THE ROLE OF THE C5A RECEPTOR IN HOST DEFENSE AGAINST LISTERIA MONOCYTOGENES

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THE ROLE OF THE C5A RECEPTOR IN HOST DEFENSE AGAINST _LISTERIA MONOCYTOSGENES_

A

DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
Graduate School of Biomedical Sciences
In Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

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August, 2014
ACKNOWLEDGEMENTS

I must begin by thanking my advisor, Dr. Rick Wetsel. As I looked for labs to do research in the summer before medical school, Rick’s lab immediately caught my eye as his interests in stem cells and immunology paralleled my own. He quickly responded to my email, invited me in to talk about it, and graciously supported me that summer. I enjoyed the experience so much that I felt compelled to pursue additional training via the MD-PhD program, and there again Rick backed my application in a way that it possible for me to be here today. His support and advice over the years has been invaluable.

I would also like to thank all of the members of my many committees, namely Drs. Brian Davis, Joseph Alcorn, David Corry, Michael Blackburn, Scott Drouin, Yeonseok Chung, and Amber Luong for their contribution to my graduate school education. Their support, advice and challenging questions have helped push my project forward over the last five years.

I am especially grateful to the other members of the Wetsel lab, specifically Mr. John Morales, Dr. Dachun Wang, and Dr. Stacey Mueller-Ortiz. Mr. Morales provided me with countless hours of invaluable experimental support. Dr. Wang was responsible for my introduction to cell culture and cloning. Dr. Mueller-Ortiz in particular deserve my highest praise. By allowing me to utilize her infection model she put me on a direct trajectory to the completion of this thesis. Furthermore, she acted as a sounding board and gave me great feedback over the last few years, and as such I would not be at this point without her.

Finally, I would like to conclude by thanking all my family and friends for their support. They have all supported me and helped me take my mind off the science from time
to time, allowing me to come back reinvigorated. My girlfriend Sheree Chen has been especially helpful in that regard. I cannot conclude without recognizing my parents and my grandparents. They taught me humility, integrity, and indulged my endless curiosity. As such, they are directly responsible for the man I am today.
THE ROLE OF THE C5A RECEPTOR IN HOST DEFENSE AGAINST *LISTERIA MONOCYTOGENES*

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*Listeria monocytogenes* (Lm) is a major cause of mortality resulting from food poisoning in the United States. While the complement component C5 is known to be protective in listeriosis, it is unknown how its cleavage fragment C5a participates. Here we show in a model of systemic Lm infection that the C5a receptor is essential for host defense. C5aR-/- mice have reduced survival and increased bacterial burden in the liver and spleen in comparison to WT mice. Surprisingly, C5aR-/- mice also have a dramatic reduction in splenocyte numbers resulting from elevated cell death as indicated by TUNEL staining and caspase 3 activity. This splenocyte depletion affected all major subsets of splenocytes, indicating a broad protective effect for C5aR. C5aR was not required for the production of protective cytokines such as TNF-α, IFN-γ and IL-6. As Type 1 IFN impedes the host response to Lm through the promotion of splenocyte death, we examined the effect of C5a and C5aR on type 1 IFN expression *in vivo* and *in vitro*. Serum levels of IFN-α and IFN-β are significantly higher in C5aR-/- mice than WT mice. The elevation of type 1 IFN in C5aR-/- mice correlated with increased expression of TRAIL, a downstream target of type 1 IFN and an important driver of splenocyte loss in listeriosis. Pre-stimulation with C5a directly represses LPS-induced IFN-β expression in the macrophage cell line J774A *in vitro*. Finally, treatment of C5aR-/- mice with a type 1 IFN receptor blocking antibody resulted in
near complete rescue of Lm-induced mortality. Thus, these findings reveal for the first time a critical role for C5aR in host defense against Lm through the suppression of type 1 IFN expression.
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GENERAL INTRODUCTION

Listeriosis

Foodborne illness has long been a scourge of mankind. While little record exists of its prevalence or death toll prior to the modern era, there is reason to believe foodborne disease has had a marked impact on human history. For example, Alexander the Great, the renowned conqueror of the ancient world, was vanquished not through military defeat but by typhoid fever (1). Although improvements in sanitation have greatly reduced its incidence, food poisoning continues to be a major problem today. As many as 1 in 6 Americans are sickened annually by contaminated food (2, 3). One of the most serious foodborne illnesses is listeriosis. The causative agent of listeriosis is the Gram positive bacillus *Listeria monocytogenes* (Lm). While Lm was first identified in 1926, its route of transmission was not recognized until the early 1980s when an outbreak of listeriosis was linked to a coleslaw manufacturing plant (4, 5). The threat of Lm to the food supply stems from several factors. First, Lm is widely dispersed in the environment. Samples of soil, ground water, and fecal material from domestic animals frequently contain Lm (6). Through these sources it can easily taint manufactured food products. Second, Lm is endowed with remarkable hardiness. It tolerates both high salinity and acidity, treatments used in food preparation to limit bacterial growth (7). Finally, in contrast to other pathogenic bacteria, Lm proliferates at temperatures as low as 4°C (8). Thus, a modest initial inoculum can result in high levels of contamination after prolonged refrigeration. For these reasons, strict protocols for food preparation are enforced by regulatory agencies in the United States and abroad. Unfortunately, breakdowns in these protocols are common and result in regular outbreaks of listeriosis. A prime example of this was a 2011 outbreak involving contaminated
cantaloupes from Jensen Farms in Colorado (9). The CDC reports that 147 people were afflicted, resulting in 33 deaths and one miscarriage. As a consequence, Lm was responsible for the deadliest outbreak of foodborne illness in U.S. history.

Healthcare providers frequently view listeriosis as an uncommon condition (9). Healthy adults are generally resistant to Lm, developing a mild gastroenteritis at most upon exposure. However, in the elderly, immunocompromised, and patients with chronic illness, listeriosis results in severe systemic disease associated with sepsis, meningitis/encephalitis, and/or death (10). The mortality rate following hospitalization is extremely high (20-30%) in comparison to more common foodborne illnesses such as salmonellosis and shigellosis (6). Because of this, listeriosis is the third leading cause of death from food poisoning in the United States and the second leading cause in the European Union (9, 10). As the population continues to age and more immunosuppressive drugs enter the market the incidence of invasive listeriosis will likely increase. A second susceptible group are pregnant women and their unborn children (6, 10). Although pregnant women seldom have significant illness themselves, Lm breaches the placental barrier and causes severe infections in the fetus, with abortion, stillbirth or neonatal sepsis/meningitis as possible outcomes. Therefore, listeriosis may cause severe illness across the full span of human life, from the unborn to the elderly.

**Life cycle of *Listeria monocytogenes***

Aside from its clinical significance, Lm has been of great importance to the scientific community as a model organism for the study of intracellular pathogenesis. Accordingly, its life cycle and virulence factors are extensively described (11, 12). Lm readily enters non-
professional phagocytes through a family of cell surface proteins called internalins. For example, the best characterized internalin, internalin A (InIA), binds E-cadherin and triggers cytoskeletal remodeling to promote bacterial internalization (13). As E-cadherin is a junctional protein expressed by epithelial cells, InIA allows Lm to penetrate the intestinal epithelial barrier. Curiously, murine E-cadherin does not act as a receptor for InIA (14). This explains the poor infectivity of Lm by gastric lavage in mice. In line with this, transgenic mice expressing human E-cadherin are more susceptible to intragastric infection than WT mice, and mutant Lm expressing a modified InIA that binds murine E-cadherin are 1000-fold more capable of infecting mice through the intragastric route (15, 16). Similarly, internalin B promotes internalization through its recognition of the host receptor tyrosine kinase Met (17). Once inside the cell, Lm secretes several virulence factors to lyse the phagosome. Of primary importance is the pore-forming molecule listeriolysin O (LLO) (18). LLO-deficient Lm strains are avirulent and cannot leave the phagosome. Lm also secretes phospholipases that, together with LLO, release bacteria into the nutrient-rich cytosol (11, 12). Once in the cytosol, Lm hijacks host actin filaments to move about the cell. This is achieved through the virulence factor ActA (19). By polymerizing actin, ActA propels Lm through the cell and ultimately allows its intercellular migration through protrusions of the host cell membrane into neighboring cells. Taken together, these factors make Lm an extremely efficient pathogen by allowing it to live within the cell and evade immune recognition.
Figure 1. Life cycle and virulence factors of *Listeria monocytogenes*. A) Lm initially enters the host cell through phagocytosis. To access the intracellular compartment of non-phagocytic cells such as those of the intestinal epithelium, Lm binds E-cadherin through internalin A (InIA) and/or Met through internalin B (InIB). This binding triggers the uptake of Lm into a phagosome. B) Once inside the phagosome, Lm secretes the pore-forming toxin listeriolysin O (LLO) and phospholipase C (PLC). LLO and PLC lyse the phagosomal membrane, releasing Lm into the cytosol where it replicates freely. C) Lm exploits the host cell’s actin cytoskeleton through the virulence factor ActA. ActA polymerizes actin monomers to propel Lm through the cytoplasm. This propulsion ultimately allows for its intercellular spread via membrane protrusions.
Host response to *Listeria monocytogenes*

Considerable work has also gone into the characterization of the host response to Lm. Much of it has focused on the adaptive immune response. A T cell response involving both CD4+ and CD8+ T cells is required for sterilizing immunity during a primary infection and for secondary responses (20, 21). In contrast, humoral immunity does not appear to make a significant contribution (22, 23). This likely reflects the bacterium’s capacity for intercellular spread without causing cell lysis. CD4+ T cells confer protection through the secretion of IFN-γ, an essential cytokine that increases the bactericidal capabilities of macrophages (21, 24). Similarly, CD8+ T cells have bactericidal activity through some combination of cytokine production and cytolytic activity (24).

While adaptive immunity is required for total clearance of Lm from the organism, a potent innate immune response must precede it to provide bacterial containment and activate lymphocytes. In fact, the earliest response occurs within minutes of its injection into the bloodstream. Tissue macrophages rapidly sterilize the blood by phagocytosing the circulating bacteria (25). Kupffer cells, the tissue macrophages of the liver, play a major role in this process, and indeed the vast majority of Lm is sequestered in this organ (26). Neutrophils also have an important early function as they quickly infiltrate the liver and participate in bacterial clearance (26-29). In contrast, neutrophils are dispensable for bacterial control in the spleen (28, 29). Cells of monocyte/macrophage lineage are paramount as their depletion or defective mobilization result in profound failure to clear the Lm from either organ (30-32). Furthermore, many acute inflammatory cytokines contribute to the early host response to Lm. Studies with neutralizing antibodies and knockout mice have revealed essential roles for TNF-α, IL-6, IL-12 and the IL-1 family (33-39). In addition
to their ability to mobilize and activate neutrophils, monocytes and macrophages, these cytokines also drive the expression of IFN-γ by NK cells, providing an early innate source of the critical macrophage activating cytokine (40, 41).

