The Role of Acanthamoeba culbertsoni Serine Proteases in Abating Microglial-Like Cell Cytokines and Chemokines

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THE ROLE OF ACANTHAMOEBA CULBERTSONI SERINE PROTEASES IN ABATING MICROGLIAL-LIKE CELL CYTOKINES AND CHEMOKINES

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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Abstract

THE ROLE OF ACANTHAMOEBA CULBERTSONI SERINE PROTEASES IN ABATING MICROGLIAL-LIKE CELL CYTOKINES AND CHEMOKINES

By Jenica L. Harrison, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2009

Major Director: Guy A. Cabral, Ph.D.
Professor, Department of Microbiology and Immunology

Acanthamoeba culbertsoni is an opportunistic free-living amoeba that is causative of granulomatous amoebic encephalitis (GAE), a chronic and often fatal central nervous system (CNS) disease that is most prevalent in immune compromised individuals. One hallmark of this disease is the formation of granulomas within the CNS, which are commonly absent in immune compromised individuals. Granulomas are usually composed of amoebae, microglia (CNS macrophages), macrophages, T cells, B cells, and neutrophils. Previous studies have demonstrated that microglia respond to Acanthamoeba by producing pro-inflammatory cytokines such as tumor necrosis factor
alpha (TNF)-α, interleukin (IL)-1α, and IL-1β. In addition, activated microglia and macrophages have been demonstrated to be cytolytic (i.e., amoebicidal) to *Acanthamoeba*. Furthermore, previous studies also indicated that *Acanthamoeba* secrete a myriad of factors including proteases. The role of these proteases during GAE has not been fully elucidated; however, it is thought that these factors may aid in the chronic persistence of *Acanthamoeba* within the CNS by modulating the host immune response.

Using two-dimensional (iso-dalt) gel electrophoresis, we demonstrated that *A. culbertsoni* secrete factors that degrade culture medium proteins. Initial gelatin zymography studies demonstrated that propagation of *A. culbertsoni* in medium with high iron content leads to augmentation of protease activity. Gelatin zymography in concert with protease inhibitors demonstrated that *A. culbertsoni* secrete proteases predominantly of the serine protease class. Using an *in vitro* co-culture model, we demonstrated that co-culture of *A. culbertsoni* with mouse microglial-like cells (BV-2 cells) results in the augmentation of *A. culbertsoni* serine protease activity and stimulation of pro-inflammatory cytokine and chemokine protein expression by microglial-like cells. However, the *A. culbertsoni*-elicited proteases were shown to degrade microglial-like cell elicited cytokines and chemokines. Collectively, our results suggest that *A. culbertsoni*-secreted serine proteases may play a role in *A. culbertsoni* CNS immune evasion by increasing *A. culbertsoni* CNS dissemination via the diminution of granuloma formation and by dampening microglial-dependent cytokine response.
Introduction

_Acanthamoeba_ belong to a group of opportunistic free-living amoebae, including _Balamuthia mandrillaris, Naegleria fowleri_, and _Sappinia diploidea_, which have the ability to cause disease in humans [Visvesvara et al. 2007]. _Acanthamoeba_ are classified in the kingdom Protista, phylum Rhizopodia, class Lobosa, order Amoebida, family Acanthamoebidae, genus _Acanthamoeba_. _Acanthamoeba_ are found worldwide and have been isolated from a variety of environmental sources including air, soil, dust, tap water, freshwater, seawater, swimming pools, air conditioning units, and contaminated contact lens cases [Marciano-Cabral and Cabral 2003]. _Acanthamoeba_ reproduce by binary fission and have two life cycle stages; the actively feeding trophozoite and the dormant cyst (Figure 1). The trophozoite stage represents the infective form of _Acanthamoeba_. Morphologically, trophozoites average 12-35 μm in size, have a nucleus with a large central nucleolus, and express spine-like projections called acanthapodia [Marciano-Cabral and Cabral 2003; Khan 2006]. In the environment the trophozoite feeds on bacteria (with a preference for gram-negative bacteria), algae, and yeast [Rodriquez-Zaragoza et al. 1994; Visvesvara et al. 2007]. A double-walled round cyst, which ranges in size from 5-20 μm, is formed during unfavorable environmental conditions such as extreme temperature or pH changes [Marciano-Cabral and Cabral 2003; Khan 2006].
Figure 1: Life Cycle Forms of *Acanthamoeba*. Scanning electron micrographs of *Acanthamoeba* (A) trophozoites displaying characteristic acanthapodia (arrows) and (B) cyst. The scale bars designate 10 µm and 1 µm in A and B, respectively. Courtesy of Dr. Francine Marciano-Cabral, Ph.D.
Grouping of *Acanthamoeba* species was originally established using cyst morphology [Page 1967; Pussard and Pons 1977]. Using this criterion, species of *Acanthamoeba* were divided into three groups based on having large cysts (Group I), wrinkled ectocysts and endocysts with a triangular, polygonal, or oval morphology (Group II), or smooth ectocytes and round endocysts (Group III). This classification scheme proved to be challenging as cyst morphology may vary depending on conditions [Sawyer and Griffin 1975]. Today, *Acanthamoeba* are grouped based on nuclear 18S ribosomal rRNA sequence differences. Using this classification scheme, 12 different genotypes (T1-T12) have been described [Stothard et al. 1998]. Most of the pathogenic *Acanthamoeba* species belong to the T4 genotype [Khan 2006]. Table 1 lists the classification of *Acanthamoeba* that have been linked to disease in humans.

Of the approximately 20 species of *Acanthamoeba* that have been identified, only twelve (*A. astronyxis*, *A. castellanii*, *A. culbertsoni*, *A. divionesis*, *A. griffini*, *A. hatchetti*, *A. healyi*, *A. lenticulata*, *A. lugdunensis*, *A. polyphaga*, *A. quina*, and *A. rhysodes*) have been linked to human disease [Marciano-Cabral and Cabral 2003; Visvesvara et al. 2007; Centers for Disease Control]. *Acanthamoeba* are associated with an eye infection known as amoebic keratitis (AK), a chronic and slow progressing central nervous (CNS) system disease called granulomatous amoebic encephalitis (GAE), and a skin infection called cutaneous acanthamoebiasis [Marciano-Cabral and Cabral 2003; Khan 2006; Visvesvara et al. 2007]. It has been reported that approximately 80% of the normal human population has serum antibodies against *Acanthamoeba*; however, *Acanthamoeba* infections are generally rare and usually are found in individuals who have a
Table 1 – Genotypes of *Acanthamoeba* Associated with Disease in Humans

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<thead>
<tr>
<th>Species Group</th>
<th>Sequence Type</th>
<th>Morphological</th>
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<tbody>
<tr>
<td><em>A. astronyxis</em></td>
<td>T7</td>
<td>I</td>
</tr>
<tr>
<td><em>A. castellanii</em></td>
<td>T4</td>
<td>II</td>
</tr>
<tr>
<td><em>A. culbertsoni</em></td>
<td>T10</td>
<td>III</td>
</tr>
<tr>
<td><em>A. divionensis</em></td>
<td>T4</td>
<td>II</td>
</tr>
<tr>
<td><em>A. griffini</em></td>
<td>T3</td>
<td>II</td>
</tr>
<tr>
<td><em>A. hatchetti</em></td>
<td>T11</td>
<td>II</td>
</tr>
<tr>
<td><em>A. healyi</em></td>
<td>T12</td>
<td>III</td>
</tr>
<tr>
<td><em>A. lenticulata</em></td>
<td>T5</td>
<td>III</td>
</tr>
<tr>
<td><em>A. lugdunensis</em></td>
<td>T4</td>
<td>II</td>
</tr>
<tr>
<td><em>A. polyphaga</em></td>
<td>T4</td>
<td>II</td>
</tr>
<tr>
<td><em>A. quina</em></td>
<td>unknown</td>
<td>II</td>
</tr>
<tr>
<td><em>A. rhysodes</em></td>
<td>T4</td>
<td>II</td>
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</table>

predisposition for *Acanthamoeba* infection (i.e., contact lens wearers and immune compromised individuals; discussed below) [Chappell et al. 2001].

AK is potentially a blinding eye infection and represents the most common disease caused by *Acanthamoeba* in humans [Clarke and Niederkorn 2006a]. *Acanthamoeba* that have been linked to AK include *A. castellanii*, *A. polyphaga*, *A. hatchetti*, *A. culbertsoni*, *A. rhysodes*, *A. griffini*, *A. quina*, and *A. lugdunensis* [Marciano-Cabral and Cabral 2003]. This disease is most commonly associated with contact lens wearers and those who have experienced corneal trauma [Niederkorn et al. 1999]. The prevalence of this disease in contact lens wearers is thought to be associated with exposure to contaminated water and/or contact lens solutions. Additionally, used contact lenses are thought to provide unique environments that facilitate *Acanthamoeba* biofilm formation on their surface, thus increasing the likelihood of infection [Khan 2006]. Adhesion of *Acanthamoeba* trophozoites to the corneal epithelium via a trophozoite expressed mannose binding protein (MBP) is the first step in AK infection [Clarke and Niederkorn 2006b]. Invasion of the cornea is facilitated by *Acanthamoeba* factors such as extracellular matrix specific binding proteins and proteases (discussed below). Symptoms of AK include photophobia, excessive tearing, and ocular pain [Marciano-Cabral and Cabral 2003]. One hallmark of this disease is the development of an ocular ring which results from immune cell infiltration. AK is diagnosed via corneal biopsy and confocal microscopy [Visvesvara et al. 2007]. This infection is most successfully treated when caught in its earliest stages. Chemotherapeutics used to treat AK include
polyhexamethylene biguanide (PHMB) or chlorhexidine digluconate (CHX) in combination with propamidine isethionate (Brolene) [Khan 2006; Visvesvara et al. 2007].

The innate immune system is thought to be the primary host resistance element involved in AK immunity. Diminution of macrophage and neutrophil responses in \textit{in vitro} and \textit{in vivo} models of AK have been demonstrated to result in an increase in disease severity; thus, these immunocytes are thought to represent the primary cells that act against corneal invading \textit{Acanthamoeba} [van Klink et al. 1996; Hurt et al. 2003c; Clarke and Niederkorn 2006a]. Although the innate immune system is primarily involved in controlling AK, secretory IgA (sIgA), a component of the adaptive immune system that is normally found in tears, also is thought to play a role in resistance to AK infection. Secretory IgA is thought to prevent the adhesion of trophozoites to corneal epithelium, to augment the action of neutrophils, and to inhibit the deleterious actions of \textit{Acanthamoeba} secreted proteases [van Klink et al. 1997; Leher et al. 1998; Hurt et al. 2003b; Said et al. 2004; Clarke and Niederkorn et al. 2006a]

\textit{Acanthamoeba} also can cause a cutaneous or disseminated infection known as cutaneous acanthamoebiasis. Cutaneous acanthamoebiasis can occur in immune competent individuals; however, it is associated more frequently with immune compromised individuals [Marciano-Cabral and Cabral 2003]. It is thought that cutaneous \textit{Acanthamoeba} infections originate from direct contact of amoeba trophozoites with broken skin or from hematogenous dissemination derived from the respiratory tract or CNS [Marciano-Cabral and Cabral 2003]. Mortality from this infection has been reported to be 73\% among individuals without \textit{Acanthamoeba} CNS infection and 100\%
among those with CNS involvement [Torno et al. 2000]. One symptom of this disease is the presence of draining non-healing skin ulcers. Skin biopsy in concert with polymerase chain reaction (PCR), indirect immunofluorescence (IIF), and/or direct isolation of amoebae from tissue are current diagnostic tools used for diagnosing this infection. Oral itraconazole, pentamidine, and 5-fluocytosine in concert with topical chlorhexidine gluconate and ketoconazole cream can be used to treat cutaneous acanthamoebiasis [Helton et al. 1993; Slater et al. 1994].

