyte precursors migrate in the presence of increased C3a or C5a. Previous studies have shown that oligodendrocyte progenitors do not migrate in response to C5a (59); however these *in vitro* studies lack many factors that may be available *in vivo* to act in concert with C3a/C5a to enhance migration. Future cuprizone studies utilizing C3aR and C5aR knockout mice should be helpful in determining whether or not these receptors are necessary on oligodendrocyte precursors for their migration to demyelinated areas. However, if not much demyelination occurs in receptor knockout mice, then the migration of oligodendrocyte precursors may not be necessary. There are also C3aR and C5aR antagonists (72, 75) that could be given to mice after demyelination to block receptor function during remyelination to determine if oligodendrocyte precursor migration relies on C3aR and C5aR signaling.

It is also possible that C3a/C5a may encourage proliferation or production of other growth factors that mature oligodendrocyte progenitors once they have reached sites of demyelination. Studies have shown that growth factors; including platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF1), brain-derived neurotrophic factor (BDNF) and NT-3 are necessary growth factors for oligodendrocyte precursor maturation and proliferation (186). It is unknown if anaphylatoxin stimulation affects levels of any of these growth factors in the CNS. Future *in vitro* studies should address if C3a/C5a stimulation promotes upregulation of growth factor production necessary for maturation and/or proliferation of oligodendrocyte precursors.

Because C3a and C5a were both shown to exacerbate demyelination pathology, concentrations of these molecules would have to be rigorously controlled to reach levels sufficient for enhanced remyelination, but not increase damage in active lesions. Several groups have hypothesized that maturing neural precursor stem cells *ex vivo* and then grafting these cells into progressive patients with demyelinated lesions may stimulate remyelination and many studies have focused on specific molecules that may mature neural precursor cells into myelin-producing, mature oligodendrocytes (186, 208). Again, it will be necessary to determine if C3a and/or C5a could potentially mature neural precursors *in vitro* before this therapy could be a viable option for treatment of MS patients.

Involvement of other complement effectors

C3a and C5a proteins are products of cascades that produce several effector proteins. Therefore, determining which complement proteins are playing a harmful versus protective role during CNS disease is ultimately necessary in understanding the overall role of complement and for testing potential therapeutic targets. Testing individual complement proteins and their role in demyelination and remyelination would involve utilizing mice that have a single complement protein or receptor knocked out. Additionally, establishing a role for complement regulatory proteins in the CNS and how they may regulate complement-mediated inflammation during demyelination will be necessary for future studies. Our lab has established a role for sCrry protein in previous studies (107) and preliminary results from our lab also indicate that the alternative pathway regulatory protein Factor H may play a role in regulating inflammation during cuprizone-induced demyelination. In addition to determining the role of complement proteins in animal models, such as the cuprizone model, it will also be necessary to analyze these proteins in MS patients during relapse, remission and progressive stages of

disease to fully understand the role of these proteins and how they apply to human disease.

REFERENCES

1. Walport MJ. 2001. Complement. First of two parts. *N Engl J Med* 344: 1058-66

2. Walport MJ. 2001. Complement. Second of two parts. *N Engl J Med* 344: 1140-4

3. Johns TG, Bernard CC. 1997. Binding of complement component Clq to myelin oligodendrocyte glycoprotein: a novel mechanism for regulating CNS inflammation. *Mol Immunol* 34: 33-8

4. Nauta AJ, Trouw LA, Daha MR, Tijsma O, Nieuwland R, Schwaeble WJ, Gingras AR, Mantovani A, Hack EC, Roos A. 2002. Direct binding of C1q to apoptotic cells and cell blebs induces complement activation. *Eur J Immunol* 32: 1726-36

5. Jiang HX, Siegel JN, Gewurz H. 1991. Binding and complement activation by C-reactive protein via the collagen-like region of C1q and inhibition of these reactions by monoclonal antibodies to C-reactive protein and C1q. *J Immunol* 146: 2324-30

6. Ying SC, Gewurz AT, Jiang H, Gewurz H. 1993. Human serum amyloid P component oligomers bind and activate the classical complement pathway via residues 14-26 and 76-92 of the A chain collagen-like region of C1q. *J Immunol* 150: 169-76

7. Garlanda C, Bottazzi B, Bastone A, Mantovani A. 2005. Pentraxins at the crossroads between innate immunity, inflammation, matrix deposition, and female fertility. *Annu Rev Immunol* 23: 337-66

8. Song WC, Sarrias MR, Lambris JD. 2000. Complement and innate immunity. *Immunopharmacology* 49: 187-98

9. Guo RF, Ward PA. 2005. Role of C5a in inflammatory responses. *Annu Rev Immunol* 23: 821-52

10. Thurman JM, Holers VM. 2006. The central role of the alternative complement pathway in human disease. *J Immunol* 176: 1305-10

11. Huber-Lang M, Sarma JV, Zetoune FS, Rittirsch D, Neff TA, McGuire SR, Lambris JD, Warner RL, Flierl MA, Hoesel LM, Gebhard F, Younger JG, Drouin SM, Wetsel RA, Ward PA. 2006. Generation of C5a in the absence of C3: a new complement activation pathway. *Nat Med* 12: 682-7

12. Amara U, Rittirsch D, Flierl M, Bruckner U, Klos A, Gebhard F, Lambris JD, Huber-Lang M. 2008. Interaction between the coagulation and complement system. *Adv Exp Med Biol* 632: 71-9

13. Mold C. 1999. Role of complement in host defense against bacterial infection. *Microbes Infect* 1: 633-8

14. van Beek J, Elward K, Gasque P. 2003. Activation of complement in the central nervous system: roles in neurodegeneration and neuroprotection. *Ann N Y Acad Sci* 992: 56-71

15. Krych-Goldberg M, Atkinson JP. 2001. Structure-function relationships of complement receptor type 1. *Immunol Rev* 180: 112-22

16. Gasque P. 2004. Complement: a unique innate immune sensor for danger signals. *Mol Immunol* 41: 1089-98

17. Gerard C, Gerard NP. 1994. C5A anaphylatoxin and its seven transmembrane-segment receptor. *Annu Rev Immunol* 12: 775-808