Although the overall direction of the innate immune response is protective during Lm infections, certain elements have detrimental effects. The anti-inflammatory cytokine IL-10 acts broadly to curtail inflammatory responses and thereby limit immunopathology (42, 43). Therefore, IL-10 is a double edge sword during infection – on one hand it can limit immune injury, but on the other hand it can dampen the immune response to pathogens. Examples of infectious models that fall on each side of the blade are plentiful (43). In listeriosis models, however, the effects of IL-10 are largely detrimental (44-46). Similarly, there is also ample evidence that the type I IFNs are harmful during systemic Lm infections (47-49). Mice deficient in the type I IFN receptor IFNAR1 or the type I IFN-inducing transcription factor IRF3 are highly protected against Lm. Type I IFNs are thought to sensitize lymphocytes and myeloid cells to cell death (50, 51). Numerous TUNEL+ lymphocytes are observed in the spleen between 48 to 72 hours post-infection (hpi) in WT mice whereas few are seen in IFNAR1/- and IRF3/- mice (47, 48, 50, 52). While leukocyte depletion is harmful in its own right, apoptotic lymphocytes trigger IL-10 expression in splenic macrophages and dendritic cells (50). Thus, type I IFNs set in motion a deleterious chain of events that inhibit the innate and adaptive responses to Lm.
The Complement System

A major element of innate immunity is the complement system. Complement consists of a large collection of secreted proteins that sequentially cleave one another to yield multiple effector molecules. This cascade of protein cleavage is triggered by pathogens via one of three pathways (53). While the pathways differ in their initiating factors and the molecules employed, all converge with the formation of a C3 convertase, a complex of complement fragments that activate C3, the cornerstone of the complement cascade. The first pathway identified, the classical pathway, is initiated by the C1 complex, a complex of C1q, C1r, and C1s. The pathway begins when C1q binds to antibody-antigen immune complexes and subsequently undergoes a conformational change. This conformational change triggers the autocatalytic activation of C1r, which in turn cleaves and activates C1s. Finally, C1s cleaves C4 and C2, forming the classical C3 convertase, C4bC2a. In the related lectin pathway, C1q is replaced by mannose-binding lectin (MBL) (55). MBL binds mannose moieties on bacterial surfaces and subsequently activates two MBL-associated serine proteases, MASP-1 and MASP-2. The complex of these molecules resembles the C1 complex and can similarly cleave C4 and C2 to generate C3 convertases. The last pathway of complement activation is called the alternative pathway (56). The alternative pathway is unique in that it does not require a recognition factor like C1q or MBL. Rather, the pathway begins with the spontaneous hydrolysis of C3. In aqueous environments, a low level of C3 hydrolysis occurs constantly. Hydrolyzed C3 interacts with factor B and triggers the latter’s cleavage by factor D to form a fluid phase C3 convertase, C3(H2O)Bb. Under homeostatic conditions, these convertases cleave only a small amount of C3, and the resulting fragments, C3a and C3b, are rapidly inactivated. However, C3b can attach to bacterial surfaces and
interact with factor B and properdin to form a stable alternative C3 convertase, C3bBb. The alternative pathway also acts as an amplification loop. C3b produced by any pathway can associate with factor B and properdin to form alternative C3 convertases. In fact, the majority of complement activation \textit{in vivo} is thought to result from this amplification (56). Finally, C3 convertases from either the classical or alternative pathways can interact with an additional C3b molecule to form C5 convertases (C4bC2aC3b or C3bBbC3b, respectively) that split C5 into C5a and C5b. C5b then concludes the complement cascade through its association with C6, C7, C8 and C9 to form the so-called membrane attack complex (MAC).
Figure 2. Overview of the Complement System. Bacteria like Lm can activate the complement system in one of three ways. In the classical pathway, antibodies (Ab) bind to the bacterial surface and undergo a conformational change allowing C1q to bind their Fc region. The activated C1 complex then cleaves C4 and C2 to generate the classical C3 convertase, C4bC2a. In the lectin pathway, mannose binding lectin (MBL) binds mannose residues on the surface of Lm. MBL can then form a complex with MASP-1 and MASP-2 that cleaves C4 and C2 similar to the C1 complex. Finally, in the alternative pathway, the spontaneous hydrolysis of C3 leads to the formation of a fluid phase C3 convertase.
C3(H2O)Bb that can cleave C3. C3b then deposits on the surface of Lm and binds factor B (fB) to form the alternative pathway C3 convertase, C3bBb. This convertase allows for the complement amplification loop. Ultimately, all pathways converge on C3 and result in the generation of three types of complement effector molecules: C3b, the anaphylatoxins C3a and C5a, and the membrane attack complex (C5b-9).
Complement activation thus results in the generation of many cleavage fragments. Of these, the major effector molecules are C3b, the MAC (C5b-9), and C3a and C5a, the complement anaphylatoxins. In addition to its role in C3 & C5 convertases, the C3b fragment is also an important opsonizing agent. Complement activation on bacterial surfaces leads to a coating of C3b. C3b is recognized by many complement receptors, including CR1 (CD35), CR3 (CD11b-CD18), and CR4 (CD11c-CD18) (57). C3b-complement receptor binding triggers the phagocytosis by cells of macrophage/monocyte lineage and neutrophils. Therefore, C3b plays an important role in the recognition and clearance of bacteria from the bloodstream and tissues. The MAC is also an essential component of host defense against bacteria. Upon inserting into the bacterial membrane, the MAC forms a transmembrane pore that causes bacterial lysis (58). Accordingly, complement deficiencies in man involving the terminal complement components C5-C9 are associated with increased susceptibility to infection, especially by the encapsulated bacteria Neisseria meningitides (59). However, Gram positive bacteria like Lm are resistant to MAC-mediated killing due to their thick protective layer of peptidoglycan (60).

The final class of complement effector molecules are the complement anaphylatoxins. First identified in 1914 by Friedberger as the product of the reaction between serum and immune complexes that induces anaphylactic shock, it is now appreciated that the anaphylactic activity of activated complement resides in C3a and C5a (61, 62). C3a and C5a act through two G-protein coupled receptors, the C3a receptor (C3aR) and the C5a receptor (C5aR), respectively (62). There is also a second C5a receptor, C5L2, which lacks an intracellular signaling domain and is thought to act as a decoy receptor (63). As anaphylaxis is an undesirable state, anaphylatoxin activity is tightly regulated. This is
achieved in the serum by carboxypeptidase N (CPN) and carboxypeptidase R (CPR) (65, 66). CPN & CPR are basic carboxypeptidases that cleave C-terminal arginine and lysine residues from peptides. This makes them powerful regulators of the anaphylatoxins as both have C-terminal arginine residues that are essential for receptor binding. The desarginated form of C3a has little to no biological activity, whereas C5a-desArg is usually described as retaining ~1-10% activity (66). However, recent studies suggest that C5a-desArg may retain nearly full activity in some cell types (66, 67).

Despite their name, C3a and C5a have a broad range of biological activities outside of anaphylaxis. This is reflected in the wide tissue and cellular distribution of their receptors. Their greatest expression is seen in cells of the myeloid lineage – monocyte, macrophages, neutrophils, mast cells, basophils, eosinophils and dendritic cells (DC) (68, 69). In the last two decades, their distribution has been significantly expanded to include many non-immune cells (70-73). C3aR and C5aR have also been identified on cells of the lymphoid lineage such as T cells, B cells and NK cells (74-77). In many of these cells, the anaphylatoxins have chemotactic activity. In particular, C5a has long been recognized as one of the strongest chemotactic agents known for neutrophils (78). Outside of chemotaxis, the anaphylatoxins also drive immune cell activation. For example, C5a primes the respiratory burst and triggers degranulation in neutrophils (79, 80). Numerous studies have also established that C5a potentiates the expression of pro-inflammatory cytokines like TNF-α, IL-6 and IL-1 in myeloid cells (81-84). Thus, the anaphylatoxins are often characterized as pro-inflammatory molecules due to their ability to recruit leukocytes, trigger immune cell activation, and enhance inflammatory cytokine production.
However, there is also evidence that the anaphylatoxins can have regulatory effects during the immune response. For some time it has been appreciated that C5a is a potent suppressor of IL-12 in monocytes and macrophages (85-87). This is surprising as IL-12 is critical for the development of cell-mediated immune responses through its role in the Th1 cell differentiation. Supporting these in vitro observations, C5aR-/ mice more effectively control the parasite Leishmania major and have a greater Th1 response than WT mice (87).

Thus, C5a holds IL-12 expression in check and thereby limits Th1 responses to pathogens, presumably in an attempt to prevent immunopathology. Outside of this, C5a can also heighten the expression of the anti-inflammatory cytokine IL-10 in LPS-treated macrophages in vitro and during endotoxemia in vivo (88). IL-10 in turn represses the expression of IL-17A, an important mobilizer of neutrophils during bacterial infections. Therefore, the anaphylatoxins cannot be categorized simply as pro-inflammatory or anti-inflammatory molecules. C3a and C5a are perhaps best described as immune modulating agents (89).

**Complement and Listeria monocytogenes**

There has long been interest in the role of complement in listeriosis. The extracellular distribution of complement has led investigators to question whether complement participates in the host response to intracellular bacteria. Furthermore, since intracellular bacteria actively seek phagocytosis, complement might even be detrimental to the host by facilitating phagocytosis through opsonization. As early as 1974 it was found that human serum enhances the killing of Lm by human monocytes (90). This enhancement was almost completely lost with the heat inactivation of serum. Since complement activity is
heat labile, this provided the first indication of an anti-listerial function for complement. Subsequently, work by Baker et al. definitively established that the cell wall of Lm activates complement (91). Incubation of the cell wall fraction with animal serum generated a chemotactic factor that induced neutrophil migration. Complement fixation was apparent as serum incubated with the cell wall fraction had reduced hemolytic activity. Furthermore, the molecular weight of the chemotactic factor was shown to be approximately 15 kDa, a value consistent with reported molecular weight of C5a. It was further demonstrated that this complement activation occurred through the alternative pathway. The production of the chemotactic factor was sensitive to EDTA, an inhibitor of both the classical and alternative pathway, but not EGTA, an inhibitor of the classical pathway alone. Curiously, protease treatment of the cell wall fraction had no effect on its ability to cause complement activation, suggesting that a non-protein component like peptidoglycan might be responsible. This study thus provided the first clear evidence that Lm activates complement and suggested that the resulting complement anaphylatoxins might participate in the host response.

I. Complement opsonizes *Listeria monocytogenes*

When bacteria enter the bloodstream they are rapidly opsonized by serum factors such as immunoglobulins and complement. In rodent models, Lm is removed from the circulation at an extremely rapid rate by Kupffer cells (KCs) in the liver (26). Presumably, opsonization drives this early clearance through the promotion of phagocytosis. Many groups have attempted to determine the relative importance of immunoglobulin and complement in this process with mixed results. Consistent with the work of Baker et al.,
several groups have determined that the opsonization of Lm occurs largely through complement activation via the alternative pathway in mouse serum (92, 93). In man, however, contrasting results were reported. For example, Croize et al. found that C3 deposits on the surface of Lm in normal human serum through alternative pathway activation (94). On the other hand, two other groups observed that the IgG fraction of human serum drives opsonization and that complement is dispensable as heat inactivation had no effect in their assays (95, 96). Finally, Bortolussi et al. found a role for both immunoglobulin and complement in Lm opsonization by human serum (97). In their experiments optimal opsonization required both the heat labile activity and the IgM (but not IgG) fraction of serum. In line with this, zymosan-treated serum lacking alternative pathway activity could opsonize Lm, whereas C4 inactivator-treated serum lacking classical pathway activity could not. It is noteworthy that in these studies low concentrations of serum (3%) were used in an attempt to mimic conditions found at sites of infection rather than in the circulation. When Bortolussi et al. tested higher concentrations of serum (10%) in their assays, heat inactivation did not eliminate opsonization. The reason for the discrepancies between these studies is not entirely clear. As this variation arose in human studies in which serum was prepared from multiple donors and in different countries, the differences may reflect the exposure status of the serum donors. In contrast, Lm exposure may be uncommon in laboratory animals, resulting in a lack of Lm-specific immunoglobulin and thus a complete complement dependence. Regardless, it is clear that complement can opsonize Lm.