GAE is a chronic and often fatal CNS disease. GAE is most prevalent in immune compromised individuals [Marciano-Cabral and Cabral 2003]. *Acanthamoeba* that are known to cause GAE include *A. culbertsoni, A. castellanii, A. astronyxis, A. polyphaga, A. healyi,* and *A. divionesis* [Visvesvara et al. 2007]. *Acanthamoeba* trophozoites gain access to the CNS via two distinct routes: through the olfactory neuroepithelium or through hematogenous spread. However, it is believed that in human GAE trophozoites most often gain access to the CNS via hematogenous spread originating from the lungs or cutaneous infection [Marciano-Cabral and Cabral 2003; Khan 2008]. Specifically, it is believed that trophozoites from the blood enter the brain parenchyma via the capillary endothelium or the cerebral spinal fluid (CSF) via the endothelial cells of the choroid plexus [Khan 2007]. Once in the CNS, microglia and invading peripheral macrophages are the primary immune cells that interact with *Acanthamoeba* causing the release of pro-inflammatory mediators [Marciano-Cabral and Cabral 2003]. GAE is characterized by the formation of granulomas (*Figure 2*); however, granulomas are rarely observed in
Figure 2: Characteristic Granuloma Associated with Granulomatous Amoebic Encephalitis (GAE). Hematoxylin and Eosin (H & E) stain of a GAE-associated granuloma illustrating hallmark infiltration of microglia and macrophages surrounding *Acanthamoeba* (arrow); bar designates 200 µm. Courtesy of Dr. Francine Marciano-Cabral, Ph.D.
immune compromised individuals [Martinez 1982]. Granulomas formed during GAE are composed of amoebae, microglia, macrophages, polymorphonuclear cells, T cells, and B cells [Marciano-Cabral and Cabral 2003; Khan 2008]. Granulomas have been demonstrated to be instrumental to slowing the progression of GAE as evidenced by the finding that immune suppression of *Acanthamoeba* infected mice, that is induced by treatment with the cannabinoid delta-9-tetrahydrocannabinol (Δ⁹-THC), results in an increased mortality rate as compared to control mice [Marciano-Cabral et al. 2001]. The increase in mortality rate of Δ⁹-THC-mediated immune suppressed mice infected with *Acanthamoeba* was subsequently linked to a decreased presence of macrophages at focal sites of *Acanthamoeba* in the brain [Cabral and Marciano-Cabral, 2004]. Symptoms of GAE include nausea, vomiting, headache, stiff neck, seizures and lethargy [Marciano-Cabral and Cabral 2003]. GAE is usually diagnosed upon autopsy. The observation of brain lesions by Magnetic Resonance Imagery (MRI), observation of cysts by brain biopsy, and use of PCR have been the principal modes for diagnosis of GAE. GAE is treated with a combination chemotherapeutics including oral itraconazole, pentamidine, sulfadiazine, fluconazole, and fluocytosine [Visvesvara et al. 2007]. Treatment can be successful if GAE is diagnosed early; however, in most cases prognosis is poor even in the presence of chemotherapeutics.

Microglia and CNS invading peripheral macrophages are thought to be the primary cells that are responsible for mounting the immune response to invading *Acanthamoeba*. Microglia, also known as “resident brain macrophages”, are one of four types of glial cells in the CNS; other glial cells include astrocytes, oligodendrocytes, and
ependymal cells. Microglia exist in various states of activation, as do macrophages. While in a resting state, microglia are in a ramified form in close association with astrocytes and are not capable of phagocytosis. Microglia respond to a variety of stimuli including cytokines such as interferon gamma (IFN)γ and TNF-α; and pathogen-derived components such as lipopolysaccharide (LPS). Once activated, microglia are capable of responding to sites of brain injury and can be characterized by their amoeboid shape, surface receptor expression, and chemokine/cytokine production. Additionally, reactive microglia are capable of migration to sites of damage via the chemokine/chemokine receptor network, phagocytosis that is promoted by TNF-α, and antigen presentation that is activated by IFNγ.

Reactive microglia express a plethora of cell surface receptors including: pattern recognition receptors for LPS (CD14/Toll receptor 4 (TLR4)) and mannose (mannose receptors); chemokine receptors CCR2, CCR3, CCR5, CXCR4, and CX3CR1; Fc receptors Fcγ R1, RII, RIII; cytokine receptors for IFNγ, TNF (TNF RI and RII), interleukin (IL)-1, and IL-12; and purinergic receptors [Vass and Lassmann et al. 1990; Peress et al. 1993; Ulvestad et al. 1994; Peterson et al. 1995; Becher and Antel 1996; Dopp et al. 1997; Harrison et al. 1998; Suzumura et al. 1998; Marzolo et al. 1999; McManus et al. 2000; Simpson et al. 2000; Aloisi 2001; Bsibsi et al. 2002; Ogata et al. 2003; Rock et al. 2004]. Reactive microglia also are capable of producing and releasing a host of immune mediators. Some of these include the chemokines IL-8, regulated on activation, normal T-cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1 α/β/γ, MIP-2, interferon gamma-inducible 10kDa protein (IP-10), and
monocyte chemoattractant protein (MCP)-1; and pro-inflammatory cytokines IL-1α, IL-1β, IL-6, IL-12, and TNF-α [Aloisi 2001; Ambrosini and Aloisi 2004; Rock et al., 2004].

Studies have highlighted the role of microglia and CNS-invading peripheral macrophages as instrumental immune effector cells during GAE, contributing to the direct control of *Acanthamoeba* spread [Marciano-Cabral et al. 1998; Benedetto et al. 2002; Benedetto et al. 2003; Marciano-Cabral et al. 2004]. It has been demonstrated that microglia elicit pro-inflammatory cytokines such as IL-1α, IL-1β, and TNF-α in response to *Acanthamoeba* [Marciano-Cabral et al. 2004, Shin et al. 2001]. Moreover, Marciano-Cabral et al. [2004] demonstrated that highly pathogenic *A. culbertsoni* stimulates the secretion of higher levels of IL-1α, IL-1β, and TNF-α from primary rat microglia as compared to when these cells are stimulated with weakly pathogenic *A. castellanii*. However, reports indicate that such cytokines are not cytolytic to *Acanthamoeba* and, rather, may serve to activate microglial/macrophage cells for contact-dependent mediated killing of *Acanthamoeba* [Marciano-Cabral et al. 1998]. Activated macrophages have been reported to have an enhanced ability to phagocytose and kill *Acanthamoeba* [Marciano-Cabral et al. 1998; Alizadeh 2007]. In addition, microglia primed with IFN-γ plus IL-1β or TNF-α have been shown to be amoebastatic or amoebicidal against *A. castellanii*, respectively [Benedetto et al. 2002; Benedetto et al. 2003].

*Acanthamoeba* use both contact-dependent and contact-independent pathogenesis mechanisms [Khan 2006]. Contact-dependent pathogenesis mechanisms include adhesion to extracellular matrix proteins, binding to mannose sugars on host cells via mannose binding protein (MBP) expressed by *Acanthamoeba* trophozoites, and
phagocytosis of host cells [Khan 2006; Rocha-Azevedo et al. 2009]. Contact-independent pathogenesis mechanisms include the hydrolysis of ATP via ecto-ATPase expression and secretion of phospholipases and proteases [Khan 2006].

The first step to *Acanthamoeba* host invasion involves recognition of, and adhesion to, host tissue-expressed mannose and extracellular matrix proteins. Recently, it has been demonstrated that highly pathogenic *Acanthamoeba* display differential adherence to extracellular matrix proteins with a preference for laminin-1 [Rocha-Azevedo et al. 2009]. This adherence is thought to be attributed to an uncharacterized 55 kDa membrane protein expressed by highly pathogenic *Acanthamoeba* [Rocha-Azevedo et al. 2009]. Studies have highlighted the role of *Acanthamoeba* MBP in binding corneal epithelial and human brain microvascular endothelial cells (HBMECs) [Morton et al. 1991; Alsam et al. 2003]. *Acanthamoeba* binding is thought to induce host cell cycle arrest which may lead to apoptosis [Alizadeh et al. 1994; Shin et al. 2000; Sissons et al. 2004b; Sissons et al. 2005]. Moreover, it has been demonstrated that damage to neurons and microglia occurs via contact-dependent mechanisms [Pettit et al. 1996; Marciano-Cabral et al. 2000; Shin et al. 2000]. *Acanthamoeba* binding also has been linked to the induction of *Acanthamoeba* signal transductional pathways leading to increased protease secretion (discussed below) and activation of actin polymerization [Taylor et al. 1995; Hurt 2003b; Clarke and Niederkorn 2006b; Khan 2006]. Actin polymerization is instrumental to morphological changes that lead to phagocytosis of host cells. Phagocytosis serves as a food acquiring process for amoebae as evidenced by the presence of food cups during this event [Pettit et al. 1996].
One contact-independent pathogenesis mechanism used by *Acanthamoeba* is the hydrolysis of ATP by membrane ecto-ATPases (ecto-nucleotidases) [Sissons et al. 2004a]. Ecto-ATPases belong to a group of enzymes known as ecto-enzymes that are characterized by the extracellular expression of their active sites [Sissons et al. 2004a; Goding 2000]. Ecto-ATPases hydrolyse adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and adenosine monophosphate (AMP); and ADP to AMP [Goding 2000]. It has been demonstrated that clinical isolates of *Acanthamoeba* express higher levels of ecto-ATPases than environmental isolates [Sissons et al. 2004a]. During *Acanthamoeba* infection hydrolyzed ATP in the form of ADP acts on host cell expressed P2Y$_2$ purinergic receptors [Mattana et al. 2002]. This ADP activation has been demonstrated to induce TNF-α secretion, caspase 3 activation, and apoptosis of monocytic cells via P2Y$_2$ receptors [Mattana et al. 2002]. Furthermore, ecto-ATPase activity increases in the presence of mannose, indicating that receptor activity is increased during host cell binding [Sissons et al. 2004a].

Other contact-independent pathogenesis mechanisms used by *Acanthamoeba* include the production and secretion of phospholipases and proteases; of these, the production of proteases has been most widely studied. It has been demonstrated that pathogenic *Acanthamoeba* secrete more phospholipase A than non-pathogenic *Acanthamoeba* [Cursons et al. 1978; Misra et al. 1983]. Pathogenic *Acanthamoeba* produce serine, cysteine, and metallo- proteases [He et al. 1990; Hadás et al. 1993a; Mitro et al. 1994; Mitra et al. 1995; Cao et al. 1998; Alfieri et al. 2000; Cho et al. 2000; Hong et al. 2000; Khan et al. 2000; Kong et al. 2000; Na et al. 2001; Hong et al. 2002;
Na et al. 2002; Alsam et al. 2005; Kim et al. 2006; Sissons et al. 2006]. However, serine proteases represent the major class of proteases secreted by *Acanthamoeba* [Khan 2006].

Serine proteases constitute a class of enzymes that have the amino acid serine located in their active site and are able to cleave peptide bonds. Highly virulent *Acanthamoeba* have been demonstrated to secrete higher levels of proteases than less virulent *Acanthamoeba* [Hadás et al. 1993b; Khan et al. 2000]. In *Acanthamoeba*’s natural environment, serine proteases are thought to play a key role in encystment and excystment [Moon et al. 2008; Dudley et al. 2008]. *Acanthamoeba* serine proteases also are believed to facilitate invasion and evasion within the infected host.