18. Gerard NP, Gerard C. 2002. Complement in allergy and asthma. *Curr Opin Immunol* 14: 705-8

19. Hawlisch H, Wills-Karp M, Karp CL, Kohl J. 2004. The anaphylatoxins bridge innate and adaptive immune responses in allergic asthma. *Mol Immunol* 41: 123-31

20. Klos A, Tenner AJ, Johswich KO, Ager RR, Reis ES, Kohl J. 2009. The role of the anaphylatoxins in health and disease. *Mol Immunol* 46: 2753-66

21. Hugli TE. 1981. The structural basis for anaphylatoxin and chemotactic functions of C3a, C4a, and C5a. *Crit Rev Immunol* 1: 321-66

22. Miwa T, Song WC. 2001. Membrane complement regulatory proteins: insight from animal studies and relevance to human diseases. *Int Immunopharmacol* 1: 445-59

23. Liszewski MK, Post TW, Atkinson JP. 1991. Membrane cofactor protein (MCP or CD46): newest member of the regulators of complement activation gene cluster. *Annu Rev Immunol* 9: 431-55

24. Tsujimura A, Shida K, Kitamura M, Nomura M, Takeda J, Tanaka H, Matsumoto M, Matsumiya K, Okuyama A, Nishimune Y, Okabe M, Seya T. 1998. Molecular cloning of a murine homologue of membrane cofactor protein (CD46): preferential expression in testicular germ cells. *Biochem J* 330 (Pt 1): 163-8

25. Kim DD, Song WC. 2006. Membrane complement regulatory proteins. *Clin Immunol* 118: 127-36

26. Kirschfink M. 1997. Controlling the complement system in inflammation. *Immunopharmacology* 38: 51-62

27. Beinrohr L, Dobo J, Zavodszky P, Gal P. 2008. C1, MBL-MASPs and C1-inhibitor: novel approaches for targeting complement-mediated inflammation. *Trends Mol Med* 14: 511-21

28. Schlaf G, Rothermel E, Oppermann M, Schieferdecker HL, Jungermann K, Gotze O. 1999. Rat complement factor I: molecular cloning, sequencing and expression in tissues and isolated cells. *Immunology* 98: 464-74

29. Molina H. 2002. The murine complement regulator Crry: new insights into the immunobiology of complement regulation. *Cell Mol Life Sci* 59: 220-9

30. Matthews KW, Mueller-Ortiz SL, Wetsel RA. 2004. Carboxypeptidase N: a pleiotropic regulator of inflammation. *Mol Immunol* 40: 785-93

31. Haas PJ, van Strijp J. 2007. Anaphylatoxins: Their role in bacterial infection and inflammation. *Immunol Res* 37: 161-75

32. Lee H, Whitfeld PL, Mackay CR. 2008. Receptors for complement C5a. The importance of C5aR and the enigmatic role of C5L2. *Immunol Cell Biol*

33. Woodruff TM, Ager RR, Tenner AJ, Noakes PG, Taylor SM. 2010. The role of the complement system and the activation fragment C5a in the central nervous system. *Neuromolecular Med* 12: 179-92

34. Rahpeymai Y, Hietala MA, Wilhelmsson U, Fotheringham A, Davies I, Nilsson AK, Zwirner J, Wetsel RA, Gerard C, Pekny M, Pekna M. 2006. Complement: a novel factor in basal and ischemia-induced neurogenesis. *Embo J* 25: 1364-74

35. Shinjyo N, Stahlberg A, Dragunow M, Pekny M, Pekna M. 2009. Complement-derived anaphylatoxin C3a regulates in vitro differentiation and migration of neural progenitor cells. *Stem Cells* 27: 2824-32

36. Lynch MA. 2009. The multifaceted profile of activated microglia. *Mol Neurobiol* 40: 139-56

37. Chan WY, Kohsaka S, Rezaie P. 2007. The origin and cell lineage of microglia: new concepts. *Brain Res Rev* 53: 344-54

38. Walton MR, Gibbons H, MacGibbon GA, Sirimanne E, Saura J, Gluckman PD, Dragunow M. 2000. PU.1 expression in microglia. *J Neuroimmunol* 104: 109-15

39. Ransohoff RM, Perry VH. 2009. Microglial physiology: unique stimuli, specialized responses. *Annu Rev Immunol* 27: 119-45

40. Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, Mehler MF, Conway SJ, Ng LG, Stanley ER, Samokhvalov IM, Merad M. 2010. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science* 330: 841-5

41. Hanisch UK, Kettenmann H. 2007. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat Neurosci* 10: 1387-94

42. Nimmerjahn A, Kirchhoff F, Helmchen F. 2005. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science* 308: 1314-8

43. Raivich G. 2005. Like cops on the beat: the active role of resting microglia. *Trends Neurosci* 28: 571-3

44. Kim SU, de Vellis J. 2005. Microglia in health and disease. *J Neurosci Res* 81: 302-13

45. Rotshenker S. 2003. Microglia and macrophage activation and the regulation of complement-receptor-3 (CR3/MAC-1)-mediated myelin phagocytosis in injury and disease. *J Mol Neurosci* 21: 65-72

46. Hanisch UK. 2002. Microglia as a source and target of cytokines. *Glia* 40: 140-55

47. Veerhuis R, Janssen I, De Groot CJ, Van Muiswinkel FL, Hack CE, Eikelenboom P. 1999. Cytokines associated with amyloid plaques in Alzheimer's disease brain stimulate human glial and neuronal cell cultures to secrete early complement proteins, but not C1-inhibitor. *Exp Neurol* 160: 289-99

48. Hauwel M, Furon E, Canova C, Griffiths M, Neal J, Gasque P. 2005. Innate (inherent) control of brain infection, brain inflammation and brain repair: the role of microglia, astrocytes, "protective" glial stem cells and stromal ependymal cells. *Brain Res Brain Res Rev* 48: 220-33