II. **CR3 and Listeria monocytogenes**
Multiple complement receptors (CR1, CR3, CR4) bind to C3b-coated bacteria and trigger their phagocytosis (57). Early work from Rosen et al. revealed a critical role for CR3 in the host response to listeriosis in vivo (98). Mice treated with an anti-CR3 monoclonal antibody (mAb) 24 hpi with Lm had massively elevated bacterial proliferation in the liver and spleen and complete mortality at an infectious dose that otherwise caused a modest sublethal infection in untreated animals. Careful histological analysis demonstrated a failure of neutrophils and monocytes to organize around infective foci in the liver. The work also ruled out a role for CR3 in early bloodstream sterilization. CR3 blockade had no effect on the kinetics of bacterial clearance from the blood, indicating that KCs do not utilize CR3 to phagocytose Lm. However, it must be recognized that as an integrin receptor, CR3 also has complement-independent functions. Multiple ligands for CR3 outside of the complement system have been identified, including ICAM-1 and extracellular matrix proteins (99). Thus, it is unclear what extent of this phenotype is complement-dependent.

Subsequent studies from Drevets et al. established that CR3 contributes to listerial phagocytosis and killing by macrophages in vitro (93, 100, 101). Incubation with serum was found to greatly enhance the phagocytosis of Lm by macrophages (93). When macrophages were treated with an anti-CR3 monoclonal antibody, the effect of serum treatment was lost. Neutralization of C3 similarly reduced phagocytosis, demonstrating that C3b-CR3 interactions were responsible for the enhanced phagocytosis. It was later found that listericidal macrophages such as protease peptone-elicited macrophages utilize CR3 for bacterial phagocytosis to a greater extent than non-listericidal macrophages such as those elicited by thioglycollate (100). Furthermore, listericidal macrophages restricted Lm to the phagosome, whereas in non-listericidal thioglycollate-elicited macrophages, Lm lysed
phagosomes and spread throughout the cytoplasm. These observations lead to the hypothesis that CR3 promotes listerial killing via phagosomal containment. To test this hypothesis, peptone-elicited macrophages were treated with anti-CR3 mAbs, and their ability to kill Lm was assessed (101). As in the case of phagocytosis, CR3 blockade dose-dependently inhibited bacterial killing. In fact, at high doses peptone-elicited macrophages were converted into permissive hosts. However, while blocking CR3 diminished listerial killing, it did not result in bacterial escape from the phagosome. Thus, complement and CR3 promote the phagocytosis and killing of Lm in murine macrophages through an undetermined mechanism independent of phagosomal containment.

III. CRIg and *Listeria monocytogenes*

As mentioned previously, Lm is rapidly cleared from the bloodstream by KCs in the liver following intravenous injection. Since complement opsonizes Lm through alternative pathway activation on the bacterial surface, its participation in this process seemed likely. However, KCs express little CR3, and blocking experiments indicated a lack of CR3 involvement (98, 102). This discrepancy was resolved in 2006 with the discovery of a fifth complement C3b receptor, the Complement Receptor of the Immunoglobulin superfamily (CRIg) (103). The expression of CRIg is highly restricted, being found only in tissue macrophages such as KCs and a subset of peritoneal macrophages. To examine the contribution of CRIg to early listerial clearance, KCs were isolated from WT and CRIg-/- mice one hour after i.v. infection. KCs from CRIg-/- mice contained substantially less Lm than WT KCs, demonstrating a role for CRIg in the clearance of opsonized Lm from the blood. This reduced KC uptake resulted in a redistribution of Lm within CRIg-/- mice, with
less Lm depositing in the liver and more in the spleen and lungs as early as 10 minutes post-injection in comparison with WT mice. CRIg-/- mice were also more susceptible to Lm-induced mortality. Thus, CRIg is at least partially responsible for the early clearance of Lm from the blood. In its absence, Lm is re-directed from the liver, a site of rapid early bacterial clearance via recruited neutrophils, to more permissive sites of infection like the spleen and lung. In addition, CRIg also contributes directly to the listericidal activity of macrophages. Kim et al. recently reported that treatment of macrophages with either an agonistic anti-CRIg antibody or C3b dimers enhances their ability to kill Lm (104). This CRIg-mediated induction of bactericidal activity was linked to elevated phagosome-lysosome fusion. CRIg associates with CLIC3, a chloride ion channel, which increases the chloride ion concentration and acidification of phagosomes and is required for CRIg-mediated killing. Therefore, at least two complement C3 receptors, CR3 and CRIg, contribute to listerial killing by macrophages.

IV. Complement and the adaptive response to Listeria monocytogenes

For many years, immunologists regarded innate and adaptive immunity as non-overlapping spheres. The involvement of innate immunity in the early containment of infections was recognized, but the adaptive response, a separate entity, was responsible for complete bacterial clearance. However, it is now widely appreciated that the two systems are fundamentally linked (105). The innate response both initiates and shapes the direction of adaptive immunity. As a key component of innate immunity, complement is thought to participate in the adaptive T cell response to pathogens (106). Early studies of complement in viral infections demonstrated a requisite role for C3 and/or C5aR in T cell activation in
response to both influenza virus and lymphocytic choriomeningitis virus (107-109).

Complement regulates adaptive immunity through its actions on both antigen presenting cells (APCs) and T cells. APCs activate T cells via the engagement of antigen-loaded MHC molecules with antigen-specific T cell receptors, co-stimulatory molecules, and T cell polarizing cytokines like IL-12. Upon exposure to antigen-specific T cells or TLR agonists, APCs mature and develop increased T cell stimulatory capacity. Consistent with a role for complement in this process, C3, C3aR, and C5aR-deficient APCs express lower levels of class II MHC and the co-stimulatory molecules CD80 and CD86 than WT APCs and have reduced T cell stimulatory capability (110-114). More remarkably, a requirement for T cell expression of C3, C5, C3aR and C5aR has also been reported (115, 116). Resting T cells express low levels of complement and complement receptors. Upon T cell activation through antigen presentation or CD3/CD28 ligation, a substantial induction of C3, C3aR and C5aR occurs (115). Like APCs, T cells deficient in C3aR and C5aR produce lower levels of the co-stimulatory molecules CD28 and CD40L following T cell activation (115).

Furthermore, C3aR and C5aR mediate the activation of Akt in response to CD28 ligation, an integral component of Th1 differentiation (115). As a consequence, the proliferation of purified C3aR-/- C5aR-/- T cells following CD3/CD28 ligation is severely impaired. C5aR-mediated activation of Akt also has a pro-survival role as it limits the induction of apoptosis in response to T cell activation (116). This was linked to the up-regulation of the anti-apoptotic molecule Bcl-2 and down-regulation of the pro-apoptotic receptor Fas (116). Thus, complement acts directly on APCs and T cells at multiple levels to promote T cell immunity.
To date, two studies have demonstrated a requirement for C3 in T cell activation during listeriosis. In the first study, Nakayama et al. discovered that C3-/- mice develop less antigen-specific CD4+ and CD8+ T cells than WT mice following Lm infection (117). Curiously, while C5aR contributes to T cell activation during viral infection and in other models, neither C5aR antagonism nor deficiency impacted T cell activation in listeriosis. To investigate the mechanism by which C3 contributes to T cell activation, the phenotype of C3-/- DCs was characterized. Again in contrast to prior work, Nakayama et al. found the maturation state of Lm-infected C3-/- DCs was comparable to WT DCs. In line with this, C3 deficiency did not impede the ability of DCs to activate CD8+ T cells in vitro. Nakayama et al. subsequently examined whether C3 directly regulates T cell proliferation in listeriosis. Adoptive transfer experiments in which C3-/- CD8+ T cells were introduced into Lm-infected WT mice revealed comparable levels of T cell activation in donor (C3-/-) and recipient (C3+/+) cells. As transfer into a C3 sufficient environment allows C3-/- T cells to activate normally it would appear that they do not have an intrinsic defect in proliferation. However, when purified CD8+ T cells were stimulated with plate-bound anti-CD3, less proliferation was observed in C3-/- CD8+ T cells than C3+/+ cells. Altogether, these results argue that C3 is required at the level of the T cell itself for optimal T cell activation during listeriosis.

Subsequent work by Verschoor et al. confirmed the requirement of C3 for optimal CD8+ T cell responses during listeriosis (118). However, their studies unearthed a substantially different mechanism. Whereas Nakayama et al. postulated that C3 acts directly on T cells to promote their activation during listeriosis, Verschoor et al. found that C3 drives T cell responses by targeting Lm to APCs in the spleen. In order for a productive Lm
infection to develop within the spleen, CD8α+ DCs must be present in the red pulp (119, 120). Shortly after i.v. injection, Lm is detected almost exclusively within these cells. The importance of complement in pathogen clearance from the circulation in other models led Verschoor et al. to examine how C3 contributes to this phenomenon. Consistent with a role for C3 in this process, C3 was required for optimal colonization of the spleen by Lm. C3-/- CD8α+ DCs were found to contain far less Lm than C3+/+ CD8α+ DCs at 1 hpi.

Remarkably, the targeting of Lm to CD8α+ DCs by C3 is dependent on platelet-Lm aggregation. Platelets bind circulating Lm through a newly identified C3b receptor, the glycoprotein GPIb. Like C3 deficiency, depletion of platelets or GPIb deficiency impaired the delivery of Lm to CD8α+ DCs. Among the various DC subsets, CD8α+ DCs are uniquely capable of cross-presenting exogenous antigens through class I MHC to CD8+ T cells. In keeping with this, the impaired uptake of Lm by CD8α+ DCs in these mice resulted in reduced CD8+ T cell expansion. Thus, C3 drives the development of an adaptive antilisterial response in vivo by diverting a small portion of Lm away from the macrophages of reticuloendothelial system to splenic CD8α+ DCs. This model therefore diverges significantly from that of Nakayama et al. and others in which a direct role for complement in T cell activation has been proposed. Regardless, C3 clearly contributes to the development of T cell immunity during listeriosis.

V. Role of the complement anaphylatoxin receptors in listeriosis

A considerable body of research now stresses the importance of C3 and complement-mediated opsonization in host defense against Lm. On the other hand, the role of the complement anaphylatoxins has been scarcely examined. To fill this void, our laboratory
recently examined the role of C3aR in a mouse model of systemic Lm infection. Very few studies have assessed the importance of C3aR during infection. Surprisingly, C3aR deficiency resulted in enhanced bacterial clearance in a mouse model of pulmonary Pseudomonas aeruginosa infection (121). In contrast, the work of Mueller-Ortiz et al. now establishes a protective role for C3aR during listeriosis (122). C3aR-/- mice fare significantly worse than WT mice following Lm infection, with reduced survival, increased bacterial burden and tissue injury. A broad analysis of serum cytokine and chemokine levels showed that C3aR does not contribute to the production of protective cytokines or chemokines during listeriosis. As discussed earlier, splenocyte apoptosis is a major feature of Lm infection and is thought to negatively regulate bacterial clearance through the induction of IL-10 expression. Since T cell-expressed C3aR reportedly promotes T cell survival under homeostatic conditions and during T cell activation, it was reasoned that C3aR might protect against Lm-induced splenocyte death (115, 116). In accordance with this, more splenocyte apoptosis was detected in Lm-infected C3aR-/- mice than infected WT mice. Furthermore, total splenocyte numbers were reduced in infected C3aR-/- mice as well. This splenocyte depletion was not restricted to T cells, as all splenocytes subsets were affected. The enhanced susceptibility of infected C3aR-/- splenocytes to cell death associated with lower levels of the anti-apoptotic molecule Bcl-2 and higher levels of the pro-apoptotic receptor Fas than in infected WT splenocytes. Thus, C3aR shifts the overall cellular status of splenocytes to a more anti-apoptotic state during listeriosis. In line with the accepted model, the increased splenocyte apoptosis in C3aR-/- mice was linked with higher serum levels of IL-10. To test whether the elevated apoptosis seen in C3aR-/- mice was responsible for the phenotype, C3aR-/- mice were pre-treated with the caspase inhibitor Z-
VAD, an inhibitor of apoptosis, before infection. In support of the hypothesis, Z-VAD reduced bacterial burden in the liver, splenocyte apoptosis and IL-10 to levels comparable to WT mice. Thus, C3aR protects the host against listeriosis by inhibiting splenocyte death. A similar phenotype was recently described in C3aR/- mice infected with the intracellular bacterium *Chlamydia psittaci* (Cp) (123). C3aR/- mice are more susceptible to pulmonary Cp infections and have significantly less T cells and B cells in their draining lymph nodes than WT mice. It is unclear whether this reduction is a consequence of elevated cell death or reduced proliferation. However, it is tempting to speculate that these data taken together may demonstrate a broad pro-survival role for C3aR in leukocytes during intracellular bacterial infections.

