THE ROLE OF COMPLEMENT C3 AS A TRIGGER FOR HUMAN MACROPHAGE DEATH DURING INFECTION WITH *FRANCISELLA TULARENSIS* STRAIN SCHU S4

By

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**ABSTRACT**

*Francisella tularensis*, a Tier 1 Select Agent, is a highly infectious Gram-negative bacterium that causes the potentially deadly disease tularemia. Macrophages are considered a primary target of *F. tularensis* and serve as a replicative niche for the pathogen. *F. tularensis* escapes the phagosome to replicate in the host cell cytosol. Macrophage death is commonly observed following *F. tularensis* infections. The mechanism and significance of macrophage death in tularemia is not well understood, though the cause has been commonly attributed to the high bacterial burdens obtained following cytosolic replication. Research presented here indicates that extensive cytosolic replication of *F. tularensis* subsp. *tularensis* strain SCHU S4 in human monocyte-derived macrophages is neither necessary nor sufficient to induce macrophage death. Serum opsonization of *F. tularensis* results in iC3b deposition on the bacterial surface, which greatly enhances uptake by macrophages. Macrophage death was observed following uptake of *F. tularensis* in the presence of human serum, but not in the presence of C3-depleted serum, even when the multiplicities of infection were adjusted to ensure equal uptake under both conditions. This revealed that C3 plays an important role in macrophage death following infection with serum-opsonized *F. tularensis*. Single-cell analysis revealed that high bacterial burdens were not required for the induction of macrophage death. Infection with the mutant SCHU S4 Δ*purMCD*, which can escape the phagosome but is deficient in cytosolic replication, resulted in macrophage death in a C3-dependent fashion like wild-type SCHU S4, supporting the conclusion that replication to high cytosolic burdens is not required for the induction of macrophage death. Infection with the mutant SCHU S4 Δ*fevR*, which cannot escape the phagosome but was taken up by macrophages at similar levels to wild-type bacteria in a C3-dependent manner, did not induce macrophage death. This revealed that engagement of
complement receptors on the macrophage surface by uptake of iC3b-opsonized *F. tularensis* did not yield a sufficient signal to trigger macrophage death. One interpretation of these findings is that phagosome escape, but not extensive cytosolic replication of C3-opsonized *F. tularensis* is required for the induction of macrophage death. The existence of a cytosolic C3 sensor which leads to NF-κB activation has been proposed by the same laboratory that identified TRIM 21 as a cytosolic antibody receptor. Here a hypothetical model is proposed in which C3 fragments enter the cytosol following phagosome escape of serum-opsonized *F. tularensis*, and that the response to cytosolic C3 in *F. tularensis*-infected macrophages is diverted to a cell death pathway resulting in host macrophage death.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMM</td>
<td>bone marrow-derived macrophage</td>
</tr>
<tr>
<td>BSL-3</td>
<td>biosafety level 3</td>
</tr>
<tr>
<td>CFU</td>
<td>colony-forming unit</td>
</tr>
<tr>
<td>CR</td>
<td>complement receptor</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>HI-HS</td>
<td>heat-inactivated human serum</td>
</tr>
<tr>
<td>HS</td>
<td>human serum</td>
</tr>
<tr>
<td>LAMP-1</td>
<td>lysosomal-associated membrane protein 1</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>LVS</td>
<td>Live Vaccine Strain</td>
</tr>
<tr>
<td>MAP</td>
<td>mitogen-activated protein</td>
</tr>
<tr>
<td>MDM</td>
<td>monocyte-derived macrophage</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>PI</td>
<td>post-infection</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>TLR2</td>
<td>Toll-like receptor 2</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

HISTORY

While responding to an outbreak of plague during the early 1900s in California (1, 2), George McCoy and his colleagues from the U.S. Public Health and Marine-Hospital Service observed a disease similar to the plague in ground squirrels but were unable to identify the etiological agent (3). The researchers initially had difficulty culturing the small bacilli, but were able to transmit the disease to a variety of rodent species using infected blood and tissue samples via subcutaneous, intraperitoneal and nasal inoculations. They also successfully transmitted the disease via fleas or contaminated food. In 1912, George McCoy and Charles Chapin, having managed to isolate and culture the Gram-negative causative agent of the plague-like disease, introduced *Bacterium tularense*, named after Tulare County, California where the disease was first observed (4, 5).

Around the same time as the identification of the bacterium, outbreaks of new diseases in humans, such as rabbit fever, deer-fly fever, squirrel plague and glandular type of tick fever, were being described by physicians and researchers across the United States (6-16). In 1921, the disease caused by *Bacterium tularense* was given the name tularemia by Edward Francis, a U.S. Public Health Service physician and researcher (13). Many of these infections for which *Bacterium tularense* was implicated or suspected to be the causative agent are chronicled by Francis in his 1925 landmark article, “Tularemia” (17). Due to his numerous contributions to the study of tularemia and its etiological agent (17-19), the bacterium was renamed *Francisella tularensis* in his honor in 1947 (20).

These early publications in *Francisella* research demonstrate many of the hallmarks associated with the bacterium and the disease it causes, including the wide range of hosts found
in nature, the many routes of entry, the high infectivity of the bacterium, and the vast array of
disease outcomes ranging from mild symptoms to death. In a 1922, Francis issued a warning to
researchers and physicians alike concerning the dangers of Francisella research and likelihood
for natural infections:

All of the men, six in number, who have been intimately connected during the last two
years with the laboratory investigations of tularemia which the Public Health Service has
been conducting have contracted this disease. Such a record of morbidity among
investigators of a disease is probably unique in the history of experimental medicine.
Fortunately, there were no fatalities... Our experience should serve as a warning against
unwarranted indifference to an infection which has claimed all of those who have
continuously worked with it in the laboratory. The ready susceptibility of man to this
infection in nature and in the laboratory, its wide prevalence in nature in a number of
rodents, and the growing number of blood-sucking insects found capable of conveying
the infection should combine to put the medical profession of the United States on the
watch for cases of this new disease of man. (21)

Francisella as a Biological Weapon and the Quest for Biodefense through Human Subject
Studies

It wasn’t long after the discovery of F. tularensis and the description of tularemia that
this infectious agent was considered for use as a biological weapon (22-25). F. tularensis was
one of the many biological agents studied by Japan’s prolific biological warfare program, which
conducted experiments between 1932 and 1945 resulting in the deaths of thousands of people (25,
26). The United States and the Soviet Union are both known to have tested, developed and
stockpiled F. tularensis as a biological weapon (23-25, 27-31). The development of biological
weapons in the United States ended by the executive order of President Richard Nixon, and all stockpiles of biological weapons were reportedly destroyed by 1973, with the exception of a small arsenal found under CIA control in 1975 (23, 25, 27-29).

In addition to the development of biological weapons, the United States also devoted research efforts for the purpose of biodefense. Research at Fort Detrick, home to the US Army Medical Research Institute of Infectious Diseases, not only brought about advances in biocontainment and biosafety (e.g. biosafety cabinets) (28, 32), but it also led to important insights regarding diagnosis and treatment of infectious diseases, decontamination protocols and vaccine development (24, 32, 33). During Operation Whitecoat (1954-1973) human volunteers, most of whom were Seventh-day Adventist conscientious objectors trained as Army Medics, were exposed to biological agents to determine human vulnerability, establish proper treatment protocols and test vaccine efficacy (24, 33). It is reported that no volunteers died during Operation Whitecoat (33). *F. tularensis* was intensely studied during Operation Whitecoat. It was even one of the biological agents aerosolized in the “Eight Ball”, a 1 million liter stainless steel sphere that allowed for the delivery of a precise dosage of organisms to both animals and humans by the respiratory route. Though research during Operation Whitecoat did not yield an approved vaccine against *F. tularensis*, it did provide valuable information on human susceptibility to *Francisella*, disease progression and treatment options (34-39).

In 1969, the World Health Organization (WHO) estimated that in a city of a developed country with a population of 5 million, 500,000 people would be exposed to a 50% infective dose of *F. tularensis* resulting in 250,000 clinical cases of tularemia (40). In a developed country with antibiotic treatments available within the first 48 hours of exposure, 60,000 people would need hospitalization and 4,500 people would die. In a city of a developing country with a population of
5 million, 125,000 clinical cases of tularemia and 30,000 deaths over 10 days would be expected. In 1997, the CDC used another model to estimate the economic impact of a bioterrorist attack using *F. tularensis* (41). It was estimated that if 100,000 people were exposed to an *F. tularensis* cloud, 82,500 cases of tularemia and 6,188 deaths would be expected in the absence of intervention, with a cost of ~$5.4 billion. If prophylaxis began in the first 48 hours, the number of deaths could be reduced to ~2,500, and the cost would be reduced to ~$2.5 billion.

*F. tularensis* is considered a potential weapon of bioterror (42). As such, the United States classifies *F. tularensis* as Tier 1 Select Agent (42 CFR 73). In humans, *F. tularensis* has a low infectious dose and is associated with high rates of morbidity and mortality when not treated promptly with antibiotics. There is still no approved vaccine. Past biological weapons programs have demonstrated that the bacterium can be aerosolized and disseminated with ease. The intentional misuse of the pathogen could threaten our public health and safety. Work with this agent requires a BSL-3 facility equipped with biocontainment, biosafety and biosecurity features.

**FRANCISELLA TULARENSIS**

*F. tularensis* is a non-motile, non-spore forming, faintly-staining Gram-negative, pleomorphic coccobacillus (0.2 x ~1 μm) (18, 43-46). It is a fastidious aerobic bacterium that requires cysteine and iron for extracellular growth, and grows best at 37°C and near neutral pH (18, 43-45). The circular genome of *F. tularensis* contains approximately 1.89 million base pairs and is AT rich, with approximately 33% GC content (47, 48).
Taxonomy, Ecology and Epidemiology

*F. tularensis* is a member of the γ-proteobacteria (47, 49). However, it is quite divergent from other γ-proteobacteria and phylogenetically distant from its closest-related genera, *Coxiella* and *Legionella* (47). In recent years, there has been an expansion in the genus *Francisella*, which now includes species *tularensis, novicida, philomiragia, noatunensis, halioticida, hispanensis* and *guangzhouensis* (49). *F. tularensis* causes tularemia in humans. All other species are considered environmental species, associated primarily with saltwater. *F. novicida* and *F. philomiragia* do not cause tularemia in healthy humans, but can serve as opportunistic pathogens in immunocompromised individuals, though this is exceptionally rare. *F. noatunensis* and *F. halioticida* are pathogens of fish and abalone, respectively. Little is known about *F. hispanensis*, which has been isolated from only a few patients in Spain and Australia (50-54), and *F. guangzhouensis*, which was isolated from water reserves of air-conditioning cooling towers in China during routine screenings for *Legionella* (55).

There are three subspecies of *F. tularensis* known as *tularensis* (type A), *holarctica* (type B) and *mediasiatica*. Type A and type B strains are the etiological agents of tularemia in humans, albeit with different severities. Tularemia was first believed to be confined to the United States (17), but cases were soon described in many countries of the northern hemisphere (18, 24, 56, 57). While the highly virulent *F. tularensis* subsp. *tularensis* is found in North America (20, 22), the less virulent type B strains can be found throughout the northern hemisphere. *F. tularensis* subsp. *mediasiatica* has never been documented to cause disease in humans, is found only in Central Asia and parts of the former Soviet Union, and can be isolated from rabbits and ticks (58, 59).
The type A strains have been divided into two subpopulations, type A1 and type A2, based on genetic analyses of 316 *F. tularensis* clinical isolates collected by or submitted to the CDC from around the United States between 1964 and 2004 (60-62). Interestingly, the two genetically distinct subpopulations also have distinct geographical localization and virulence tendencies. It was found that the type A1 strains are located primarily in the central and eastern United States (60, 61, 63), with an average mortality rate of 14% (61). The geographical distribution of type A1 strains overlap with distribution of the eastern cottontail rabbit (*Sylvilagus floridanus*), the Lone Star Tick (*Amblyomma americanum*) and the American dog tick (*Dermacentor variabilis*) (60). The type A1 strains can be further divided into two genetic clusters, A1a and A1b, with average mortality rates of 4% and 24%, respectively (63). There is no geographic distinction that can be made between strains of A1a or A1b. The difference in mortality rates suggest that there may be an intrinsic characteristic that contributes to the virulence of A1b strains that is not present in A1a strains. Type A2 strains are found primarily in the western United States (60, 61), and are less virulent that A1 strains. None of the clinical A2 strains analyzed resulted in a human death (61, 63). The geographic distribution of type A2 strains overlapped with the distribution of the mountain cottontail rabbit (*Sylvilagus nuttallii*), the Rocky Mountain wood tick (*Dermacentor andersoni*) and the deer fly (*Chrysops discalis*) (60).

Type B strains were found throughout the United States, but tended to cluster around major waterways and areas with high rainfall (61). The CDC reported that the average mortality rate of the 74 type B clinical isolates from the United States included in their study was 7% (61). This reported fatality rate seems high for what is considered to be a less virulent subspecies of *F. tularensis*. The overall fatality rate reported for the clinical isolates studied, including type A
and type B strains was 9% (61). This is much higher than the reported fatality rate (~2%) for tularemia cases in the United States between 1985 and 1992 (24). A bias may exists in the clinical samples. Perhaps the isolation of *F. tularensis* occurs more frequently from patients with more severe disease states or with greater success after death during autopsies.

Despite over 100 years of study, the ecology of tularemia is still not completely understood. Many factors complicate our understanding of the ecology of *F. tularensis*. *F. tularensis* has an immensely wide range of hosts with detection of the bacterium in over 250 animal species including man, rabbits, lemmings, muskrats, other mammals; ticks, deerflies, mosquitos, other arthropods; birds; fish; amphibians; and even protozoa (22, 64). Additionally, the findings derived from the study of clinical isolates suggested that the ecology of type A strains may be different from type B strains. As noted above, type A strains may be more associated with rabbit and insect vectors while type B strains may be more associated with water sources (60, 61). These factors make it difficult to tease out the life cycle and routes of transmission of the bacterium in nature. Rabbits have historically been considered a primary reservoir for *F. tularensis* in the United States and have even been implicated in the spread of *F. tularensis* from the central United States to the east coast (18, 61, 65). However, recent findings suggest that wild cottontails are far too susceptible to type A *F. tularensis* to serve as a reservoir for the pathogen and should be thought of as a dead-end host (66-68). Contact with infected rabbits was once considered to be the origin of many cases of tularemia, but most human *F. tularensis* infections in the United States are currently associated with insect bites (69). This may be due to a change in human behavior with fewer people hunting and handling wild rabbits nowadays. There is also evidence that the environment itself may actually serve as the reservoir for *F. tularensis*. Though *F. tularensis* does not form spores, it has been shown to enter a viable
but non-culturable state when held at 8°C for extended periods of time in sterile tap water (70). Numerous outbreaks of waterborne tularemia coupled with the detection of *F. tularensis* in natural waters (64, 71-74) also serves as evidence that *F. tularensis* can persist in the environment. Persistence of *F. tularensis* in the environment is also demonstrated by outbreaks of pneumonic tularemia, often associated with dust generated by lawnmowers and farming activity [Martha’s Vineyard, MA, 2000-2010, (68, 75, 76); Castilla and León, Spain, 2007, (77); Jämtland County, Sweden, 2010 (78)]. Additionally, genomic evidence of clinical isolates suggests that *F. tularensis* can survive in the environment for decades with incredibly low mutation rates and is likely dispersed long distances by wind (78, 79).

In the United States, there was a peak number of 2,291 cases of tularemia reported in 1939 (80). The number of reported tularemia cases has since declined (58, 80, 81). The number of tularemia cases reported each year in the United States have recently been between 100 and 300 cases (82). Some infections with *F. tularensis* may go undiagnosed or misdiagnosed due to the diffuse nature of the symptoms, the lack significant virulence that some strains possess, and the empiric treatment of unidentified infections with antibiotics. A decline in hunting and trade practices, and perhaps even an overall decrease in the awareness of tularemia, may also have contributed to the decline of reported tularemia cases in the United States. Over half of the reported cases between 1990 and 2010 occurred in just 5 centrally located states (Missouri, Arkansas, Oklahoma, Kansas and South Dakota) (69, 83). Incidence of tularemia is also high in Massachusetts (Martha’s Vineyard) (69, 83). In 2015, the CDC noted a spike in the number of cases reported in Colorado, Nebraska, South Dakota and Wyoming for unknown reasons (84).

In Europe, incidence of tularemia usually occurs in patterns of endemic outbreaks, with between 1,000 and 2,500 cases of tularemia reported each year (85). The number of tularemia
cases reported in Europe has dramatically increased since the early 1990s, and this is likely due to an increase in mandatory reporting (85). Between 1992 and 2012, nearly half of all tularemia cases reported in Europe occurred in Sweden and Finland (85). It is possible that the disparity of cases that occurred in Sweden and Finland is due to better reporting in these nations.