49. Gasque P, Dean YD, McGreal EP, VanBeek J, Morgan BP. 2000. Complement components of the innate immune system in health and disease in the CNS. *Immunopharmacology* 49: 171-86

50. Gasque P, Singhrao SK, Neal JW, Wang P, Sayah S, Fontaine M, Morgan BP. 1998. The receptor for complement anaphylatoxin C3a is expressed by myeloid cells and nonmyeloid cells in inflamed human central nervous system: analysis in multiple sclerosis and bacterial meningitis. *J Immunol* 160: 3543-54

51. Heese K, Hock C, Otten U. 1998. Inflammatory signals induce neurotrophin expression in human microglial cells. *J Neurochem* 70: 699-707

52. Nolte C, Moller T, Walter T, Kettenmann H. 1996. Complement 5a controls motility of murine microglial cells in vitro via activation of an inhibitory G-protein and the rearrangement of the actin cytoskeleton. *Neuroscience* 73: 1091-107

53. Dong Y, Benveniste EN. 2001. Immune function of astrocytes. *Glia* 36: 180-90

54. Pekny M, Wilhelmsson U, Bogestal YR, Pekna M. 2007. The role of astrocytes and complement system in neural plasticity. *Int Rev Neurobiol* 82: 95-111

55. Barnum SR. 1995. Complement biosynthesis in the central nervous system. *Crit Rev Oral Biol Med* 6: 132-46

56. Magnus T, Chan A, Linker RA, Toyka KV, Gold R. 2002. Astrocytes are less efficient in the removal of apoptotic lymphocytes than microglia cells: implications for the role of glial cells in the inflamed central nervous system. *J Neuropathol Exp Neurol* 61: 760-6

57. Lacy M, Jones J, Whittemore SR, Haviland DL, Wetsel RA, Barnum SR. 1995. Expression of the receptors for the C5a anaphylatoxin, interleukin-8 and FMLP by human astrocytes and microglia. *J Neuroimmunol* 61: 71-8

58. Levi-Strauss M, Mallat M. 1987. Primary cultures of murine astrocytes produce C3 and factor B, two components of the alternative pathway of complement activation. *J Immunol* 139: 2361-6

59. Armstrong RC, Harvath L, Dubois-Dalcq ME. 1990. Type 1 astrocytes and oligodendrocyte-type 2 astrocyte glial progenitors migrate toward distinct molecules. *J Neurosci Res* 27: 400-7

60. Jauneau AC, Ischenko A, Chan P, Fontaine M. 2003. Complement component anaphylatoxins upregulate chemokine expression by human astrocytes. *FEBS Lett* 537: 17-22

61. Jauneau AC, Ischenko A, Chatagner A, Benard M, Chan P, Schouft MT, Patte C, Vaudry H, Fontaine M. 2006. Interleukin-1beta and anaphylatoxins exert a synergistic effect on NGF expression by astrocytes. *J Neuroinflammation* 3: 8

62. Ludwin SK. 1997. The pathobiology of the oligodendrocyte. *J Neuropathol Exp Neurol* 56: 111-24

63. Baumann N, Pham-Dinh D. 2001. Biology of oligodendrocyte and myelin in the mammalian central nervous system. *Physiol Rev* 81: 871-927

64. McTigue DM, Tripathi RB. 2008. The life, death, and replacement of oligodendrocytes in the adult CNS. *J Neurochem* 107: 1-19

65. Zeis T, Schaeren-Wiemers N. 2008. Lame ducks or fierce creatures? The role of oligodendrocytes in multiple sclerosis. *J Mol Neurosci* 35: 91-100

66. Hosokawa M, Klegeris A, Maguire J, McGeer PL. 2003. Expression of complement messenger RNAs and proteins by human oligodendroglial cells. *Glia* 42: 417-23

67. Nataf S, Levison SW, Barnum SR. 2001. Expression of the anaphylatoxin C5a receptor in the oligodendrocyte lineage. *Brain Res* 894: 321-6

68. Davoust N, Jones J, Stahel PF, Ames RS, Barnum SR. 1999. Receptor for the C3a anaphylatoxin is expressed by neurons and glial cells. *Glia* 26: 201-11

69. Nataf S, Stahel PF, Davoust N, Barnum SR. 1999. Complement anaphylatoxin receptors on neurons: new tricks for old receptors? *Trends Neurosci* 22: 397-402

70. Walker DG, McGeer PL. 1992. Complement gene expression in human brain: comparison between normal and Alzheimer disease cases. *Brain Res Mol Brain Res* 14: 109-16

71. Rus H, Cudrici C, David S, Niculescu F. 2006. The complement system in central nervous system diseases. *Autoimmunity* 39: 395-402

72. Fonseca MI, Ager RR, Chu SH, Yazan O, Sanderson SD, LaFerla FM, Taylor SM, Woodruff TM, Tenner AJ. 2009. Treatment with a C5aR antagonist decreases pathology and enhances behavioral performance in murine models of Alzheimer's disease. *J Immunol* 183: 1375-83

73. Wyss-Coray T, Yan F, Lin AH, Lambris JD, Alexander JJ, Quigg RJ, Masliah E. 2002. Prominent neurodegeneration and increased plaque formation in complement-inhibited Alzheimer's mice. *Proc Natl Acad Sci U S A* 99: 10837-42

74. Woodruff TM, Costantini KJ, Crane JW, Atkin JD, Monk PN, Taylor SM, Noakes PG. 2008. The complement factor c5a contributes to pathology in a rat model of amyotrophic lateral sclerosis. *J Immunol* 181: 8727-34

75. Jacob A, Bao L, Brorson J, Quigg RJ, Alexander JJ. 2010. C3aR inhibition reduces neurodegeneration in experimental lupus. *Lupus* 19: 73-82

76. Jacob A, Hack B, Bai T, Brorson JR, Quigg RJ, Alexander JJ. 2010. Inhibition of C5a receptor alleviates experimental CNS lupus. *J Neuroimmunol* 221: 46-52