While C3aR clearly participates in the host response to Lm, the role of C5aR in listeriosis remains uncertain. Since C5a can repress IL-12 expression and inhibit the Th1 response to the intracellular parasite *Leishmania major*, C5aR might be detrimental in listeriosis (67, 85-87). However, there is also reason to suspect a protective function for C5aR. Several decades ago it was found that A/J mice, a mouse strain naturally deficient in C5, are more susceptible to Lm (124, 125). Of the two C5 fragments, the loss of C5b is an unlikely candidate for this susceptibility due to the resistance of Gram positive bacteria to MAC-mediated lysis (60). Thus, it seems probable that the loss of C5a is responsible for the increased susceptibility of A/J mice. These studies therefore sought to determine the function of C5aR in host defense against Lm by subjecting WT and C5aR/- mice to a model of systemic Lm infection.
CHAPTER ONE

The C5 Anaphylatoxin Receptor (C5aR) provides protection in a mouse model of *Listeria monocytogenes* infection
INTRODUCTION

The CDC estimates that *Listeria monocytogenes*, the causative agent of the foodborne illness listeriosis, is responsible for approximately 1600 hospitalizations and 260 deaths annually in the United States (9). Accordingly, Lm is the third leading cause of death from food poisoning and a major public health problem (7, 8, 10). Invasive listeriosis generally occurs in the elderly, patients suffering from chronic illness, and the immune compromised (10). This illustrates the importance of the immune response in host defense against Lm, as all three groups suffer from immune defects. While it is known that many components of innate and adaptive immunity are essential in this process, it is unclear what role the complement anaphylatoxin C5a and its receptor C5aR play. Studies in the 1980s demonstrated that mice with a natural deficiency in C5 such as the A/J strain are more susceptible to intravenous or intraperitoneal infection with Lm than C5 sufficient strains (124, 125). More recently, this increased susceptibility was extended to intragastric inoculation, the natural route of Lm infection (126). Of the two C5 cleavage fragments, C5a and C5b, only the absence of C5a is likely to contribute to impaired resistance in C5 deficient mice. The membrane attack complex, a lytic pore assembled by C5b on bacterial surfaces, cannot penetrate the thick, peptidoglycan-rich cell wall of Gram positive bacteria like Lm (60). However, it must be recognized that while C5 deficiency is a major feature of the A/J strain, other genetic differences exist between it and C5 sufficient mouse strains like C57BL/6. For example, while A/J mice are more susceptible to *Staphylococcus aureus* than C57BL/6 mice, this susceptibility was linked to polymorphisms outside of the *Hc* (C5) locus (127). Thus, the listeriosis phenotype described in A/J mice could be independent of C5
deficiency. Alternatively, polymorphisms outside of the Hc locus could obscure a detrimental contribution by C5.

C5aR is widely assumed to have anti-microbial functions. Indeed, the first phenotype described in C5aR/- mice was an enhanced susceptibility to pulmonary infections with *Pseudomonas aeruginosa* (128). Furthermore, many bacteria produce virulence factors that either block the production of C5a or antagonize C5aR (129). *Serratia marcescens*, a cause of hospital acquired infections, and many *Streptococcus* spp. produce proteases and peptidases that degrade C5a (130, 131). *Staphylococcus* spp. have two unique virulence factors that target C5a. CHIPS, the Chemotaxis inhibitory protein of *S. aureus*, binds to phagocytes and specifically blocks neutrophil chemotaxis towards C5a and formylated peptides (132, 133). They also produce SSL-7, Staphylococcal superantigen-like protein 7, which binds C5 and prevents its cleavage (134). Finally, it was recently reported that the Vi capsular polysaccharide of *Salmonella typhi* inhibits C3 activation and thereby prevents the development of a C5a-dependent neutrophil chemotactic response towards the bacteria (135). The evolution of virulence factors to combat C5a illustrates the importance of C5a in the host response to bacteria.

However, multiple infectious disease models have unveiled detrimental roles for the receptor in host defense. These studies take two forms. On one hand, C5a can drive the development of excessive inflammation in the host, resulting in immunopathology and a failure of clearance. The first example of this was in sepsis. In the cecal ligation and puncture (CLP) model, the cecum is punctured, releasing its bacteria-rich contents into the peritoneum (136). This results in excessive inflammation that triggers the development of a sepsis-like disease in rodents. Early neutralization of C5a dampens the inflammatory
response and thereby rescues rodents from CLP-induced mortality (137). Additionally, bacterial counts in the spleen and liver are significantly reduced as well. Excessive inflammation is believed to cause immune paralysis in phagocytes. C5a can directly mediate immune paralysis in neutrophils, as high levels of C5a inhibit the respiratory burst and production of TNF-α in response to LPS (138, 139). Thus, C5a contributes to the development of an excessive inflammatory response that inhibits bacterial clearance. Similar findings were recently reported in a mouse model of cerebral malaria (140).

On the other hand, C5a can limit bacterial clearance by exerting regulatory activity. C5a suppresses the release of the IL-12 family of cytokines by monocytes and macrophages in response to bacterial products (67, 85-87). As IL-12 is a key driver of Th1 polarization, this activity likely limits the development of cell-mediated immunity, a critical component of the host response to intracellular pathogens. Indeed, C5aR-/- mice have a more robust Th1 response to the intracellular parasite *Leishmania major* (87). The increase in activated CD4+ and CD8+ T cells seen in C5aR-/- mice correlated with enhanced clearance of the parasite from the inoculation site. More recently, the gingival pathogen *Porphyromonas gingivalis* (Pg) was shown to actively exploit the regulatory activity of C5a for immune evasion (141). Pg secretes gingipains, cysteine proteases that cleave C5 to generate C5a. This activity was long considered paradoxical because of C5a’s assumed anti-microbial function. However, the resulting cross-talk between C5aR and TLR2 inhibits not only IL-12 expression but also the production of nitric oxide, a potent anti-microbial factor (67, 141). Accordingly, treatment of macrophages with C5a reduces their ability to kill Pg. As a Th1 response is critical for the host response to Lm, this regulatory function of C5a/C5aR could be detrimental in listeriosis. Therefore, in these studies we set out to determine the role of
C5aR in listeriosis by putting WT and C5aR/− mice through an intravenous Lm infection model.
MATERIALS AND METHODS

Mice

The C5aR-/ mice used for these studies have been previously described (142). They were backcrossed for over ten generations onto the C57BL/6 background. Age-matched C57BL/6 mice from our colony served as WT controls. All mice were housed in HEPA-filtered Techniplast cages in a barrier facility. Male mice between 11 to 14 weeks of age were used in these studies. All mouse protocols followed institutional guidelines for animal care and welfare.

Bacterial infection

Listeria monocytogenes ATCC strain 13932 (MicroBioLogics, Inc.), a clinical isolate, was used for all studies. Bacteria were cultured in Bacto brain heart infusion (BHI) broth at 37°C to mid-logarithmic phase, pelleted by centrifugation, washed with PBS, and resuspended in PBS. Mice were infected i.v. with 1 X 10^5 bacteria in 100 µl PBS. Control mice received 100 µl PBS. The number of bacteria present in the inoculum was verified by culturing serial dilutions of the inoculum on Bacto BHI agar plates.

Survival study

Mice were infected i.v. with 5 X 10^4 Lm and were observed every 6 hours. Mice that showed signs of severe morbidity were euthanized. Survival curves were generated using GraphPad Prism software, and statistical significance was assessed using the Logrank test.
Bacterial burden in the liver and spleen

Following exsanguination from the inferior vena cava, the liver and spleen were dissected from mice either 24 hpi or 72 hpi, rinsed in PBS and then placed in 2 ml HBSS. Organs were homogenized using a PRO200 homogenizer (ProScientific) on medium speed and were then placed on ice. Serial dilutions were plated on BHI agar plates to determine bacterial numbers per organ. Data are expressed as mean CFU per organ ± SEM.

Spleen histology

The whole spleen was dissected at 72 hpi, rinsed in PBS and fixed in 10% neutral buffered formalin for at least 24 h at 4°C. Organs were dehydrated, embedded in paraffin, cut into 5-μm sections and stained with either hematoxylin & eosin or the DeadEnd™ Colorimetric TUNEL System (Promega) for identification of apoptotic cells. Brightfield images were taken using Spot Advanced software and a Zeiss Axioskop microscope (Carl Zeiss) equipped with a SPOT-RT digital camera (Diagnostic Instruments). For spleen histology a 20X objective was used for a final magnification of 200x.

Measurement of caspase 3 activity

Caspase 3 activity was measured in spleen homogenates using the CaspACE Assay System (Promega). Briefly, dissected spleens were cut in half. One half was used to enumerate the number of cells in the spleen, while the other half was homogenized as described above.
After clearing the homogenate by centrifugation, caspase 3 activity was measured as per manufacturer’s instructions. The measured activity was normalized by the number of cells per spleen (per $10^7$ cells) and is reported as mean absorbance (A450) ± SEM.

**Immunophenotyping**

Spleens were dissociated into single cell suspensions using a GentleMACS Dissociator (Miltenyi Biotec). Suspensions were filtered successively through 100 and 40 µM filters. Erythrocytes were then lysed with ACK lysis buffer (Lonza). Total live cell numbers were determined by counting cells with a hemocytometer and trypan blue exclusion. Fc receptors were blocked by incubation with an anti-CD16/32 antibody (BD Pharmingen). Cells were subsequently stained with antibodies for CD4 (GK1.5), CD8 (53–6.7), CD19 (6D5), NK1.1 (PK136), CD11b (M1/70), CD11c (N418), Ly6G (1A8), Ly6C (HK1.4), and/or TRAIL (N2B2) (Biolegend). During the final wash step DAPI (Invitrogen) was added as a viability dye. A minimum of 50,000 events were collected on a FACSaria (BD Biosciences) flow cytometer. Data analysis was done using the Kaluza program (Beckman Coulter). Dead cells were excluded from the analysis by gating on DAPI negative cells. Data are expressed as mean cell number per organ ± SEM.