**Prototypic Species of *Francisella***

*F. tularensis* subsp. *tularensis* strain SCHU S4 is the prototypic type A strain. SCHU was first isolated in 1941 by Lee Foshay from the skin ulcer of a patient in Ohio (86-88). Strain SCHU S4 was derived from Foshay’s original strain SCHU in a 1951 experiment that linked colony morphology to pathogenicity (87). SCHU S4, smooth (S) variant clone #4, was described as a smooth, blue colony with a watery consistency. SCHU S4 was highly virulent in mice, with an absolute lethal dose of 1-10 organisms following peritoneal injection of the bacteria (87). In 1960, Operation Whitecoat experiments conducted on volunteer inmates of the Ohio State Penitentiary revealed that as few as 10 SCHU S4 bacteria delivered intracutaneously (38) or by the respiratory route (37) were sufficient to cause disease in humans. SCHU S4 belongs to the subclass A1a (88). In 2014, the virulence of SCHU S4 in mice was compared to more recent clinical isolates of type A1a, A1b, A2 and B strains (88). C57BL/6 J mice were infected intradermally with 10 to 20 bacteria and the temperature of each mouse was the monitored. After the temperature fell below the normal range following the febrile illness, termed the hypothermic drop point (89), mice were euthanized. Based solely on the time to drop point, it was reported that SCHU S4 is significantly less virulent than “modern” clinical isolates of type A strains and exhibited virulence properties more like those of type B strains (88). This suggests that what has been considered for decades to be the prototypic pathogenic type A model organism, may not represent the virulence of other type A strains. It must be noted here that
virulence in mice does not necessarily correlate to virulence in humans. For example, mice are far more susceptible to *F. tularensis* LVS and *F. novicida* than humans (90). This lone report by Mullins, et al. (88) should not deter researchers from the use of SCHU S4, as SCHU S4 is well characterized and consistency throughout research is important. However, researchers should acknowledge the limitations of a model organism and consider the use of other type A strains in addition to SCHU S4 for experiments that require the challenge of highly pathogenic strains, such as for the development of vaccines and therapeutics.

In 1956, the United States and Soviet Union embarked on a “medical exchange mission” that made available to the United States a freeze-dried *F. tularensis* attenuated live vaccine, named strain 15, developed by Professor N. G. Olsufiev of the Gamaleia Institute, Moscow, USSR (91). When plated on agar, researchers noted two colony variants, grey and blue. The blue variant was more virulent, and after being passed five times in mice the recovered strain was designated the live vaccine strain (LVS) (92). *F. tularensis* subsp. *holartica* Live Vaccine Strain (LVS) may be considered the prototypic type B strain, but not without acknowledging that it was selected as a vaccine candidate for its uncharacterized attenuation. LVS is exempt from Select Agent regulation and can be studied under BSL-2 conditions, making it a popular model organism for studying *F. tularensis*. LVS was never approved as a vaccine for widespread use, in part because the nature of its attenuation is not well understood. Additionally, human serum agglutinin titers following vaccination with LVS were not good predictors of protection against challenge with the more virulent SCHU S4 strain (37, 39). It should also be noted that the pathophysiological responses induced by LVS in mice are more inflammatory than those of type A strains and other type B strains (93). Regardless, LVS has been widely studied in tularemia
research because it maintains its virulence in mice and is much safer to work with than type A strains.

During Operation Whitecoat, researchers tested the efficacy of both intracutaneous and respiratory LVS vaccination protocols in human volunteers (35, 37, 39). During the first trial, 80% of non-vaccinated controls fell ill 4 to 7 days following respiratory challenge with 10 to 50 SCHU S4 bacteria (37). Intracutaneous vaccination with LVS reduced to 17% the incidence of volunteer inmates from the Ohio State Penitentiary that fell ill following low dose respiratory challenge with SCHU S4 (37). However, intracutaneous LVS vaccination did not protect volunteers from large dose respiratory challenges of approximately 20,000 SCHU S4 bacteria (35). During respiratory vaccination studies (39), it was found that inhalation of $10^4$ LVS bacteria resulted in a very mild systemic illness in 30% of the volunteers. None of the volunteers that received $10^4$ LVS bacteria via the respiratory route developed a fever, though nearly all of them had pea-sized cervical lymph nodes. Inhalation of $10^6$ or $10^8$ LVS bacteria caused more severe illness in 80-90% of the volunteers (39). Of the volunteers that received $10^8$ LVS bacteria via the respiratory route, 80% had temperatures $>100^\circ$F that typically began 3 days after exposure and 7% reached the experimental threshold ($103^\circ$F) requiring antibiotic intervention. When non-vaccinated controls were challenged with 25,000 SCHU S4 bacteria, 94% had temperatures $>100^\circ$F, and 89% required antibiotic treatment (39). The onset of illness following exposure to the challenge dose of SCHU S4 typically occurred on day 3 for non-vaccinated controls and on day 4 for vaccinated volunteers. Respiratory vaccination with $10^4$ LVS bacteria did not provided effective protection against a respiratory challenge of 25,000 SCHU S4 bacteria (39). Similar to the intracutaneous LVS vaccinated controls, approximately 60% of the volunteers required antibiotic intervention. While the majority of the volunteers that received the
larger doses of aerosolized LVS vaccine did have fevers > 100°F, none of them reached the experimental threshold requiring antibiotic intervention following challenge with SCHU S4 (39). As mentioned earlier, researchers noted that antibody titers following immunization with LVS did not correlate with protection against the virulent SCHU S4 strain (37, 39). Cell-mediated immune responses were not studied in these early human trials. Current tularemia vaccine research has yet to find a good correlate for protection in humans (34, 94), but clinical investigations into LVS continue (95). For more details on current tularemia vaccine research, please note these reviews (34, 94).

*F. novicida* strain U112 was isolated from a water sample in Utah (96). It was originally classified as its own species for several reasons (96, 97): *F. novicida* was less fastidious than *F. tularensis*; unlike *F. tularensis*, *F. novicida* fermented sucrose; *F. novicida* was less virulent in rabbits than *F. tularensis*; and *F. novicida* did not cross-react with serum from rabbits immunized with *F. tularensis* (96). *F. novicida* was later classified as a subspecies of *F. tularensis* based solely on global genetic similarities (52), despite the objection from many prominent researches in the field (98). In 2014, Kingry and Petersen (48) reviewed the many phenotypic and genomic differences between *F. tularensis* and *F. novicida*, hopefully ending the debate on the classification of *F. novicida* as a separate species from *F. tularensis*. *F. novicida* does not cause tularemia in healthy humans, but has been widely studied in the laboratory for several reasons. First, *F. novicida* strain U112 is exempt from Select Agent regulation, and unlike in humans, it does cause illness in mice. It is also attractive for studies that require genetic manipulations, as such tasks are more easily accomplished in *F. novicida*. However, immune responses to *F. novicida* are not suitable representations of *F. tularensis*-induced tularemia pathogenesis in humans (48, 93). For example *F. novicida* induces activation of the
inflammasome, pro-inflammatory cytokine release, and rapid mouse macrophage death (99-102), whereas type A *F. tularensis* does not appear to induce inflammatory responses, at least not in vitro in human macrophages nor in vivo during the early stages of infection (101, 103-105). In the literature, *F. novicida* has often mistakenly been referred to as *F. tularensis*, so great care must be taken when interpreting results because immune responses to *F. novicida* vary greatly from those to *F. tularensis*.

**TULAREMIA**

*I know of no other infection of animals communicable to man that can be acquired from sources so numerous and so diverse. In short, one can but feel that the status of tularemia, both as a disease in nature and of man, is one of potentiality.*

– R. R. Parker, 1933 (56, 106)

**Routes of Entry and Clinical Manifestations**

The clinical presentation of tularemia (18, 22, 24, 43, 44) is most often characterized with a sudden onset of fever 3 to 6 days post exposure, but the incubation period can range from 1 to 21 days. Sudden fever is often accompanied by other non-specific symptoms including headaches, chills, malaise, anorexia and fatigue. Without antibiotics, the febrile illness with chronic debility can persist for months, and relapse is not uncommon (18). Of note, there has never been a report of person-to-person transmission of tularemia.

The clinical presentation of tularemia varies with the pathogen’s route of entry. Ulceroglandular is the most common form of tularemia, accounting for ~80% of the cases (44), and presents with a papule or ulcer at the site of inoculation with regional lymph node swelling. If not treated, the infection can persist for months and even progress to septicemia or pneumonic
tularemia in 10-15% of cases (44). Ulceroglandular tularemia is rarely fatal, but it is estimated that if left untreated approximately 5% of cases could result in death (44). Ulceroglandular tularemia is most often contracted by insect bites (ticks, deerflies or mosquitoes) or by handling infected animals. Glandular tularemia presents with regional lymph node swelling without the obvious ulcer. Contamination of the eye results in oculoglandular tularemia, which presents with conjunctivitis with preauricula lymph node swelling. Ingestion of *F. tularensis* can result in oropharyngeal or intestinal tularemia. It is most often associated with cervical lymph node swelling, but may also present with lesions of the mouth or lips, tonsillitis, pharyngitis, abdominal pain and occasionally diarrhea. Pneumonic tularemia is the most deadly form of the disease with mortality rates as high as 60% in the absence of treatment (107). Pneumonic tularemia can arise from inhalation of *F. tularensis* or the spread of infection from another site. It typically presents with a dry cough, dyspnea, and chest pain. Throughout the literature, the term typhoidal tularemia has been used to describe a systemic illness that lacks early localizing signs and symptoms.

**Pathology**

Pathology in human tularemia (44, 108, 109) includes microabscesses and granulomas with central necrosis in the lymph nodes, liver, lungs and spleen. A review of tularemia cases that occurred in Tennessee between 1949 and 1976 revealed that radiological findings in pneumonic tularemia are quite variable (110). Many patients had immune infiltrates in the lung tissue, fewer had pleural effusion, and only one had hilar lymphadenopathy. Histological examination revealed edema, fibrin deposition, cellular debris and neutrophil infiltration of lung tissue and alveolar spaces (109). The precise cause of death from tularemia is unknown, but septic shock resulting in organ failure has been suggested (24, 111).
Review of available histological reports of *F. tularensis* infections have revealed that non-human primate, rabbit and mouse models are appropriate models of human tularemia, provided that the challenge strains are “fully virulent” type A and type B strains of *F. tularensis*, as opposed to the use of LVS or *F. novicida* (93). Extensive caspase-3-mediated macrophage death was observed in necrotic foci found in the liver and spleens of mice following infection with type A *F. tularensis* (112). Death of infected macrophages resulted in the loss of containment of the pathogen within the hepatic granulomas and allowed for the dissemination of the pathogen into hepatocytes throughout the liver (112).

By contrast, infections with LVS and *F. novicida* appear to induce tissue damage by activating pro-inflammatory responses (93). LVS induces far less cell death and necrosis in mouse tissues than is observed following infection with type A *F. tularensis* (93, 112, 113). Rather, inflammatory foci continue to grow in size while remaining viable nearly a week post-infection (93, 113). Despite the low levels of cell death, both activated caspase-1 and caspase-3 were observed in the livers of LVS-infected mice (112). *F. novicida* induces activation of caspase-1 and the inflammasome (93, 99, 114). LVS and *F. novicida* also induce the release of pro-inflammatory cytokines that likely contribute to the tissue damage observed in mice (93).

**Diagnosis and Treatment**

Tularemia has classically been diagnosed by a positive serological test weeks after the onset of disease (18). Care must be taken when interpreting serological tests as cross-reactions with several other pathogens, including *Brucella, Salmonella, Yersinia* and *Legionella*, have been reported (18, 44). Additionally, culture of the bacterium from clinical samples can confirm diagnosis. However, isolation of *F. tularensis* from clinical samples is not always successful (or even attempted due to potential hazards (43)) and can take several days due to the fastidious
growth of the pathogen. Additional diagnostic tests include PCR or direct ELISA of clinical samples for the detection of *F. tularensis* (44).

Mortality rates due to tularemia have greatly declined since the availability of antibiotic treatment. In the United States, fatality rates have fallen from 5-15% to just below 2% (24). The antibiotic of choice is streptomycin, especially for severe cases, and should be given for 10 days (24, 110). Streptomycin is the only antibiotic approved by the FDA for the treatment of tularemia, but gentamicin may serve as an alternative. Tetracyclines (including doxycycline) may be used in less severe cases, but they have been associated with relapse and should be given for 2 to 3 weeks (115). Ciprofloxacin also appears to be effective in the treatment of tularemia (116). Experiments in mice also suggest that nanoparticles with be a more efficacious mode of treatment in the future as they are readily internalized by macrophages, the primary target of *F. tularensis* (117, 118).

**INTRACELLULAR LIFE CYCLE**

*F. tularensis* is considered to be a facultative intracellular pathogen. The bacterium possesses the ability to infect a wide range of cells, including macrophages (119, 120), dendritic cells (121), neutrophils (122, 123), alveolar epithelial cells (124), endothelial cells (125), hepatocytes(126), erythrocytes (127) and B cells (128). Macrophages have long been considered the primary targets of infection by *F. tularensis* and they provide the pathogen with a niche for high levels of replication. Macrophages are among the first cells infected following respiratory infection by type A and type B *F. tularensis* (129-131). Many receptors on the macrophage surface have been implicated in the uptake of *F. tularensis*, including the mannose receptor, the scavenger receptor, IgG-Fc receptors, nucleolin and complement receptors CR3 and CR4 (132-
139). Serum opsonization greatly enhances uptake of *F. tularensis*, particularly via CR3 (132, 134, 135, 137, 138). For a more in depth discussion on the role of complement during *F. tularensis* infection of human macrophages, please see Chapter 3 of this dissertation.

*F. tularensis* is taken up by human monocyte-derived macrophages (MDMs) by a unique actin dependent process called “looping phagocytosis” (132). Looping phagocytosis is distinct from conventional phagocytosis, micropinocytosis and coiling phagocytosis and involves uptake of *F. tularensis* by asymmetric pseudopodia that very loosely encompass the bacterium. Just moments after uptake, the *F. tularensis* bacterium is found in a relatively tight fitting phagosome (132). The *Francisella*-containing phagosome transiently acquires early endosomal antigen-1 (EEA-1), followed by late endosomal markers CD63 and lysosome-associated membrane protein-1 (LAMP-1) (140, 141). *Francisella*-containing phagosomes do not appear to mature to phagolysosomes, as they do not acquire the lysosomal protease cathepsin D (140). *F. tularensis* escapes the macrophage phagosome at a rate that varies with host species, e.g. by 1 h in mouse macrophages and by 8 h in human macrophages (140-142). While there has been some controversy over the need for phagosome acidification prior to escape (141, 143, 144), it appears that acidification of the phagosome is not required for escape of *F. tularensis* into the cytosol of human macrophages (144). Once inside the macrophage cytosol, *F. tularensis* replicates to high numbers (141, 145). The doubling time of type A strain SCHU S4 in mouse bone marrow-derived macrophages (BMM) is estimated to be ~1 h (141). The capacities for phagosome escape and intracellular replication appear to be essential for *F. tularensis* virulence, as strains that lack these abilities are attenuated in mice (142, 146, 147).
Macrophage Death

As mentioned above, macrophage death is a hallmark of tularemia (93, 112, 119, 148, 149). The mechanism of macrophage death is not well understood following infection with *F. tularensis*. Caspase-1-mediated pyroptosis occurs following macrophage infection with *F. novicida* strain U112 (99, 100, 150-154). Unlike *F. novicida*, it has been demonstrated that type A *F. tularensis* does not activate caspase-1 (101, 103). Type A *F. tularensis* has been found to induce caspase-3-mediated apoptotic death of macrophages in vivo, as well as in cultured mouse and human macrophages (93, 112, 155-158). Caspase-3 mediated macrophage death appears to end the containment of type A *F. tularensis* within microgranulomas in mice, thereby facilitating the dissemination of the pathogen (112). Macrophage death is not the only mechanism of dissemination that has been observed. It has also been demonstrated in both mouse BMMs and human MDMs that *F. tularensis* strain SCHU S4 can be passed from an infected macrophage to an uninfected macrophage via a process called trogocytosis, which involves the temporary fusion of plasma membranes allowing for cytosolic exchange before the cells eventually separate (159).

Anecdotally, extensive cytosolic replication of the bacterium is routinely cited as the cause of macrophage death when describing the lifecycle of *F. tularensis*. Apart from the observation that *F. tularensis* can achieve high intracellular burdens before macrophage death occurs, there is little evidence to cite a threshold cytosolic burden as the trigger of macrophage death. Perhaps the only evidence suggesting that replication of *F. tularensis* is required for the induction of macrophage death comes from the antibiotic treatment of J774.A1 macrophage-like cells infected with LVS (160). Macrophage death could be prevented if cultures were treated with ciprofloxacin within the first 12 h of infection, but delaying treatment until 15 h post-infection (PI) failed to prevent macrophage death. The authors suggested that replication of the
bacterium during the first 12 h of infection was not sufficient to trigger macrophage death, but by 15 h PI the level of bacterial replication was such that macrophage death could not be prevented. It is known that *F. tularensis* gene expression changes while inside the macrophage (146).

Perhaps you simply need live, biologically active bacteria at 15 h PI, rather than replication to a threshold burden by 15 h PI. Our recent findings (161) suggest that cytosolic replication of type A *F. tularensis* is neither necessary nor sufficient to induce human macrophage death.

Apart from the potential to increase dissemination of *F. tularensis*, the significance and cause of macrophage death is also not well understood. For some infections, early host cell death can serve the host by eliminating the pathogen’s replicative niche and clearing the infected cell (162, 163). This does not appear to be the case for *F. tularensis* because the bacterium is able to undergo many rounds of replication before macrophage death occurs.