77. Compston A, Coles A. 2002. Multiple sclerosis. *Lancet* 359: 1221-31

78. Ingram G, Hakobyan S, Robertson NP, Morgan BP. 2009. Complement in multiple sclerosis: its role in disease and potential as a biomarker. *Clin Exp Immunol* 155: 128-39

79. Trapp BD, Nave KA. 2008. Multiple sclerosis: an immune or neurodegenerative disorder? *Annu Rev Neurosci* 31: 247-69

80. Lassmann H, Bruck W, Lucchinetti CF. 2007. The immunopathology of multiple sclerosis: an overview. *Brain Pathol* 17: 210-8

81. Siffrin V, Vogt J, Radbruch H, Nitsch R, Zipp F. 2010. Multiple sclerosis - candidate mechanisms underlying CNS atrophy. *Trends Neurosci* 33: 202-10

82. McFarland HF, Martin R. 2007. Multiple sclerosis: a complicated picture of autoimmunity. *Nat Immunol* 8: 913-9

83. Ozawa K, Suchanek G, Breitschopf H, Bruck W, Budka H, Jellinger K, Lassmann H. 1994. Patterns of oligodendroglia pathology in multiple sclerosis. *Brain* 117 (Pt 6): 1311-22

84. Rodriguez M. 2007. Effectors of Demyelination and Remyelination in the CNS: Implications for Multiple Sclerosis. *Brain Pathol* 17: 219-29

85. Lucchinetti C, Bruck W, Parisi J, Scheithauer B, Rodriguez M, Lassmann H. 2000. Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann Neurol* 47: 707-17

86. Sospedra M, Martin R. 2005. Immunology of multiple sclerosis. *Annu Rev Immunol* 23: 683-747

87. Rodriguez M. 2003. A function of myelin is to protect axons from subsequent injury: implications for deficits in multiple sclerosis. *Brain* 126: 751-2

88. Foote AK, Blakemore WF. 2005. Inflammation stimulates remyelination in areas of chronic demyelination. *Brain* 128: 528-39

89. Stadelmann C, Bruck W. 2008. Interplay between mechanisms of damage and repair in multiple sclerosis. *J Neurol* 255 Suppl 1: 12-8

90. Ercolini AM, Miller SD. 2006. Mechanisms of immunopathology in murine models of central nervous system demyelinating disease. *J Immunol* 176: 3293-8

91. Kipp M, Clarner T, Dang J, Copray S, Beyer C. 2009. The cuprizone animal model: new insights into an old story. *Acta Neuropathol* 118: 723-36

92. Gasque P, Singhrao SK, Neal JW, Gotze O, Morgan BP. 1997. Expression of the receptor for complement C5a (CD88) is up-regulated on reactive astrocytes, microglia, and endothelial cells in the inflamed human central nervous system. *Am J Pathol* 150: 31-41

93. Muller-Ladner U, Jones JL, Wetsel RA, Gay S, Raine CS, Barnum SR. 1996. Enhanced expression of chemotactic receptors in multiple sclerosis lesions. *J Neurol Sci* 144: 135-41

94. Barnett MH, Prineas JW. 2004. Relapsing and remitting multiple sclerosis: pathology of the newly forming lesion. *Ann Neurol* 55: 458-68

95. Terenyi N, Nagy N, Papp K, Prechl J, Olah I, Erdei A. 2009. Transient decomplementation of mice delays onset of experimental autoimmune encephalomyelitis and impairs MOG-specific T cell response and autoantibody production. *Mol Immunol* 47: 57-63

96. Nataf S, Carroll SL, Wetsel RA, Szalai AJ, Barnum SR. 2000. Attenuation of experimental autoimmune demyelination in complement-deficient mice. *J Immunol* 165: 5867-73

97. Davoust N, Nataf S, Holers VM, Barnum SR. 1999. Expression of the murine complement regulatory protein crry by glial cells and neurons. *Glia* 27: 162-70

98. Liu J, Miwa T, Hilliard B, Chen Y, Lambris JD, Wells AD, Song WC. 2005. The complement inhibitory protein DAF (CD55) suppresses T cell immunity in vivo. *J Exp Med* 201: 567-77

99. Boos L, Campbell IL, Ames R, Wetsel RA, Barnum SR. 2004. Deletion of the complement anaphylatoxin C3a receptor attenuates, whereas ectopic expression of C3a in the brain exacerbates, experimental autoimmune encephalomyelitis. *J Immunol* 173: 4708-14

100. Reiman R, Gerard C, Campbell IL, Barnum SR. 2002. Disruption of the C5a receptor gene fails to protect against experimental allergic encephalomyelitis. *Eur J Immunol* 32: 1157-63

101. Reiman R, Campos Torres A, Martin BK, Ting JP, Campbell IL, Barnum SR. 2005. Expression of C5a in the brain does not exacerbate experimental autoimmune encephalomyelitis. *Neurosci Lett* 390: 134-8

102. Bullard DC, Hu X, Schoeb TR, Axtell RC, Raman C, Barnum SR. 2005. Critical requirement of CD11b (Mac-1) on T cells and accessory cells for development of experimental autoimmune encephalomyelitis. *J Immunol* 175: 6327-33

103. Vanguri P, Koski CL, Silverman B, Shin ML. 1982. Complement activation by isolated myelin: activation of the classical pathway in the absence of myelin-specific antibodies. *Proc Natl Acad Sci U S A* 79: 3290-4

104. Seil FJ. 1977. Tissue culture studies of demyelinating disease: a critical review. *Ann Neurol* 2: 345-55

105. Liu WT, Vanguri P, Shin ML. 1983. Studies on demyelination in vitro: the requirement of membrane attack components of the complement system. *J Immunol* 131: 778-82

106. Soane L, Cho HJ, Niculescu F, Rus H, Shin ML. 2001. C5b-9 terminal complement complex protects oligodendrocytes from death by regulating Bad through phosphatidylinositol 3-kinase/Akt pathway. *J Immunol* 167: 2305-11