**Statistical analysis**

Statistical analysis was done with GraphPad Prism 5. All values are expressed as mean values with the SEM as error bars. For experiment involving two groups, data was analyzed via unpaired two-tailed t test. In experiment involving multiple groups one-way ANOVA
with the Tukey post-test was used to determine significance. Survival curves were analyzed by the log-rank (Mantel-Cox) test. P values < 0.05 were considered significant.
RESULTS

C5aR deficiency results in increased susceptibility to Lm

We began our assessment of the role of C5aR in host defense against Lm with a survival experiment. WT and C5aR/- mice were injected i.v. with $4 \times 10^4$ CFU of Lm and then followed for two weeks. At this dose no mortality was observed in WT mice (9 of 9 survived). In contrast, approximately 60% of C5aR/- mice succumbed to the infection within the first week (3 of 7 survived, $p = 0.0103$) (Fig. 3). To determine if C5aR contributes to the control of Lm we infected WT and C5aR/- mice and then harvested livers and spleens at 24 and 72 hpi. At 24 hpi a modest two-fold elevation of Lm was observed in the spleens of C5aR/- mice compared with WT mice ($p = 0.0276$) (Fig. 4A). No difference in bacterial burden was observed in the liver between the two genotypes at this time (Fig. 4A). The liver results argue against a role for C5aR in early neutrophil recruitment during listeriosis. Neutrophils infiltrate the liver within hours of Lm injection, and their depletion results log-fold increases in bacterial burden at 24 hpi (24-29). Accordingly, the lack of a difference in CFUs in the liver at 24 hpi suggests that C5a, a potent neutrophil chemoattractant, is either unneeded or redundant in this regard. Indeed, recent data suggests that the related formyl peptide receptors are responsible for early neutrophil recruitment (143). By 72 hpi a marked difference in CFUs was observed in both organs. C5aR/- mice had approximately 6-fold more bacteria in their spleens ($p < 0.0001$) and 26-fold more in their livers ($p = 0.0010$) than WT mice (Fig. 4B). This elevation of bacterial burden roughly coincides with the onset of mortality in C5aR/- mice. Therefore, C5aR is required for the containment and survival of Lm infection.
Figure 3. Survival of WT and C5aR-/- mice during Lm infection. WT and C5aR-/- mice were infected i.v. with $5 \times 10^4$ Lm and followed for two weeks. $n = 9$ for WT and $n = 7$ for C5aR-/- mice, $p = 0.0110$ by Log-rank test.
Figure 4. Bacterial burden of WT and C5aR-/- mice during Lm infection. WT and C5aR-/- mice were infected i.v. with $1 \times 10^5$ Lm, and at (A) 24 hpi and (B) 72 hpi spleens and livers were dissected, homogenized and CFU per organ was determined. The data is presented as mean CFU per organ ± SEM. $n = 10$ per group per time point, n.s. = not significant, * $p = 0.0276$, ** $p = 0.0010$, *** $p < 0.0001$ by t-test.
Greater spleen pathology in C5aR-/- mice

In order to better understand the phenotype, we next examined the histology of the spleen in infected WT and C5aR-/- mice. Lm initially enters the spleen through CD8α+ dendritic cells in the red pulp (119, 120). From there Lm migrates into the white pulp via dendritic cells and causes lymphocyte and myeloid cell depletion (47, 48, 52). By H&E staining, no obvious differences could be seen between the spleens of PBS-treated WT and C5aR-/- mice (Fig. 5). However, by 72 hpi the appearance of the genotypes was markedly different. In comparison with Lm-infected WT spleens, infected C5aR-/- spleens were strikingly hypocellular (Fig. 5). This was most apparent in the splenic follicles of C5aR-/- mice as they lacked the typical densely packed appearance still observable in WT mice.

The depletion of splenocytes during Lm infections is thought to result from cell death and is associated with the appearance of TUNEL+ cells between 48 to 72 hpi (47, 48, 52). We therefore did TUNEL staining to further characterize the differences in pathology between the genotypes. In PBS-treated WT and C5aR-/- mice, little to no TUNEL staining was seen (Fig. 6). Consistent with prior reports, Lm infection resulted in the appearance of TUNEL+ cells in WT mice at 72 hpi. In spleens of C5aR-/- mice, a dramatic increase in the amount of TUNEL staining was observed (Fig. 6). Thus, the TUNEL assay suggests that a greater degree of cell death occurs in spleens of Lm-infected C5aR-/- mice. We also examined caspase 3 activity in spleen homogenates from PBS treated and Lm-infected WT and C5aR-/- mice. Caspase 3 is the key executioner caspase that initiates apoptosis. Therefore, its activation is used as an indicator of apoptosis. PBS-treated animals had little caspase 3 activity (Fig. 7). Similar to the results of TUNEL staining, Lm-infected C5aR-/- mice had substantially more caspase 3 activity on a per cell basis than Lm-infected WT mice.
(p < 0.0001). Taken together, these results suggest that C5aR protects against spleen pathology during Lm infection by preventing splenocyte death.
Figure 5. Histological examination of the spleens of WT and C5aR-/− mice during Lm infection. WT and C5aR-/− mice were infected i.v. with PBS or 1 × 10⁵ Lm, and their spleens were removed at 72 hpi, formalin fixed and paraffin embedded. 5-µm sections were stained with hematoxylin & eosin and examined under a 20x objective for a total magnification of 200x. Images are representative of 4 mice per group.
Figure 6. TUNEL staining of spleens from WT and C5aR-/- mice during Lm infection. WT and C5aR-/- mice were infected i.v. with PBS or 1 X 10^5 Lm, and their spleens were removed at 72 hpi, formalin fixed and paraffin embedded. 5-μm sections were subjected to TUNEL staining and examined under a 20x objective for a total magnification of 200x. Images are representative of 4 mice per group.
Figure 7. Caspase 3 activity in spleen homogenates from WT and C5aR-/- mice during Lm infection. Spleen homogenates were prepared from PBS treated and Lm-infected WT and C5aR-/- mice at 72 hpi, and caspase 3 activity was measured. Data is presented as mean caspase 3 activity per 10^7 cells ± SEM. n = 4 per condition and genotype, *** p < 0.0001 by ANOVA with Tukey post-test.
Massive splenocyte depletion occurs in Lm-infected mice

To expand on our histological observations, we assessed the total number of viable cells in the spleens of infected and uninfected mice. No significant difference in splenocyte numbers was observed between uninfected WT and C5aR−/− mice (Fig. 8A). At 24 hpi splenocyte numbers were not significantly different from uninfected mice, and no difference existed between the genotypes. However, by 72 hpi there was a 43% reduction in splenocyte numbers in WT mice vs. uninfected WT controls (p = 0.0097). In line with the histological data, infected C5aR−/− mice had approximately 80% fewer splenocytes than infected WT mice at this time point (p = 0.0007) (Fig. 8A). Similarly, spleen weight was significantly reduced in infected C5aR−/− mice versus WT mice at 72 hpi (p = 0.0003) (Fig. 8B).

The spleen consists of many types of immune cells that play different roles in the course of an infection. C5aR is thought to be expressed by many of them, albeit with considerable variation in expression levels. Recent work indicated a direct role for T cell-expressed C5aR in promoting T cell survival both in vitro and in vivo (115, 116). Therefore, it seemed important to determine if the cell loss was specific for particular subsets of splenocytes. To test this we dissociated spleens, counted the number of viable cells, and then used cell surface staining along with a viability dye to identify the major subsets of live lymphocytes and myeloid cells. Similar numbers of B cells (CD19+), CD4+ & CD8+ T cells, NK cells (NK1.1+), neutrophils (CD11b+, Ly6G+/Ly6C+), monocytes (CD11b+, Ly6G−/Ly6C+) and dendritic cells (CD11c+) were observed in uninfected WT and C5aR−/− mice (Fig. 9A) and in mice at 24 hpi (Fig. 9B). This provided further evidence that C5aR is not critical for early immune cell recruitment during listeriosis. By 72 hpi every cell type
examined was significantly reduced in C5aR-/− mice relative to their WT counterparts (p ≤ 0.0444) (Fig. 9C). C5aR thus broadly protects against splenocyte depletion in listeriosis.
Figure 8. Total splenocyte numbers and spleen weight in WT and C5aR-/− mice during Lm infection. (A) WT and C5aR-/− mice were infected i.v. with 1 X 10⁵ Lm, and their spleens were removed 24 hpi and 72 hpi for determination of total viable cell counts. Spleens from uninfected animals were used as controls. Data is presented as mean cells per spleen ± SEM. n = 3 per genotype for controls, 6 per time point and genotype otherwise. (B) WT and C5aR-/− mice were infected i.v. with 1 X 10⁵ Lm, and their spleens were removed at 72 hpi. Spleens were fixed for 24 h in formalin and then dried and weighed. Spleens from uninfected animals were used as controls. Data is presented as mean weight per spleen ±
SEM. $n = 3$ per genotype for controls, 4 per infected genotype. * $p \leq 0.0097$, ** $p \leq 0.0007$, *** $p = 0.0003$ by ANOVA with Tukey post-test.
Figure 9. Splenocyte subset analysis in WT and C5aR/- mice during Lm infection. WT and C5aR/- mice were infected i.v. with $1 \times 10^5$ Lm, and their spleens were removed 24 hpi and 72 hpi for determination of total viable cell counts. Spleens from uninfected animals were used as controls (A). Splenocytes were stained with various markers and the viability dye DAPI to determine the number of live splenocyte cell types at 24 hpi (B) and 72 hpi (C). Myeloid cells were defined as follows: Neu = neutrophils, CD11b+ Ly6G+ Ly6C+, Mo = monocytes, CD11b+ Ly6G- Ly6C+, DC = dendritic cells, CD11c+. Data is presented as
mean cells per spleen ± SEM. $n = 3$ per genotype for controls, 6 per time point and genotype otherwise. * $p \leq 0.0444$, ** $p \leq 0.0074$, *** $p = 0.0003$ by t-test.
DISCUSSION

In this study, we have shown for the first time that the C5a receptor is essential for host defense against the intracellular pathogen *Listeria monocytogenes*. Significant mortality is observed within the first week of infection in C5aR-/ mice at a dose that otherwise causes a sublethal infection in WT mice. The elevated mortality was associated with increased bacterial burdens in the spleen at 24 hpi and in both the liver and spleen at 72 hpi, indicating that C5aR is required for bacterial containment. Remarkably, C5aR deficiency also resulted in a significant increase in splenocyte depletion. Histological examination of the spleen revealed a striking hypocellular appearance in C5aR-/ mice at 72 hpi that was not seen in WT mice. As elevated TUNEL staining and caspase 3 activity were detected in C5aR-/ spleens, it would appear that this hypocellularity results from increased cell death. The hypocellular appearance of the spleen was confirmed by performing cell counts in dissociated spleens. C5aR-/ mice had only 1/5th the number of splenocytes that WT mice had at 72 hpi. This reduction in splenocyte numbers was also paralleled by a 50% reduction in spleen weight in C5aR-/ mice in comparison with WT mice. Finally, we looked to see if the elevated splenocyte loss in C5aR-/ mice was associated with a specific splenocyte subset. In fact, all splenocyte subsets examined were similarly affected. The protection afforded splenocytes by C5aR during listeriosis is therefore a broad one.

C5a can drive the recruitment of neutrophils to sites of inflammation. Hence, it is reasonable to expect that the absence of C5aR might impair their recruitment during listeriosis. However, the data present here suggests that is not the case. Neutrophils are critical participants in early bacterial clearance in the liver (25-29). An influx of neutrophils can be detected as early as 1 hpi (143). Following neutrophil depletion, log-fold elevations
in bacterial CFUs are seen in liver at 24 hpi (29). Therefore, if C5aR is an important driver of neutrophil recruitment, one would expect to see elevated bacterial counts in C5aR-/livers at this time. The lack of a difference between WT and C5aR-/ mice at 24 hpi suggests C5aR plays little role in neutrophil recruitment to the liver. Indeed, while C5a is a powerful neutrophil recruiting factor, there are other molecules with similar potency. For example, formylated peptides, small peptides shed by bacteria, activate two related G-protein coupled receptors, the formyl peptide receptors (FPR1 and FPR2). It is known that Lm secretes formylated peptides and that mice deficient in FPR1 are highly susceptible to Lm infection (145, 146). More recently, it was discovered that FPR1 and FPR2 are almost entirely responsible for early neutrophil recruitment in listeriosis (143). Additionally, the chemokines MIP-2 and IL-8 are produced in the liver following Lm infection, and their neutralization was found to impair neutrophil recruitment (147). C5aR may therefore be redundant in this regard.

While our study is the first comprehensive examination of the role of C5aR in listeriosis, Nakayama et al. previously assessed the contribution of C5aR to T cell activation in a similar model (117). The two studies may seem contradictory at a superficial level. In their work, C5aR was found to be dispensable for T cell activation during listeriosis. In contrast, we found that C5aR deficiency resulted in a large reduction in T cell numbers in the spleen. However, this is an apples to oranges comparison. Here we examined total T cell numbers at 24 and 72 hpi, whereas Nakayama et al. compared the number of activated, antigen specific T cells at 168 hpi. Accordingly, these observations are not necessarily incompatible. For example, antigen-specific T cells may be protected against the T cell depletion seen in C5aR-/mice. Alternatively, antigen-specific T cells may rebound from...
their early depletion in C5aR-/- mice and expand to levels comparable to WT mice by 168 hpi. Future investigations will hopefully reconcile these observations.