Many pathogens have the ability to modulate host cell death (163-165). Some pathogens are capable of inducing host cell death to prevent inflammation and recruitment of effector cells to the site of infection. Some pathogens are capable of repressing host cell death to maintain their replicative niche or avoid extracellular detection. Whether or not *F. tularensis* is capable of modulating the life or death responses in macrophages remains to be determined. *F. tularensis* does prolong the lifespan of neutrophils, presumably to maintain its replicative niche (166). The mechanism by which *F. tularensis* is capable of extending the neutrophil lifespan is unknown, but it could be conferred by intracellular or extracellular live bacteria (166). Additionally, recent evidence suggests that macrophage death is not induced by *F. tularensis* alone, but may result from the interplay of signaling that arises from cytosolic *F. tularensis* and complement C3 peptides (161, 167). Further research is required to elucidate the role type A *F. tularensis* plays macrophage death.
IMMUNE EVASION

*F. tularensis* is quite capable of “flying under the radar,” so to speak. One stealth strategy employed by *F. tularensis* is the failure to activate TLR4 (168-170), which may be mediated by the lack of binding between *F. tularensis* LPS (lipopolysaccharide) and host LPS-binding protein (171). TLR4, along with MD-2, CD14 and LPS-binding protein, serves as a pattern recognition receptor to activate macrophages and stimulate secretion of inflammatory cytokines in response to LPS, a major component of Gram-negative bacterial outer membranes (172). *F. tularensis* LPS has an unusual structure compared other Gram-negative bacteria (173, 174), including a lipid A structure that is tetra-acylated with 16-18 carbon acyl chains (compared to *E. coli* lipid A which is hexa-acylated with 12-14 carbon acyl chains). It has also been reported that TLR4 does not provided any survival advantage to mice infected with *F. tularensis* (175, 176).

*F. tularensis* lipoproteins do engage TLR2 (177). TLR2 signaling is required for NF-κB activation and pro-inflammatory cytokine production in LVS-infected mouse models (177-182). TLR2−/− mice appear to lack protective innate immune responses and are more susceptible to LVS infection (180, 182).

*F. tularensis* is also capable of evading complement-mediated lysis (122, 183), a trait that appears to be conferred at least in part by its LPS and cell wall structure (183-186) and the binding of Factor H (187) to promote the conversion of C3b to iC3b on the bacterial surface (184, 187). Complement iC3b on the bacterial surface mediates uptake of *F. tularensis* by macrophages via CR3, which has been shown to down-regulate TLR-2-mediated pro-inflammatory signaling (138), likely contributing to the pathogen’s immune evasion strategy. A
more detailed discussion on the interactions between \emph{F. tularensis} and the complement system can be found in Chapter 3.

\emph{F. tularensis} also appears to actively suppress inflammatory responses as part of its immune evasion strategy. Following pulmonary infection of mice, \emph{F. tularensis} strain SCHU S4 initially suppresses inflammatory cytokine secretion and the activation of macrophages and dendritic cells while also inducing secretion of the anti-inflammatory cytokine TGF-\(\beta\) (104, 111, 188). \emph{F. tularensis} LVS is capable of actively suppressing macrophage inflammatory responses to \emph{Escherichia coli} LPS in mouse macrophages (189, 190). LVS also suppresses the activation of NF-\(\kappa\)B and phosphorylation of the mitogen-activated protein kinase p38 in the J774 mouse macrophage cell line (189, 190). Additionally, LVS can downregulate TLR4- and TLR2-mediated pro-inflammatory and interferon responses in murine BMM by suppressing K63 ubiquitination and the formation of TRAF6 and TRAF3 complexes (191). In mouse macrophages, SCHU S4 promotes the degradation of host mRNA encoding inflammatory cytokines (105). The mechanism(s) by which \emph{F. tularensis} suppresses pro-inflammatory signaling are not entirely known, but it often appears that type six secretion or phagosome escape of the bacterium is required (105, 189-191).

Type A and type B strains of \emph{F. tularensis} also evade the detrimental effects of reactive oxygen species by inhibiting the formation or activity of the NADPH oxidase complex in human neutrophils and macrophages (192, 193). \emph{F. tularensis} also expresses enzymes that help protect it from reactive oxygen species, such as the catalyase katG, peroxidases and superoxide dismutases (194-196).
**VIRULENCE FACTORS**

*F. tularensis* virulence is not completely understood. Genomic analysis has not revealed any genes encoding type III, IV or V secretion systems in *F. tularensis* (47, 197). *F. tularensis* may employ type 1 secretion systems, which are conventionally associated with multi-drug efflux and toxin secretion in Gram-negative bacteria (198). TolC is the outer membrane protein involved in type 1 secretion. *F. tularensis* has two TolC homologs, TolC and FtlC, which localize to the outer membrane and play a role in drug resistance (199, 200). TolC has been shown to contribute to LVS virulence in mice, but less so for SCHU S4 (157, 201, 202). There are still no known virulence factors secreted by TolC or FtlC.

The Francisella pathogenicity island (FPI) is approximately 33.9 kb in length, and is duplicated in *F. tularensis* strains (47), but only one copy of the FPI is present in *F. novicida*. Many of the investigations into FPI functions and virulence have been completed in *F. novicida* strain U112 because of the convenience this strain offers when preparing genetic mutants.

The FPI and other virulence genes are regulated by several transcription factors that are required for phagosome escape, including FevR (*Francisella* effector of virulence regulation), MglA (macrophage growth locus A) and SspA (stringent starvation protein A) (203-206). A model of *F. tularensis* virulence gene regulation has recently been proposed (207). MglA and SspA form a heterodimer that interacts with RNA polymerase (208). FevR binds the MglA–SspA complex, but not to either individual protein alone (209). Regulation of *Francisella* virulence is thought to be stress induced as ppGpp (guanosine-tetr phosphat e), which functions as a general stress signal, complexes with MglA–SspA to allow for high affinity interactions with FevR (207, 209, 210). It is suggested that the MglA–SspA–ppGpp–FevR complex allows FevR to bind its DNA promoter sequence stabilizing the RNA polymerase and facilitating
transcription of the FPI and other virulence genes (207). What triggers production of ppGpp is still unknown.

The FPI encodes a type six secretion system (T6SS) that is required for phagosome escape and intra-macrophage growth (211, 212). T6SSs are similar to bacteriophage contractile sheaths and are found in approximately a quarter of Gram-negative bacteria (213). The T6SS is anchored to the membrane by a baseplate complex of proteins. The shaft of the needle is composed of Hcp proteins, topped with the needle point VgrG protein trimer, and PAAR protein tip. Secreted effectors are expected to be loaded on to the PAAR tip and inside the Hcp needle. The Hcp tubule is surrounded by a contractile sheath, composed of VipA and VipB, which drives the needle through the bacterial membranes into the target cell. ClpV ATPase is responsible for disassembly and recycling of the contracted sheath.

In Francisella, IglC (intracellular growth locus C) is the Hcp homolog which composes the T6SS needle shaft (214). IglC is perhaps the most widely studied FPI protein because it was the first to be discovered and is required for T6SS activity and phagosome escape. The FPI also encodes a VgrG protein (212). IglG serves as the PAAR protein (215). It is suspected that IglF is a virulence factor loaded onto IglG for secretion (215). IglA and IglB bind to assemble the contractile sheath (211, 214). PdpB and DotU are baseplate proteins (212). Francisella lacks ClpV, but ClpB is required to disassemble the contracted sheaths (216). T6SS assembly appears near the poles of F. novicida cells and is dependent on iglF, iglG, iglI and iglJ (216). Other FPI genes, pdpC, pdpD, pdpE and anmK are not required for assembly of the T6SS (216). However pdpC and pdpD are required for phagosome escape in mouse macrophages and might be T6SS secreted virulence effectors (216).
The mechanism by which the T6SS results in degradation of the phagosome is still unknown. Many LVS and SCHU S4 FPI mutants remain trapped in the phagosome, do not replicate in host cells, and exhibit reduced virulence in mice (142, 205, 217-226). Expression of the FPI and T6SS genes, particularly iglC, are upregulated during intracellular growth of LVS and SCHU S4 (146, 227). Additionally, deletion of fevR or mglA prevents phagosome escape and intracellular replication (204, 221, 228), although the range of other effects are uncertain. FPI deletion mutants are often used in research to help elucidate immune evasion and virulence features of *F. tularensis*.

**STUDY RATIONALE**

Aerosol release of highly virulent strains is considered to be the primary deployment mechanism of *F. tularensis* in a bioterror attack (24). Understanding the microbiology and pathogenicity of highly virulent *F. tularensis* strains is important for public safety. Hence, this study employed the prototypic type A *F. tularensis* strain SCHU S4. Macrophages are typically important protectors during bacterial infections (229). However, macrophages do not efficiently kill *F. tularensis*. Macrophages are considered the primary targets of *F. tularensis* and are the predominant cell type infected immediately following exposure to *F. tularensis* via the respiratory route. Not only do macrophages serve as an important replicative niche for the pathogen, but macrophage death is a hallmark of tularemia and results in the loss of containment of type A *F. tularensis* allowing for dissemination. Little is known about what triggers macrophage death in tularemia. It is the aim of this study to identify the prompts that lead to macrophage death following infection with type A *F. tularensis*. 
THE COMPLEMENT SYSTEM

The research presented in this dissertation will demonstrate the importance of the complement system, in particular complement component C3, in determining the fate of human macrophages following infection with \textit{F. tularensis} subsp. \textit{tularensis} strain SCHU S4. Please allow for a brief introduction of extracellular complement activation and cell surface complement receptors [excerpt from (167)].

\cite{Brock2017}.

\textbf{Extracellular Complement Activation and Regulation}

For detailed descriptions of complement activation, the reader is referred to several excellent reviews \cite{230-232}. There are three pathways of complement activation (Figure 1), all of which result in the proteolytic cleavage of complement component C3, an abundant serum protein. The classical and lectin pathways both generate a C3 convertase composed of the peptides C4b and C2a. Through the classical pathway, IgM and IgG antibodies, when bound to their respective antigens, bind C1q and initiate the assembly of the C1qr2s2 complex. This complex cleaves C4 and C2 to produce a C3 convertase, designated C4bC2a. In the lectin pathway, the binding of certain carbohydrate patterns on microorganisms by either serum mannose-binding lectin (MBL) or ficolin proteins recruits and activates mannose-binding lectin-associated serine proteases (MASPs), which cleave C4 and C2 to generate the same C3 convertase. The alternative pathway is constitutively active with a low level “tick-over” of C3 in which an internal thioester bond is spontaneously hydrolyzed yielding C3(H2O). Hydrolyzed C3 has a conformation similar to C3b \cite{233} and can bind complement factor B (FB). Constitutively active factor D (FD) then cleaves FB, yielding an alternative pathway C3 convertase designated
Figure 1. Pathways of extracellular complement activation.
C3(H₂O)Bb. Like the C4b2a convertase, the alternative pathway convertase can cleave C3 to produce a bioactive short C3a peptide and a lengthier C3b peptide (Figure 2). C3a can serve as an anaphylatoxin. One of the primary functions of C3b is as a potent opsonin, which is attributed to its exposed thioester bond. Unless C3 has been spontaneously hydrolyzed by water, the C3b thioester can react with amine or hydroxyl groups. Formation of amide or ester linkages assures covalent attachment of C3b to nearby target surfaces, which makes them stable ligands for complement receptor-mediated uptake.

The alternative pathway also serves to amplify complement activation initiated by any of the three pathways by utilizing C3b as a focus for the formation of additional C3 convertases. Thus, C3b bound to a surface can form a complex with FB, which when cleaved by FD, becomes the new alternative pathway C3 convertase C3bBb. This amplification process results in increased localized activation of complement in proximity to susceptible microbial surfaces.

The binding of C3b to C3 convertases changes their specificity to C5-cleaving enzymes. Proteolysis of C5 produces the peptides C5a (another anaphylatoxin and potent chemotactic factor) and C5b, which initiates formation of the membrane attack complex. C5b binds C6, which then complexes with C7 and inserts into lipid membranes. The C5b67 complex then interacts with C8, followed by recruitment of multiple copies of C9, which polymerize to form a membrane pore, followed by rapid cell lysis.

Complement activation is tightly regulated by a variety of serum and membrane-bound proteins that control the three complement pathways [reviewed in (231, 234)]. The primary outcome is to limit host tissue damage that would result from unabated complement activation. Only two of these regulatory components will be mentioned here. Factor I (FI) is a serum protein that, along with certain cofactors, cleaves the alpha chain of C3b yielding inactivated
Figure 2. Simple schematic of C3 processing. Adapted from Nishida, et al. (235).
C3b (iC3b) (Figure 2). The formation of iC3b terminates amplification of the complement cascade by preventing formation of additional C3 convertases and also halts C5 cleavage, which diminishes assembly of the membrane attack complex. However, iC3b retains its opsonic activity, albeit with different complement receptor specificity than that of C3b. Factor H (FH) serves as a co-factor for FI and also competes with FB binding to C3b, preventing formation of the alternative pathway C3 convertase.

**Cell Surface Receptors for C3**

In addition to fluid phase complement factors, there are many membrane bound complement receptors that are important for complement regulation and complement-mediated clearance, phagocytosis and cellular signaling. Here we will discuss membrane bound complement receptors that have been shown to have important implications for *F. tularensis* virulence and host defense. For a comprehensive review of complement receptors, the reader is referred to two important reviews (234, 236). Complement receptor 1 (CR1 or CD35) is expressed on leukocytes and erythrocytes and binds C3b (Table 1). In addition to its role in complement-mediated opsonophagocytosis, CR1 serves as a membrane-bound cofactor for FI, leading to conversion of C3b to iC3b. CR3 is a heterodimer of the membrane proteins CD11b and CD18 and is expressed on neutrophils, macrophages, follicular dendritic cells, eosinophils, basophils, NK cells and platelets. CR3 shows high affinity for iC3b, which facilitates phagocytosis of iC3b-bearing particles. While we will focus primarily on the opsonic activities of these receptors, CR3 also plays a role in leukocyte trafficking to sites of infection and regulating cellular responses initiated by certain Toll-like receptors. CR4 is comprised of heterodimers of CD11c and CD18 and is expressed on monocytes and macrophages. CR4 also binds iC3b with high affinity.
Table 1. Human complement receptors with known involvement in the uptake of serum-opsonized *F. tularensis*.

<table>
<thead>
<tr>
<th>Complement Receptor</th>
<th>Preferred Ligand*</th>
<th>Cell expression*</th>
<th>Function in <em>F. tularensis</em> infection</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR1 (CD35)</td>
<td>C3b, C4b</td>
<td>Leukocytes (including neutrophils and macrophages) and erythrocytes</td>
<td>Uptake of serum-opsonized <em>F. tularensis</em> by human neutrophils</td>
<td>(137)</td>
</tr>
<tr>
<td>CR3 (CD11b, CD18)</td>
<td>iC3b</td>
<td>Neutrophils, macrophages, follicular dendritic cells, eosinophils, basophils, NK cells and platelets</td>
<td>Uptake of serum-opsonized <em>F. tularensis</em> by human neutrophils, macrophages and dendritic cells. Crosstalk with TLR2 – inhibition of TLR2-mediated inflammatory signaling</td>
<td>(121, 132, 137, 138)</td>
</tr>
<tr>
<td>CR4 (CD11c, CD18)</td>
<td>iC3b</td>
<td>Monocytes and macrophages</td>
<td>Uptake of serum-opsonized <em>F. tularensis</em> by macrophages and dendritic cells</td>
<td>(121, 137)</td>
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*Receptor ligand specificity and cell expression obtained from the cited reviews (234, 236).*
Apart from the involvement of complement receptors in the uptake of *F. tularensis* by phagocytic cells, little research has investigated the role of other cell surface complement receptors and regulators. For example, tissue macrophages also express the complement receptor CR1g, which binds the beta chain of C3, allowing the receptor to phagocytize both C3b- and iC3b-opsonized particles. CR1g is important for the clearance of pathogens (237, 238), but its role during *F. tularensis* infection has not been investigated. Mature B cells express CR2 (or CD21), which binds iC3b, C3dg and C3d peptides derived from FI-mediated cleavage of iC3b. One report suggests that subsets of mouse B cells employ CR2 along with the B cell receptor for uptake of *F. tularensis* (239). CD46, CD55 and CD59 are expressed by most host cell types, regulate complement activation and protect host tissues from complement-mediated damage by aiding in the inactivation of C3, disrupting the C3 convertases or preventing the formation of the membrane attack complex, respectively. Whether or not these complement regulators play a role in *F. tularensis* virulence or host defense is unknown.
CHAPTER 2: COMPLEMENT C3 AS A PROMPT FOR HUMAN MACROPHAGE DEATH DURING INFECTION WITH FRANCISELLA TULARENSIS STRAIN SCHU S4


ABSTRACT

Tularemia is caused by the Gram-negative bacterial pathogen *Francisella tularensis*. Infection of macrophages and their subsequent death are believed to play important roles in the progression of disease. Because complement is a particularly effective opsonin for *Francisella*, we asked whether complement-dependent uptake of *F. tularensis* strain SCHU S4 affects the survival of primary human macrophages during infection. Complement component C3 was found to be an essential opsonin in human serum not only for greatly increased uptake of SCHU S4 but also for the induction of macrophage death. Single-cell analysis also revealed that macrophage death did not require a high intracellular bacterial burden. In the presence of C3, macrophage death was observed at 24 hours post-infection in a quarter of the macrophages that contained only 1 to 5 bacterial cells. Macrophages infected in the absence of C3 rarely underwent cell death, even when they contained large numbers of bacteria. The need for C3, but not extensive replication of the pathogen, was confirmed by infections with SCHU S4 ΔpurMCD, a mutant capable of phagosome escape but of only limited cytosolic replication. C3-dependent *Francisella* uptake alone was insufficient to induce macrophage death, as evidenced by the failure of the phagosome escape-deficient mutant SCHU S4 ΔfevR to induce cell death despite opsonization with C3. Together, these findings indicate that recognition of C3-opsonized *F. tularensis*, but not extensive cytosolic replication, plays an important role in regulating macrophage viability during intracellular infections with type A *F. tularensis*.
INTRODUCTION

*Francisella tularensis* is the causative agent of the zoonotic life-threatening disease tularemia. Among the subspecies responsible for disease in human beings, *F. tularensis* subsp. *tularensis* (type A) is the most virulent and causes high morbidity and mortality when delivered via the respiratory route (24, 35, 75, 107). Very low minimum infectious doses have been reported with pulmonary challenge in humans (35, 37). In mice, alveolar macrophages are among the earliest cells infected following respiratory challenge (129-131). *F. tularensis* rapidly disrupts the phagosome and enters the cytosol of cultured macrophages where it replicates to high intracellular numbers (140-142). Likewise, phagocytic cell death and bacteremia follow infection of mice with *F. tularensis* and lead to secondary colonization and pathology in the spleen, liver and draining lymph nodes (93, 112, 148, 240). Indeed, one of the hallmark histopathological features of disseminated tularemia caused by the *F. tularensis* subsp. *tularensis* is the appearance of infected clusters of macrophages and myeloid cells (microgranulomas), which rapidly transform into necrotic foci (93, 112, 148, 149). Infection of macrophages with the laboratory strain *F. tularensis* subsp. *tularensis* SCHU S4 does not appear to activate caspase-1-mediated pyroptosis (101, 103), as has been reported for *Francisella novicida* strain U112 (99, 100). *F. novicida* infections in humans are exceedingly rare and are most often associated with immunocompromised humans (48). In contrast, strains of *F. tularensis*, including type A strains, that are pathogenic in otherwise healthy humans have been found to induce caspase-3-mediated apoptotic death in mouse and human macrophages (93, 112, 155-158).