Many studies have linked *Acanthamoeba* serine proteases to host invasion. *Acanthamoeba* serine proteases have been demonstrated to degrade ECM components such as collagen, laminin, elastin [Kong et al. 2000; Na et al. 2001; Na et al. 2002; Hurt et al. 2003a; Sissons et al. 2006]. In addition, *Acanthamoeba* binding to corneal epithelial cells via MBP has been shown to increase the expression of a 133kDa *Acanthamoeba* serine protease called mannose-induced protease (MIP-133); and, subsequent studies found this protease to be cytolytic to these cells [Hurt et al. 2003a and Hurt et al. 2003b]. Using an *in vitro* blood-brain barrier (BBB) model, Alsam et al. [2003] demonstrated that, although *Acanthamoeba* binding to HBMECs can be blocked *in vitro* with exogenous mannose, HBMEC cell cytotoxicity increased in the presence of exogenous mannose. Subsequent studies demonstrated that conditioned medium from *A. castellanii* increased BBB permeability and that this effect was prevented in the presence of the serine protease inhibitor phenylmethylsulphonylfluoride (PMSF) [Alsam et al.
Moreover, it has been demonstrated that conditioned medium from *Acanthamoeba* degrades zonula occludens 1 (ZO-1) and occludin tight junction proteins of HBMEC monolayers; this action is believed to be mediated by *Acanthamoeba* serine proteases [Khan 2007].

*Acanthamoeba* serine proteases also have been implicated as having a functional role in evasion of host cell defenses. Previous studies have indicated that *Acanthamoeba* serine proteases are able to degrade the immunoglobulins secretory IgA (sIgA), IgG, IgM; the cytokines IL-1α and IL-1β; and other proteins such as plasminogen, hemoglobin, fibrinogen, and albumin [Kong et al. 2000; Na et al. 2001; Na et al. 2002; Sissons et al. 2006]. However, all of these studies were performed using exogenous substrates (i.e., recombinant protein substrates) and, though they give important insight to the potential functional role of serine proteases in host immune evasion, there is still a paucity of information with regards to how *Acanthamoeba* specifically use their serine proteases for immune evasion.

The goal of the present study was to determine the functional relevance of *A. culbertsoni*-elicited serine proteases in CNS immune evasion. We hypothesized that serine proteases provide an effective means of host immune evasion for *Acanthamoeba* by dampening microglial immune response through the degradation of elicited cytokines and chemokines. In order to test our hypothesis we proposed four specific aims: i) to characterize proteases elicited by *A. culbertsoni*, ii) to determine the cytokine and chemokine profile elicited by microglial-like cells in response to *A. culbertsoni*, iii) to determine if *A. culbertsoni*-elicited factors could degrade constitutive and LPS-stimulated
cytokines and chemokines produced by microglial-like cells, and iv) to determine if pro-inflammatory cytokines and chemokines elicited by microglial-like cells in the presence of A. culbertsoni could be degraded by A. culbertsoni serine proteases.

We demonstrate that A. culbertsoni secrete serine proteases, the levels of which are augmented in the presence of microglial-like cells. A. culbertsoni induced the gene expression of a plethora of pro-inflammatory cytokines and chemokines by microglial-like cells as assessed by Multiprobe Ribonuclease Protection Assay (RPA) and cytokine/chemokine protein microarray. However, we demonstrate that the cytokines and chemokines elicited from microglial-like cells were degraded by A. culbertsoni serine proteases. While it is likely that A. culbertsoni use a multifactorial process to elicit pathogenesis during GAE, these studies suggest that A. culbertsoni serine proteases aid in A. culbertsoni CNS immune evasion by specifically targeting microglial facilitated granuloma formation and anti-microbial activity.
Materials and Methods

**Reagents.** Dulbecco’s Modified Eagle’s Medium (DMEM; with phenol red, without L-glutamine), 100X penicillin/streptomycin, 1M HEPES, 100X nonessential amino acids (NEAA), 200 mM L-glutamine, and 100X Modified Eagle’s Medium (MEM) vitamins were purchased from Cellgro/Mediatech, Inc. (Herndon, VA). Fetal Bovine Serum (FBS) and heat-inactivated (HI) FBS were purchased from both Cellgro/Mediatech, Inc. and KSE Scientific (Durham, NC). Neurobasal™-A medium (without phenol red), B-27 supplement, 1X phosphate buffered saline (PBS; without MgCl₂ and CaCl₂; pH 7.4), and TRIzol® reagent were purchased from Invitrogen (Carlsbad, CA). Lipopolysaccharide (LPS), phenylmethylsulphonylfluoride (PMSF), trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), and glucose were purchased from Sigma-Aldrich (St. Louis, MO). HyClone® 100X penicillin G/streptomycin/amphotericin B, Remel oxoid neutralized liver digest, and Remel proteose peptone were purchased from Thermo Fisher Scientific (Lenexa, KS). Bacto™ yeast extract was purchased from Becton, Dickinson and Company (Franklin Lakes, NJ). Dextrose was purchased from J.T. Baker Chemicals (Phillipsburg, NJ).

**Amoebae.** *Acanthamoeba culbertsoni* (ATCC # 30171) were acquired from the American Type Culture Collection (ATCC, Manassas, VA). Axenic (i.e., culture free of
other contaminating microorganisms) amoebae were maintained either in oxoid medium (0.55% w/v oxoid neutralized liver digest, 0.3% w/v dextrose, 0.5% w/v proteose peptone, 0.25% w/v yeast extract, 1% FBS, and 0.1% hemin) or proteose peptone, yeast, glucose (PYG) medium (2% w/v proteose peptone, 0.2% w/v yeast extract, and 0.1M glucose) at 37°C.

**Microglia.** Immortalized primary mouse microglial-like BV-2 cells were a gift from Dr. Michael McKinney of the Mayo Clinic (Jacksonville, FL). BV-2 cells were maintained in complete DMEM (i.e., DMEM supplemented with 10% HI-FBS, 100 U/ml penicillin G, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 0.01M HEPES, 1X NEAA, 2mM L-glutamine, and 1X MEM vitamins) at 37°C with 5% CO₂.

**Conditioned Medium.** Microglial conditioned medium (MCM) was generated by culturing 10⁶ BV-2 cells in T25 cm² (Greiner, Monroe, NC) tissue culture flasks in complete DMEM overnight at 37°C with 5% CO₂. The following day, cultures were washed with sterile PBS and 5ml complete Neurobasal™-A medium (serum free, supplemented with 1X B-27 supplement, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.5 mM L-glutamine) were added. Neurobasal™-A medium is specifically formulated to mimic the serum-free environment of the brain. Cells were cultured an additional 9-24 h at 37°C with 5% CO₂. The supernatant (i.e., conditioned medium) was collected by centrifugation at 1700 x g for 10 min at 4°C. Conditioned medium from LPS stimulated BV-2 cells was generated in a similar manner except that BV-2 cells were initially cultured overnight in 60 mm x 15 mm petri dishes at 37°C with 5% CO₂. Cells
were washed with PBS, complete Neurobasal™-A medium (CNBA) containing 100 ng/ml LPS was added, and cultures were incubated 8 h at 37°C with 5% CO₂. Supernatants (LPS/MCM) were collected by centrifugation (1700 x g, 10 min, 4°C) and stored at -80°C until assayed for cytokines and chemokines.

*Acanthamoeba culbertsoni* conditioned medium (ACM) was generated from cultures of 10⁹ *A. culbertsoni* (ACM-HI) and 10⁶ *A. culbertsoni* (ACM-LO). For generation of ACM-HI, axenic *A. culbertsoni* were cultured in Erlenmeyer flasks containing 1L oxoid or PYG medium at 37°C with shaking for 4 days to yield approximately 10⁹ amoebae. The amoebae were pelleted by centrifugation at 6090 x g for 20 min at 4°C, 5ml CNBA were added to the pellet and the pellet was incubated at 37°C for 24 h. The amoebae were re-pelleted by centrifugation (1700 x g, 10 min, 4°C). The supernatant (i.e., conditioned medium) was removed and centrifuged at (1700 x g, 10 min, 4°C) to remove debris, and the conditioned medium (i.e., supernatant) was stored at -80°C until used in experiments. The protein concentration of ACM-HI was determined by the Bradford method [1976], dilutions of this conditioned medium were used in select experiments. ACM-LO was prepared by incubating 10⁶ axenic *A. culbertsoni*, which was originally propagated in oxoid medium, in 5ml CNBA in T25 cm² flasks at 37°C with 5% CO₂ for 24 h. The supernatant was then removed, centrifuged (1700 x g, 10 min, 4°C) and stored -80°C.

Conditioned medium from co-cultures of BV-2 cells and *A. culbertsoni* (MCM/ACM) was generated by first culturing 10⁶ BV-2 cells in complete DMEM in T25 cm² flasks overnight at 37°C with 5% CO₂. The next day, the cells were washed with
PBS, CNBA was added, and $10^6$ *A. culbertsoni* (originally propagated in oxoid medium) was added to the BV-2 cell cultures. Co-cultures were incubated at 37°C with 5% CO$_2$ for 9-24 h. Culture supernatants were then harvested by centrifugation at (1700 x g, 10 min, 4°C) and stored at -80°C.

**Two-Dimensional (Iso-Dalt) Gel Electrophoresis (2D-PAGE).** Two-dimensional (Iso-Dalt) gel electrophoresis was used to separate proteins in CNBA (background control) and ACM. Briefly, amoebae were cultured in Erlenmeyer flasks containing 1L PYG medium at 37°C with shaking for 4 days to yield approximately $10^9$ amoebae. The amoebae were pelleted by centrifugation at 6090 x g for 20 min at 4°C, 5ml CNBA were added to the pellet and the pellet was incubated at 37°C for 24 h. The amoebae were re-pelleted by centrifugation (1700 x g, 10 min, 4°C). The supernatant (i.e., conditioned medium) was removed and centrifuged at (1700 x g, 10 min, 4°C) to remove debris, and the conditioned medium (i.e., supernatant) was stored at -80°C until used in experiments. In order to remove interfering salts before performing the isoelectric focusing first-dimension, samples were dialyzed overnight against ultrapure deionized water using Slide-A-Lyzer 3.5K molecular weight cutoff dialysis cassettes (Pierce Biotechnology, Inc, Rockford, IL). Protein concentrations were determined by the Bradford method [Bradford, 1976] and 500μg of CNBA protein and 1000μg of ACM protein were added to ReadyPrep™ rehydration/sample buffer (8 M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte® 3/10 ampholyte, and trace bromophenol blue) (Bio-Rad, Hercules, CA) in a total volume of 200 μl. A carbonic anhydrase carbamylated isoelectric point marker (20μl) (GE Healthcare, Piscataway, NJ) was added to the CNBA protein sample before
performing first dimension isoelectric focusing. The protein samples were applied to ReadyStrip™ IPG pH 3-10 (Bio-Rad) strips that had been previously rehydrated with ReadyPrep™ rehydration/sample buffer overnight at room temperature. The samples were allowed to passively rehydrate for 2 h at room temperature. The proteins were isoelectrically focused for 45,900 and 59,000 volt hours for CNBA and ACM, respectively, using a PROTEAN® IEF cell (Bio-Rad). Electrophoresis in the second dimension was performed in 10-20% gradient SDS-PAGE gels using the Criterion™ gel electrophoresis system (Bio-Rad). The gels were stained with Bio-Safe™ Coomassie Blue G-250 (Bio-Rad). Gels were scanned on a Microtek ScanMaker 9800XL/TMA 1600 flatbed scanner (Microtek, Santa Monica, CA) interfaced with a Compaq computer (Hewlett-Packard Company, Houston, TX) using SilverFast scanning software.

**Gel Zymography.** Gel zymography was performed to measure protease activity in conditioned media. Briefly, 10μl 2X Novex® tris-glycine SDS sample buffer (Invitrogen) were added to each 10μl sample prior to loading. In select experiments, samples were incubated (37°C for 30 min.) with PMSF and E-64 prior to electrophoresis in order to assess for serine and cysteine protease activity, respectively. Samples were loaded into pre-cast Novex® 10% gelatin zymogram gels (Invitrogen) and gels were electrophoresed (125V, 90 min) in Novex® tris-glycine SDS buffer. Following electrophoresis gels were incubated (30 min, room temperature) in 1X Novex® zymogram renaturing buffer (Invitrogen) with gentle rocking, and then equilibrated (30 min, room temperature) in 1X Novex® zymogram developing buffer, pH 7.5 (Invitrogen) with gentle rocking. Following the addition of fresh 1X Novex® zymogram developing buffer, pH 7.5, gels
were incubated at 37ºC overnight. Protease digestion was viewed as areas of clearing on a blue background following staining with SimplyBlue™ Safestain (Invitrogen). Gels were scanned on a Microtek ScanMaker 9800XL/TMA 1600 flatbed scanner interfaced with a Compaq computer using SilverFast scanning software.