Relatively few studies to date have examined the role of C5aR in the host response to intracellular bacteria. Outside of Lm, it is well-established that C5aR protects mice during Mycobacterium bovis bacillus Calmette-Guerin (BCG) infections (148-153). This protection is conferred in several ways. First, C5a enhances cytokine and chemokine production in BCG infected macrophages (148). This in turn appears to enhance their bactericidal activity (148, 150). Second, C5a is needed for optimal T cell activation by DCs, as C5-/- DCs produce less IL-12p70 and have reduced CD40 expression relative to WT cells (151). Finally, C5aR is required for the formation of granulomas, dense collections of macrophages and lymphocytes that limit the dissemination of BCG (149, 152, 153). C5aR may make similar contributions during listeriosis. In contrast, C5aR is not significant during pulmonary infections with Chlamydia psittaci (Cp) (154). While C3-/- and C3aR-/- mice have reduced survival and impaired bacterial clearance, C5-/- and C5aR-/- mice are indistinguishable from WT mice (123, 154). Curiously, several aspects of the phenotype of C3aR-/- mice in this model are similar to that of C3aR-/- and C5aR-/- mice during listeriosis. Of particular note, Cp-infected C3aR-/- mice have less B cells and CD4+ T cells in lung-draining lymph nodes than Cp-infected WT mice (123). This may point to a broad role for the anaphylatoxin receptors in lymphocyte survival during intracellular bacterial infections. Why C5aR is protective during Lm infection but not Cp infection is unclear. However, the importance of C5aR can vary between tissues. For example, C5aR is required for the clearance of Pseudomonas aeruginosa from the lung but is dispensable for its clearance in the
peritoneum (128). C5aR may therefore be required during systemic infections but be dispensable during pulmonary infections with intracellular bacteria.

A series of papers recently proposed that C3/C5 and C3aR/C5aR are important factors in T cell survival and activation (115, 116). Likewise, C5a prolongs the survival of neutrophils during sepsis (155). C5aR may therefore protect mice against listeriosis by providing pro-survival signals that limit Lm-induced splenocyte death. Protection of lymphocytes would be particularly beneficial. One of the paradoxes of listeriosis is that while lymphocytes are needed for complete bacterial clearance, their presence during the early phase of infection is detrimental. Splenectomized mice and lymphocyte deficient mouse strains (e.g. SCID, RAG-/-) have reduced bacterial loads during the first few days of infection in comparison with lymphocyte sufficient mice (156, 50). Reconstitution of lymphocyte deficient mice with bone marrow from lymphocyte sufficient mice increases their susceptibility (50). The susceptibility conferred by lymphocytes is thought to be a consequence of lymphocyte apoptosis. Apoptotic lymphocytes inhibit innate immunity through the induction of the anti-inflammatory cytokine IL-10 in macrophages (157). In keeping with this, almost no IL-10 is detectable in the spleens of RAG-/- mice at 72 hpi, whereas substantial amounts are seen in WT mice (50). Therefore, if C5a/C5aR directly provides pro-survival signals to lymphocytes during listeriosis it could account for the results seen in this model.

However, it will be important to examine how C5aR modulates cytokine production before continuing to explore this hypothesis. For example, if C5aR is required for optimal production of protective cytokines like IFN-γ or TNF-α, then the elevated cell death might be downstream of a failure to contain bacterial growth. Similarly, since type 1 IFN impairs
the host response by enhancing splenocyte death, C5aR could protect splenocytes through the inhibition of type 1 IFN expression. Resolution of these questions will allow for the discovery of the mechanism by which C5aR protects the host during listeriosis
The C5 Anaphylatoxin Receptor (C5aR) protects against *Listeria monocytogenes* infection by inhibiting Type 1 IFN expression
INTRODUCTION

The early host response to Listeria monocytogenes (Lm) is dominated by the innate immune system. While a Th1 response is required for sterilizing immunity, numerous studies have demonstrated an essential role for innate immune cells and cytokines during the first few days of an infection, a period preceding the activation of adaptive immunity. Deficiency or neutralization of many pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-6, IL-1β, and IL-12 results in enhanced susceptibility to listeriosis with elevated bacterial loads by 72 hours post-infection (hpi) (33-39). While the production of cytokines is largely driven by bacterial recognition through Toll-like receptors and cytoplasmic DNA sensors, there is reason to believe that the complement anaphylatoxin C5a might participate in their induction. Many studies have demonstrated that C5a can potentiate the production of TNF-α, IL-6, and IL-1β in response to bacterial products in monocytes and macrophages in vitro (81-83). These observations have subsequently been validated in vivo following the development of complement knockout mice. For example, mice lacking DAF, a complement regulator that inhibits the formation of C3 convertases on the cell surface, have substantially higher serum levels of TNF-α, IL-6, and IL-1β following LPS injection than their wild-type (WT) counterparts (84). This increase is almost entirely dependent on C5a, as cytokine levels in mice doubly deficient in DAF and C5aR are not significantly different from WT mice. Similarly, C5aR deficiency or blockade is associated with reduced cytokine production in mouse models of sepsis, liver injury, Porphyromonas gingivalis-induced periodontitis, and renal ischemia-reperfusion injury (67, 158, 159, 160). Thus, C5a could protect the host against Lm by contributing to the early induction of protective cytokines.
However, not all of innate immunity is protective during listeriosis. A large amount of evidence now shows that the type 1 IFNs, IFN-α and IFN-β, are detrimental in mouse models of Lm infection. Mice deficient in either the type 1 IFN receptor IFNAR or the type 1 IFN-inducing transcription factor IRF3 are highly resistant to listeriosis (47-49). This resistance is thought to stem from type 1 IFN’s ability to promote cell death in lymphocytes and myeloid cells (47, 48, 51). Type 1 IFN do not trigger cell death themselves but rather increase the sensitivity of cells to insults from bacteria and bacterial products. This results from the induction of numerous pro-death genes including TRAIL, DAXX, PKR, and p53 (47, 161, 162). Consistent with this, treatment of lymphocytes with type 1 IFN increases their susceptibility to LLO-induced apoptosis, and Lm-infected macrophages undergo necrotic cell death in a type 1 IFN-dependent fashion (48, 51). Furthermore, few TUNEL+ splenocytes are observed in the spleens of Lm-infected IFNAR-/- and IRF3-/- mice in contrast with the considerable TUNEL staining seen in WT mice (47, 48). Although the loss of effector immune cells is likely detrimental in and of itself, lymphocyte apoptosis causes additional immune suppression through the induction of IL-10 expression in macrophages (157). Indeed, mice lacking either IFNAR or lymphocytes produce little IL-10 during listeriosis (50). An alternative hypothesis to explain the protection afforded by C5aR in this model would therefore be that C5a/C5aR negatively regulates type 1 IFN expression. Thus, in these studies we sought to examine how C5aR regulates cytokine production during Lm infection.
MATERIALS AND METHODS

Reagents

LPS (E. coli 0111:B4) was obtained from Sigma-Aldrich. Purified human C5a (<0.1 EU endotoxin/µg) was purchased from Complement Research Technologies.

Mice

The C5aR-/- mice used for these studies have been previously described (30). They were backcrossed for over ten generations onto the C57BL/6 background. Age-matched C57BL/6 mice from our colony served as WT controls. All mice were housed in HEPA-filtered Techniplast cages in a barrier facility. Male mice between 11 to 14 weeks of age were used in these studies. All mouse protocols followed institutional guidelines for animal care and welfare.

Bacterial infection

Listeria monocytogenes ATCC strain 13932 (MicroBioLogics, Inc.), a clinical isolate, was used for all studies. Bacteria were cultured in Bacto brain heart infusion (BHI) broth at 37°C to mid-logarithmic phase, pelleted by centrifugation, washed with PBS, and resuspended in PBS. Mice were infected i.v. with 1 X 10^5 bacteria in 100 µl PBS. Control mice received 100 µl PBS. The number of bacteria present in the inoculum was verified by culturing serial dilutions of the inoculum on Bacto BHI agar plates.
Cytokine measurements

Most cytokines and chemokines were measured in sera or clarified liver homogenates taken at 24 & 72 hpi by the Milliplex Mouse Cytokine/Chemokine 22-plex kit (Millipore #MPXMCYTO70KPMX22) on the Luminex 200 system. Serum IFN-α and IFN-β levels at 24 hpi were measured using the VeriKine Mouse IFN Alpha ELISA kit and VeriKine Mouse IFN Beta ELISA kit (R&D Systems), respectively, as per manufacturer’s instructions. IFN-β was measured in cell culture supernatants using the LEGEND MAX Mouse IFN-β ELISA kit (Biolegend).

In vitro experiments

J774A cells were obtained from frozen stocks in our lab and were cultured in 10% FBS supplemented DMEM. The night before experiments 2x10^5 cells were plated per well in 48 well tissue culture treated plates (Corning). C5a was added to wells approximately 1 hour before treatment with 100 ng/ml LPS. For Lm infections bacteria were added at a multiplicity of infection (MOI) of 1 and then plates were centrifuged for 5 minutes at approximately 100g to deposit the bacteria onto the cells. The infection was allowed to progress for 1 hour. The cell membrane impermeable antibiotic gentamicin (Sigma) was then added to a final concentration of 10 µg/ml to limit extracellular bacterial replication. Supernatants were removed at the time indicated and centrifuged for 15 minutes at 10,000g to remove dead cells and bacteria.

Survival study
Mice were infected i.v. with $5 \times 10^4$ Lm and were observed every 6 hours. Mice that showed signs of severe morbidity were euthanized. For rescue experiments mice were injected i.p. with 1 mg of either the IFNAR blocking antibody, MAR1-5A3 (BioXCell) or an isotype control antibody, MOPC-21 (BioXCell), 4 hours before infection. Survival curves were generated using GraphPad Prism software, and statistical significance was assessed using the Logrank test.