The uptake of *F. tularensis* by macrophages can be mediated by a number of opsonic receptors, including the mannose receptor, the scavenger receptor, IgG-Fc receptors, nucleolin
and complement receptors CR3 and CR4 (132-139). Opsonization of *F. tularensis* with C3 results in particularly efficient phagocytosis by human macrophages (132, 134, 135, 138).

Several reports have suggested that the outcome of macrophage infection with *F. tularensis* is influenced by the receptor that mediates pathogen uptake (132, 133, 136, 138, 241). Clemens et al. (241) reported that the morphology of uptake of two *F. tularensis* Live Vaccine Strain O-antigen mutants by human macrophages differed when they were opsonized with C7-depleted serum compared to heat-inactivated human serum (HI-HS). They suggested that C3 opsonization of O-antigen mutants mediates greater physical interaction between the pathogen and macrophage surfaces, altering the morphology of phagocytosis. Geier and Celli (136) reported that CR3-mediated uptake of strain SCHU S4 by mouse bone marrow-derived macrophages resulted in somewhat impaired phagosome escape compared to that with non-opsonized bacteria. Dai et al (138) reported that CR3-mediated uptake of serum-opsonized SCHU S4, but not non-opsonized SCHU S4, inhibited Toll-like receptor 2 (TLR2)-dependent cytokine production, the phosphorylation of ERK1/2 and p38 mitogen-activated protein (MAP) kinases, and the activation of NF-κB in human macrophages. These findings indicate that the responses to C3-opsonized *F. tularensis* can be significantly different from those initiated by non-opsonized bacteria.

Given the importance of C3-mediated uptake in shaping the macrophage response to *F. tularensis*, we have asked whether C3 opsonization of *F. tularensis* subsp. *tularensis* alters macrophage survival. Specifically, we wished to determine whether opsonization of strain SCHU S4 with complement influenced cell death induction in infected primary human monocyte-derived macrophages (MDM).
RESULTS

Complement component C3 promotes uptake of SCHU S4 and induction of human macrophage death following Francisella infection.

As an initial test of the effects of heat-sensitive serum components, SCHU S4 bacteria were opsonized in medium containing either fresh-frozen human serum (HS) or heat-inactivated human serum (HI-HS) and used to infect human MDM. The uptake of bacteria and induction of macrophage death (measured as lactate dehydrogenase [LDH] release into culture supernatants) increased with increasing HS concentration (Figure 3A). Both half-maximum uptake and LDH release in HS were achieved at multiplicities of infection (MOIs) of 20 to 30 (Figure 4). Heat inactivation of HS significantly reduced bacterial uptake to that seen in the absence of serum (data not shown) and diminished macrophage death to levels observed in uninfected cells (Figure 3B). To determine whether the death-promoting effects of HS simply reflected greater initial bacterial uptake than occurred in the presence of HI-HS, MDM were infected under the two opsonization conditions at MOIs that yielded comparable initial bacterial burdens (Figure 3C). Despite comparable numbers of intracellular bacteria throughout the course of infection, significantly higher levels of cell death were seen in cultures infected in the presence of HS than in those infected in the presence of HI-HS.

The heat-sensitive opsonic effect of serum on Francisella has been associated with the action of C3 peptides, namely, iC3b, produced during complement activation and deposited on the bacterial surface (184, 187). To determine the role of serum C3 in MDM death during Francisella infection, SCHU S4 bacteria were opsonized in HS, C3-depleted HS, or control C5-depleted HS and used to infect MDM (Figure 5A). Following gentamicin treatment, all cultures were maintained in autologous HI-HS-containing medium to limit the opsonin effects to the
Figure 3. Heat-labile components in human serum promote SCHU S4 uptake and the induction of macrophage death. (A) MDM were infected with SCHU S4 (MOI = 230) in the presence of various concentrations of HS. Bacterial burdens and LDH release were measured at 3 and 24 h PI. *, significant differences compared to samples infected in 7.5% HS at the same time PI. This experiment was performed twice. (B) MDM were infected with SCHU S4 (MOI = 80) in the presence of 7.5% HS or HI-HS. *, significant differences in bacterial burden between groups or significant differences in LDH release compared to uninfected MDM at each time point. The results shown are representative of three independent experiments. (C) Bacterial burdens and LDH release were measured at the indicated times PI in HS at an MOI = 7 or in HI-HS at an MOI = 730. *, significant differences comparing HS to HI-HS cultures. Results are representative of three independent experiments.
Figure 4. Bacterial burden and MDM death as a function of MOI. MDM were infected with SCHU S4 with various MOIs in the presence of HS. Data labels indicate the actual MOI. Bacterial burden and LDH release were measured at 3 and 48 h PI. The results shown are representative of three independent experiments.
uptake phase of infection. Serum lacking C3 failed to enhance bacterial uptake or promote macrophage death, resulting in an infection equivalent to that seen in HI-HS. The addition of purified C3 protein to replenish C3-depleted serum restored its ability to enhance uptake of *Francisella* and promote macrophage death. Heat inactivation of the C3-replenished serum reversed this effect. Depletion of C5 resulted in intracellular bacterial numbers and cell death levels similar to those observed in HS-containing cultures. These findings provide strong evidence that both bacterial uptake and the induction of macrophage death during infection with HS-opsonized SCHU S4 are C3 dependent.

As has been previously shown (184, 187), opsonization of SCHU S4 with HS deposited iC3b on the surface of SCHU S4, which was not seen in the absence of serum (Figure 5B). Minimal C3 deposition on bacteria was observed after treatment with HI-HS, with only minor conversion to iC3b. Opsonization with C3-depleted serum failed to deposit any detectable C3 peptides. Replenishing C3-depleted serum with purified C3 restored the deposition of iC3b on the surface of SCHU S4. Whereas some C3b deposition occurred with heat-inactivated C3-replenished serum, there was no conversion to iC3b. This indicated that a strong correlation existed between the deposition of iC3b on the surface of SCHU S4 and the ability of opsonized bacteria to promote macrophage death.

**High levels of intracellular *F. tularensis* alone are neither sufficient nor necessary for the induction of C3-dependent macrophage death.**

We next asked whether C3 promoted cell death solely by promoting high cytosolic bacterial burdens. Infections of MDM with SCHU S4 were initiated in HS or C3-depleted HS after adjusting the MOIs to achieve equivalent initial bacterial burdens under the two conditions (Figure 6). Despite similar intracellular bacterial numbers throughout the course of infection,
Figure 5. Complement component C3 mediates enhanced bacterial uptake and macrophage death induction. MDM were infected with SCHU S4 (MOI = 80) in the presence of the indicated sera. *, significant differences in bacterial burden or LDH release when comparing test sera to HS at the indicated times PI. The results shown are representative of three independent experiments. (B) Deposition of C3 peptides on SCHU S4 (8 x 10^7 bacteria per lane) was detected via Western blotting. Purified C3 and iC3b proteins serve as markers. These results are representative of three independent experiments.
MDM infected with HS-opsonized SCHU S4 showed a significantly higher level of cell death than MDM infected with bacteria opsonized with C3-depleted HS (Figure 6A).

Because LDH release measures death among a population of cells and the intracellular burden likely varies among individual macrophages, a single-cell microscopic analysis of bacterial load and cell viability was performed. MDM monolayers were infected in HS or C3-depleted HS with MOIs that yielded comparable initial burdens. Inspection of MDM monolayers 3 h post-infection (PI) revealed no significant differences in the overall percentage of infected cells under these two infection conditions. The two infection conditions also yielded similar relative frequency distributions of the numbers of bacteria per MDM following uptake at 3 h PI (Figure 6B).

The replication of the pathogen within macrophages is illustrated in Figure 6A and by comparing the relative frequency distribution at 3 h PI to that at 24 h PI (Figure 6B and 6C). Macrophages containing more than 100 bacteria each were observed under both infection conditions at 24 h PI (Figure 6C). The percentage of uninfected cells did not decrease greatly from 3 to 24 h PI under either condition, indicating that secondary infection rates were low in these cultures. Infections initiated in HS and C3-depleted HS maintained similar relative frequency distributions of intracellular bacteria at 24 h PI, with the exception that there were more macrophages with greater than 100 bacteria under HS than under C3-depleted HS infection conditions (Figure 6C).

Despite these similar bacterial burdens, only cultures infected in the presence of C3 showed significant levels of macrophage death as measured by positive cytosolic staining with the fixable viability dye Zombie Red (Figure 6D and 6E). Analysis of single cell death events in C3-opsonized cultures was particularly informative. Over 25% of MDM that contained only 1 to
Figure 6. Macrophage death following Francisella infection requires C3 but not a high intracellular bacterial burden. To ensure similar levels of bacterial uptake, MDM were infected at a low MOI with SCHU S4 in the presence of HS or at a high MOI with SCHU S4 in the presence of C3-depleted serum. The results shown are averages from 3 independent experiments for which the average MOI was 24 in HS and 890 in C3-depleted HS. (A) *, significant differences in bacterial burden between the two uptake conditions or in LDH release compared to MDM infected in C3-depleted serum at each time point. (B to F) MDM on coverslips were stained with Zombie Red (red) and rabbit antiserum to Francisella (green) and then mounted in Prolong Gold with DAPI (blue). Individual MDM were enumerated based on their bacterial load and incidence of Zombie Red-positive staining for each uptake condition. (B and C) Relative frequency distributions for bacterial load at 3 h PI (B) and 24 h PI (C). *, significant differences in the percentage of MDM containing the indicated range of bacterial counts comparing HS and C3-depleted HS. (D) Representative images of Zombie Red-stained MDM infected in HS or C3-depleted HS. Scale bars, 20 μm. (E) Percentages of Zombie Red-positive cells found under each condition at 24 h PI. *, significant differences compared to uninfected MDM. (F) *, significant differences between the percentage of Zombie Red-positive cells in HS and C3-depleted HS found in each bacterial load category at 24 h PI.
5 bacteria were dead at 24 h PI (Figure 6D and 6F), indicating that high bacterial burdens were not necessary for cell death induction. Conversely, approximately half of the MDM that contained more than 100 bacteria each remained viable at 24 h PI (Figure 6D, left panel and 6F). Although MDM death during infection with C3-opsonized bacteria correlated with the number of intracellular bacteria, cell death in these cultures did not require a high bacterial burden, nor did a large number of intracellular bacteria ensure that macrophage death would result. Additionally, cell death in infected HS cultures was rarely observed in MDM that lacked bacteria, indicating that bystander cell death was a rare event.

In comparison to HS cultures, MDM infected in the absence of C3 showed a similar range of bacterial burdens at 24 h PI (Figure 6C) but little evidence of cell death (Figure 6D, right panel and 6E). Even in macrophages that contained very high bacterial loads (> 100 bacteria per MDM) following infection in C3-depleted HS, the frequency of cell death was not above that observed in uninfected cells (Figure 6F).

These findings led to the prediction that a mutant F. tularensis strain with limited capacity for cytosolic replication would still induce C3-dependent macrophage death. To test this hypothesis, infections of MDM with the replication-deficient SCHU S4 ΔpurMCD mutant were compared to wild-type (WT) SCHU S4 infections (Figure 7). Deletion of the purMCD genes, which are required for purine synthesis, has no effect on the ability of SCHU S4 to escape the phagosome in mouse (242) and human (Figure 8B and 8C) macrophages but has been shown to limit cytosolic replication (147, 242). Exposure of SCHU S4 ΔpurMCD to HS deposited iC3b on the bacterial surface, just as it did with WT SCHU S4 (Figure 8A). There was also no difference in the uptake of the WT and ΔpurMCD bacteria in HS-containing cultures (Figure 7A). As with WT SCHU S4, increasing the MOI in C3-depleted HS resulted in equivalent initial
Figure 7. Replication-deficient mutant SCHU S4 ΔpurMCD induces wild-type levels of C3-dependent macrophage death. MDM were infected with WT or ΔpurMCD SCHU S4 at a low MOI in the presence of HS or at a high MOI in the presence of C3-depleted serum. The results shown are averages from 3 independent experiments. The average MOI for WT was 22 in HS and 890 in C3-depleted HS. The average MOI for SCHU S4 ΔpurMCD was 17 in HS and 672 in C3-depleted HS. (A) *, significant differences in bacterial burden compared to the WT in HS or significant differences in LDH release compared to uninfected MDM at each time point. ns, not significant. (B to F) MDM infected in HS were stained with Zombie Red (red) and rabbit antiserum to Francisella (green) and then mounted in Prolong Gold with DAPI (blue). Individual MDM infected with either WT or ΔpurMCD SCHU S4 were enumerated based on their bacterial load and incidence of Zombie Red-positive staining. (B and C) Relative frequency distributions for bacterial load at 3 h PI (B) and 24 h PI (C). *, significant differences between the percentage of WT SCHU S4- and SCHU S4 ΔpurMCD-infected macrophages containing the indicated range of bacterial counts. (D) Representative images of Zombie Red stained MDM infected with either WT SCHU S4 or SCHU S4 ΔpurMCD in the presence of HS. Scale bars, 20 μm. (E) Percentage of Zombie Red-positive cells found at 24 h PI under each infection condition. *, significant differences compared to uninfected MDM. ns, not significant. (F) There were no significant differences between the percentages of Zombie Red-positive cells in WT SCHU S4- and SCHU S4 ΔpurMCD-infected MDM found in each bacterial load category at 24 h PI.
Figure 8. WT and mutant strains of SCHU S4 support similar levels of C3-deposition and traffic to the expected cellular locations in human macrophages.  (A) A Western blot was used to detect C3 deposition on WT and mutant strains of SCHU S4 when opsonized with HS, C3-depleted HS, or C3-replenished HS (4 x 10^7 bacteria per lane). Purified C3 and iC3b proteins serve as markers.  (B, C) MDM were infected with SCHU S4 WT or mutant strains in the presence of HS and analyzed for LAMP-1 co-localization by confocal microscopy.  The MOI were as follows:  WT = 49, ΔfevR = 31, ΔfevR + pfevR = 41, ΔpurMCD = 47.  Representative images (B) are presented as maximum projections of 1 μm Z-series stacks spanning the entire monolayer.  Scale bars represent 20 μm.  The average percent LAMP-1 positive bacteria in each infected MDM is shown in panel C.  * denotes significant differences compared to WT.  This complete experiment was performed once.
bacterial burdens for SCHU S4 ΔpurMCD in HS and C3-depleted HS (Figure 7A). While the ΔpurMCD mutant failed to replicate to the same extent as WT bacteria, it did induce the same level of macrophage death in a C3-dependent manner (Figure 7A).

These relationships were confirmed by single-cell confocal analysis of cell death events revealed by Zombie Red staining. There was no difference at 3 h PI in the relative frequency distributions of bacteria per MDM between WT and ΔpurMCD SCHU S4 following uptake in HS (Figure 7B). However, there was a significantly higher percentage of MDM infected with WT bacteria that achieved high bacterial burdens (> 100 bacteria per MDM) at 24 h PI than those infected with SCHU S4 ΔpurMCD (Figure 7C). Over 80% of the macrophages infected with C3-opsonized SCHU S4 ΔpurMCD contained fewer than 50 bacteria at 24 h PI. Nonetheless, macrophages infected in the presence of HS with either WT or ΔpurMCD SCHU S4 showed equivalent high levels of cell death (Figure 7D, 7E and 7F). Similar to WT infections, nearly 25% of the MDM infected with just 1 to 5 ΔpurMCD bacteria were dead at 24 h PI (Figure 7F). Thus, a mutant strain of SCHU S4 that lacked the ability to replicate to a high intracellular density was still able to promote host cell death when opsonized with C3-containing HS.

**Complement-mediated uptake alone is not sufficient for the induction of macrophage death following Francisella infection.**

To determine whether complement receptor engagement provides a sufficient signal for initiating MDM death, we opsonized the phagosome escape-deficient mutant SCHU S4 ΔfevR with HS or C3-depleted HS and used these bacteria to infect MDM. Deletion of the fevR gene eliminates the ability of SCHU S4 to escape the phagosome (221). Exposure of SCHU S4 ΔfevR
or the complemented mutant to HS deposited iC3b on the bacterial surface, just as it did with WT SCHU S4 (Figure 8A). Fluorescence confocal microscopy verified the expected intracellular localization of each strain 8 h PI and demonstrated a persistent co-localization between SCHU S4 ΔfevR and the lysosomal marker LAMP-1 (Figure 8B and 8C) (221). Complementation of the mutant with a fevR-expressing plasmid restored its ability to escape the phagosome.