**Light Microscopy.** Light microscopy images of BV-2 cell cultures, *A. culbertsoni* cultures, and BV-2/ *A. culbertsoni* co-cultures were acquired using an Olympus CK2 inverted microscope (Opelco, Washington, DC) in concert with an attached XV-GP230 digital video camera (Panasonic, Yokohoma, Japan). A Dell Dimension XPS1450 computer (Dell, Inc., Round Rock, Texas) programmed with Videum 100 hardware and Window NT software (Winnov, Sunnyvale, CA) was used to capture images.

**Multiprobe Ribonuclease Protection Assay (RPA).** BV-2 cells (1.5 x 10^6) were co-cultured (6 h) with *A. culbertsoni* in 60 mm culture dishes at 37ºC with 5% CO₂. Total RNA was prepared from cell cultures using TRIzol® reagent (Invitrogen) according to the manufacturer’s instructions, subjected to isopropanol precipitation, and dissolved directly in 1X hybridization buffer (BD Biosciences/PharMingen, San Diego, CA). A Riboquant RPA was used to assess for levels of mouse chemokine mRNA (mCK-5c probe template set; BD Biosciences/PharMingen). The ribo-probes were labeled with ³²P [UTP] (MP Biomedicals, Aurora, OH) to a specific activity of greater than 3,000 Ci/mmol. The isolated RNA samples then were hybridized with the probe overnight at 56ºC and the protected fragments were resolved on a 6% polyacrylamide gel containing 6M urea. Imaging of the protected fragments was performed using a 445 SI Phosphorimager
The pixel intensity of each band was quantified using ImageQuant 4.1 software (Molecular Dynamics) and the amount of chemokine mRNA was normalized for loading by dividing the pixel value for the chemokine expression band by the sum of the pixel values for the mRNAs of the housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and a ribosomal protein, L32.

**Cytokine/Chemokine Protein Microarray.** The RayBio® Mouse Cytokine Antibody Array III (RayBiotech, Inc., Norcross GA) was used as a semi-quantitative measure of cytokines and chemokines in culture supernatants according to the manufacturer’s instructions. Briefly, arrays were blocked (30 min, room temperature) in manufacturer supplied blocking buffer. After blocking, 1ml of culture supernatant was added. Following incubation overnight at 4°C, the membranes were washed with manufacturer provided wash buffers and incubated (1.5 h) with biotin-conjugated anti-cytokine antibodies. After washing to remove unbound biotin-conjugated anti-cytokine antibodies, the membranes were incubated (2 h) with horseradish peroxidase (HRPO)-conjugated strepavidin, washed, and protein was visualized by chemilumenescent reaction in concert with a Kodak X-OMAT 2000A processing instrument (Kodak, Rochester, NY) and Kodak BioMAX XAR film. Film was scanned on a Microtek ScanMaker 9800XL/TMA1600 flatbed scanner and densitometry was performed using Quantity One® software (Bio-Rad, Hercules, CA).
**Enzyme-linked Immunosorbent Assay (ELISA).** BV-2 cells were co-cultured with *A. culbertsoni* for 9-24 h (37°C with 5% CO₂), culture supernatant (MCM/ACM) was collected and incubated (37°C with 5% CO₂) an additional 12-48 h. DuoSet mouse sandwich ELISA kits (R & D Systems, Inc., Minneapolis, MN) were used to quantify levels of TNF-α, MIP-1α, and MIP-2 in MCM/ACM samples. For ELISA, Corning Costar® EIA plates (Corning, Inc., Lowell, MA) were coated with 0.8 µg/ml goat anti-mouse TNF-α, 0.4 µg/mL goat anti-mouse MIP-1α, or 2.0 µg/ml rat anti-mouse MIP-2, incubated overnight at room temperature, washed (0.05% Tween-20 in PBS, pH 7.4), and blocked (1 h) with reagent diluent (1% BSA in PBS, pH 7.4, 0.2 µm filtered). Following addition of samples or standards (100 µl), plates were incubated (2 h) at room temperature, washed, and incubated (2 h, room temperature) with 150 ng/ml biotinylated goat anti-mouse TNF-α, 100 ng/ml biotinylated goat anti-mouse MIP-1α, or 75 ng/ml biotinylated goat anti-mouse MIP-2. The plates then were washed, and streptavidin conjugated HPRO was added. Following incubation (20 min, room temperature) a 1:1 mixture of manufacturer provided substrate reagent (H₂O₂ and Tetramethylbenzidine (TMB)) was added (20 min, room temperature). After addition of stop solution (2 N H₂SO₄), the optical density of each sample was determined using a SpectraMax 250 spectrophotometer (MDS Analytical Technologies, Sunnyvale, CA) in concert with SoftMax Pro software (MDS Analytical Technologies). All wells were read at 450nm with a correction wavelength of 570nm. Standards consisted of two-fold dilutions of recombinant mouse TNF-α (2000 pg/ml to 31.25 pg/ml), MIP-1α (500 pg/ml to 7.81 pg/ml), or MIP-2 (1000 pg/ml to 15.63 pg/ml).
**Data Analysis.** For cytokine/chemokine protein microarrays, background was subtracted and the density of duplicate cytokine and chemokine array spots (i.e., the sum of pixels per array spot), which corresponded to the relative amount of cytokines or chemokines in each supernatant sample, was normalized to the average density of the standards (i.e., internal positive controls consisting of biotin-conjugated IgG) included in each membrane. Density values were graphed as the mean value intensity and a fold difference of ≥ 2.1 as compared to control was considered significant. Error bars represent the ± S.D. of duplicate cytokine or chemokine spots. For ELISA, the percent maximum response was calculated by comparing the optical density of samples that were incubated for an additional 12-48 h to optical density of the starting co-culture supernatant (i.e., 9 h or 24 h co-culture supernatant); the optical density of the co-culture supernatant at 9 h or 24 h was considered the 100% value. Samples were performed in triplicate and the average optical density of each sample was used to calculate percent maximum response.
Results

*Acanthamoeba culbertsoni* Secrete Degradative Enzymes.

Two-dimensional (iso-dalt) gel electrophoresis (2D-PAGE) was used to visualize and compare proteins of CNBA versus ACM. For this study, supernatant form *A. culbertsoni* (i.e., ACM) was generated from $10^9$ amoebae which were propagated in PYG medium, pelleted, and incubated in the presence of CNBA; the resulting supernatant was designated as ACM-HI. The proteomic profile of CNBA revealed a large protein spot at the approximate molecular weight (kDa) of 62, which is consistent with the approximate molecular weight predicted for albumin (Figure 3). A carbonic anhydrase pI marker, which was included as an internal standard within the CNBA two-dimensional gel, had a molecular weight of 26 kDa and a pI range approximately 4.8-6.7 (Figure 3). ACM-HI exhibited a proteomic profile that was distinct from that of CNBA (Figure 4). Notably, the ACM-HI proteomic profile displayed a complete absence of the 62 kDa albumin spot (Figure 4). These results are consistent with the presence of *A. culbertsoni* degradative enzymes in ACM-HI.
Figure 3: Visualization of Complete Neurobasal™-A Medium Proteins. Complete Neurobasal™-A Medium proteins (500 µg) were separated based on relative charge (pI) (pH 3-10) in the first dimension and relative molecular weight in the second dimension (10-20% SDS-PAGE). Proteins were visualized using Coomassie G-250 protein stain. The top arrow indicates a major protein spot at 62 kDa. The bottom arrow indicates a carbonic anhydrase pI marker at 26 kDa and pI range of ~4.8-6.7 that was included as an internal standard. Complete Neurobasal™-A Medium was used to generate conditioned medium from BV-2 cell cultures, A. culbertsoni cultures, and BV-2 cell plus A. culbertsoni co-cultures.
Figure 4: Visualization of *A. culbertsoni*-Conditioned Medium Proteins. *Acanthamoeba culbertsoni* conditioned medium (ACM) was generated from approximately $10^9$ amoebae (i.e., ACM-HI) which were propagated in proteose peptone, yeast, glucose (PYG) medium. Following propagation, amoebae were pelleted and incubated in the presence of complete Neurobasal™-A medium (24 h). Supernatant (i.e., conditioned medium) was harvested and proteins (1000 µg) were separated based on relative charge (pI) (pH 3-10) in the first dimension and relative molecular weight in the second dimension (10-20% SDS-PAGE). Following electrophoresis, proteins were visualized using Coomassie G-250 protein stain.
Acanthamoeba culbertsoni Secrete Serine Proteases.

Gelatin zymography was employed in order to determine whether differences in the two-dimensional (iso-dalt) gel electrophoresis proteomic profiles of CNBA versus ACM-HI were attributed to the presence of A. culbertsoni protease activity. Gelatin zymography is a technique that allows for the detection of serine, cysteine, and metallo-proteases. Initially, the proteolytic profiles of ACM-HI generated from amoebae propagated in either oxoid or PYG medium were compared (Figure 5). Acanthamoeba culbertsoni propagated in oxoid or PYG medium exhibited three common protease bands of approximately 187, 97, and 58 kDa (Figure 5). Propagation of A. culbertsoni in oxoid medium resulted in protease activity that was more robust as compared to amoebae propagated in PYG medium. CNBA did not show evidence of protease activity (Figure 5). These results indicate that components of the oxoid medium influence A. culbertsoni protease levels. Oxoid medium was used to propagate amoebae in all subsequent experiments.

Titration of ACM-HI revealed protease activity at positions corresponding to 187, 97, 90, and 58 kDa (Figure 6). Protease activity located at positions corresponding 187 and 58 kDa were the most robust. Protease activity located at positions corresponding to 97 and 90 kDa was significantly reduced at 0.5 µg as compared to the starting concentration at 1.5 µg (Figure 6).

Protease inhibitors in concert with gelatin zymography were used to identify the class of proteases secreted by A. culbertsoni. Initially, ACM-HI (1.0 µg) was incubated with either the serine protease inhibitor PMSF (2.0-0.125 mM) or anhydrous ethanol
Figure 5: A Comparison of the Effect of Propagation Medium on *A. culbertsoni* Protease Activity. *A. culbertsoni* (10⁹) were propagated in either oxoid (OX) or PYG media. Then amoebae were pelleted and cultured in the presence of complete Neurobasal™-A medium (background control, C) for 24 h. Supernatants (i.e., *A. culbertsoni*-conditioned medium; ACM) were collected and 1.0 µg of ACM protein was assessed by gelatin zymography. Arrows indicate protease activity at positions corresponding to 187, 97, and 58 kDa. Relative molecular weight was calculated on the basis of the mean center point for each respective band on gelatin zymograms.
Figure 6: Titration of Protease Activity in $10^9$ A. culbertsoni-Conditioned Medium.
The protease activity of 1.5-0.25 µg of $10^9$ A. culbertsoni-conditioned medium (ACM-HI) was assessed by gelatin zymography. Arrows indicate protease activity at positions corresponding to 187, 97, 90, and 58 kDa. Relative molecular weight was calculated on the basis of the mean center point for each respective band on gelatin zymograms.
(PMSF vehicle) (Figure 7). All ACM-HI protease activity was sensitive to PMSF indicating that *A. culbertsoni* secrete serine proteases (Figure 7). The PMSF vehicle did not have an effect on protease activity. Specifically, ACM-HI serine protease activity was completely inhibited by PMSF at concentrations as low as 1.0 mM. As expected, lower concentrations of PMSF (i.e., 0.500-0.125 mM) resulted in slight recovery of protease activity at 187 kDa. Based on these results, 1.0 mM PMSF was used in all subsequent experiments requiring the use of protease inhibitors. ACM-HI (0.75 µg) was also incubated with the cysteine protease inhibitor E-64 (10-1 µM) at pH 7.5 (Figure 8). Gelatin zymogram studies were also performed on ACM-HI (1.0 µg) which was incubated in the presence of 10 µM E-64 at pH 5.0 (data not shown). Gelatin zymography revealed that ACM-HI protease activity was not sensitive to E-64 (Figure 8 and data not shown). This result suggests that *A. culbertsoni* cysteine protease activity was not detectable under the experimental conditions used.