107. Briggs DT, Martin CB, Ingersoll SA, Barnum SR, Martin BK. 2007. Astrocyte-specific expression of a soluble form of the murine complement control protein Crry confers demyelination protection in the cuprizone model. *Glia* 55: 1405-15

108. Szalai AJ, Hu X, Adams JE, Barnum SR. 2007. Complement in experimental autoimmune encephalomyelitis revisited: C3 is required for development of maximal disease. *Mol Immunol* 44: 3132-6

109. Floris S, Blezer EL, Schreibelt G, Dopp E, van der Pol SM, Schadee-Eestermans IL, Nicolay K, Dijkstra CD, de Vries HE. 2004. Blood-brain barrier permeability and monocyte infiltration in experimental allergic encephalomyelitis: a quantitative MRI study. *Brain* 127: 616-27

110. de Vries HE, Kuiper J, de Boer AG, Van Berkel TJ, Breimer DD. 1997. The blood-brain barrier in neuroinflammatory diseases. *Pharmacol Rev* 49: 143-55

111. Pettinelli CB, McFarlin DE. 1981. Adoptive transfer of experimental allergic encephalomyelitis in SJL/J mice after in vitro activation of lymph node cells by myelin basic protein: requirement for Lyt 1+ 2- T lymphocytes. *J Immunol* 127: 1420-3

112. Zamvil SS, Steinman L. 1990. The T lymphocyte in experimental allergic encephalomyelitis. *Annu Rev Immunol* 8: 579-621

113. Gold R, Linington C, Lassmann H. 2006. Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research. *Brain* 129: 1953-71

114. Bakker DA, Ludwin SK. 1987. Blood-brain barrier permeability during Cuprizone-induced demyelination. Implications for the pathogenesis of immune-mediated demyelinating diseases. *J Neurol Sci* 78: 125-37

115. Kondo A, Nakano T, Suzuki K. 1987. Blood-brain barrier permeability to horseradish peroxidase in twitcher and cuprizone-intoxicated mice. *Brain Res* 425: 186-90

116. Mason JL, Jones JJ, Taniike M, Morell P, Suzuki K, Matsushima GK. 2000. Mature oligodendrocyte apoptosis precedes IGF-1 production and oligodendrocyte progenitor accumulation and differentiation during demyelination/remyelination. *J Neurosci Res* 61: 251-62

117. Matsushima GK, Morell P. 2001. The neurotoxicant, cuprizone, as a model to study demyelination and remyelination in the central nervous system. *Brain Pathol* 11: 107-16

118. Venturini G. 1973. Enzymic activities and sodium, potassium and copper concentrations in mouse brain and liver after cuprizone treatment in vivo. *J Neurochem* 21: 1147-51

119. Groebe A, Clarner T, Baumgartner W, Dang J, Beyer C, Kipp M. 2009. Cuprizone treatment induces distinct demyelination, astrocytosis, and microglia cell invasion or proliferation in the mouse cerebellum. *Cerebellum* 8: 163-74

120. Hiremath MM, Saito Y, Knapp GW, Ting JP, Suzuki K, Matsushima GK. 1998. Microglial/macrophage accumulation during cuprizone-induced demyelination in C57BL/6 mice. *J Neuroimmunol* 92: 38-49

121. Plant SR, Arnett HA, Ting JP. 2005. Astroglial-derived lymphotoxin-alpha exacerbates inflammation and demyelination, but not remyelination. *Glia* 49: 1-14

122. Linares D, Taconis M, Mana P, Correcha M, Fordham S, Staykova M, Willenborg DO. 2006. Neuronal nitric oxide synthase plays a key role in CNS demyelination. *J Neurosci* 26: 12672-81

123. McMahon EJ, Cook DN, Suzuki K, Matsushima GK. 2001. Absence of macrophage-inflammatory protein-1alpha delays central nervous system demyelination in the presence of an intact blood-brain barrier. *J Immunol* 167: 2964-71

124. Arnett HA, Wang Y, Matsushima GK, Suzuki K, Ting JP. 2003. Functional genomic analysis of remyelination reveals importance of inflammation in oligodendrocyte regeneration. *J Neurosci* 23: 9824-32

125. Wessels MR, Butko P, Ma M, Warren HB, Lage AL, Carroll MC. 1995. Studies of group B streptococcal infection in mice deficient in complement component C3 or C4 demonstrate an essential role for complement in both innate and acquired immunity. *Proc Natl Acad Sci U S A* 92: 11490-4

126. Morell P, Barrett CV, Mason JL, Toews AD, Hostettler JD, Knapp GW, Matsushima GK. 1998. Gene expression in brain during cuprizone-induced demyelination and remyelination. *Mol Cell Neurosci* 12: 220-7

127. Arnett HA, Mason J, Marino M, Suzuki K, Matsushima GK, Ting JP. 2001. TNF alpha promotes proliferation of oligodendrocyte progenitors and remyelination. *Nat Neurosci* 4: 1116-22

128. Sidman RL, Abervine, J. B. & Pierce, E. T. 1971. *Atlas of the Mouse Brain and Spinal Cord*. Cambridge, MA: Harvard University Press

129. Ligon KL, Alberta JA, Kho AT, Weiss J, Kwaan MR, Nutt CL, Louis DN, Stiles CD, Rowitch DH. 2004. The oligodendroglial lineage marker OLIG2 is universally expressed in diffuse gliomas. *J Neuropathol Exp Neurol* 63: 499-509

130. Cudrici C, Niculescu F, Jensen T, Zafranskaia E, Fosbrink M, Rus V, Shin ML, Rus H. 2006. C5b-9 terminal complex protects oligodendrocytes from apoptotic cell death by inhibiting caspase-8 processing and up-regulating FLIP. *J Immunol* 176: 3173-80

131. Circolo A, Garnier G, Fukuda W, Wang X, Hidvegi T, Szalai AJ, Briles DE, Volanakis JE, Wetsel RA, Colten HR. 1999. Genetic disruption of the murine complement C3 promoter region generates deficient mice with extrahepatic expression of C3 mRNA. *Immunopharmacology* 42: 135-49