Similar to the case for WT SCHU S4, the uptake of the ΔfevR mutant was greatly increased by opsonization with HS compared to C3-depleted HS, indicating that uptake of both strains was C3 dependent (Figure 9). As expected, the escape-deficient mutant showed impaired intracellular replication, and this trait could be genetically complemented with a fevR-expressing plasmid. Although the SCHU S4 ΔfevR strain was taken up in a C3-dependent manner, it failed to induce MDM death (Figure 9), indicating that complement receptor-mediated uptake by itself was not a sufficient signal for host cell death induction. Complementation with a fevR-expressing plasmid restored the ability of the mutant to access the cytosol and induce C3-dependent MDM death.

Neither the presence of large numbers of cytosolic C3-deficient bacteria nor the initial uptake of C3-opsonized bacteria was by itself sufficient to induce MDM death. For this reason, we tested a combination of these signals (Figure 10). MDM were first infected with WT bacteria in C3-depleted HS at a high MOI to achieve high cytosolic burdens. Following gentamicin treatment and removal of residual extracellular bacteria, the macrophages were infected with C3-opsonized WT or SCHU S4 ΔfevR bacteria, and MDM viability was measured. Challenging pre-infected MDM with C3-opsonized WT SCHU S4 led to MDM death. Challenging the same pre-infected macrophages with C3-opsonized SCHU S4 ΔfevR did not affect the viability of the
Figure 9. The phagosome escape deficient mutant SCHU S4 ΔfevR fails to induce macrophage death despite C3 opsonization. MDM were infected with WT SCHU S4 or the mutant strains in the presence of HS or C3-depleted HS. The MOIs were as follows: WT, 87; ΔfevR, 86; ΔfevR + pfevR, 96. Significant differences (*) in bacterial burden and LDH release compared to the WT in HS are shown for 48 h PI. The results shown are representative of three independent experiments.
Figure 10. Engagement of cell surface complement receptors is not sufficient for the induction of macrophage death, despite high cytosolic bacterial load. MDM were first infected at a high MOI with WT SCHU S4 in the presence of C3-depleted HS. This was followed by a second infection with either WT or ΔevR SCHU S4 in the presence of HS or C3-depleted HS. Significant differences (*) in bacterial burden or LDH release are shown compared to the primary infection of WT in C3-depleted serum alone (no secondary infection). ns, not significant. The results shown are averages from 3 independent experiments in which the average MOI for the primary infection with WT in C3-depleted HS was 1,889. The average MOI in the secondary infection was 189 for WT and 97 for ΔevR SCHU S4.
macrophages. Thus, even in MDM that contained large numbers of intracellular *F. tularensis*, subsequent binding and uptake of C3-opsonized bacteria did not ensure macrophage death. Only when C3 peptides were present on the WT SCHU S4 strain did MDM death result.

**DISCUSSION**

Because C3 is important for the uptake of *F. tularensis* (121, 132-135, 184, 187) and macrophage death is a common consequence of *Francisella* infection (93, 99, 112, 148, 160), we undertook this study to determine whether complement component C3 contributes to the induction of cell death in primary human macrophages during type A *F. tularensis* infections. A number of findings reported here are consistent with bacterial surface-bound iC3b having a major role in initiating macrophage death during type A *Francisella* infections. Unlike HS, C3-depleted HS failed to promote cell death even when similar levels of bacterial uptake were attained by adjusting the MOIs. Uptake and cell death induction were fully restored when C3-depleted serum was supplemented with purified C3 protein. Replenishing C3-depleted serum also restored the deposition of iC3b on the surface of SCHU S4, and heat inactivation of C3-replenished serum abolished both macrophage death and iC3b deposition. In contrast, C5-depleted serum was similar to HS in its ability to promote both bacterial uptake and host cell death, indicating that this downstream complement component was not involved. Our findings are consistent with the conclusion that iC3b plays an important role in controlling the viability of infected human macrophages in addition to its well-characterized effect on the uptake of type A *F. tularensis* by these cells (132, 133, 135, 138). Though we have not directly examined other opsonins found in normal human serum, the results with C3-depleted serum suggest that they lack the ability to promote macrophage death following infection with type A *F. tularensis*. 
The results of the current study predict that macrophage death would not occur following infection with type A *F. tularensis* in culture medium lacking complement activity. For example, opsonization with fetal bovine serum, which contains very low levels of C3 and little hemolytic complement activity (243), would not be expected to support this form of macrophage death.

We were surprised to find that cell death measured by single-cell analysis occurred independent of the presence of large numbers of cytosolic SCHU S4 bacteria. Rather, macrophage death appeared to be dependent solely on the presence of complement component C3 during initial infection. Cell death was rarely observed in macrophages infected in C3-depleted serum, whether the MDM contained a low or an extremely high bacterial load. Despite a correlation between higher bacterial burden and cell death frequency when SCHU S4 was opsonized with C3, over 25% of the macrophages that contained just 1 to 5 bacterial cells were dead at 24 h PI and approximately half of the MDM that contained more than 100 bacteria were still viable. These findings indicate that a high burden was neither sufficient nor necessary for C3-dependent macrophage death. Considered together, our results indicate that C3 is an important trigger for macrophage death independent of its role as an opsonin. Given that uninfected bystander cells in infected cultures did not die, even in the presence of C3, it seems reasonable to conclude that both infection and C3 are required to induce the death of a given macrophage.

The finding that C3-opsonized WT SCHU S4 could induce cell death without achieving high intracellular bacterial burdens led us to predict that a mutant strain with a limited capacity for intracellular replication would nonetheless behave like WT SCHU S4 as long as it bore iC3b. Both LDH release assays and single-cell microscopic analysis of macrophages infected with the
cytosolic replication-deficient mutant SCHU S4 ΔpurMCD confirmed the prediction that a high bacterial burden resulting from extensive cytosolic replication is not required for macrophage death.

*F. tularensis* infections in macrophages proceed from bacterial uptake and escape from endocytic vesicles to a phase of significant cytosolic replication. Because the death of human macrophages infected with type A *F. tularensis* is delayed until after 15 h PI in vitro, it has been reasonable to assume that bacterial replication is required to signal macrophage death and bacterial release. The findings that minimally infected MDM frequently underwent cell death and that other MDM with high bacterial burdens remained viable at 24 h PI question this presumed connection between pathogen replication and cell death.

A number of published studies using receptor blocking antibodies or small interfering RNA (siRNA) knockdown techniques have implicated complement receptor CR3 and its ligand iC3b in the uptake by human MDM of both *F. tularensis* subsp. holarctica and *F. tularensis* subsp. *tularensis* (132, 135, 137, 138, 184, 187). For this reason, we next asked whether signaling through complement receptors played an essential role in MDM death following infection with type A *Francisella*. Similar to the case for WT SCHU S4, the SCHU S4 ΔfevR phagosome escape-deficient mutant was taken up by MDM in a C3-dependent fashion. However, unlike the WT, the mutant failed to induce macrophage death, despite the presence of iC3b on its surface. This suggested that complement receptor engagement, although important for bacterial uptake, was not a sufficient signal for macrophage death induction.

We then asked whether the combination of complement receptor binding of C3-opsonized SCHU S4 and cytosolic *F. tularensis* constituted sufficient signals for the cell death response. MDM were first infected with a high MOI of WT SCHU S4 in the absence of C3 and
then challenged with either C3-opsonized WT SCHU S4 or C3-opsonized SCHU S4 ΔfevR, both of which were capable of binding to complement receptors for uptake in a C3-dependent fashion. Secondary infection with C3-opsonized WT bacteria induced death as expected. Secondary infection with C3-opsonized SCHU S4 ΔfevR failed to induce cell death, despite WT levels of uptake that were C3 dependent. Reversing the order of these two infections did not alter the results (data not shown). Thus, the presence of large numbers of cytosolic SCHU S4 lacking surface iC3b plus engagement of cell surface complement receptors was not sufficient to induce macrophage death, even when the two signals were combined. The fact that FevR controls the expression of a number of genes in *F. tularensis* (146, 204) does not alter the fact that surface complement receptor engagement, even in the presence of cytosolic bacteria, did not signal for cell death. Only when MDM were infected with WT SCHU S4 bearing surface iC3b did macrophage death result.

Apoptosis is a form of programmed cell death which is non-inflammatory and non-lytic, whereas pyroptosis is associated with the assembly of inflammasomes, the activation of inflammatory caspases (e.g., caspase-1), the release of the pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18, and cell lysis (244, 245). *Francisella novicida* has been reported to activate inflammasomes in both mouse and human cells (99-102). Type A *F. tularensis* does not appear to activate inflammasomes (101, 103). Rather, virulent type A *F. tularensis* has been more closely associated with macrophage apoptosis, as evidenced by the expression of activated caspase-3 in tissues of infected mice (112).

CR3-mediated uptake of serum-opsonized SCHU S4 by human MDM has been reported to inhibit TLR2-induced NF-κB activation and nuclear translocation, as well as to suppress the release of pro-inflammatory cytokines, including IL-1β (138). Consistent with this report, we
found that, unlike the pro-inflammatory *F. novicida* U112 strain (99, 101), neither C3-opsonized WT SCHU S4 nor C3-opsonized ΔpurMCD SCHU S4 stimulated significant IL-1β release (Figure 11). Thus, our findings extend earlier evidence (101, 103, 138) and suggest that C3-opsonized *F. tularensis* also fails to induce macrophage death by pyroptosis. It may indeed inhibit aspects of this pro-inflammatory form of cell death.

It is known that *F. tularensis* is very responsive to its environment (246). For example, growth of *F. tularensis* in different bacterial media can alter the virulence of the bacterium, both in vitro and in mice (247-250). Additionally, *F. tularensis* is capable of responding to increased concentrations of spermine (250), limiting concentrations of iron (251), and changes to mammalian temperatures (252), which is understood to reflect its adaptation to the host and intra-macrophage environment. It is possible that C3 deposition, the binding of Factor H (187), or C3b inactivation leads *F. tularensis* to alter its expression of important virulence properties.

It is also possible that C3 activation alters an essential aspect of the pathogen-host cell interaction. Clemens et al. (241) reported a different form of looping phagocytosis by macrophages infected with the *F. tularensis* Live Vaccine Strain opsonized with C3 than was seen with non-opsonized bacteria. Geier and Celli (136) have reported that uptake of C3-opsonized SCHU S4 via CR3 in mouse bone marrow-derived macrophages delayed and partially decreased phagosomal escape by the bacterium compared to its intracellular trafficking in the absence of the receptor. Additional studies will be necessary to determine whether either of these effects contributes to the high level of cell death seen in infections with C3-opsonized type A *F. tularensis*.

The experiments with sequentially infected macrophages (Figure 10) suggest that MDM death may require the entry of bacteria bearing C3 peptides into the cytosol. Tam et al. (253)
Figure 11. Neither WT nor ΔpurMCD SCHU S4 induced release of the inflammatory cytokine IL-1β. MDM were infected with WT SCHU S4 (MOI = 36), ΔpurMCD SCHU S4 (MOI = 50) or Francisella novicida strain U112 (MOI = 35) in the presence of HS. IL-1β release into culture supernatants was measured by ELISA at 24 h PI. The results shown are representative of three independent experiments.
reported that infection of a variety of nonimmune cells with a number of microbes capable of
carrying C3 peptides into the cytosol led to the activation of signaling pathways that were not
activated in the absence of C3 opsonization. The results of an array of experiments, including
transfection of C3-coated beads, were consistent with the conclusion that cytosolic C3 activated
intracellular signaling pathways associated with host defense. Although the authors postulated
the existence of a cytosolic C3-sensing system similar to the TRIM21 system, which recognizes
cytosolic IgG (254, 255), no such sensor has yet been described. These findings add to a
growing list of potential intracellular functions associated with complement (253, 256) and may
have relevance to the pathogenesis of a number of intracellular microbes capable of reaching the
cytosol.

Although the mechanism by which C3 facilitates the death of human macrophages
infected with type A Francisella remains unclear, the results presented here clearly demonstrate
that complement component C3 can play a pivotal role in determining the fate of human
macrophages during Francisella infections. As Francisella will likely encounter the
complement system during in vivo infections, a better understanding of complement-Francisella
interactions is needed. Like Francisella, other intracellular microbial pathogens are likely to
activate the complement system prior to host cell entry and may utilize C3 to regulate important
features of disease pathogenesis.

MATERIALS AND METHODS

Ethics statement. Protocols for the collection and handling of the blood and identifying data
from healthy human donors were approved by University of Kansas Medical Center Institutional
Review Board. The University of Kansas Medical Center is an approved Select Agent entity
(Registration No.C20070606-0662) responsible for oversight of all the research described here with type A *F. tularensis* strains.

**Reagents.** Histopaque-1077, gentamicin, protease inhibitor cocktail (P8340) and RPMI 1640 medium with L-glutamine were obtained from Sigma-Aldrich (St. Louis, MO). 10× Dulbecco’s phosphate buffered saline (DPBS) and a solution containing both penicillin (10,000 U/mL) and streptomycin (10,000 μg/mL) (17-602E) were obtained from Lonza (Walkersville, MD). Human fibronectin was purchased from Corning (Bedford, MA). Chocolate agar plates were obtained from Remel (Lenexa, KS). Rabbit antiserum to *F. tularensis* was purchased from Becton, Dickinson and Company (Sparks, MD). Alexa Fluor 488-conjugated donkey anti-rabbit IgG and the Zombie Red Fixable Viability Kit were purchased from BioLegend (San Diego, CA). Horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (ZyMax 81-1620) and ProLong Gold Antifade with DAPI (4’,6’-diamidino-2-phenylindole) were obtained from Life Technologies (Eugene, OR). HyGLO™ Quick Spray chemiluminescent HRP substrate was from Denville Scientific (Holliston, MA). Pooled human AB serum was from Atlanta Biologicals (Flowery Branch, GA). Commercial human serum (HS) with intact complement activity and C3-depleted HS were purchased from either Quidel (San Diego, CA) or Complement Technology, Inc. (Tyler, TX). C3-depleted serum was verified to be devoid of C3 via Western blotting. C5-depleted HS and goat polyclonal antiserum to human C3 (A304) were purchased from Quidel. Purified C3 protein (A113c) and purified iC3b protein (A115) were purchased from Complement Technology, Inc. To prepare C3-replenished HS, purified C3 protein was added to C3-depleted HS to obtain a final concentration of 1 mg/mL C3.
To prepare autologous HS, whole blood was allowed to clot for 30 minutes at 37°C and then centrifuged at 530 × g for 10 min at 4°C. The serum fraction was immediately collected, centrifuged again, and stored as aliquots at -80°C. When required, heat-inactivated HS (HI-HS) was prepared immediately prior to use by incubation at 56°C for 30 minutes.

**Bacterial strains.** Wild-type (WT) *F. tularensis* type A strain SCHU S4 was provided by Kevin King (Midwest Research Institute, Kansas City, MO), prepared under National Institute of Allergy and Infectious Diseases, National Institutes of Health contract SHHSM266200400002C. SCHU S4 mutant strains used in these experiments were kindly provided by Catharine Bosio (Hamilton, MT) with permission of Jean Celli (Pullman, WA). Mutant strains SCHU S4 ∆fevR (*Francisella* effector of virulence regulation, ∆FTT0383) (146), the complemented SCHU S4 ∆fevR + pfevR (∆0383pFNLT60383) (146), and SCHU S4 ∆purMCD (242) have been described previously. *Francisella novicida* strain U112 was kindly provided by Lee-Ann Allen (Iowa City, IA).

Stocks of bacterial strains were prepared by growth in supplemented Mueller-Hinton broth and stored as aliquots at -80°C as previously described (257). Complemented SCHU S4 ∆fevR + pfevR was grown in the presence of 10 μg/mL kanamycin, and SCHU S4 ∆purMCD was grown in the presence of 2.5% fetal bovine serum and 1.0% Proteose Peptone. At 2 to 3 days prior to infection, bacterial stocks were rapidly thawed and streaked for isolation on chocolate agar. Inoculated chocolate agar plates were incubated at 37°C with 5% CO₂ for 2 to 3 days. For growth of complemented SCHU S4 ∆fevR + pfevR, chocolate agar plates were supplemented with 25 μg of kanamycin. On the day of infection, 1 to 6 colonies were suspended in RPMI and the indicated serum was added as a source of opsonins at a concentration of 7.5%
(vol/vol), unless otherwise noted. No difference in bacterial viability was noted for WT or mutant strains following opsonization with HS, HI-HS, or C3-depleted HS.

**Monocyte-derived macrophages.** For the preparation of monocyte-derive macrophages (MDM), peripheral blood mononuclear cells were purified as described by Clemens et al. (140) from venous blood of healthy human donors without a history of tularemia. Diluted, heparinized blood was centrifuged (530 × g for 26 min at 25°C) over Histopaque-1077. Cells at the plasma-Histopaque interface were collected, washed and incubated in Teflon beakers (Savillex, Eden Prairie, MN) at 37°C in a humid atmosphere with 5% CO₂ for 5 to 7 days in RPMI medium containing 20% pooled human AB serum, 100 units/mL penicillin and 100 μg/mL streptomycin. Supernatant containing non-adherent cells was then removed and adherent MDM were eluted in RPMI containing 10 mM HEPES following incubation of the beakers on ice for 30 minutes. MDM were > 93% positive for CD14 and > 93% positive for CD11b by flow cytometry. Fifty thousand MDM were cultured per well in 96-well plates and incubated overnight to allow for adherence. Alternatively, 2 × 10⁵ MDM were plated in 24-well plates with or without fibronectin-coated coverslips. Non-adherent cells were removed the next day prior to infection.