Protease activity in ACM-HI, conditioned medium from 10⁶ amoebae (ACM-LO), and conditioned medium from co-cultures of BV-2 cells (mouse microglial-like cells) plus *A. culbertsoni* (MCM/ACM) were compared using gelatin zymography. ACM-HI, ACM-LO, and MCM/ACM exhibited three common protease bands corresponding to 187, 97, and 58 kDa (Figure 9). In addition, all protease bands were serine proteases as evidenced by their sensitivity to PMSF. The similarities in protease activity from ACM-LO and MCM/ACM indicate that co-culture of *A. culbertsoni* with BV-2 cells did not elicit differential patterns of protease expression. However, an overall increase in protease activity was observed in MCM/ACM as compared to ACM-LO,
Figure 7: *Acanthamoeba culbertsoni* Secret Proteases that Are Inhibited by the Serine Protease Inhibitor Phenylmethlysulphonylfluoride (PMSF). Conditioned medium (ACM-HI) (1.0 µg) from $10^9 A. culbertsoni$ was subjected to no treatment (0), incubated (30 min, 37°C) in the presence of 5% anhydrous ethanol that served as the vehicle (V) for PMSF, or incubated (30 min, 37°C) in the presence of the serine protease inhibitor PMSF (2.0-0.125 mM). Arrows indicate protease activity at positions corresponding to 187, 97, 90, and 58 kDa. Relative molecular weight was approximated based on comparable gelatin zymograms.
Figure 8: *Acanthamoeba culbertsoni* Secrete Proteases that Are Not Inhibited by the Cysteine Protease Inhibitor trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64). $10^9$ *A. culbertsoni*-conditioned medium (ACM-HI) (0.75 µg) was subjected to no treatment (0) or incubated (30 min, 37°C) in the presence of the cysteine protease inhibitor E-64 (10-1 µM). Arrows indicate protease activity at positions corresponding to 187, 97, 90, and 58 kDa. Relative molecular weight was calculated on the basis of the mean center point for each respective band on gelatin zymograms.
Figure 9: A Comparison of the Proteolytic Activity in Three Different Types of *A. culbertsoni*-Conditioned Media. Equal volumes of $10^9$ *A. culbertsoni*-conditioned medium (ACM-HI), $10^6$ *A. culbertsoni*-conditioned medium (ACM-LO), and conditioned medium from co-culture of $10^6$ *A. culbertsoni* plus $10^6$ BV-2 cells (MCM/ACM) were subjected to no treatment (0), incubated (30 min, 37°C) in the presence of 5% anhydrous ethanol that served as the vehicle (V) for PMSF, or incubated (30 min, 37°C) in the presence of the serine protease inhibitor PMSF (P) (1.0 mM), and protease activity was assessed using gelatin zymography. Arrows indicate protease activity at positions corresponding to 187, 97, and 58 kDa. Relative molecular weight was calculated on the basis of the mean center point for each respective band on gelatin zymograms.
indicating that co-culture of *A. culbertsoni* with microglial-like cells augments *A. culbertsoni* protease activity.

Gelatin zymography also was used to compare protease activity in ACM-LO and MCM/ACM from 9-24 h cultures (Figure 10). Protease activity in ACM-LO and MCM/ACM from 9 h culture was less intense as compared to the protease activity at 24 h, indicating a time-related accumulation of *A. culbertsoni* proteases in culture supernatant. Protease activity in MCM/ACM was more intense than that of ACM-LO at all time points studied, indicating that co-culture with BV-2 cells augments *A. culbertsoni* protease activity (Figure 10).

**Acanthamoeba culbertsoni** Stimulate Microglial-Like Cells to Produce and Secrete Cytokines and Chemokines.

In order to determine whether BV-2 cells were inducibly responsive to *A. culbertsoni* in terms of expressing chemokines, BV-2 cells were co-cultured (6 h) with *A. culbertsoni* and the levels of select chemokine mRNAs were evaluated by RPA (Figure 11). BV-2 cells maintained in the absence of *A. culbertsoni* did not elicit discernable levels of chemokine mRNAs. In contrast, *A. culbertsoni* stimulated BV-2 cells to produce mRNAs for monocyte chemoattractant protein (MCP)-1, interferon gamma-inducible 10kDa protein (IP-10), macrophage inflammatory protein (MIP)-2, MIP-1α, and MIP-1β. Of these, the most robust induction levels were those for MIP-1α. *Acanthamoeba* did not express mRNAs for cytokines nor internal controls included in the RPA (data not shown).
Figure 10: *Acanthamoeba culbertsoni*-Conditioned Medium Proteolytic Activity Increases in a Time-Related Manner and Is Augmented During Co-culture With BV-2 Cells. The proteolytic activity of conditioned medium from $10^6$ *A. culbertsoni* (ACM-LO) and conditioned medium from co-culture of $10^6$ *A. culbertsoni* plus $10^6$ BV-2 cells (MCM/ACM) (9-24 h cultures) was assessed by gelatin zymography. ACM-LO is labeled “A” and MCM/ACM is labeled “M/A”. Arrows indicate protease activity located at positions corresponding to 187, 97, and 58 kDa. Relative molecular weight was calculated on the basis of the mean center point for each respective band on gelatin zymograms.
Figure 11: *Acanthamoeba culbertsoni* Induces Chemokine mRNA Expression by BV-2 Cells. A Multiprobe Ribonuclease Protection (RPA) assay was used to measure chemokine mRNA expression by BV-2 cells following co-culture (6 h) with *A. culbertsoni*. (A) RPA: lanes labeled “P” represent the undigested probes for LTN, RANTES, MIP-1α, MIP-1β, MIP-2, IP-10, MCP-1, TCA-3, Eotaxin and the constitutively expressed internal controls L32 and GAPDH. Lanes labeled “M + A” and “M” represent samples from BV-2 cells plus *A. culbertsoni* and BV-2 cells alone, respectively. (B) Graphical depiction of RPA results. Courtesy of Dr. Erinn Raborn, Ph.D.
In order to determine the profile of cytokines/chemokines at the protein level that were elicited in response to *A. culbertsoni*, culture supernatants from BV-2 cell cultures maintained in the absence (MCM) or presence of *A. culbertsoni* (MCM/ACM) (9-24 h) were examined using cytokine/chemokine protein microarray (Figures 12 and 13). A 9 h period post co-culture was selected for assessment since it represents a 3 h differential time interval post assessment of mRNA elicitation and thereby putatively allowed for the production of a protein product (Figure 12). A 24 h period post co-culture time was selected in order to assess time-related differences in cytokine and chemokine expression as compared to 9 h culture supernatants (Figure 13). BV-2 cell cultures maintained (9 h) in the absence of *A. culbertsoni* constitutively expressed relatively high levels of MCP-1, MIP-1α, and MIP-1γ as well as moderate levels of MIP-2, platelet factor 4 (PF-4), P-Selectin, soluble tumor necrosis factor receptor (sTNF R) I and sTNF RII (Figure 12). BV-2 cells maintained (9 h) in co-culture with *A. culbertsoni* expressed decreased levels of MIP-1α (2.1 fold decrease), MIP-1γ (2.1 fold decrease), PF-4 (2.3 fold decrease), sTNF RI (3.4 fold decrease), and sTNF RII (2.6 fold decrease) (Figure 12). There was also a decrease in MCP-1 levels (2.0 fold decrease) observed in supernatants from co-cultures of BV-2 cells and *A. culbertsoni* (Figure 12). However, augmented levels of granulocyte-colony stimulating factor (G-CSF) (2.3 fold increase), IL-12p40/70 (2.5 fold increase), TNF-α (2.6 fold increase), and MIP-3α (4.1 fold increase) were observed from co-culture supernatants as compared to supernatants for BV-2 cell cultures maintained in the absence of *A. culbertsoni* for 9 h (Figure 12). There was also an increase in IL-1α levels (1.5 fold increase) observed in supernatants from co-cultures of BV-2 cells and
Figure 12: Cytokine and Chemokine Protein Expression by BV-2 Cells following 9 h Co-culture with *A. culbertsoni*. The RayBio® Mouse Cytokine Antibody Array III arrays were used to screen for cytokines and chemokines in supernatants from BV-2 cell culture (9 h, MCM) and BV-2 cell plus *A. culbertsoni* co-culture (9 h, MCM/ACM). (A) Membrane arrays comparing cytokine and chemokine proteins of MCM versus MCM/ACM. (B) Graphical depiction of select cytokines and chemokines in MCM versus MCM/ACM. The relative amount of each cytokine and chemokine was normalized using the average density of the standards (Std.) represented in each membrane. Cytokines and chemokines are labeled: (1) G-CSF, (2) IL-1α, (3) IL-1β, (4) IL-12p40/70, (5) MCP-1, (6) MIP-1α, (7) MIP-1γ, (8) MIP-2, (9) MIP-3β, (10) MIP-3α, (11) PF-4, (12) P-Selectin, (13) RANTES, (14) TNF-α, (15) sTNF RI, and (16) sTNF RII. * indicates a ≥ 2.1 fold increase as compared to MCM, + indicates a ≥ 2.1 fold decrease as compared to MCM.
Figure 13: Cytokine and Chemokine Protein Expression by BV-2 Cells following 24 h Co-culture with *A. culbertsoni*. The RayBio<sup>®</sup> Mouse Cytokine Antibody Array III arrays were used to screen for cytokines and chemokines in supernatants from BV-2 cell culture (24 h, MCM) and BV-2 cell plus *A. culbertsoni* co-culture (24 h, MCM/ACM). (A) Membrane arrays comparing cytokine and chemokine proteins of MCM versus MCM/ACM. (B) Graphical depiction of select cytokines and chemokines in MCM versus MCM/ACM. The relative amount of each cytokine and chemokine was normalized using the average density of the standards (Std.) represented in each membrane. Cytokines and chemokines are labeled: (1) G-CSF, (2) IL-1α, (3) IL-1β, (4) IL-12p40/70, (5) MCP-1, (6) MIP-1α, (7) MIP-1γ, (8) MIP-2, (9) MIP-3β, (10) MIP-3α, (11) PF-4, (12) P-Selectin, (13) RANTES, (14) TNF-α, (15) sTNF RI, and (16) sTNF RII. * indicates a ≥ 2.1 fold increase as compared to MCM, + indicates a ≥ 2.1 fold decrease as compared to MCM.
*A. culbertsoni* (Figure 12). Levels of MCP-1, MIP-1α, MIP-1γ, MIP-2, MIP-3β, MIP-3α, P-Selectin, sTNF RI, and sTNF RII in 24 h supernatants from BV-2 cells maintained in the absence of *A. culbertsoni* were similar to those of BV-2 cells co-cultured with *A. culbertsoni* (Figure 13). Augmented levels of G-CSF (4.5 fold increase), IL-12p40/70 (3.7 fold increase), and TNF-α (4.7 fold increase) were observed from co-culture supernatants as compared to supernatants from BV-2 cell cultures maintained in the absence of *A. culbertsoni* for 24 h (Figure 13). An increase in IL-1α levels (1.7 fold increase) was also observed in supernatants from co-cultures of BV-2 cells and *A. culbertsoni* (Figure 13). In addition, a decrease in PF-4 (2.2 fold decrease) and normal T-cell expressed and secreted (RANTES) (2.1 fold decrease) was observed from co-culture supernatants as compared to supernatants for BV-2 cell cultures maintained in the absence of *A. culbertsoni* for 24 h (Figure 13). Notably, there was an overall decrease in MIP-3α (5.0 fold decrease) and TNF-α (3.8 fold decrease) levels in 24 h co-culture supernatants as compared to 9 h co-culture supernatants (Figures 12 and 13).