132. Calida DM, Constantinescu C, Purev E, Zhang GX, Ventura ES, Lavi E, Rostami A. 2001. Cutting edge: C3, a key component of complement activation, is not required for the development of myelin oligodendrocyte glycoprotein peptide-induced experimental autoimmune encephalomyelitis in mice. *J Immunol* 166: 723-6

133. Gavrilyuk V, Kalinin S, Hilbush BS, Middlecamp A, McGuire S, Pelligrino D, Weinberg G, Feinstein DL. 2005. Identification of complement 5a-like receptor (C5L2) from astrocytes: characterization of anti-inflammatory properties. *J Neurochem* 92: 1140-9

134. Mukherjee P, Thomas S, Pasinetti GM. 2008. Complement anaphylatoxin C5a neuroprotects through regulation of glutamate receptor subunit 2 in vitro and in vivo. *J Neuroinflammation* 5: 5

135. Osaka H, Mukherjee P, Aisen PS, Pasinetti GM. 1999. Complement-derived anaphylatoxin C5a protects against glutamate-mediated neurotoxicity. *J Cell Biochem* 73: 303-11

136. Torkildsen O, Brunborg LA, Myhr KM, Bo L. 2008. The cuprizone model for demyelination. *Acta Neurol Scand Suppl* 188: 72-6

137. Cianflone K, Xia Z, Chen LY. 2003. Critical review of acylation-stimulating protein physiology in humans and rodents. *Biochim Biophys Acta* 1609: 127-43

138. Patel MM, Goyal BR, Bhadada SV, Bhatt JS, Amin AF. 2009. Getting into the brain: approaches to enhance brain drug delivery. *CNS Drugs* 23: 35-58

139. Flierl MA, Stahel PF, Rittirsch D, Huber-Lang M, Niederbichler AD, Hoesel LM, Touban BM, Morgan SJ, Smith WR, Ward PA, Ipaktchi K. 2009. Inhibition of complement C5a prevents breakdown of the blood-brain barrier and pituitary dysfunction in experimental sepsis. *Crit Care* 13: R12

140. Jacob A, Hack B, Chiang E, Garcia JG, Quigg RJ, Alexander JJ. 2010. C5a alters blood-brain barrier integrity in experimental lupus. *Faseb J* 24: 1682-8

141. Yao J, Harvath L, Gilbert DL, Colton CA. 1990. Chemotaxis by a CNS macrophage, the microglia. *J Neurosci Res* 27: 36-42

142. Neumann H, Kotter MR, Franklin RJ. 2009. Debris clearance by microglia: an essential link between degeneration and regeneration. *Brain* 132: 288-95

143. Mason JL, Suzuki K, Chaplin DD, Matsushima GK. 2001. Interleukin-1beta promotes repair of the CNS. *J Neurosci* 21: 7046-52

144. Marriott MP, Emery B, Cate HS, Binder MD, Kemper D, Wu Q, Kolbe S, Gordon IR, Wang H, Egan G, Murray S, Butzkueven H, Kilpatrick TJ. 2008. Leukemia inhibitory factor signaling modulates both central nervous system demyelination and myelin repair. *Glia* 56: 686-98

145. Mason JL, Xuan S, Dragatsis I, Efstratiadis A, Goldman JE. 2003. Insulin-like growth factor (IGF) signaling through type 1 IGF receptor plays an important role in remyelination. *J Neurosci* 23: 7710-8

146. Rollins TE, Siciliano S, Kobayashi S, Cianciarulo DN, Bonilla-Argudo V, Collier K, Springer MS. 1991. Purification of the active C5a receptor from human polymorphonuclear leukocytes as a receptor-Gi complex. *Proc Natl Acad Sci U S A* 88: 971-5

147. Skokowa J, Ali SR, Felda O, Kumar V, Konrad S, Shushakova N, Schmidt RE, Piekorz RP, Nurnberg B, Spicher K, Birnbaumer L, Zwirner J, Claassens JW, Verbeek JS, van Rooijen N, Kohl J, Gessner JE. 2005. Macrophages induce the inflammatory response in the pulmonary Arthus reaction through G alpha i2 activation that controls C5aR and Fc receptor cooperation. *J Immunol* 174: 3041-50

148. Amatruda TT, 3rd, Gerard NP, Gerard C, Simon MI. 1993. Specific interactions of chemoattractant factor receptors with G-proteins. *J Biol Chem* 268: 10139-44

149. Rabiet MJ, Huet E, Boulay F. 2007. The N-formyl peptide receptors and the anaphylatoxin C5a receptors: an overview. *Biochimie* 89: 1089-106

150. Braun L, Christophe T, Boulay F. 2003. Phosphorylation of key serine residues is required for internalization of the complement 5a (C5a) anaphylatoxin receptor via a beta-arrestin, dynamin, and clathrin-dependent pathway. *J Biol Chem* 278: 4277-85

151. Cain SA, Monk PN. 2002. The orphan receptor C5L2 has high affinity binding sites for complement fragments C5a and C5a des-Arg(74). *J Biol Chem* 277: 7165-9

152. Kalant D, Cain SA, Maslowska M, Sniderman AD, Cianflone K, Monk PN. 2003. The chemoattractant receptor-like protein C5L2 binds the C3a des-Arg77/acylation-stimulating protein. *J Biol Chem* 278: 11123-9

153. Okinaga S, Slattery D, Humbles A, Zsengeller Z, Morteau O, Kinrade MB, Brodbeck RM, Krause JE, Choe HR, Gerard NP, Gerard C. 2003. C5L2, a nonsignaling C5A binding protein. *Biochemistry* 42: 9406-15

154. Bamberg CE, Mackay CR, Lee H, Zahra D, Jackson J, Lim YS, Whitfeld PL, Craig S, Corsini E, Lu B, Gerard C, Gerard NP. 2010. The C5a receptor (C5aR) C5L2 is a modulator of C5aR-mediated signal transduction. *J Biol Chem* 285: 7633-44

155. Johswich K, Klos A. 2007. C5L2--an anti-inflammatory molecule or a receptor for acylation stimulating protein (C3a-desArg)? *Adv Exp Med Biol* 598: 159-80