**Statistical analysis**

Statistical analysis was done with GraphPad Prism 5. All values are expressed as mean values with the SEM as error bars. For experiment involving two groups, data was analyzed via unpaired two-tailed t test. In experiment involving multiple groups one-way ANOVA with the Tukey post-test was used to determine significance. Survival curves were analyzed by the log-rank (Mantel-Cox) test. P values < 0.05 were considered significant.
RESULTS

C5aR is not required for the expression of protective inflammatory cytokines and chemokines

Many cytokines are required during the first 72 hours of Lm infection to marshal the innate immune response (33-39). Outside of the aforementioned cytokines, the cytokine G-CSF also plays a critical role during listeriosis as a growth factor driving myelopoiesis (163). Additionally, multiple chemokines including MCP-1, MIP-1α, and IP-10 act to recruit immune cells to sites of infection (30, 32, 164-166). As C5a potentiates inflammatory cytokine and chemokine expression in many models, we anticipated that the susceptibility of C5aR-/- mice to Lm might be linked to deficient production of these protective molecules during Lm infection. We therefore examined serum cytokine and chemokine levels in WT and C5aR-/- mice at 24 and 72 hpi through the Luminex platform. Contrary to our expectations, the expression of most cytokines and chemokines were either similar or elevated in C5aR-/- mice compared with WT mice as early as 24 hpi (Fig. 10A,B). While bacterial numbers in the spleen were increased at this time point in C5aR-/- mice and could contribute to the phenomena, cytokine and chemokine levels in the liver showed a similar pattern despite equivalent bacterial burdens in the organ at that point (Fig. 11A,B). Thus, C5aR is not required and may actually inhibit early inflammatory cytokine production during listeriosis.
Figure 10. Serum cytokine and chemokine levels in WT and C5aR-/- mice during Lm infection. WT and C5aR-/- mice were infected i.v. with 1 X 10^5 Lm or PBS, and serum was isolated from the mice 24 hpi and 72 hpi. Cytokine (A) and chemokine (B) levels were measured using the Luminex platform. Sera from PBS injected animals had little to no detectable cytokines and chemokines, and no differences were observed between the genotypes (data not shown). Data is presented as mean pg/ml ± SEM. n = 10-12 mice per genotype per time point. * p ≤ 0.0388, ** p ≤ 0.0086, *** p < 0.0001 by t-test.
Figure 11. Liver cytokine and chemokine levels in WT and C5aR/- mice during Lm infection. WT and C5aR/- mice were infected i.v. with 1 X 10^5 Lm or PBS, and their livers were homogenized at 24 hpi and 72 hpi. Cytokine (A) and chemokine (B) levels were measured using the Luminex platform. Homogenates from PBS injected animals had little to no detectable cytokines and chemokines, and no differences were observed between the genotypes (data not shown). Data is presented as mean pg/ml ± SEM. n = 10-12 mice per genotype per time point. * p ≤ 0.0388, ** p ≤ 0.0086, *** p < 0.0001 by t-test.
**C5a represses type 1 IFN expression in vivo**

The phenotype of Lm-infected C5aR−/− mice is in many ways reminiscent of the phenotype of IFNAR−/− mice (47-49). IFNAR−/− mice have significantly enhanced bacterial clearance at 72 hpi but show little difference in bacterial counts at 24 hpi relative to WT mice, indicating that the detrimental effects of type 1 IFN manifest during second and third days of infection. Similarly, in C5aR−/− mice bacterial counts were only modestly elevated at 24 hpi whereas at 72 hpi a large difference was observed. IFNAR−/− mice are protected against splenocyte death, whereas C5aR−/− mice have greatly enhanced splenocyte depletion. These parallels led us to suspect that C5a/C5aR might inhibit the type 1 IFN pathway in listeriosis. We therefore examined type 1 IFN expression in WT and C5aR−/− mice. There are two major types of type 1 IFN, IFN-α and IFN-β. In Lm models IFN-α expression is partially dependent on IFN-β, and type 1 IFN expression peaks at 24 hpi (167-169). At this time point both IFN-α and IFN-β were significantly elevated in the serum of C5aR−/− mice (p ≤ 0.0004) (Fig. 12). As in prior reports serum IFN-β levels were quite low (38, 39). However, a consistent difference was seen between WT and C5aR−/− mice.
Figure 12. Serum type 1 interferon expression in WT and C5aR-/− mice during Lm infection. WT and C5aR-/− mice were infected i.v. with 1 × 10⁵ Lm or PBS, and serum was isolated from the mice 24 hpi and 72 hpi. IFN-α (A) and IFN-β (B) were measured by ELISA. Sera from PBS injected animals had no detectable type 1 IFN (data not shown). Data is presented as mean pg/ml ± SEM. n = 9-11 per genotype, *** p ≤ 0.0004 by t-test.
An important target downstream of type 1 IFN in listeriosis is the TNF-related apoptosis-inducing ligand (TRAIL). A member of the TNF superfamily, TRAIL is a type 1 IFN response gene (170). Indeed, in the spleens of IFNAR-/- mice the induction of TRAIL expression in response to Lm infection is highly blunted (47). While TRAIL expression can be induced in a variety of immune cells, in listeriosis its expression is observed primarily on the surface of NK cells (171). TRAIL triggers cell death by binding to the death receptors DR4 and DR5, two receptors that are widely expressed among immune cells (171). TRAIL-/- mice resemble IFNAR-/- mice in their enhanced containment of Lm and reduced splenocyte depletion (171, 172). Thus, we examined TRAIL expression on NK1.1+ NK cells by flow cytometry to determine whether the elevation of type 1 IFN observed in C5aR-/- mice was biologically significant. In PBS-treated animals little to no TRAIL+ NK cells were observed (Fig. 13A). By 72 hpi a small fraction of NK cells were TRAIL+ in WT animals. The percentage of TRAIL+ NK cells was significantly higher in C5aR-/- mice (p < 0.0001) (Fig. 13B). Altogether, these data show that C5aR inhibits type 1 IFN expression and its downstream target TRAIL during listeriosis.
Figure 13. Natural killer cell expression of TRAIL in WT and C5aR−/− mice during Lm infection. WT and C5aR−/− mice were treated with PBS or infected and their spleens

A

PBS

72 hpi

B

% TRAIL+ NK cells

WT PBS  C5aR−/− PBS  WT Lm  C5aR−/− Lm

*p < 0.05, **p < 0.01, ***p < 0.001
removed at 72 hpi. Splenocytes were stained with the viability dye DAPI, TRAIL-PE, and NK1.1-APC to determine the percentage of live TRAIL+ NK cells. Representative histograms of DAPI-, NK1.1+ cells are shown (B). The percentage of live TRAIL+ NK cells in mice from two independent experiments are shown in a scatter plot (C). $n = 6$ per group, ** $p = 0.0055$, *** $p < 0.0001$ by ANOVA with Tukey post-test.
C5a negatively regulates LPS-induced IFN-β expression in vitro

While C5a potentiates the expression of many pro-inflammatory cytokines, it has been known for some time that C5a selectively inhibits the induction of IL-12 expression in macrophages in response to the bacterial product LPS (67, 86, 87). As C5a/C5aR inhibits the expression of type 1 IFN in vivo during listeriosis, we were curious if C5a could similarly repress type 1 IFN expression in vitro. To examine this, we utilized J774A cells, a murine macrophage cell line. When J774A cells were stimulated concurrently with LPS and C5a, C5a had no effect on their production of IFN-β (data not shown). However, when J774A cells were pre-incubated with C5a for 1 hour and then stimulated with LPS, a clear inhibition of IFN-β expression was observed (Fig. 14A). This inhibition was observed even at C5a concentrations as low as 1 ng/ml (Fig. 14A). As pre-incubation with C5a was required for the inhibition of IFN-β, we subsequently did a time course experiment to examine the kinetics of this inhibition (Fig. 14B). Inhibition of IFN-β expression could be seen after a pre-incubation as short as 30 minutes but did not reach a maximal level until 2 hours. This state of IFN-β repression was maintained as long as 8 hours after stimulation with C5a, but by 12 hours the effect had dissipated. Thus, exposure to C5a induces a limited period of reduced LPS responsiveness in terms of IFN-β production in macrophages (Fig. 14B). We also examined the ability of C5a to repress Lm-induced IFN-β production in macrophages. Infection of J774A cells with Lm resulted in the release of much greater amounts of IFN-β than observed in response to LPS. While C5a caused a modest reduction of Lm-induced IFN-β expression it was not significant (Fig. 14C).
Figure 14. C5a represses LPS-induced IFN-β expression in J774A macrophages

(A) J774A cells were pre-treated with a dose range of C5a for 1 h and then stimulated with 100 ng/ml LPS for 20 h. IFN-β was measured in supernatants by ELISA. (B) J774A cells were incubated with medium with or without 50 nM C5a for the time indicated and then stimulated with 100 ng/ml LPS for 20 h. IFN-β was then measured in supernatants by ELISA. The percent inhibition was calculated by dividing the difference in the concentration of IFN-β between C5a treated wells and untreated wells by the concentration of IFN-β in untreated wells. (C) J774A cells were pre-treated with a dose range of C5a for 1 h and then infected with Lm at a MOI of 1 for 20 h. IFN-β levels in supernatants were determined by ELISA. Data is presented as mean pg/ml or % inhibition ± SEM and are representative of at
least 2 independent experiments. ns = not significant, *** p < 0.0001 by ANOVA with Tukey post-test.
Blockade of the type 1 interferon receptor rescues C5aR-/− mice

If dysregulation of type 1 IFN expression is indeed responsible for the mortality observed in Lm infected C5aR-/− mice then inhibition of the type 1 IFN axis should rescue them. To test this, we administered either an isotype antibody or an IFNAR blocking antibody (MAR1-5A3) i.p. 4 hours before i.v. infection (173). Similar to our earlier survival study, at a dose of 5x10^4 CFU, WT mice showed no mortality (12 of 12 survived) (Fig. 15). C5aR-/− mice given MAR1-5A3 were almost completely rescued (13 of 14 survived), whereas an isotype antibody failed to rescue them (3 of 14 survived) (p < 0.0001). Taken together, our data suggests that C5a protects mice during listeriosis by directly repressing type 1 IFN expression.
Figure 15. Blocking IFNAR rescues C5aR-/− mice from Lm-induced mortality. 4 hours before infection C5aR-/− mice were administered 1 mg of either the IFNAR blocking antibody MAR1-5A3 or an isotype antibody MOPC-21 i.p. in PBS. WT and C5aR-/− mice were then infected i.v. with 5 × 10⁴ Lm and followed for two weeks. n = 12-14 mice per condition, p < 0.0001 by Log-rank test.
DISCUSSION

In this chapter we provide evidence that the complement anaphylatoxin receptor C5aR protects mice against Lm through the inhibition of type 1 IFN expression. In the previous chapter it was shown that the absence of C5aR in mice during listeriosis resulted in a dramatic elevation of splenocyte depletion in comparison with WT mice. As type 1 IFN greatly enhances lymphocyte apoptosis and macrophage necrosis in listeriosis, we examined serum IFN-α & β levels at 24 hpi and found that both are significantly elevated in C5aR-/- mice in comparison with WT mice. This elevation was associated with increased expression of TRAIL, a type 1 IFN-response gene and a major driver of Lm-induced splenocyte depletion, in splenic NK cells in C5aR-/- mice. We subsequently demonstrated that C5a directly suppresses IFN-β expression induced by the TLR4 agonist LPS in the macrophage cell line J774A, cells representative of one of the major sources of type 1 IFN during listeriosis. Finally, we showed that blockade of IFNAR rescued C5aR-/- mice from Lm-induced mortality, thereby demonstrating that the elevation of type 1 IFN seen in C5aR-/- mice is responsible for their increased susceptibility to Lm.

In the last ten years it has been suggested that the complement anaphylatoxins C3a and C5a directly promote the survival of a wide variety of immune and non-immune cells including neutrophils, T cells, neurons, and tumor cells through their receptors C3aR and C5aR (115, 116, 155, 174, 175). In vitro, constitutive signaling through the anaphylatoxin receptors is required for optimal T cell survival (115). Similarly, T cell activation and expansion in vivo requires C3aR and C5aR signaling at the level of the T cell, in part because the anaphylatoxin receptors inhibit activation-induced T cell apoptosis (115, 116). In support of this hypothesis, the absence or antagonism of C3aR and C5aR reduces T cell
activation and expansion in many mouse models (109, 115, 116, 176). However, it is as of yet unclear whether the direct pro-survival activity of the anaphylatoxins in vitro completely accounts for these observations in vivo. For example, C3a and C5a potently modulate the expression of numerous cytokines that influence cellular activation and survival. Outside of T cells, the anaphylatoxins also promote cellular survival and regeneration in murine hepatocytes and embryonic chick retinas in vivo (177, 178). While C3aR and/or C5aR are expressed by both cell types, the protection afforded by the anaphylatoxins is achieved indirectly through the induction of multiple cytokines including IL-6, TNF-α, and IL-8. Here we have discovered an additional means by which C5aR can have pro-survival activity through an indirect mechanism. Instead of bolstering the expression of protective cytokines as in liver or retina injury models, C5aR protects against Lm-induced splenocyte loss through the inhibition of type 1 IFN expression.