Light microscopy was used to observe the morphology of BV-2 cells, *A. culbertsoni*, and BV-2 cell plus *A. culbertsoni* co-cultures maintained in CNBA for 9-24 h (Figure 14). BV-2 cells maintained in the absence of *A. culbertsoni* (9-24 h) were attached to the plastic substratum and displayed a ramified morphology, consistent with these microglial-like cells being in a resting state (Figure 14 A-C). *A. culbertsoni* maintained in the absence of BV-2 cells (9-24 h) were observed attached to the plastic substratum and in their trophozoite form with multiple vacuoles (Figure 14 D-F). BV-2 cells co-cultured with *A. culbertsoni* (9-24 h) were observed in a rounded (i.e., amoeboid)
Figure 14: Light Microscopy Images of BV-2 Cells, A. culbertsoni, and BV-2 Cells Plus A. culbertsoni Co-cultures. 10^6 BV-2 cells, 10^6 A. culbertsoni, and 10^6 BV-2 cells plus 10^6 A. culbertsoni were cultured for 9 h (A, D, G), 15 h (B, E, H), and 24 h (C, F, I). (A-C) BV-2 cell cultures, (D-F) A. culbertsoni cultures, and (G-I) BV-2 cell plus A. culbertsoni co-cultures. Arrows in A-C indicate BV-2 cell processes associated with ramified morphology. Arrows in D-F indicate vacuoles associated with A. culbertsoni trophozoites. Arrows in G-H indicate BV-2 cells. 40x magnification.
form and detached from the plastic substratum, which is consistent with these cells being in a reactive or cytotoxic state (Figure 14 G-I). Notably, BV-2 cells maintained 24 h in the presence of A. culbertsoni were observed to be over 90% detached from the plastic substratum (Figure 14 I). A. culbertsoni morphology in co-culture with BV-2 cells was similar to that for cultures of A. culbertsoni maintained in the absence of BV-2 cells (Figure 14 G-I).

*Acanthamoeba culbertsoni* Elicited Factors Degrade Constitutively-Expressed Microglial-Like Cell Cytokines and Chemokines.

In order to determine further the effect of *A. culbertsoni* on cytokines and chemokines elicited from BV-2 cells, BV-2 cells were cultured in the absence (8 and 18.5 h) or presence (4-18.5 h) of 0.70 mg/ml of ACM-HI and levels of cytokines and chemokines were assessed by cytokine/chemokine protein microarray (Figure 15). A concentration of 0.70 mg/ml ACM-HI represented a 1:2.5 dilution (dilution factor calculated based on the average ACM-HI concentration in all experiments represented) of the original ACM-HI medium. Supernatant from BV-2 cells maintained in the absence of ACM-HI for 8 h elicited MCP-1, MIP-1α, MIP-1γ, MIP-2, PF-4, P-Selectin, regulated on activation, RANTES, sTNF RI, and sTNF RII (Figure 15). The relative amount of all of these immune factors was increased in supernatants from BV-2 cells maintained in the absence of ACM-HI for 18.5 h (Figure 15). In addition, eotaxin-2 also was elicited by BV-2 cells in the absence of ACM-HI following culture for 18.5 h (Figure 15). In contrast, supernatant from BV-2 cells maintained in the presence of ACM-HI for 4-8 h
Figure 15: Conditioned Medium from *A. culbertsoni* Degrades Constitutively Expressed Cytokines and Chemokines Elicited by BV-2 Cells. The RayBio® Mouse Cytokine Antibody Array III arrays were used to screen for cytokines and chemokines in supernatants from BV-2 cell cultures maintained in Neurobasal™-A medium (control medium; BV-2) for 8-18.5 h or in ACM-HI (BV-2 + ACM-HI) (0.70 mg/ml) for 4-18.5 h. Chemokines are labeled: (5) MCP-1, (6) MIP-1α, (7) MIP-1γ, (8) MIP-2, (11) PF-4, (12) P-Selectin, (13) RANTES, (15) sTNF RI, (16) sTNF RII, and (17) Eotaxin-2. Internal standards are labeled “Std”.
exhibited overall diminished levels of constitutively expressed cytokines and chemokines (Figure 15). In addition, culturing BV-2 cells in the presence of ACM-HI for 18.5 h resulted in the complete depletion of constitutively expressed cytokines and chemokines (Figure 15).

Morphological studies using light microscopy revealed that BV-2 cells maintained for 18.5 h in the absence of ACM-HI displayed a ramified morphology (Figure 16). However, BV-2 cells exposed to ACM-HI for 4-8 h displayed an amoeboid morphology consistent with BV-2 cells being in an active state or exhibiting incipient cytotoxicity. Maintenance of BV-2 cells in the presence of ACM-HI for 18.5 h resulted in an amoeboid morphology that exhibited membrane blebbing in > 50% of BV-2 cells, ruptures in cell membranes, and extrusion of cytosol (Figure 16). These observations are in agreement with factors elicited from A. culbertsoni, present in high concentration, being cytotoxic to BV-2 cells.

In order to further confirm if factors elicited from A. culbertsoni could deplete BV-2 cell pro-inflammatory cytokines and chemokines, BV-2 cells were exposed to 100 ng/ml LPS for 8 h, culture supernatant was collected (LPS/MCM), then incubated in the absence or presence of ACM-HI (0.70 mg/ml) for an additional 8 h. The presence of cytokine and chemokine proteins in resulting supernatants was assessed by cytokine/chemokine protein microarray (Figure 17). A concentration of 0.70 mg/ml ACM-HI represented a 1:2.5 dilution (dilution factor calculated based on the average ACM-HI concentration in all experiments represented) of the original ACM-HI medium to LPS/MCM. LPS treatment of BV-2 cells resulted in a robust induction of cytokines
Figure 16: Effect of $10^9$ A. culbertsoni-Conditioned Medium (ACM-HI) on BV-2 Cell Morphology. BV-2 cells were cultured in Neurobasal™A medium (control medium) or treated with ACM-HI (0.70 mg/ml) for 4-18.5 h. Arrows indicate areas of cytosol extrusion at 18.5 h. 40x magnification.
Figure 17: Conditioned Medium from *A. culbertsoni* Degrades Cytokines and Chemokines Elicited from Lipopolysaccharide (LPS) Stimulated BV-2 Cells. BV-2 cells were stimulated (8 h) with 100 ng/ml LPS and culture supernatant (LPS/MCM) was collected. Then, LPS/MCM was incubated with ACM-HI (0.70 mg/ml) (B) or an equal volume of complete Neurobasal™-A medium (control) for 8 h (A). Cytokines and chemokines are labeled: (1) G-CSF, (4) IL-12p40/70, (5) MCP-1, (6) MIP-1α, (7) MIP-1γ, (8) MIP-2, (13) RANTES, (14) TNF-α (15) sTNF RI, (16) sTNF RII, and (18) GM-CSF, (19) IL-6.
and chemokines. BV-2 cell stimulation with LPS induced the production of G-CSF, granulocyte macrophage-colony stimulating (GM-CSF), interleukin-6 (IL-6), IL-12p40/70, MCP-1, MIP-1\(\alpha\), MIP-1\(\gamma\), MIP-2, RANTES, TNF-\(\alpha\), sTNF RI, and sTNF RII (Figure 17). Thus, the profile for LPS induction of cytokines and chemokines from BV-2 cells differed from that resulting from induction with *A. culbertsoni*. In particular, robust levels of IL-6 were elicited in the presence of LPS and not in the presence of *A. culbertsoni*. Incubation of LPS/MCM with ACM-HI (0.70 mg/ml) for 8 h resulted in nearly complete depletion of cytokines and chemokines (Figure 17).

*Acanthamoeba culbertsoni* Serine Proteases Are Associated with the Degradation of *A. culbertsoni*-Stimulated Microglial-Like Cell Cytokines and Chemokines.

In order to determine whether *A. culbertsoni* proteases could exert a similar effect on cytokines and chemokines elicited from BV-2 cells in the presence of *A. culbertsoni*, BV-2 cells and *A. culbertsoni* were co-cultured for 24 h (1:1 ratio), the culture supernatants (presumably containing amoebae-elicited proteases plus cytokines and chemokines produced by BV-2 cells) were collected and incubated for an additional 72 h, and the presence of cytokine and chemokine proteins were assessed by protein microarray (Figure 18). Using this approach, a decrease in MIP-1\(\alpha\) (8.3 fold decrease), PF-4 (2.1 fold decrease), and RANTES (2.1 fold decrease) was observed from co-culture supernatants as compared to supernatants for BV-2 cell cultures maintained in the absence of *A. culbertsoni* (Figure 18). Levels of TNF-\(\alpha\) and sTNF RI were also decreased (1.8 fold decrease and 1.7 fold decrease, respectively) (Figure 18).
Figure 18: *Acanthamoeba culbertsoni* Proteases Degrade *A. culbertsoni* Induced Microglial Chemokines from BV-2 Cells. BV-2 cells ($10^6$) were cultured alone or co-cultured with *A. culbertsoni* ($10^6$) for 24 h. The supernatant was harvested and incubated an additional 72 h and the relative amount of cytokines and chemokines present was measured using RayBio® Mouse Cytokine Antibody Array III assay. (A) Membrane array for culture supernatant from BV-2 cells (MCM). (B) Membrane array for culture supernatant from BV-2 cells co-cultured with *A. culbertsoni* (MCM/ACM). (C) Graphical depiction comparing select chemokines. Chemokines are labeled: (5) MCP-1, (6) MIP-1α, (7) MIP-1γ, (8) MIP-2, (11) PF-4, (13) RANTES, (14) TNF-α, and (15) sTNF RI. + indicates a ≥ 2.1 fold decrease as compared to MCM.
Protease activity associated with culture supernatants from Figure 18 was assessed by gelatin zymography. BV-2 cells maintained in the absence of *A. culbertsoni* did not exhibit protease activity (Figure 19). In contrast, *A. culbertsoni* co-cultured with BV-2 cells (24 h) exhibited protease activity at zymogram gel positions that corresponded to 187, 97, and 58 kDa. This protease activity remained stable following an additional 72 h of incubation at 37ºC (Figure 19).