156. Sayah S, Jauneau AC, Patte C, Tonon MC, Vaudry H, Fontaine M. 2003. Two different transduction pathways are activated by C3a and C5a anaphylatoxins on astrocytes. *Brain Res Mol Brain Res* 112: 53-60

157. Sayah S, Ischenko AM, Zhakhov A, Bonnard AS, Fontaine M. 1999. Expression of cytokines by human astrocytomas following stimulation by C3a and C5a

anaphylatoxins: specific increase in interleukin-6 mRNA expression. *J Neurochem* 72: 2426-36

158. Francis K, Lewis BM, Akatsu H, Monk PN, Cain SA, Scanlon MF, Morgan BP, Ham J, Gasque P. 2003. Complement C3a receptors in the pituitary gland: a novel pathway by which an innate immune molecule releases hormones involved in the control of inflammation. *Faseb J* 17: 2266-8

159. McCarthy KD, de Vellis J. 1980. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J Cell Biol* 85: 890-902

160. Nikcevich KM, Piskurich JF, Hellendall RP, Wang Y, Ting JP. 1999. Differential selectivity of CIITA promoter activation by IFN-gamma and IRF-1 in astrocytes and macrophages: CIITA promoter activation is not affected by TNF-alpha. *J Neuroimmunol* 99: 195-204

161. Anderson RD, Haskell RE, Xia H, Roessler BJ, Davidson BL. 2000. A simple method for the rapid generation of recombinant adenovirus vectors. *Gene Ther* 7: 1034-8

162. Osaka H, McGinty A, Hoepken UE, Lu B, Gerard C, Pasinetti GM. 1999. Expression of C5a receptor in mouse brain: role in signal transduction and neurodegeneration. *Neuroscience* 88: 1073-82

163. Ma X, Reynolds SL, Baker BJ, Li X, Benveniste EN, Qin H. 2010. IL-17 enhancement of the IL-6 signaling cascade in astrocytes. *J Immunol* 184: 4898-906

164. Gao YJ, Ji RR. 2008. Activation of JNK pathway in persistent pain. *Neurosci Lett* 437: 180-3

165. Yoshida H, Metoki N, Ishikawa A, Imaizumi T, Matsumiya T, Tanji K, Ota K, Ohyama C, Satoh K. 2010. Edaravone improves the expression of nerve growth factor in human astrocytes subjected to hypoxia/reoxygenation. *Neurosci Res* 66: 284-9

166. Kawasaki T, Kitao T, Nakagawa K, Fujisaki H, Takegawa Y, Koda K, Ago Y, Baba A, Matsuda T. 2007. Nitric oxide-induced apoptosis in cultured rat astrocytes: protection by edaravone, a radical scavenger. *Glia* 55: 1325-33

167. Hesselgesser J, Horuk R. 1999. Chemokine and chemokine receptor expression in the central nervous system. *J Neurovirol* 5: 13-26

168. Bsibsi M, Persoon-Deen C, Verwer RW, Meeuwsen S, Ravid R, Van Noort JM. 2006. Toll-like receptor 3 on adult human astrocytes triggers production of neuroprotective mediators. *Glia* 53: 688-95

169. Stark JL, Lyons JA, Cross AH. 2004. Interferon-gamma produced by encephalitogenic cells induces suppressors of cytokine signaling in primary murine astrocytes. *J Neuroimmunol* 151: 195-200

170. Qin H, Niyongere SA, Lee SJ, Baker BJ, Benveniste EN. 2008. Expression and functional significance of SOCS-1 and SOCS-3 in astrocytes. *J Immunol* 181: 3167-76

171. Albright AV, Shieh JT, Itoh T, Lee B, Pleasure D, O'Connor MJ, Doms RW, Gonzalez-Scarano F. 1999. Microglia express CCR5, CXCR4, and CCR3, but of these, CCR5 is the principal coreceptor for human immunodeficiency virus type 1 dementia isolates. *J Virol* 73: 205-13

172. Flynn G, Maru S, Loughlin J, Romero IA, Male D. 2003. Regulation of chemokine receptor expression in human microglia and astrocytes. *J Neuroimmunol* 136: 84-93

173. Han Y, Wang J, Zhou Z, Ransohoff RM. 2000. TGFbeta1 selectively up-regulates CCR1 expression in primary murine astrocytes. *Glia* 30: 1-10

174. Biancotti JC, Kumar S, de Vellis J. 2008. Activation of inflammatory response by a combination of growth factors in cuprizone-induced demyelinated brain leads to myelin repair. *Neurochem Res* 33: 2615-28

175. Brett FM, Mizisin AP, Powell HC, Campbell IL. 1995. Evolution of neuropathologic abnormalities associated with blood-brain barrier breakdown in transgenic mice expressing interleukin-6 in astrocytes. *J Neuropathol Exp Neurol* 54: 766-75

176. Campbell IL, Abraham CR, Masliah E, Kemper P, Inglis JD, Oldstone MB, Mucke L. 1993. Neurologic disease induced in transgenic mice by cerebral overexpression of interleukin 6. *Proc Natl Acad Sci U S A* 90: 10061-5

177. Juttler E, Tarabin V, Schwaninger M. 2002. Interleukin-6 (IL-6): a possible neuromodulator induced by neuronal activity. *Neuroscientist* 8: 268-75

178. John GR, Lee SC, Brosnan CF. 2003. Cytokines: powerful regulators of glial cell activation. *Neuroscientist* 9: 10-22

179. Gruol DL, Nelson TE. 1997. Physiological and pathological roles of interleukin-6 in the central nervous system. *Mol Neurobiol* 15: 307-39

180. Marz P, Heese K, Dimitriades-Schmutz B, Rose-John S, Otten U. 1999. Role of interleukin-6 and soluble IL-6 receptor in region-specific induction of astrocytic differentiation and neurotrophin expression. *Glia* 26: 191-200