**Infection.** Two- to 3-day colonies of individual SCHU S4 strains were suspended in RPMI, and serum was added to a concentration of 7.5% (unless otherwise noted). Bacteria were opsonized for 30 to 60 minutes at 37°C and then used to infect MDM in medium containing like serum. Following 2 h of infection at 37°C, the medium was removed and replaced with like medium containing 50 μg/mL gentamicin for 1 h to kill residual extracellular bacteria. All cultures were then washed 3 times in RPMI with 10 mM HEPES and placed in medium containing 7.5%
autologous HI-HS to minimize re-infection of the monolayer and restrict differences in opsonization to the uptake phase.

The multiplicity of infection (MOI) was determined following opsonization by dilution plating on chocolate agar. Macrophage bacterial burdens were determined by lysing MDM in 0.01% sodium dodecyl sulfate (SDS) in sterile water for 1 minute followed by dilution in DPBS and plating on chocolate agar.

Measurement of macrophage death. The CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI) was used to determine the percent lactate dehydrogenase (LDH) release. Culture supernatants were collected at the indicated times post-infection (PI) and stored at 4°C until assayed. LDH release was calculated as a proportion of total LDH released upon lysis of uninfected cell monolayers with detergent supplied in the assay kit. Each supernatant was assayed in triplicate.

Single-cell analysis by confocal microscopy. MDM on fibronectin-coated coverslips were infected as described above. For identification of dying macrophages at 24 h PI, coverslips were stained with the Zombie Red fixable viability amine-reactive dye according to the manufacturer’s instructions. Zombie Red was used at a concentration of 1:100 in DPBS for 20 minutes at room temperature, protected from light. The coverslips were then washed twice with kit wash buffer and once with DPBS. Coverslips were treated with 4% paraformaldehyde in DPBS for 1 h and then stored at 4°C in 70% ethanol. After verifying sterility, coverslips were washed in DPBS and incubated at room temperature for 1 h with a rabbit antiserum to *Francisella*. Following DPBS washes, coverslips were stained with Alexa Fluor 488-conjugated
donkey anti-rabbit IgG in DPBS. Coverslips were mounted in ProLong Gold with DAPI and imaged using the ACS APO 40×/1.15 numerical aperture oil objective of a Leica TCS SPE confocal microscope. Images are presented as maximum projections of 1 μm Z-series stacks spanning the entire monolayer.

For at least 200 individual macrophages per sample, the presence or absence of Zombie Red staining and the number of bacteria within each cell were recorded. Only those MDM with diffuse cytosolic Zombie Red staining were scored as positive.

**C3 deposition.** Deposition of C3 peptides onto the surface of SCHU S4 strains was monitored by Western blotting following a slight modification of the procedure described by Clay et al. (184). Several 2- to 3-day colonies of bacteria were suspended in RPMI medium containing 7.5% serum in 1.5-mL polypropylene microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA) that had been pre-blocked with 1% bovine serum albumin (BSA). Bacterial inputs were enumerated by serial dilution plating to ensure equal loading for gel electrophoresis. Following 1 h of opsonization at 37°C, bacterial suspensions were centrifuged at 12,000 × g for 5 min at 4°C. The bacteria were washed twice and resuspended in DPBS containing a protease inhibitor cocktail. Following heat treatment (97°C for 5 minutes) in Laemmli buffer, samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% acrylamide gels (Bio-Rad, Hercules, CA) and transferred to Immobilon®-P polyvinylidene fluoride membranes (Millipore, Billerica, MA). Membranes were blocked with 3% BSA in Tris-buffered saline containing 0.05% Tween 20 (TBS-T), which was also used for antibody dilutions. Membranes were then incubated with goat polyclonal antiserum to human C3 for 2 h at room temperature and washed 5 times in TBS-T. The membranes were then incubated in
HRP-conjugated rabbit anti-goat antiserum for 1 h at room temperature and then washed 5 times in TBS-T. Chemiluminescent HRP substrate was used for detection.

**Cellular localization by confocal microscopy.** For detection of *Francisella* co-localization with LAMP-1 at 8 hours PI, MDM on fibronectin-coated coverslips were infected as described in Methods. Coverslips were treated with 4% paraformaldehyde in DPBS for 1 hour and then stored at 4°C in 70% ethanol. After verifying sterility, coverslips were treated with 0.5 M ammonium chloride in DPBS to quench auto-fluorescence. Coverslips were then washed in DPBS and incubated at room temperature for 1 hour with rabbit antiserum to Francisella and mouse monoclonal IgG anti-human lysosomal-associated membrane protein-1 (LAMP-1) (H4A3, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). Following DPBS washes, coverslips were stained with Alexa Fluor 488-conjugated donkey anti-rabbit IgG and Alexa Fluor 555-conjugated donkey anti-mouse IgG (Life Technologies, Eugene, OR) in DPBS. Coverslips were mounted in ProLong Gold with DAPI and imaged using the ACS APO 63×/1.30 numerical aperture oil objective of a Leica TCS SPE confocal microscope. Images are presented as maximum projections of 1 μm Z-series stacks spanning the entire monolayer.

For determining percent co-localization of bacteria with LAMP-1, at least 75 infected MDM were analyzed per SCHU S4 strain. Bacteria in each cross-section through an infected MDM were scored as not co-localized (Alexa Fluor 488 positive only; green) or co-localized (Alexa Fluor 488 and Alexa Fluor 555 positive; yellow) with LAMP-1. The average percent LAMP-1 positive bacteria in each infected MDM was calculated.
IL-1β ELISA. MDM were infected as described in the Methods. At 24 hour PI, culture supernatants were collected, 0.22 μm filter sterilized and stored at -20°C. IL-1β concentrations were determined using the Human IL-1β ELISA Set II (BD, San Diego, CA) according to the manufacturer’s instructions.

Statistical analysis. Error bars in graphed data represent standard deviations of triplicate measurements. Data were analyzed using a Student’s t-test when comparing 2 groups or an analysis of variance (ANOVA) followed by Tukey’s post-hoc test when comparing 3 or more groups. A P value of < 0.001 was selected for comparisons within a single representative experiment and a P value of < 0.01 for comparisons of means of three independent experiments. Significant differences are indicated with an asterisk (*). In some cases, differences are identified as not significant (ns) between two indicated groups.
CHAPTER 3: *FRANCISELLA TULARENSIS* CONFRONTS THE COMPLEMENT SYSTEM

Brock, S.R. and M.J. Parmely. Frontiers Cellular and Infection Immunity, Volume 7, Article 523, December 2017, https://doi.org/10.3389/fcimb.2017.00523. Reused under Creative Commons Attribution 4.0 International Public License. An excerpt from this article introducing the complement system can be found in Chapter 1.

**ABSTRACT**

*Francisella tularensis* has developed a number of effective evasion strategies to counteract host immune defenses, not the least of which is its ability to interact with the complement system to its own advantage. Following exposure of the bacterium to fresh human serum, complement is activated and C3b and iC3b can be found covalently attached to the bacterial surface. However, the lipopolysaccharide and capsule of the *F. tularensis* cell wall prevent complement-mediated lysis and endow the bacterium with serum resistance. Opsonization of *F. tularensis* with C3 greatly increases its uptake by human neutrophils, dendritic cells and macrophages. Uptake occurs by an unusual looping morphology in human macrophages. Complement receptor 3 is thought to play an important role in opsonophagocytosis by human macrophages, and signaling through this receptor can antagonize Toll-like receptor 2-initiated macrophage activation. Complement C3 also determines the survival of infected human macrophages and perhaps other cell types. C3-opsonization of *F. tularensis* subsp. *tularensis* strain SCHU S4 results in greatly increased death of infected human macrophages, which requires more than complement receptor engagement and is independent of the intracellular replication by the pathogen. Given its entry into the cytosol of host cells, *F. tularensis* has the potential for a number of other complement-mediated interactions. Studies on the uptake C3-opsonized adenovirus have suggested the existence of a C3 sensing system that initiates cellular responses to cytosolic C3b present on invading microbes. Here we propose that C3 peptides enter the cytosol of human macrophages following phagosome escape of *F.*
Francisella tularensis and are recognized as intruding molecular patterns that signal host cell death. With the discovery of new roles for intracellular C3, a better understanding of tularemia pathogenesis is likely to emerge.

INTRODUCTION

Francisella tularensis is the bacterial pathogen responsible for the infectious disease tularemia. While tularemia is relatively rare, infection via the respiratory route can be particularly life-threatening when not treated with appropriate antibiotics in a timely fashion (24, 35, 75, 107). There are two subspecies of F. tularensis that account for the majority of infections in immunocompetent human beings. F. tularensis subsp. tularensis (type A) is considered the more virulent and will be the primary focus of this article. F. tularensis subsp. holarctica (type B) is also pathogenic in humans, but is less often associated with severe morbidity or mortality. Francisella novicida causes a tularemia-like disease in mice, but rarely infects human beings where disease is restricted to the immunocompromised (48). Following exposure to type A and type B F. tularensis by the pulmonary route, macrophages are among the first cells infected (129-131) and serve as an early and continuing replicative niche for the pathogen. Many receptors on the macrophage surface have been implicated in the uptake of F. tularensis (132-139), but complement receptors, especially CR3, have consistently been found to be the primary mediators of enhanced uptake of serum-opsonized F. tularensis by human macrophages (132, 137, 138). Once inside the cell, F. tularensis escapes the macrophage phagosome at a pace that varies with host species and replicates in the cytosol to high numbers (140-142).

Macrophage death is a common outcome following in vivo infection with F. tularensis and partially explains the appearance of necrotic foci in the livers, lungs, spleens and lymph
nodes in several mammalian species (93). The mechanisms and significance of macrophage death depend on the (sub)species of Francisella studied (99, 100, 112). For example, F. novicida is a highly proinflammatory pathogen, which induces rapid cell death in mouse macrophages that limits the ability of the bacteria to replicate in the host (99, 100). When describing the lifecycle of type A and type B F. tularensis, it is not uncommon to attribute macrophage death to an uncharacterized signal associated with the extensive cytosolic replication of the pathogen. For example, Lai et al. (160) reported on the effects of antibiotic treatment of J774.A1 macrophage-like cells infected with the F. tularensis Live Vaccine Strain (LVS). Treating cultures with ciprofloxacin within the first 12 hours of infection prevented both the replication of the bacteria and host cell death measured 24 hour post-infection (PI). If ciprofloxacin treatment was delayed until 15 hours PI, host cell death at 24 hours PI was similar in magnitude to that of untreated, infected control cells. The authors concluded that intracellular bacterial replication was required for the induction of macrophage death. Recent studies performed in our laboratory with type A F. tularensis (161) have questioned this interpretation. Intracellular replication of the SCHU S4 strain did not appear to be required for the induction of death in primary human macrophages.

During the course of these studies, we found that complement C3 played an important role in determining the survival of infected macrophages. Accordingly, in this article we review what is known about the interactions between F. tularensis and the complement system, discuss recent findings about the functions of intracellular complement, and propose new ways of thinking about the complement system in tularemia. Our primary focus will be on F. tularensis subsp. tularensis, although our use of the designation F. tularensis reflects an effort to include
relevant studies performed with subsp. *holartica* strains. We acknowledge this comes with the risk that future studies may prove some conclusions to be too inclusive.

**INTRACELLULAR ACTIONS OF COMPLEMENT**

Based on phylogenetic studies and the presence of C3-like proteins in porifera (sponges), Elvington et al. (258) have suggested that complement proteins served first to protect the intracellular space before evolving into a system for defending against pathogens at the cell membrane or in intercellular or intravascular domains of higher organisms. Only recently have we begun to appreciate the extent to which complement mediates important intracellular functions [reviewed in (259, 260)].

Many cell types produce C3 (261) and maintain intracellular stores of the protein (256). Elvington et al. (262) recently showed that intracellular C3 derives from a C3(H₂O) recycling pathway in which hydrolyzed, but not native, C3 is taken up from the extracellular environment. After being loaded with C3(H₂O), Farage B lymphoma cells released ~80% of the C3(H₂O) back into the culture medium, suggesting that the cells processed the remainder as a source of bioactive C3 peptides (256, 262). Complement receptors CR1, CR2, CR3 and CD46 did not appear to be involved in the uptake of C3(H₂O) in this recycling pathway (262).

Liszewski and colleagues (256) have extensively documented mechanisms of C3 activation within cells. For example, cathepsin L can cleave C3 to form C3a and C3b within the lysosomes of human T cells. The resulting C3a mediates the tonic intracellular activation of its complement receptor C3aR on the lysosome membrane, leading to baseline mTOR activation necessary for T cell homeostasis. Inhibition of cathepsin L or siRNA inhibition of C3aR expression led to T cell apoptosis. Activation of T cells through their antigen receptors resulted
in the transport of vesicles containing C3aR, C3 and cathepsin L to the cell surface. In an autocrine fashion, cleavage of C3 at the cell surface and binding of C3a and C3b to their respective receptors, C3aR and CD46, polarized the T cell to a Th1 phenotype (256, 263).

Intracellular activation of C3 is not limited to T cells. It has also been demonstrated in a variety of primary human cell types including monocytes, neutrophils, and B cells, as well as cultured human fibroblasts, ME-180 epithelial cells and umbilical vein endothelial cells (256). The proteases responsible for intracellular C3 cleavage vary among cell types. While cathepsin L mediates activation of C3 in T cells and monocytes, it is not responsible for the C3 activation observed in lung epithelial cells (256). Both cathepsin L and cathepsin B contribute to C3 cleavage within human intestinal epithelial cells (264). Factor H and FI can be taken up by cells and mediate intracellular cleavage of C3(H₂O) (262). Factor H has also been shown to interact with cathepsin L to increase cleavage of endogenous C3 yielding iC3b (265). Clearly, additional studies are needed to form a complete understanding of the significance of intracellular complement activation and its potential relationship with host defense.

Tam and colleagues (253) demonstrated that the presence of C3 peptides in the cytosol may also serve as molecular patterns that initiate danger signaling. A variety of C3-opsonized microbes, including both RNA and DNA non-enveloped viruses and the ΔsifA mutant of Salmonella, activated a NF-κB-driven luciferase reporter when present in the cytosol. The reporter was not activated when cytosolic entry was prevented or when pathogens were not opsonized with C3. Latex beads opsonized with a mixture of purified C3, FB and FD also activated NF-κB when transfected into HEK293T cells, suggesting that recognition of microbial patterns was not essential for this response. Signaling initiated by cytosolic C3 was independent of the signaling intermediates MyD88, TRIF, RIG-I, MDA5, Syk and STING, but appeared to
involve the mitochondrial antiviral signaling (MAVS) protein and TNF receptor-associated factor (TRAF). Cytosolic C3 sensing was observed in a variety of non-immune mammalian cell lines indicating that the proposed C3-detection pathway may be active in a number of cell types. However, it remains unknown whether macrophages sense cytosolic C3 in this manner. It should also be noted that these findings have not, as yet, been confirmed by other investigators and that a putative cytosolic C3 sensor has not yet been identified. This laboratory has identified tripartite motif-containing 21 (TRIM21) as a cytosolic sensor for IgG and IgM that also leads to the activation of NF-κB and interferon regulatory factors (254, 255, 266). The notion that C3 peptides mediate similar intracellular surveillance is quite provocative and certainly worthy of further study.

COMPLEMENT ACTIVATION BY *F. TULARENSIS*

In the conventional sense, *F. tularensis* is relatively serum resistant, meaning that it can survive in human serum (HS) without succumbing to the lytic effects of complement (122, 183). Serum resistance appears to be conferred by the lipopolysaccharide (LPS) and cell wall structure of *F. tularensis*, as evidence by the increased activation of complement (especially via the classical pathway) and susceptibility to serum-mediated lysis of LPS and capsule mutants (183-186). It is possible that mutations in other *F. tularensis* genes similarly alter the density of iC3b deposition following serum opsonization. Additionally, growth of *F. tularensis* in different culture media, which can alter the expression of high molecular weight surface carbohydrates, can affect the extent of C3 deposition on the bacterial surface (247). During opsonization of *F. tularensis* in HS, FH can bind to serum-opsonized *F. tularensis* (187), promoting the conversion of C3b to iC3b. This prevents the assembly of the membrane attack complex on the bacterial
F. tularensis may also express a surface CD59-like peptide (267), which may further hinder formation of the membrane attack complex by binding C8 or C9.

Several reports indicate that both the classical and alternative pathways are activated by F. tularensis (184, 187) and include the observation that C1q is required for C3 deposition on the bacterium under certain conditions (137, 184, 268). Natural IgM antibodies appear to play a role in complement activation by F. tularensis via the classical complement pathway (137, 185). Schwartz et al. showed that human serum (HS) from donors without a history of tularemia contained IgM antibodies that reacted with F. tularensis and mediated C3 deposition during the first 30 minutes of opsonization. Ben Nasr and Klimpel (187) reported that, although the classical pathway was activated, they were unable to detect binding of serum IgM to F. tularensis. Balagopal et al. (133) used immunofluorescence and ELISA to detect antibodies bound to F. novicida following opsonization of the bacteria with HS. The differences between these reports may reflect the bacterial strains that were studied or the use of different techniques to detect antibody binding. Regardless, there appears to exist sufficient evidence to conclude that the classical pathway can mediate C3 opsonization of F. tularensis.