In order to determine if *A. culbertsoni* serine proteases were specifically linked to *A. culbertsoni* stimulated BV-2 cell cytokine and chemokine degradation, BV-2 cells were co-cultured with *A. culbertsoni* for 9-24 h, supernatants were collected and incubated an additional 12-48 h in the presence or absence of PMSF. The levels of TNF-α, MIP-1α, and MIP-2 proteins in resulting supernatants were assessed by ELISA. Previous assessment of cytokines and chemokines using protein microarrays indicated that TNF-α expression was optimal at 9 h and that MIP-1α and MIP-2 levels were stable up to 24 h. Thus, 9 h and 24 h co-culture times were chosen for assessment of TNF-α and for MIP-1α and MIP-2, respectively. TNF-α, MIP-1α and MIP-2 were chosen due to their roles in: i) recruiting microglia/macrophages (MIP-1α), T cells (MIP-1α), and neutrophils (MIP-2) and ii) activating microglia/macrophages for amoebicidal activity (TNF-α). TNF-α, MIP-1α and MIP-2 present in co-culture supernatants in the absence of PMSF were degraded in a time-related manner (Figures 20-22). Specifically, TNF-α was decreased by approximately 80% in co-culture supernatants maintained for an additional 12 h as compared to levels in initial co-culture supernatants (Figure 20).
Figure 19: *Acanthamoeba culbertsoni* Protease Activity Persists in Co-culture Supernatants Following Incubation for 72 h. BV-2 cells (10^6) were cultured alone or co-cultured with *A. culbertsoni* (10^6) for 24 h. The supernatant was harvested, incubated an additional 72 h, and protease activity of an equal volume of each supernatant was assessed using gelatin zymography. Supernatants from BV-2 cells alone and BV-2 cells co-cultured with *A. culbertsoni* are designated as “M” and “M/A”, respectively. Supernatants from 24 h cultures were considered as the starting time and are labeled 0 h.
Figure 20: *Acanthamoeba culbertsoni* Serine Proteases Degrade TNF-α. Enzyme linked immunosorbent assay (ELISA) was used to measure TNF-α produced by BV-2 cells ($10^6$) co-cultured with *A. culbertsoni* ($10^6$) for 9 h. The culture supernatant was collected and incubated an additional 12-48 h with or without the serine protease inhibitor PMSF (1.0 mM). The percent maximum response was calculated by comparing the optical density of samples that were incubated for an additional 12-48 h to optical density of the starting co-culture supernatant (i.e., 9 h co-culture supernatant). The optical density of the co-culture supernatant at 9 h was equivalent to 1695 pg/ml TNF-α and was considered the 100% value. Samples were performed in triplicate and the average optical density of each sample was used to calculate percent maximum response.
Figure 21: *Acanthamoeba culbertsoni* Serine Proteases Degrade MIP-1α. Enzyme linked immunosorbent assay (ELISA) was used to measure MIP-1α produced by BV-2 cells ($10^6$) co-cultured with *A. culbertsoni* ($10^6$) for 24 h. The culture supernatant was collected and incubated an additional 12-48 h with or without the serine protease inhibitor PMSF (1.0 mM). The percent maximum response was calculated by comparing the optical density of samples that were incubated for an additional 12-48 h to optical density of the starting co-culture supernatant (i.e., 24 h co-culture supernatant). The optical density of the co-culture supernatant at 24 h was equivalent to 3983 pg/ml MIP-1α and was considered the 100% value. Samples were performed in triplicate and the average optical density of each sample was used to calculate percent maximum response.
Figure 22: *Acanthamoeba culbertsoni* Serine Proteases Degrade MIP-2. Enzyme linked immunosorbent assay (ELISA) was used to measure MIP-2 produced by BV-2 cells (10^6) co-cultured with *A. culbertsoni* (10^6) for 24 h. The culture supernatant was collected and incubated an additional 12-48 h with or without the addition of the serine protease inhibitor PMSF (1.0 mM). The percent maximum response was calculated by comparing the optical density of samples that were incubated for an additional 12-48 h to optical density of the starting co-culture supernatant (i.e., 24 h co-culture supernatant). The optical density of the starting co-culture supernatant was equivalent to 5247 pg/ml MIP-2 and was considered the 100% value. Samples were performed in triplicate and the average optical density of each sample was used to calculate percent maximum response.
Furthermore, TNF-α levels in co-culture supernatants were shown to be decreased by approximately 97% following an additional 48 h incubation as compared to levels observed in initial co-culture supernatants. An approximate 88% and 99% reduction in MIP-1α levels was observed in co-culture supernatants maintained for an additional 12 h and 48 h, respectively, as compared to levels in initial co-culture supernatants (Figure 21). In addition, MIP-2 levels were diminished by approximately 50% and 96% in co-culture supernatants maintained for an additional 12 h and 48 h, respectively, as compared to levels in initial co-culture supernatants (Figure 22). Degradation of TNF-α, MIP-1α, and MIP-2 in co-culture supernatants was reversed in the presence of PMSF, consistent with A. culbertsoni serine proteases as playing a role in their degradation. Specifically, in the presence of PMSF an approximate 60% and 70% difference in TNF-α and MIP-1α levels, respectively, was observed in co-culture supernatants incubated for an additional 12 h as compared to co-culture supernatants maintained an additional 12 h in the absence of PMSF (Figures 20 and 21). The presence of PMSF in co-culture supernatant resulted in the full recovery of MIP-2 levels at all time points studied (Figure 22). Although there was an overall increase in TNF-α and MIP-1α levels in co-culture supernatants maintained in the presence of PMSF as compared to co-culture supernatants maintained in the absence of PMSF, a time-related decrease of TNF-α and MIP-1α also was observed in co-culture supernatants maintained for an additional 12-48 h in the presence of PMSF (Figures 20 and 21). These observations may represent a natural breakdown of these factors within the extracellular milieu; or, they may represent the degradation of these factors by other A. culbertsoni elicited enzymes.
Discussion

Acanthamoeba are free-living amoebae that are found worldwide and can cause opportunistic infections in humans. Acanthamoeba have been isolated from a variety of environmental sources including air, soil, dust, tap water, freshwater, seawater, swimming pools, air conditioning units, and contaminated lens cases [Marciano-Cabral and Cabral 2003]. Acanthamoeba have two life cycle stages; the trophozoite and the cyst. The trophozoite stage represents the feeding as well as the infective form of Acanthamoeba [Rodriquez-Zaragoza et al. 1994; Marciano-Cabral and Cabral 2003; Visvesvara et al. 2007]. The cyst stage represents a dormant phase that is formed under adverse environmental conditions such as extreme temperature or pH changes [Marciano-Cabral and Cabral 2003].

Acanthamoeba secrete a plethora of enzymes including phospholipases and proteases. It has been demonstrated that pathogenic Acanthamoeba secrete more phospholipase A and proteases than non-pathogenic Acanthamoeba [Cursons et al. 1978; Misra et al. 1983; Hadás et al. 1993b; Khan et al. 2000]. Additionally, it has been demonstrated that pathogenic Acanthamoeba produce serine, cysteine, and metallo-proteases [He et al. 1990; Hadás et al. 1993a; Mitro et al. 1994; Mitra et al. 1995; Cao et al. 1998; Alfieri et al. 2000; Cho et al. 2000; Hong et al. 2000; Khan et al. 2000; Kong et al. 2000; Na et al. 2001; Hong et al. 2002; Na et al. 2002; Alsam et al. 2005; Kim et al.
In our studies a comparison of two-dimensional (iso-dalt) gel electrophoresis proteomic profiles of complete Neurobasal™-A medium versus A. culbertsoni-conditioned complete Neurobasal™-A medium (i.e., A. culbertsoni-conditioned medium) suggested that A. culbertsoni secrete factors that are able to degrade albumin and putatively other proteins during in vitro culture. Our findings are consistent with studies by Kong et al. [2000] that demonstrated that a serine protease from A. healyi degrades albumin. Using gelatin zymography, we detected A. culbertsoni-secreted serine proteases corresponding to positions at 187, 97, 90, and 58 kDa. However, protease activity located at the position corresponding to 90 kDa was not always observed in our gelatin zymography assays of A. culbertsoni-conditioned medium. Thus, it is possible that protease activity detected at this position could represent a breakdown product. Our gelatin zymography results are consistent with those obtained from previous studies that demonstrated that pathogenic T4 genotype Acanthamoeba secrete serine proteases at positions corresponding to 188, 97, and 55 kDa [Cao et al. 1998; Serrano-Luna et al. 2006]. We propose that the A. culbertsoni serine proteases of the approximate molecular weights of 187, 97, and 58 kDa identified in our studies likely represent serine proteases similar to that of pathogenic Acanthamoeba T4 genotype identified at 188, 97, and 55 kDa, respectively. Differences between the relative molecular weights of serine proteases identified in our studies and those of others are likely due to variations in the construction of zymography gels. Thus, our results suggest that the T10 genotype A. culbertsoni used in our studies may secrete similar serine proteases as pathogenic T4 genotype Acanthamoeba; suggesting that certain proteases may convey pathogenicity.
Using the cysteine protease inhibitor E-64 we did not observe cysteine protease activity in A. culbertsoni-conditioned medium. Studies by Alferi et al. [2000] indicate that Acanthamoeba cysteine proteases are most active at pH 4.0-5.0. Our gelatin zymogram studies were performed at pH 5.0 and 7.5. Furthermore, Serrano-Luna et al. [2006] were not able to detect cysteine protease activity in Acanthamoeba conditioned medium using E-64 at pH 3.0 - 9.0; but, they were successful at detecting cysteine protease activity using the cysteine protease inhibitor N-ethylmaleimide (NEM) [Serrano-Luna et al. 2006]. Thus, their results suggest that E-64 may not be the optimal inhibitor to use for the detection of cysteine protease activity in Acanthamoeba-conditioned medium. Using gelatin zymography, we also demonstrated that A. culbertsoni propagation medium does not influence the proteolytic profile of A. culbertsoni; however, levels of protease activity secreted from A. culbertsoni were affected. Our results suggest that the higher iron content in oxoid medium, which contains neutralized liver digest, as compared to that in PYG medium influences the level of protease activity elicited from A. culbertsoni. Our results are consistent with those of Melo-Braga et al. [2003] that demonstrated that iron-depletion reduces cysteine protease activity elicited from the bovine pathogen Tritrichomonas foetus. Collectively, our results indicate that A. culbertsoni secrete predominantly serine proteases and highlight the importance of the selection of medium used for the growth of Acanthamoeba in various studies since these may affect levels of secreted proteases. Acanthamoeba serine proteases are thought to serve a functional role in encystment and excystment of amoeba while they inhabit the environment [Dudley et
al. 2008; Moon et al. 2008]. However, it is also thought that serine proteases may play a role in *Acanthamoeba* host invasion and immune evasion.

Only a select group of *Acanthamoeba* species, including *A. culbertsoni*, have been linked to disease in humans. *Acanthamoeba* are known to cause three types of infections in humans; AK, cutaneous acanthamoebiasis, and GAE [Marciano-Cabral and Cabral 2003; Khan 2006; Visvesvara et al. 2007]. AK is a potentially blinding eye infection that is most frequently associated with contact lens wearers and it the most common disease caused by *Acanthamoeba* [Clarke and Niederkorn 2006a]. Cutaneous acanthamoebiasis is an *Acanthamoeba* skin infection that can result in hematogenous spread of *Acanthamoeba* throughout the host. Cutaneous acanthamoebiasis is commonly associated with immune compromised individuals. *Acanthamoeba* can also cause a chronic and slow progressing CNS disease called GAE.

GAE is most prevalent in immune compromised individuals [Marciano-Cabral and Cabral 2003]. It is believed that in humans GAE is most often caused by the entry of trophozoites into the CNS via hematogenous spread originating from the lungs or cutaneous infection [Marciano-Cabral and Cabral 2003; Khan 2008]. However, *Acanthamoeba* trophozoites may also gain access to the CNS via the olfactory neuroepithelium. Studies by Alsam et al. [2005], using a human brain endothelial cell model, implicated *Acanthamoeba* serine proteases in the induction of blood brain barrier permeability; thus, demonstrating a potential role of *Acanthamoeba* serine proteases in CNS invasion leading to GAE. GAE is characterized by the formation of granulomas within the CNS; however, granulomas are rarely observed in immune compromised
individuals [Martinez 1982]. Granulomas are usually composed of amoebae, microglia, macrophages, T cells, B cells, and neutrophils.