While generally thought of as a pro-inflammatory molecule, C5a can also adopt a regulatory role in certain contexts. The first demonstration of this came over a decade ago in two papers showing that C5a inhibits the expression of IL-12 in human macrophages in response to LPS and Staphylococcus aureus (85, 86). Since then this inhibitory activity has been extended to include most members of the IL-12 cytokine family and additional stimuli such as CD40 activation and the intracellular bacteria Porphyromonas gingivalis (67, 87). Beyond the IL-12 family, C5a also represses the production of IL-17A in LPS-activated macrophages in vitro and in a mouse model of endotoxemia in vivo (179). Furthermore, the inhibitory effects of C5a are not limited to cytokines, as C5aR also suppresses the expression of the chemokines CCL17 and CCL22 in DCs during allergic asthma models (180). Pathogens even exploit the regulatory activity of C5a for their own gain (141).
gingival pathogen *Porphyromonas gingivalis* actively cleaves C5 to trigger crosstalk between C5aR and TLR2 that in turn inhibits the release of nitric oxide. While the effects of C5a/C5aR on a variety of cytokines has been examined, to date no one has looked at how they regulate the type 1 IFNs. This dissertation therefore adds type 1 IFNs to the scope of C5a/C5aR’s regulatory functions for the first time. Since the primary source of type 1 IFN during listeriosis are splenic CD11b+ myeloid cells, we also examined the ability of C5a to inhibit IFN-β expression in the macrophages cell line J774A (167-169). Pre-treatment of J774A cells with C5a potently inhibited LPS-induced IFN-β expression but did not have a consistent inhibitory effect on Lm-induced IFN-β expression. Notably, Lm induced the release of substantially more IFN-β in J774A cells than LPS. This greater production may therefore have obscured the suppressive effects of C5a during Lm infection *in vitro*. Regardless, these experiments show that C5a can directly repress type 1 IFN expression in macrophages and may hint at the mechanism in listeriosis.

It is increasingly appreciated that type 1 IFN is harmful during intracellular bacterial infections (161, 162). Studies with the intracellular pathogens *Salmonella typhimurium*, *Chlamydia muridarium*, *Brucella abortus* and Lm have illustrated that type 1 IFN broadly promotes macrophage and lymphocyte death (47, 48, 144, 181, 182). Beyond this, type 1 IFN can inhibit the immune response in other ways. For example, type 1 IFN appears to dampen the responsiveness of macrophages to IFN-γ during Lm infections (183). Although type 1 IFN induces IL-10 expression during listeriosis through lymphocyte apoptosis, in other models type 1 IFN directly induces IL-10 expression in macrophages and lymphocytes (161). Furthermore, type 1 IFN suppresses the expression of IL-17, a key anti-bacterial cytokine, in both innate γδ T cells and Th17 cells (184-186). C5aR may therefore have
developed a regulatory role for type 1 IFN in order to limit their detrimental effects during intracellular bacterial infections.
SUMMARY

In this dissertation, we have identified the complement anaphylatoxin receptor C5aR as a major component of host defense against the intracellular bacteria *Listeria monocytogenes*. Following intravenous infection with Lm, C5aR-/- mice had elevated mortality and bacterial burden relative to WT mice. The worsened outcomes in C5aR-/- mice were also associated with increased spleen pathology. Histological examination revealed a markedly hypocellular appearance of the spleens of Lm-infected C5aR-/- mice in comparison with WT mice. Consistent with this, cell counts revealed that infected C5aR-/- mice have 1/5th as many splenocytes as WT mice at 72 hpi. All splenocyte subsets examined – neutrophils, monocytes, dendritic, T cells, B cells, and NK cells – were reduced in number in C5aR-/- mice. The increased splenocyte depletion is a consequence of elevated cell death, as the spleens of C5aR-/- mice have more TUNEL staining and caspase 3 activity than those of WT mice.

Splenocyte loss is a major feature of listeriosis in the mouse. A key contributor to this process are the type 1 IFNs. Since C5aR-/- mice have elevated splenocyte depletion during listeriosis, we hypothesized that C5aR protects against Lm by inhibiting the expression of type 1 IFNs. Indeed, the serum of Lm-infected C5aR-/- mice contained more IFN-α & β than that of WT mice. To test the importance of this increase in type 1 IFN expression, we also assessed the expression of a downstream target of type 1 IFN, TRAIL, in Lm-infected WT and C5aR-/- mice. More TRAIL+ NK cells were observed in C5aR-/- mice than WT mice, demonstrating that the elevation of IFN-α & β in C5aR-/- mice has a biologically significant effect. We also determined that C5a can directly repress IFN-β expression in macrophages in response to LPS. Finally, we discovered that blockade of the
type 1 IFN receptor IFNAR rescues C5aR-/− mice from Lm-induced mortality. Therefore, the up-regulation of type 1 IFN in C5aR-/− mice is indeed responsible for their worse outcomes during listeriosis. This dissertation therefore provides the first evidence that C5a/C5aR negatively regulates type 1 IFN expression. A model to explain the results of these studies is provided in Figure 16.

**Figure 16. Proposed Model of C5aR-Mediated Protection against Lm.** A) Lm triggers the release of type 1 IFN from infected myeloid cells. This type 1 IFN then enhances splenocyte death, which in turn decreases bacterial clearance. B) Lm activates the alternative pathway and thereby generates C5a. C5a inhibits the release of type 1 IFN from Lm-infected myeloid cells either directly or indirectly through other C5aR expressing cells. As a consequence, C5a reduces splenocyte loss and increases bacterial clearance.
As with any scientific study, many questions arise from this work. Chief among them is how C5a inhibits type 1 IFN expression at the cellular level. In many cases C5a appears to modulate cytokine expression through the activation of the p42/p44 MAP kinase ERK and/or the Akt kinase (84). Indeed, multiple studies have shown that C5a negatively regulates members of the IL-12 family through either ERK or Akt (67, 87). Outside of C5a/C5aR, PGE2, a prostaglandin that signals through G-protein coupled receptors, inhibits the induction of type 1 IFN expression in macrophages in response to LPS and influenza virus through the activation of Akt (187, 188). Akt is therefore an especially attractive target. Furthermore, it is unclear why C5a-mediated inhibition of type 1 IFN expression requires pre-incubation with C5a. In most studies in which C5a was found to repress cytokine expression, C5a was either administered concurrently with the primary stimulus (e.g. LPS) or shortly before (e.g. 10 minutes prior) (67, 85-87). The observation that maximal inhibition of LPS-induced type 1 IFN requires a 2 to 8 hour pre-incubation with C5a therefore differs from earlier studies. By deciphering the signaling pathways involved in this inhibition further insight into this process may be gained. It is additionally unclear why C5a did not inhibit Lm-induced IFN-β. As mentioned in the Results and Discussion sections of Chapter 2, the amount of IFN-β released by Lm-infected J774A cells was much greater than that released by LPS-treated cells. It is therefore possible that this high level of induction might have overwhelmed the suppressive effects of C5a. Future experiments utilizing lower doses of Lm may reveal C5a-mediated inhibition of Lm-induced IFN-β. Furthermore, as the duration of pre-incubation with C5a influences the degree of inhibition seen in LPS-treated J774A cells, it may be necessary to optimize this aspect for Lm
infection experiments as well. Alternatively, C5a may repress IFN-β during listeriosis indirectly as proposed in Figure 16.

Another intriguing question is whether or not C5a suppresses type 1 IFN expression during viral infections. Unlike in intracellular bacterial infections, type 1 IFN have a well-established protective role in antiviral responses (161, 162). Therefore, C5aR might act to the detriment of the host during viral infections. However, this is not necessarily so. During viral infections, the major source of systemic type 1 IFN is the plasmacytoid dendritic cell (189). In contrast, during Lm infection, macrophages and myeloid dendritic cells produce type 1 IFN, whereas plasmacytoid dendritic cells do not (164-166). It is important to note that the expression of C5aR on plasmacytoid dendritic cells is controversial. Human plasmacytoid dendritic cell reportedly express C5aR, whereas murine cells do not (190, 191). Accordingly, C5a may not impact the production of type 1 IFN during viral infections in the mouse. Alternatively, in viral infections in which myeloid cells are a major source of type 1 IFN such as pulmonary influenza infections, C5a may act to keep the antiviral response in check as to limit type 1 IFN-mediated immunopathology (192). Future studies are needed to test this hypothesis.

While the data shown here establishes a suppressive role for C5a in type 1 IFN expression, it is possible that C5a may regulate the type 1 IFN axis in other ways. For some time it has been known that C5a represses the induction of IRF-1 and IRF-8 by LPS in macrophages (87). The Interferon Regulatory Factors are transcription factors induced by IFNs in addition to other microbial products. Optimal expression of many IFN response genes require IRF-1 and/or IRF-8 activity. For example, IRF1-/- mice fail to generate nitric oxide in response to IFN-γ (193). Therefore, C5a may provide additional inhibition to the
type 1 IFN axis downstream of IFNAR activation. In line with this hypothesis, C5a was recently found to repress the expression of IL-27 in response to IFN-α in macrophages (194). This repression was associated with reduced induction of IRF-1, a critical driver of IL-27 expression. A broader look at the subset of IFN response genes that are repressed by C5a may further clarify the mechanisms by which C5a/C5αR protect against Lm and perhaps other intracellular bacteria.

Animal models have provided us with immense insight into the molecular basis of disease. However, it is increasingly appreciated that they have major limitations. For example, while the neutralization of TNF-α rescues rodents in sepsis models, anti-TNF-α monoclonal antibodies and soluble TNF receptors have little benefit in septic patients. These failings are likely a consequence of several factors (195). First, in many cases there are significant discrepancies between animal models and human disease. These discrepancies frequently arise from the complexity of disease in man. Listeriosis primarily affects the immunocompromised – either the elderly, patients undergoing treatment with immune suppressants, or those with chronic illness – yet virtually all animal studies utilize immune competent mice. Second, there are substantial differences between mice and men. The natural route of Lm infection in man is via the intestinal tract. As discussed earlier, differences in the structure of murine and human E-cadherin make mice highly resistant to oral Lm infection. As a consequence, most mouse studies involve infection via the intravenous or intraperitoneal route. Although all routes of infection result in systemic infections, there is evidence that the shape of the immune response can differ greatly between routes. While type 1 IFN is detrimental during intravenous Lm infections, a recent report demonstrated that it protects the mouse from oral infections (196). Third, most animal
studies make use of single or a few strains of highly inbred mice, whereas the human population is genetically diverse. Certain mouse strains have highly biased immune responses that influence their susceptibility to Lm. For example, Balb/c mice have strong Th2 and weak Th1 responses, whereas the opposite is seen in C57BL/6 mice. The choice of strain therefore creates a bias towards particular aspects of immunity that may not generalize to the human population.

There are several ways in which these concerns could be dealt with. First, it is possible to reconstitute the immune system of immunodeficient mice with human cells via hematopoietic stem cell transplantation (197). These humanized mice contain human immune cells of all hematopoietic lineages and thus allow for in vivo studies of the human immune system in models that cannot be done in man. Second, ex vivo studies with human monocytes could clarify if C5a can repress IFN-β expression in man as seen in the mouse. Finally, an intragastric infection model could be developed using a transgenic Lm strain with modified internalin A that binds to murine E-cadherin. This would allow the mouse model to better resemble the natural route of infection in listeriosis. These studies would therefore demonstrate how the results of this dissertation translates into the human system.

With that said, some predictions from experimental animal work in listeriosis have been confirmed in man. In the mouse, neutralization of TNF-α or deficiency of the TNF receptor results in profound susceptibility to Lm infection. In line with this, the risk of developing listeriosis is elevated in patients undergoing treatment with the TNF-α blockers infliximab, entanercept and adalimumab (198). As there is considerable interest in bringing C5aR antagonists to the clinic for the treatment of a variety of diseases such as macular degeneration, transplant rejection and arthritis, the work has important implications for the
use of C5/C5aR antagonists in man. Patients undergoing treatment with these agents should avoid unpasteurized food products and unwashed produce, and physicians should be aware of their patients’ increased risk of listeriosis.


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VITA

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