The classical pathway may be particularly important when serum opsonization occurs for periods of less than 30 minutes. Longer periods of incubation with serum may allow significant alternative pathway amplification and C3b deposition (187). We have found that uptake of F. tularensis SCHU S4 by human macrophages over 3 hours is significantly reduced in C3-depleted HS compared to HS (161). However, there is no difference in the level of SCHU S4 uptake during a 3-hour incubation in C1q-depleted HS compared to HS (Brock and Parmely, unpublished). Likewise, Ben Nasr and Klimpel (187) reported that EGTA chelation of Ca^{2+} ions necessary for classical pathway activation in HS delayed the deposition of iC3b on F. tularensis.
if opsonization was limited to 30 minutes. After 45 minutes of opsonization, there was no
difference between the levels of iC3b deposition that occurred in HS and EGTA-treated HS (187). Conversely, treatment of HS with EDTA, which chelates both Ca\textsuperscript{2+} and Mg\textsuperscript{2+} and blocks both classical and alternative pathways, prevented any detectable iC3b deposition on SCHU S4 for at least 1 hour (187). Thus, it appears that both the classical and alternative pathways can mediate complement activation during serum opsonization of \textit{F. tularensis}.

**COMPLEMENT-MEDIATED UPTAKE OF \textit{F. TULARENSIS}\**

For a more comprehensive summary on the role of various cell surface receptors in the uptake of \textit{F. tularensis}, the reader is referred to an excellent review by Moreau and Mann (269). Our focus here will be limited to the receptors involved in complement-dependent uptake of \textit{F. tularensis}.

Complement component C3 was first shown to be important for optimal uptake of \textit{F. tularensis} by human monocyte-derived macrophages (MDMs) by replenishing C3-depleted serum with C3 protein (132). This resulted in a C3 concentration-dependent uptake of bacteria. The importance of C3 in the uptake of \textit{F. tularensis} has since been confirmed by several other groups (138, 161). Antibody blocking of CR3 with anti-CD11b and anti-CD18 reduced the uptake of HS-opsonized \textit{F. tularensis} by human MDM (132). Additional studies on blocking of complement receptors with antibodies have demonstrated that CR3 and CR4 are the predominant receptors involved in the uptake of HS-opsonized \textit{F. tularensis} by human macrophages (135, 137). However, blocking antibodies often show relatively modest effects in this context. The use of siRNA to inhibit expression of CR3 in human MDM has also demonstrated that CR3 is an important receptor for the uptake of serum-opsonized SCHU S4 (138) and is consistent with the
observation that C3 deposited on *F. tularensis* during HS opsonization is rapidly converted to iC3b. Inactivated C3b, not C3b, is the primary ligand for CR3 and CR4. CR1 does not appear to play a significant role in the uptake of serum-opsonized *F. tularensis* by human MDM, based on antibody blocking of CR1 (137).

Another experimental approach for determining important receptor-ligand interactions in C3-mediated uptake of *F. tularensis* has involved heat inactivation of HS to block complement activation or selective depletion of individual complement components, both of which yield greater effects on uptake than receptor blocking with antibodies. Perhaps blocking antibodies lack the affinity required to compete with high affinity natural ligands. Alternatively, incomplete blocking by antibodies to CRs may indicate that other receptors also mediate uptake of serum-opsonized *F. tularensis*. For example, Class A scavenger receptors have been shown to bind iC3b (270) and have been implicated in the uptake of serum-opsonized *F. tularensis* (134, 136). Balagopal et al. (133) suggested that Fcγ-receptors on human MDM could also contribute to uptake of serum-opsonized *F. novicida*. A role for CRIg in the uptake of serum-opsonized *F. tularensis* has not been investigated. CRIg is a complement receptor expressed on tissue macrophages which binds the beta chain of C3, allowing the receptor to phagocytize both C3b- and iC3b-opsonized particles. The receptor has been shown to be important for the clearance of pathogens (237, 238). Thus, although our knowledge of all the receptors that mediate the uptake of serum-opsonized *F. tularensis* may be incomplete, iC3b and CR3 likely play dominate roles in *Francisella* opsonophagocytosis by macrophages.

Complement C3-mediated uptake of *F. tularensis* is not restricted to macrophages. Ben Nasr et al. showed that C3 is also required for increased uptake of *F. tularensis* by human monocyte-derived dendritic cells. Opsonization with C3-depleted HS resulted in levels of
uptake similar to those observed with un-opsonized bacteria (121, 187). Blocking with antibodies to CD11b and CD11c identified CR3 and CR4 as important for enhanced uptake by dendritic cells (121). By contrast, blocking Fc receptors had little effect (121). In similar receptor blocking studies, Schwartz and colleagues (2012) found that CR1 (CD35) and CR3 (CD11b) mediated uptake of HS-opsonized *F. tularensis* by human neutrophils (137). These studies illustrate that different cells utilize a range of complement receptors to phagocytize serum-opsonized *F. tularensis*.

**EFFECTS OF COMPLEMENT ON *F. TULARENSIS* INFECTION OF MACROPHAGES**

Complement C3-opsonization appears to have more effects than simply increasing the number of *F. tularensis* bacteria that are phagocytized. Clemens et al. showed that both non-opsonized and HS-opsonized *F. tularensis* LVS were taken up by a unique process, referred to as “looping phagocytosis,” which involved spacious, asymmetric pseudopod loops (132, 241). An O-antigen-deficient LVS mutant was also phagocytized via looping in the absence of serum. However, the morphology of uptake of this serum-sensitive O-antigen mutant was altered in the presence of C7-deficient serum, which allowed for opsonization but prevented complement-mediated bacteriolysis (241). C7-deficient serum promoted uptake of the mutant in very tight loops. As serum-sensitive O-antigen mutants support increased C3-deposition (184), the authors suggested that an increased interaction between bacterial surface bound C3 peptides and macrophage complement receptors likely led to closer physical interactions at the host-microbe interface (241). An important unanswered question is whether this morphological change leads to different signaling in the host cell.
Geier and Celli (136) demonstrated that CR3 was important in the uptake by mouse bone marrow-derived macrophages (BMM) of HS-opsonized SCHU S4. Uptake of HS-opsonized SCHU S4 delayed the maturation of the phagosome as measured by the expression of LAMP-1. Baudino et al. have also reported a delay in phagosome maturation associated with the uptake of C3-opsonized apoptotic cells (271). Uptake via CR3 decreased the proportion of SCHU S4 bacteria that escaped phagosomes measured at 30 minutes PI (136). However, differences between phagosome escape of HS-opsonized bacteria by wild-type BMM and CD11b-deficient BMM were lost by 45 min PI, suggesting the effect was only temporary.

Geier and Celli (136) also concluded that HS-opsonization restricted the replication of the pathogen measured at 12 hours PI. Our own studies with human macrophages indicate that intracellular replication rates of SCHU S4 in human macrophages are not affected by C3-opsonization (161). SCHU S4 bacteria taken up in HS did not evidence any impaired ability to replicate to high densities within human primary macrophages. It should be noted that the time required for maximum F. tularensis escape from phagosomes appears to be greater in human THP-1 cells and primary macrophages (140) than is observed in murine BMM (136), and this may explain some of the differences between these studies. Similarly, the percentage of HS-opsonized bacteria that ultimately do escape the phagosome appears to be higher in human macrophages (typically ~80%) (140, 161) than mouse macrophages (typically ~55%) (136). Another distinction between these mouse and human studies is the use of human serum as the source of opsonins in both cases. This approach assumes that human C3 interacts with human and mouse complement receptors in a similar manner and that signaling from both species of receptors is also the same.
Dai et al. (138) reported that the binding of C3-opsonized SCHU S4 to CR3 altered the human macrophage response to infection by suppressing inflammatory cytokine production induced by TLR2. By comparing infection of MDM with SCHU S4 in C3-depleted and C3-replenished human serum, the investigators found that the presence of C3-opsonization decreased the phosphorylation of MAP kinases ERK and p38 and decreased levels of secreted TNF, IL-6 and IL-1β. Serum opsonization of SCHU S4 also resulted in less NF-κB phosphorylation and nuclear translocation. Using siRNA to inhibit expression of CD11b or TLR2, they demonstrated that TLR2 activated pro-inflammatory responses to *F. tularensis* and that CR3 inhibited TLR2 signaling. CR3 inhibition of TLR2 signaling was mediated through phosphorylation of Lyn kinase.

These studies indicate that the binding and uptake of C3-opsonized *F. tularensis* has a number of effects beyond the promotion of phagocytosis. C3 mediates a different morphology of uptake, significant changes in early host cell signaling pathways, subtle changes in intracellular trafficking and even altered survival of infected macrophages (161), which will now be discussed in more detail.

**C3 CONTROLS MACROPHAGE SURVIVAL DURING INFECTION WITH TYPE A *F. TULARENSIS***

While studying infections of human MDM with *F. tularensis* SCHU S4, we observed that large numbers of macrophages in infected cultures died by 24 hours PI and that cell death was C3-dependent (161). Death was rare among macrophages that had been infected in the presence of heat-inactivated or C3-depleted serum. Single cell analysis by confocal microscopy revealed that a high cytosolic bacterial burden was not required for C3-dependent macrophage death.
Many cells that bore only a few bacteria died as long as uptake had been facilitated by the presence of fresh HS. Conversely, half of macrophages that contained more than 100 bacteria did not die by 24 hours PI when bacteria were taken up in a C3-dependent fashion. Some MDM in cultures that had been infected with C3-opsonized SCHU S4 lacked any detectable bacteria, and very few of these bystander cells died. Acknowledging that differences in the extent of bacterial uptake existed between the two opsonization conditions, we equalized initial uptake of the pathogen in HS and C3-depleted HS by adjusting the multiplicities of infection (MOI). When initial uptake levels were equivalent, similar bacterial growth occurred under the two conditions, but macrophage death was only seen in the presence of C3. We concluded that high bacterial burden was neither necessary nor sufficient for cell death induction, which was confirmed by infections with the replication-deficient SCHU S4 ΔpurMCD mutant. Despite its limited intracellular replication, the HS-opsonized ΔpurMCD mutant strain escaped the phagosome and induced cell death at levels equivalent to those seen in wild type SCHU S4-infected cultures. C3-dependent uptake alone did not explain the induction of macrophage death, as shown by the failure of the phagosome escape-deficient mutant SCHU S4 ΔfevR to induce death of MDM, despite C3 opsonization. These findings suggest that two conditions need to be met for macrophage death. First, the cells must contain the pathogen. Second, uptake must occur in a C3-dependent fashion. While we do not yet know all of the details of this process, C3 appears to be emerging as an important factor in the induction of macrophage death that is so commonly seen in tularemia (93).

The experiments of Tam et al. (253) reviewed above provide a potential context for understanding how complement promotes macrophage death following infection with type A F. tularensis. This group demonstrated that cytosolic C3 peptides, likely in the form of C3b, can
activate NF-κB in a number of cell types. If this cellular response was initiated by the sensing of a cytosolic C3 peptide, as postulated by the authors, it would provide a reasonable hypothesis for explaining what we have observed during *F. tularensis* infections of human macrophages. Accordingly, we suggest that C3 peptides, including iC3b, are recognized in the cytosol of macrophages as molecular patterns and that the response to them is directed toward cell death, rather than NF-κB activation, by type A *F. tularensis* (Figure 12). This pathogen has a well-established anti-inflammatory phenotype, which includes its ability to inhibit NF-κB activation (101, 103-105, 189-191, 272, 273). C3-dependent uptake of *F. tularensis* by CR3 further inhibits NF-κB activation and pro-inflammatory gene expression in human macrophages (138).

Our prediction that C3 peptides induce macrophage death after SCHU S4 entry into the cytosol rests, in part, on studies with the phagosome escape SCHU S4 ΔfevR mutant. Strains deficient in FevR have been used by others to determine the importance of phagosome escape in various aspects of infection of and immunity to *F. tularensis* (146, 221). However, it should be noted that FevR is a global transcriptional regulator and controls the expression of a number of *F. tularensis* genes.

Our hypothesis would predict that C3 peptides enter the cytosol with *F. tularensis*. Human serum-opsonized *F. tularensis* bears covalently attached C3b and iC3b when it is taken up by cells, although the fate of these peptides during their extended stay in the phagosome is unknown. Phagosome escape by SCHU S4 in human macrophages is not complete until 8 hours PI. In this context, it remains unclear whether the pathogen contributes more to macrophage death induction than simply transporting the relevant C3 peptides into the cytosol, but we expect that it does. Tam and colleagues were able to elicit a NF-κB response in HEK293T cells by simply transfecting the cells with latex beads opsonized with the purified complement
Figure 12. Hypothetical model of serum-opsonized *F. tularensis*-induced human macrophage death. Human serum-opsonized *F. tularensis* delivers C3 peptides into the cytosol of macrophages upon phagosome escape of the pathogen. Cytosolic *F. tularensis* and sensing of cytosolic C3 peptides trigger macrophage death.
components C3, FB and FD (253). This would suggest that the NF-κB response does not require a microbial component and that cytosolic C3 peptides may be sufficient for this response. Tam et al. (253) did not report on the viability of the host cell following the transfection of C3 peptides into the cytosol. Thus, it remains to be determined if cytosolic C3 or cytosolic C3 fragments alone are sufficient to trigger macrophage death. Because both *F. tularensis* itself and CR3 engagement are capable of inhibiting NF-κB activation (138, 189, 191), we propose that the response to cytosolic C3 in *F. tularensis*-infected macrophages is diverted to a cell death pathway (Figure 12). This would explain the requirement for C3-opsonization and align our findings with the C3 sensing model proposed by Tam and colleagues.

Testing the hypothesis that cell death is initiated by the combined effects of C3 peptides and *F. tularensis* may be best undertaken by the direct delivery of these components into the cytosol of macrophages by methods such as those described by Meyer et al. (274) or Wu et al. (275). These experimental approaches would allow one to isolate the effects of the cytosolic microenvironment from those stages of infection preceding phagosome escape and evaluate more precisely the nature of C3, the bacterial components and the host cell recognition process that combines to trigger macrophage death.

What role does CR3, which mediates the uptake of C3-opsonized SCHU S4 by human macrophages, play in signaling cell death? Two observations may be relevant. Dai et al. (138) showed that human MDM infected with C3-opsonized SCHU S4 produced decreased amounts of IL-1β, a finding we confirmed in our own studies (161). Release of IL-1β requires inflammasome-mediated caspase-1 activation, which is not a characteristic of type A *F. tularensis* (101, 103). Thus, it is unlikely that CR3 binding of C3-opsonized SCHU S4 induces caspase-1-mediated pyroptosis in human macrophages as has been reported for mouse
macrophages infected with *F. novicida* (99, 100, 102). A second finding is also relevant. When we first infected human MDM at high MOI with SCHU S4 opsonized with C3-depleted serum and then infected these cells with C3-opsonized SCHU S4 Δ*fevR* mutant bacteria, the infected macrophages remained viable. Secondary infection with C3-opsonized wild type SCHU S4 resulted in macrophage death. Likewise, infection with C3-opsonized SCHU S4 Δ*fevR* alone also did not induce macrophage death, whereas C3-opsonized wild type SCHU S4 alone did. This illustrates that CR3 engagement is not a sufficient death signal, even in macrophages infected with high numbers of intracellular bacteria lacking C3 peptides. A reasonable explanation for these findings is that macrophage death requires both cytosolic *F. tularensis* and cytosolic C3 peptides. While it cannot be ruled out that CR3 signaling (138) contributes to macrophage death, there is also no reason to assume that CR3-mediated uptake of C3-opsonized *F. tularensis* is required for cell death induction. CR3 may simply be the most efficient receptor for assuring a high frequency of infected cells.

Although a putative C3 sensor remains to be characterized, two likely ligands – C3b and iC3b – are predicted by available information. First, Tam and his colleagues implied that the ligand was C3b by the few components – C3, FB and FD – that were required for opsonizing latex beads capable of activating NF-κB following their transfection into cells (253). Second, when *F. tularensis* SCHU S4 is opsonized with HS, iC3b is the predominant peptide covalently attached to the organism (161, 184, 187). This is consistent with the high levels of uptake of C3-opsonized *F. tularensis* by human macrophages being mediated by CR3 (132, 137, 138), which shows high affinity for iC3b (Table 1).

Previous studies of mice infected with type A *F. tularensis* revealed a caspase-3-dependent pathway of macrophage death (93, 112). However, to date, we have been unable to
determine the cell death pathway activated by C3-opsonized SCHU S4 in human MDM. The extended period of time between infection with *F. tularensis* and macrophage death has suggested to some that a causal relationship exists between achieving a sufficient intracellular bacterial burden and cell death induction. However, recently published findings (161) described above are inconsistent with this interpretation. If the kinetics of cell death reflects an apoptotic process, which can take up to 24 hours (276), then the delay in the appearance of overt signs of cell death (eg., LDH release) may reflect the variability in apoptosis induction among individual cells, the complex nature of signaling pathways or the lengthy degradative processes necessary for loss of membrane integrity leading to LDH release.

*Francisella tularensis* is likely to encounter the complement system quite early in infection, given the range of cells that produce complement components and the high concentrations of these components in body fluids, especially plasma, alveolar fluids and inflammatory exudates (261, 277-279). Complement activation by extracellular microbial pathogens has traditionally been viewed as benefiting the host by mediating clearance, leukocyte chemotaxis and altered vascular permeability at sites of infection. However, this view needs to be balanced by recent reports that *F. tularensis* utilizes complement to its own advantage by avoiding many complement effector mechanisms, regulating innate immune cell activation and controlling host cell viability to promote its own survival, intracellular replication and dissemination. Caution is urged in considering therapeutic approaches to infection that might affect complement activation by *F. tularensis*. Clearly, the pathogen has a complicated and largely mysterious relationship with the complement system that deserves additional study to appreciate fully its role in tularemia pathogenesis.
CHAPTER 4: SUMMARY AND FUTURE STUDIES

The research presented here illustrates a novel role of C3 in the induction of primary human macrophage death following infection with *F. tularensis* subsp. *tularensis* strain SCHU S4. It was found that engagement of cell surface complement receptors was not sufficient for the induction of macrophage death following infection with SCHU S4, even when there were high cytosolic bacterial burdens. Additionally, it was discovered that cytosolic replication and high cytosolic burdens of SCHU S4 were neither sufficient nor required for the induction of macrophage death. The apparent need for cytosolic C3-opsonized SCHU S4 and the recent report proposing the existence of a cytosolic C3 sensor (253) led to our proposal of a new hypothetical model of macrophage death following infection with C3-opsonized type A *F. tularensis*. We propose that C3 peptides enter the cytosol are sensed by a cytosolic receptor following phagosome escape of type A *F. tularensis*, and that type A *F. tularensis* directs the cytosolic C3 signaling pathway towards cell death rather than NF-κB activation, ultimately prompting host cell death.