181. Frei K, Malipiero UV, Leist TP, Zinkernagel RM, Schwab ME, Fontana A. 1989. On the cellular source and function of interleukin 6 produced in the central nervous system in viral diseases. *Eur J Immunol* 19: 689-94

182. Boos L, Szalai AJ, Barnum SR. 2005. C3a expressed in the central nervous system protects against LPS-induced shock. *Neurosci Lett* 387: 68-71

183. Liu L, Belkadi A, Darnall L, Hu T, Drescher C, Cotleur AC, Padovani-Claudio D, He T, Choi K, Lane TE, Miller RH, Ransohoff RM. 2010. CXCR2-positive neutrophils are essential for cuprizone-induced demyelination: relevance to multiple sclerosis. *Nat Neurosci* 13: 319-26

184. Ward PA. 2010. The harmful role of c5a on innate immunity in sepsis. *J Innate Immun* 2: 439-45

185. Tanaka Y, Adams DH, Hubscher S, Hirano H, Siebenlist U, Shaw S. 1993. T-cell adhesion induced by proteoglycan-immobilized cytokine MIP-1 beta. *Nature* 361: 79-82

186. Sher F, Balasubramaniyan V, Boddeke E, Copray S. 2008. Oligodendrocyte differentiation and implantation: new insights for remyelinating cell therapy. *Curr Opin Neurol* 21: 607-14

187. Ransohoff RM. 1999. Mechanisms of inflammation in MS tissue: adhesion molecules and chemokines. *J Neuroimmunol* 98: 57-68

188. Losy J, Niezgoda A, Wender M. 1999. Increased serum levels of soluble PECAM-1 in multiple sclerosis patients with brain gadolinium-enhancing lesions. *J Neuroimmunol* 99: 169-72

189. Mulligan MS, Schmid E, Till GO, Hugli TE, Friedl HP, Roth RA, Ward PA. 1997. C5a-dependent up-regulation in vivo of lung vascular P-selectin. *J Immunol* 158: 1857-61

190. Albrecht EA, Chinnaiyan AM, Varambally S, Kumar-Sinha C, Barrette TR, Sarma JV, Ward PA. 2004. C5a-induced gene expression in human umbilical vein endothelial cells. *Am J Pathol* 164: 849-59

191. Sellebjerg F, Sorensen TL. 2003. Chemokines and matrix metalloproteinase-9 in leukocyte recruitment to the central nervous system. *Brain Res Bull* 61: 347-55

192. Speidl WS, Kastl SP, Hutter R, Katsaros KM, Kaun C, Bauriedel G, Maurer G, Huber K, Badimon JJ, Wojta J. 2010. The complement component C5a is present in human coronary lesions in vivo and induces the expression of MMP-1 and MMP-9 in human macrophages in vitro. *Faseb J*

193. Discipio RG, Schraufstatter IU. 2007. The role of the complement anaphylatoxins in the recruitment of eosinophils. *Int Immunopharmacol* 7: 1909-23

194. Szczucinski A, Losy J. 2007. Chemokines and chemokine receptors in multiple sclerosis. Potential targets for new therapies. *Acta Neurol Scand* 115: 137-46

195. Simpson J, Rezaie P, Newcombe J, Cuzner ML, Male D, Woodroofe MN. 2000. Expression of the beta-chemokine receptors CCR2, CCR3 and CCR5 in multiple sclerosis central nervous system tissue. *J Neuroimmunol* 108: 192-200

196. O'Barr SA, Caguioa J, Gruol D, Perkins G, Ember JA, Hugli T, Cooper NR. 2001. Neuronal expression of a functional receptor for the C5a complement activation fragment. *J Immunol* 166: 4154-62

197. Bogestal YR, Barnum SR, Smith PL, Mattisson V, Pekny M, Pekna M. 2007. Signaling through C5aR is not involved in basal neurogenesis. *J Neurosci Res* 85: 2892-7

198. Lindner M, Fokuhl J, Linsmeier F, Trebst C, Stangel M. 2009. Chronic toxic demyelination in the central nervous system leads to axonal damage despite remyelination. *Neurosci Lett* 453: 120-5

199. Sun HS, French RJ, Feng ZP. 2009. A method for identifying viable and damaged neurons in adult mouse brain slices. *Acta Histochem* 111: 531-7

200. Schraufstatter IU, Trieu K, Sikora L, Sriramarao P, DiScipio R. 2002. Complement c3a and c5a induce different signal transduction cascades in endothelial cells. *J Immunol* 169: 2102-10

201. Rieckmann P, Michel U, Albrecht M, Bruck W, Wockel L, Felgenhauer K. 1995. Cerebral endothelial cells are a major source for soluble intercellular adhesion molecule-1 in the human central nervous system. *Neurosci Lett* 186: 61-4

202. Hess DC, Bhutwala T, Sheppard JC, Zhao W, Smith J. 1994. ICAM-1 expression on human brain microvascular endothelial cells. *Neurosci Lett* 168: 201-4

203. Hess DC, Thompson Y, Sprinkle A, Carroll J, Smith J. 1996. E-selectin expression on human brain microvascular endothelial cells. *Neurosci Lett* 213: 37-40

204. Wong D, Dorovini-Zis K. 1995. Expression of vascular cell adhesion molecule-1 (VCAM-1) by human brain microvessel endothelial cells in primary culture. *Microvasc Res* 49: 325-39

205. Chang A, Nishiyama A, Peterson J, Prineas J, Trapp BD. 2000. NG2-positive oligodendrocyte progenitor cells in adult human brain and multiple sclerosis lesions. *J Neurosci* 20: 6404-12

206. Wolswijk G. 1998. Chronic stage multiple sclerosis lesions contain a relatively quiescent population of oligodendrocyte precursor cells. *J Neurosci* 18: 601-9

207. Chang A, Tourtellotte WW, Rudick R, Trapp BD. 2002. Premyelinating oligodendrocytes in chronic lesions of multiple sclerosis. *N Engl J Med* 346: 165-73

208. Blakemore WF. 2008. Regeneration and repair in multiple sclerosis: the view of experimental pathology. *J Neurol Sci* 265: 1-4