Microglia, resident brain macrophages, and invading peripheral macrophages have been indicated as the primary immune cells that elicit a direct response to CNS invading Acanthamoeba [Marciano-Cabral and Cabral 2003]. It has been reported that primary rat microglia release pro-inflammatory mediators, such as IL-1α, IL-1β, and TNF-α in response to Acanthamoeba [Marciano-Cabral et al. 2004]. Reports also indicate that such cytokines are not cytolytic to Acanthamoeba and, rather, may serve to activate microglial/macrophage cells for contact-dependent mediated killing of Acanthamoeba [Marciano-Cabral et al. 1998]. Indeed, activated microglia and macrophages have been demonstrated to be cytolytic (i.e., amoebicidal) to Acanthamoeba [Benedetto et al. 2002; Benedetto et al. 2003]. We demonstrated that BV-2 microglial-like cells produce mRNAs for MCP-1, MIP-1α, MIP-1β, and MIP-2 in response to A. culbertsoni. These observations suggest that exposure of the microglial-like cells to Acanthamoeba resulted in de novo gene induction. The results were extended at the protein level wherein MCP-1, MIP-1α, and MIP-1γ were constitutively produced by BV-2 cells at relatively high levels at early time periods (i.e., 9 h). Furthermore, we demonstrated that A. culbertsoni augmented the protein level of G-CSF, IL-1α, IL-12p40/70, TNF-α, and MIP-3α secreted by BV-2 cells. It is important to note that in our studies we did not observe an A. culbertsoni-dependent induction of BV-2 cell-elicited IL-1β expression as demonstrated in previous studies [Shin et al. 2001; Marciano-Cabral et al. 2004]. Thus, differences between our results and those of others are likely due to
the differences in microglial cell type and/or culture conditions used. G-CSF stimulates the growth and activation of neutrophils [Fitzgerald et al. 2001]. IL-1α, IL-1β, and TNF-α are microglial, macrophage, and T cell activating cytokines. Furthermore, TNF-α plays an instrumental role in the activation of microglial and macrophage anti-microbial activity via the activation of phagocytosis. IL-12 serves as an important signal for the induction of the Th1 cell phenotype and also serves as a stimulatory signal for Th1 cytokine secretion; and microglia are thought to represent the main source of IL-12 within the CNS [Stalder et al. 1997; Aloisi 2001]. MIP-3α stimulates the recruitment of activated and memory T cells, dendritic cells, and B cells [Dieu et al. 1998; Tanaka et al. 1999]. Overall, our results suggest that microglia elicit a variety of cytokines and chemokines in response to A. culbertsoni which are capable of autocrine and paracrine immune cell activation facilitating the formation of granulomas via the recruitment of immune cells, including microglia, macrophages, T cells, B cells, and neutrophils, to focal sites of Acanthamoeba within the CNS. It is important to note that we also observed a decrease in sTNF RI and s TNF RII levels in supernatants from BV-2 cells stimulated with A. culbertsoni for 9 h as compared to supernatants from un-stimulated BV-2 cells. sTNF RI and sTNF RII represent soluble forms of TNF RI and TNF RII, respectively, and their role in CNS immunity has not been fully elucidated; however, studies have indicated a link between increased levels sTNF RI in cerebrospinal fluid to the clinical onset of multiple sclerosis [Tsukada et al. 1993]. In contrast to the induction of pro-inflammatory factors observed at 9 h co-culture, we observed a decrease in levels of MCP-1, MIP-1α, MIP-1γ and PF-4 at 9 h. MCP-1 and MIP-1α stimulate the
chemotaxis of microglia, macrophages, dendritic cells, and T helper 1 (Th1) cells [Peterson et al. 1997; Dieu et al. 1998; Aloisi 2001]. MIP-1\(\gamma\) serves as a chemotactic signal for dendritic and memory T cells. PF-4 recently has been reported to induce the migration of microglia via the CXCR3 chemokine receptor [de Jong et al. 2008]. In addition, we detected a decrease in PF-4 and RANTES levels in supernatants from BV-2 cells stimulated with \textit{A. culbertsoni} for 24 h as compared to supernatants from un-stimulated BV-2 cells. RANTES induces the migration of microglia, macrophages, T cells, and dendritic cells [Aloisi 2001]. We also observed an overall decrease in the levels of TNF-\(\alpha\) and MIP-3\(\alpha\) protein at 24 h. The decreases in the aforementioned cytokines and chemokines could represent changes in the transcriptional or translational regulation of these BV-2 cell-elicited factors in the presence of \textit{A. culbertsoni} or, alternatively, could reflect the degradation of these factors within the extracellular milieu.

Our studies implicate proteases that are secreted by \textit{Acanthamoeba} as playing a major role in the decrease in levels of chemokines and cytokines in the extracellular milieu. In this context, previous studies have demonstrated that of \textit{Acanthamoeba} serine proteases are able to degrade immune modulating factors such as sIgA, IgG, IgM, IL-1\(\alpha\), and IL-1\(\beta\), implicating these enzymes as playing a role in host immune evasion [Kong et al. 2000; Na et al. 2001; Na et al. 2002]. However, in these studies recombinant exogenous substrates were used. Our studies are novel in that they demonstrated that proteases elicited by \textit{Acanthamoeba} degrade cytokines and chemokines natively secreted by microglial-like cells. That is, we observed that constitutively-expressed as well as LPS-stimulated cytokines and chemokines from BV-2 cells were degraded in the
presence of conditioned medium from *A. culbertsoni* cultures. Constitutively expressed BV-2 cell cytokines and chemokines which were degraded in the presence of *A. culbertsoni*-elicited factors included eotaxin-2, MCP-1, MIP-1α, MIP-1γ, MIP-2, PF-4, P-Selectin, RANTES, sTNF RI and sTNF RII. In addition, LPS-stimulated cytokines and chemokines were degraded. LPS-stimulated BV-2 cell cytokines and chemokines which were degraded in the presence of *A. culbertsoni*-elicited factors included G-CSF, GM-CSF, IL-6, IL-12p40/p70, MCP-1, MIP-1α, MIP-1γ, MIP-2, RANTES, TNF-α, sTNF RI, and sTNF RII. It is important to note that the pattern of cytokines and chemokines elicited by LPS-stimulated BV-2 cells was different than that of *A. culbertsoni*-stimulated BV-2 cells. In particular, LPS caused BV-2 cells to elicit a high level of IL-6 which was not detected when BV-2 cells were stimulated with *A. culbertsoni*. Moreover, MIP-3α and MIP-3β were elicited by *A. culbertsoni*-stimulated BV-2 cells and not by LPS-stimulated BV-2 cells. Furthermore, our studies suggest that *A. culbertsoni* serine proteases are specifically linked to the degradation of microglial cytokines and chemokines (e.g. TNF-α, MIP-1α, and MIP-2). Our results parallel findings of others that demonstrate that the serine proteases cathepsin G, elastase, and proteinase 3 degrade human MIP-1α; and that a Group A streptococcal serine protease degrades MIP-2 [Ryu et al. 2005; Hidalgo-Grass et al. 2006]. Furthermore, studies by Ferrante and Bates [1988] indicate that *A. culbertsoni* elicit an elastase; thus, it can be postulated that this serine protease may also be linked to the degradation of microglial-secreted cytokines and chemokines. Collectively, our results suggest that *A. culbertsoni*-elicited factors degrade endogenously produced BV-2 cell-elicited cytokines and chemokines, indicating a mode
by which *A. culbertsoni* applies a contact-independent mechanism to dampen microglial facilitated CNS immune cell responsiveness which, in turn, may lead to the downstream diminution of granuloma formation and microglial/macrophage anti-microbial activity.

We also detected a time-related augmentation in the level of serine protease activity from *A. culbertsoni* in the presence of BV-2 cells. The augmentation in *A. culbertsoni* serine protease activity in conditioned medium may be linked to *A. culbertsoni* binding to microglial-like cells via trophozoite expressed mannose-binding protein (MBP), and is consistent with reports that binding of *Acanthamoeba* to mannose on human brain microvascular endothelial cells via *Acanthamoeba*-expressed MBP increases the amount of secreted protease [Alsam et al. 2003; Alsam et al. 2005]. One mode by which *A. culbertsoni* serine proteases affect microglial responsiveness may be through activation of microglial-expressed receptors called protease-activated receptors (PARs), the activation of which results in the release of microglial cytokines and chemokines. PARs are a group of G-protein coupled receptors that are activated by serine and cysteine proteases. To date, four PARs have been described; PAR1, PAR2, PAR3, and PAR4. PARs are expressed by a variety of cells including platelets, endothelial cells, epithelial cells, monocytes, T cells, natural killer (NK) cells, astrocytes, neurons, and microglia [Balcaitis et al. 2003; Steinhoff et al. 2005]. We have confirmed published reports that BV-2 cells express mRNA for PAR1, PAR3, and PAR4 (data not shown) [Balcaitis et al. 2003]. PARs are activated when proteases recognize and cleave a specific amino acid sequence located at the N-terminus of the receptor; the resulting N-terminus acts as a tethered ligand by binding back on the receptor’s extracellular loop
region. PAR1 can be activated by thrombin, Factor Xa, plasmin, and activated protein C [Ossovskaya and Bunnett 2004; Steinhoff et al. 2005]. PAR2 can be activated by trypsin, tryptase, Factor VIIa, Factor Xa, and exogenous proteases such as gingipain-R of Porphyromonas gingivalis [Ossovskaya and Bunnett 2004; Steinhoff et al. 2005]. PAR3 is believed to act as a cofactor for PAR4; and, PAR3 and PAR4 can be activated by thrombin and cathepsin G [Nakanishi-Matsui, 2000; Steinhoff et al. 2005]. Activation of PARs has been linked to inflammation [Steinhoff et al. 2005]. Specifically, it has been suggested that PAR1 activation leads to the production of IL-1, IL-6, and MCP-1 by monocytes [Colotta et al. 1994; Naldini et al. 2000; Naldini et al. 2002; Steinhoff et al. 2005; Li et al. 2006]. PAR1 activation has also been linked to IL-8 production in macrophages [Zheng et al. 2007]. Activation of monocyte expressed PAR2 has been demonstrated to induce IL-1β, IL-6, and IL-8 production [Johansson et al. 2005]. PAR4 has been linked to IL-6 production by monocytes [Li et al, 2006]. Thus, we propose that it is possible that A. culbertsoni serine proteases act through BV-2 microglial-like cell-expressed PARs leading to the secretion of cytokines and chemokines observed in our studies. However, further studies using specific PAR agonists and antagonists/blockers would be required in order to establish a functional linkage between in A. culbertsoni-elicited serine protease and microglial-cell cytokine/chemokine production.

Proteases acting through PAR receptors have been linked to cell apoptosis [Steinhoff et al. 2005]. For example, PAR1 thrombin activation has been demonstrated to induce neuronal cell apoptosis [Smirnova et al. 1998]. These observations are consistent with our results from our light microscopy studies which demonstrated that
conditioned medium from *A. culbertsoni* is toxic to BV-2 cells suggestive of apoptosis. Additionally, these findings are in agreement with those that demonstrated that *Acanthamoeba* elicited factors induce morphological changes and lysis of epithelial cells [Mattana et al. 1997].

In summary, while it is likely that *Acanthamoeba* uses a multiplicity of contact-dependent and contact-independent mechanisms to elicit pathology within the CNS during GAE, our results suggest that *Acanthamoeba* have developed strategies via the elicitation of serine proteases to aid immune evasion in that compartment. Specifically, we postulate that serine proteases released by *Acanthamoeba* initially act on microglia to induce the production of a plethora of cytokines and chemokines, possibly mediated through the activation of PAR receptors on microglia. These cytokines and chemokines elicited by microglia in response to *Acanthamoeba* serve to activate and recruit additional microglia, macrophages, T cells, and neutrophils to focal sites of infection leading to the formation of granulomas. Within these granulomas it is postulated that microglia and macrophages are activated by T cell-elicited IFN-γ in combination with T cell/microglial/macrophage-elicited TNF-α, leading to microglial/macrophage-dependent amoebicidal activity [Benedetto et al. 2002; Benedetto et al. 2003]. However, under conditions of high *Acanthamoeba* burden when relatively high concentrations of *Acanthamoeba* may be present, we suggest that the attendant higher concentration of *Acanthamoeba* serine proteases leads to the degradation of microglial cytokines and chemokines. This diminution, in turn, may lead to a decrease in microglial/macrophage activation resulting in the decrease of granuloma formation and microglial/macrophage
anti-microbial activity. The resultant outcome is dissemination of *Acanthamoeba* within the CNS and progressive neuropathology. Additionally, our results suggest that *Acanthamoeba* proteases induce microglial cell apoptosis, thus, representing a means by which these opportunistic pathogens may process microglia as a source of food. In conclusion, our results suggest that *Acanthamoeba* serine proteases provide a multi-tailed approach for *Acanthamoeba* to effectively evade host CNS immunity. Furthermore, it can be postulated that appropriately designed therapeutics which block the potential activation of microglial expressed PARs by *Acanthamoeba* elicited serine proteases used in combination with current therapeutic approaches could prove to be an effective means of benefiting the clinical outcome of patients with GAE.
Literature Cited


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