Given the emerging roles of intracellular complement activity and the important role that C3 is known to play in the uptake of *F. tularensis* by macrophages and their subsequent death, it is clear that further research is needed to fully understand the role of complement C3 in tularemia. Additionally, the suggestion that cytosolic sensing of C3 can alter the outcome of *F. tularensis* infections may have far wider implications in the study of intracellular pathogens as a whole. Our results call for investigations into the potential role of C3 signaling during infection by other intracellular pathogens capable of reaching the host cell cytosol. Our findings also shed a light on the need for careful consideration of infection conditions and culture media used during in vitro infections in general. To date, the identity of a cytosolic C3 sensor has not been
reported. As there is only one report suggesting the existence of a cytosolic C3 sensor, more studies are needed to investigate this theory and to disclose the identity of the putative C3 sensor.

**FUTURE STUDIES INTO THE REQUIREMENTS FOR MACROPHAGE DEATH INDUCTION AND THE MECHANISM OF HOST CELL DEATH**

The research findings presented above have failed to determine all the requirements for cell death induction. While it has been proposed that cytosolic C3 is required for the induction of macrophage death, this hypothesis remains to be tested by analyzing the direct delivery of C3 peptides and *F. tularensis* into the cytosol. It must be determined if cytosolic C3 peptides alone are sufficient to elicit a response from primary human macrophages. Direct delivery of C3 peptides and *F. tularensis* into the cytosol could be accomplished by microinjection (274) or BLAST technology (275), provided the required equipment is available. Such capabilities would provide the tools necessary to determine the combination of signaling events required for macrophage death induction. In the absence of such technology, preliminary experiments attempting to deliver C3-opsonized latex beads into the cytosol of human macrophages by electroporation or detergent (N,N-Dimethyldodecylamine N-oxide, [LDAO])-mediated membrane permeabilization were completed with limited success. Additional studies are required to tease out the cellular location, contributing triggers and the nature of the signaling events necessary for cell death induction following infection with HS-opsonized type A *F. tularensis*.

Additionally, the mechanism of macrophage death following infection with HS-opsonized type A *F. tularensis* remains to be elucidated. Considering the proposed signaling pathways involved in cytosolic C3 sensing proposed by Tam et al. (253) and the suppressive capabilities of *F. tularensis*, a likely mechanism of macrophage death might be proposed.
As noted earlier when discussing the findings of Tam et al. (253), the NF-κB activation in response to cytosolic C3 did not appear to involve signaling via any known pattern-recognition receptors including TLRs, Fc receptors, or cytosolic DNA detection. Using siRNA knockdown, Tam et al. did determine that MAVS, TRAF proteins, p62 and TANK-binding kinase 1 (TBK1) were necessary components of the cytosolic C3 sensing pathway required for NF-κB activation. Aggregation of MAVS was not detected as a result of C3-sensing (253). TRAF6 appeared to be the primary TRAF protein involved in the cytosolic C3 signaling pathway, but simultaneous knockdown of TRAF2, -3, -5 and -6 yielded the greatest inhibition of C3-sensing (253).

The pathway described by Tam et al. likely involves formation of the TRADDosome for NF-κB activation (280, 281). In this signaling pathway, MAVS serves as a scaffold for formation of the TRADDosome. TRAF proteins, like TRAF6 or TRAF3, and TNF receptor type 1-associated death domain protein (TRADD) bind MAVS. TRADD recruits receptor-interacting protein kinase 1 (RIPK1). p62 is known to interact with RIPK1 and TRAF6 to play an important role in NF-κB activation (282). RIPK1 binds Fas-associated protein with death domain (FADD), completing formation of the TRADDosome. Interactions between TRADD, RIPK1 and FADD are mediated via death effector domains. TRAF ubiquitination of RIPK1 allows for recruitment of NF-κB essential modulator (NEMO). NEMO serves as a scaffold to form the inhibitor of κB kinase (IKK) complex, binding IKKα and IKKβ, which is responsible for phosphorylation of IκB and activation of NF-κB. Alternatively, NEMO can interact with NAP1, TBK1 and IKKe to phosphorylate interferon regulatory factors (IRFs). FADD can also recruit caspase-8 and its inhibitor FLICE-like inhibitory protein (FLIP) to the TRADDosome, sometimes resulting in cell death (281, 283, 284).
Very recently, LVS was shown to suppress K63 ubiquitination and the formation of TRAF6 and TRAF 3 complexes, including the phosphorylation and recruitment of TBK1 to the TRAF3 complex in murine BMM (191). LVS was even able to suppress the immune response (as measured by an interferon-β luciferase reporter) induced by known activators Pam3CSK4, LPS and cyclic-di-GMP, which activate the TLR2-MYD88, TLR4-TICAM1 and STING pathways respectively (191). LVS suppression of pro-inflammatory and interferon responses required live bacterial cells and type six secretion system activity and/or a cytosolic presence (191). The method by which LVS mediates this immune suppression are still unknown. However, the new data suggests that *F. tularensis* has the capacity to suppress the inflammatory response to cytosolic C3 described by Tam et al. (2014).

While it has been demonstrated that extensive caspase-3 mediated macrophage death occurs in mice following intranasal infection with type A *F. tularensis* (112), the mechanism by which macrophages die following infection with *F. tularensis* in the presence of C3 is still unknown. Additional studies are needed to elucidate the cell death pathway. Perhaps in addition to probing for caspase-3 activation in infected MDM, another interesting pathway to investigate in light of recent findings would be caspase-8 activation. At the TRADDosome, caspase-8 can cleave RIPK1 resulting in inhibition of NF-κB and IRF3 activation, and can ultimately mediate cell death (280, 281, 283, 285). Perhaps, *F. tularensis* inhibits the cytosolic C3 sensing pathway in such a manner that caspase-8 from the TRADDosome is able to initiate macrophage death.
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APPENDIX

C3-DEPENDENT UPTAKE OF *F. TULARENSIS* IN HUMAN MACROPHAGES

We have demonstrated that complement component C3 is required for enhanced uptake of *F. tularensis* and the induction of macrophage death (Figure 5). We have also shown that both are also dependent on the concentration of HS in the culture medium (Figure 3). Here we show that enhanced uptake of *F. tularensis* and subsequent macrophage death is dependent on the concentration of C3 present during uptake (Figure 13). Infection of MDMs with C3-opsonized SCHU S4 in the presence of C3-depleted serum that was replenished with varying concentration of purified C3 protein revealed that bacterial uptake and LDH release is dependent on the concentration of C3 present in the culture medium. Note that the concentrations of replenished C3 protein ranged from zero to 1 mg/mL, the concentration typically found in normal human serum.
Figure 13. Enhanced Uptake of C3-opsonized SCHU S4 and subsequent MDM death is dependent on the concentration of C3 present during uptake. SCHU S4 was pre-opsonized at 37°C for 50 minutes culture medium containing 7.5% HS or 7.5% C3-depleted HS that had been replenished with purified protein to 1 mg/mL C3. MDMs were then infected (MOI = 40) in the presence of the indicated serum conditions as described in Chapter 2. Note that MDMs infected in the presence of HS were infected with SCHU S4 pre-opsonized in 7.5% HS. MDMs infected in the presence of C3-depleted HS replenished with the various concentrations of C3 were all infected with SCHU S4 pre-opsonized in 7.5% C3-depleted HS replenished to 1 mg/mL C3. Bacterial burdens and LDH release were measured at 3 and 48 h PI. This experiment was completed once.
ANTIBODY BLOCKING OF COMPLEMENT RECEPTOR-MEDIATED UPTAKE OF C3-OPSONIZED *F. TULARENSIS*

As described in Chapter 3, antibody blocking of complement receptors CR3, CR4 and CR1 on human macrophages have been used to implicate CR3 as the predominant receptor mediating the uptake of C3-opsonized bacteria. We have been able to replicate this published data for uptake of SCHU S4 by human MDMs (137), which is presented as intracellular CFU per 100 MDM (Figure 14A). While the data presented in this manner appears compelling, the effects of antibody blocking are actually quite modest. This is demonstrated by presenting the exact same data as overall intracellular CFU (Log\(_{10}\)) as typically done in the literature, rather than by intracellular CFU per 100 MDM (Figure 14B). These modest effects may reflect that natural ligands ability to outcompete the blocking antibodies for receptor affinity. Alternatively, these results may indicate that other receptors are responsible for the uptake of serum-opsonized *F. tularensis*. It has been suggested that Class A scavenger receptors (134, 136) and Fc\(\gamma\)-receptors (133) may play a role in the uptake of serum-opsonized *Francisella*. As mentioned in Chapter 3, the role for CR1g, a complement receptor important for the clearance of C3-opsonized particles, in the uptake of serum-opsonized *F. tularensis* has yet to be investigated. Additionally, a yet unidentified mechanism may mediate the uptake of C3-opsonized *F. tularensis*. The observation presented here should not discount the role of CR3 in the uptake iC3b-coated *F. tularensis*, but it does warrant further investigation into other possible mechanisms of C3-mediated uptake of the pathogen.
Figure 14. Antibody blocking of complement receptor-mediated uptake of SCHU S4 by MDMs. MDMs were pre-blocked on ice for 20 minutes with 15 μg/mL of α-CD11b (CR3; LEAF™ Purified anti-mouse/human CD11b, BioLegend; Catalog # 101231), α-CD11c (CR4; LEAF™ Purified anti-human CD11c, BioLegend; Catalog # 301616), and α-CD35 (CR1; Azide free anti-human CD35, Acris; Catalog # BM2364) as indicated in RPMI. HS was then added to MDM cultures at the indicated concentrations. MDMs were infected with unopsonized SCHU S4 (MOI = 140) at 37°C for 1 hour. Culture medium was then removed and replaced with gentamicin containing medium. At 2 hours PI, cells were washed three times and bacterial burdens were determined as described previously. In panel A, the bacterial burden is shown as the number of intracellular CFU per 100 MDM. In panel B, the bacterial burden is shown as the Log_{10} of the number of intracellular CFU. Note that ΔHS is the same as HI-HS. This experiment is representative of two independent experiments.
THE PRESENCE OF C3 IN CULTURE MEDIUM DURING UPTAKE OF F. TULARENSIS MAY BE REQUIRED FOR INDUCTION OF MACROPHAGE DEATH

Preliminary data from Figure 13 suggests that pre-opsonization of SCHU S4 with C3 followed by infection in presence of C3-depleted HS does not lead to enhanced pathogen uptake or macrophage death. This finding is confirmed by data presented in Figure 15. Pre-opsonization of SCHU S4 in HS followed by infection in the absence of serum or the presence of HI-HS or C3-depleted serum does not result in the optimal level of uptake that can be achieved by infection in the presence of HS. Nor does infection with iC3b-coated SCHU S4 in the absence of serum or presence of HI-HS or C3-depleted HS result in macrophage death. It appears that the presence of C3 in the culture medium during the uptake of type A F. tularensis is required for the induction of macrophage death.

One possibilities that we considered was that activation of complement and the production of C3a that would occur with opsonization of SCHU S4 during the uptake phase would alter the macrophage response. Given some of the newly described consequences of C3aR engagement (256, 286), we briefly sought to explore a role for C3aR in the enhanced uptake of C3-opsonized F. tularensis or the induction of macrophage death. Very preliminary unpublished data shows that the addition of purified C3a to culture medium during infection did not enhance the uptake of HS-opsonized SCHU S4 by macrophages in the presence of C3-depleted HS, nor did it result in human macrophage (data not shown). We currently find it unlikely that C3aR signaling is responsible for the induction of macrophage death following infection F. tularensis in HS.

It is possible that the amount of C3 deposited on the bacterial surface during pre-opsonization is not sufficient to induce macrophage death following phagosome escape of F.
*tularensis*. Perhaps the required levels of cytosolic C3 for macrophage death induction can only be obtained when C3 present in the culture medium is taken up with *F. tularensis* in spacious pseudopod loops and subsequently released into the cytosol following pathogen escape from the phagosome. We have shown that levels of both uptake and MDM death rise with increasing concentrations of HS (Figure 3), and in particular C3 (Figure 13), present in culture medium during infection with SCHU S4. Combining this fact with our data presented in Figure 15, it is clear that C3 must also be present in the extracellular milieu during the initial infection with *F. tularensis* to trigger human macrophage death. Considering the rapid C3(H$_2$O) loading described by Elvington et al. (262), it is possible that iC3b-coated *F. tularensis* is taken up inside and escapes from C3-loaded vesicles which then prompts macrophage death. Complement receptors CR1, CR2, CR3 and CD46 were not involved in this C3(H$_2$O) recycling pathway. Perhaps the yet unidentified mechanism for C3(H$_2$O) loading is also responsible for the uptake of serum-opsonized *F. tularensis* that cannot be accounted for by antibody blocking of CR1, CR3 and CR4 by macrophages described above (Figure 14). Further investigations are required to test these hypotheses and elucidate the mechanism of cytosolic C3-prompted macrophage death following phagosome escape of type A *F. tularensis*. 
Figure 15. Complement component C3 in the culture medium is required for optimal uptake of HS-opsonized SCHU S4 by MDM and the induction of macrophage death. SCHU S4 was opsonized in HS and used to infect MDM in the presence of the indicated serum conditions. Bacterial burdens and LDH release were measured at 3 and 48 h PI. The data shown are averages of two experiments with and the average MOI was 125.
MECHANISM OF MACROPHAGE DEATH FOLLOWING INFECTION WITH HS-OPSONIZED F. TULARENSIS

As described previously, caspase-3 activation has been observed in F. tularensis-infected macrophages. However the mechanism of cell death induced by C3-opsonized SCHU S4 in human MDMs has not been characterized. Here we show caspase-3 activation in MDMs and TUNEL-positive cells at 24 h PI with SCHU S4 at a low MOI in HS, but not in MDMs infected at a high MOI in HI-HS (Figure 16). This experiment using immunocytochemistry has been completed once. We have been unable to detect activated caspase-3 in MDMs following infection with C3-opsonized SCHU S4, or even following in stauroporine treatment, using immunofluorescence and Western blotting techniques. It is possible that we have simply not overcome technical difficulties in these procedures or worked out the optimal time for activated caspase-3 detection. It is also possible that macrophage death following infection with C3-opsonized F. tularensis proceeds by another mechanism. Experiments should also be completed to determine if activation of caspase-8 occurs following phagosome escape of C3-opsonized F. tularensis as predicted by our hypothetical model. Clearly, further research is needed to fully elucidate the mechanism of human macrophage death in tularemia.
Figure 16. Caspase-3 activation and DNA-fragmentation in MDMs following infection with HS-opsonized SCHU S4. MDMs on coverslips were infected with SCHU S4 as described in Chapter 2. MOIs in HS and HI-HS are noted in the figure. At the indicated times PI, cells were stained for cleaved caspase-3 (Anti-cleaved caspase-3, Cell Signaling; Catalog # 9661) followed by the Histostain®-SP Rabbit Primary AEC staining Kit (red; Invitrogen, Catalog # 956143) or stained for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using the TACS®2 TdT DAB (diaminobenzidine) Kit (brown; Trevigen, Catalog # 4810-30-R).
EXTRACELLULAR RELEASE OF *F. TULARENSIS* BY HUMAN MACROPHAGES

In addition to measuring the intracellular bacterial burden in human macrophages, we also monitored the release of the pathogen into the culture supernatant (Figure 17). We found that the extracellular bacterial burdens increased over time at similar rates for infections in both HS and HI-HS. Observations in made regarding macrophage death and dissemination of type A *F. tularensis* (112) would have led to the prediction that under circumstances where macrophage death occurs, release of the pathogen will occur resulting in an increased extracellular bacterial burden that would track with the increase of LDH release. We would have also predicted that in the absence of macrophage death, little increase in extracellular bacterial burden would be observed. As expected that the number of bacteria in the extracellular milieu increased following infection in the presence of HS where macrophage death is commonly observed. It was somewhat unexpected to find such a comparable increases in the extracellular bacterial burden following infection in HI-HS where macrophage death is not observed. It is important to note that in the absence of MDMs, we saw no appreciable growth of SCHU S4 in the culture medium over the course of 48 hours (data not shown). This suggests that the increase in extracellular burden over time is not due to the replication of residual bacteria in the culture medium that were not completely removed following gentamicin treatment and washing of the monolayer after infection. It is possible that there is some other mechanism of pathogen egress from the macrophage apart from host cell death.
Figure 17. Extracellular release of SCHU S4 occurs in both the absence and presence of macrophage death. Macrophages were infected with SCHU S4 as described in Chapter 2 in HS or HI-HS with the indicated MOIs. At the indicated times PI, a sample of the culture supernatant was diluted in DPBS and plated on chocolate agar to determine the extracellular bacterial burden. Intracellular bacterial burdens and LDH release were measured also measure at the indicated times PI. This data is representative of three independent experiments.