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Understanding Virulence in the Brucellae and Francisellae: Towards Efficacious Treatments for Two Potential Biothreat Agents

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Auspices Statement

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LDRD Project Tracking Code: 08-FS-011
Amy Rasley, Principal Investigator

Abstract

Francisella tularensis, Yersinia pestis and *Brucellae* species are highly infectious pathogens classified as select agents by the Centers for Disease Control and Prevention (CDC) with the potential for use in bioterrorism attacks. These organisms are known to be facultative intracellular pathogens that preferentially infect human monocytes. As such, understanding how the host responds to infection with these organisms is paramount in detecting and combating human disease. We have compared the ability of fully virulent strains of each pathogen and their non-pathogenic near neighbors to enter and survive inside the human monocytic cell line THP-1 and have quantified the cellular response to infection with the goal of identifying both unique and common host response patterns. We expanded the scope of these studies to include experiments with pathogenic and non-pathogenic strains of *Y. pestis*, the causative agent of plague. Nonpathogenic strains of each organism were impaired in their ability to survive intracellularly compared with their pathogenic counterparts. Furthermore, infection of THP-1 cells with pathogenic strains of *Y. pestis* and *F. tularensis* resulted in marked increases in the secretion of the inflammatory chemokines IL-8, RANTES, and MIP-1β. In contrast, *B. melitensis* infection failed to elicit any significant increases in a panel of cytokines tested. These differences may underscore distinct strategies in pathogenic mechanisms employed by these pathogens.

Introduction/Background

Francisella tularensis subspecies *tularensis, Brucella suis, B. melitensis, B. abortus* and *Y. pestis* are classified as high priority pathogens and select agents by the Centers for Disease Control and Prevention (1, for complete list see: http://www.selectagents.gov/Select%20Agents%20and%20Toxins%20List.html). *Brucella* species are the etiologic agents responsible for the disease brucellosis, a zoonosis of worldwide importance. Impacts of the disease stem from losses due to reproductive failure in food animals and the loss of human productivity (1, 2, 3). Therefore, brucellosis not only jeopardizes the food supply but also causes undulant fever, a long, debilitating disease in humans. As such, *Brucella* species are recognized as potential agricultural, civilian, and military bioterrorism agents (1). The *Brucella* species have been differentiated primarily based on their host specificity, and although the three *Brucella* species listed above have been associated with human disease, *B. abortus* is most commonly associated with cattle, *B. melitensis* with goats, and *B. suis* with pigs (each of the other 5 *Brucella* species are similarly specific for different mammalian hosts) (2). The genetic basis underlying the different and specific host associations are

unknown. Since this specificity is likely defined by the interplay between host and bacterium, expressed *Brucella* surface exposed proteins and the ability of the host to detect these structures may be important in this host-pathogen interaction process. Pathogenic *Brucella* spp. express a Type IV secretion system that has been shown to be required for virulence (4).

Francisella tularensis is the causative agent of tularemia, a zoonotic disease that is readily transmissible via airborne methods, with an extraordinarily low infectious dose (1-10 organisms) (5). There are currently four recognized subspecies of *F. tularensis*, subspecies *tularensis* (Type A), *holarctica* (Type B), *novicida* and *mediasiatica*. The Type A strains are known to be highly virulent for mammals, including humans, compared with Type B strains although the reason for this disparity is currently unknown (6-9). The *novicida* subsp. has been associated with some incidences of human disease (10), although it is not considered to be pathogenic for immunocompetent individuals, while the virulence of subsp. *mediasiatica* strains has not yet been fully explored.

Y. pestis is the etiological agent of plague and is responsible for three of the most destructive human pandemics in recorded history (11). *Y. pestis* infection is often associated with high mortality rates if untreated and exhibits a short incubation period before the onset of morbidity and mortality (12). There is no widely available and effective vaccine for *Y. pestis* and multi-drug resistant strains are emerging. *Y. pestis* is a recognized biothreat agent based on the wide distribution of the bacteria in research laboratories around the world and on the knowledge that methods exist to produce and aerosolize large amounts of bacteria (1). We hypothesize that a greater understanding of molecular mechanisms of pathogenesis will ultimately lead to novel strategies to develop biothreat countermeasures and novel therapeutics.

The outcome of infection with these organisms depends largely on their ability to evade host immune defenses. A very effective way of doing this, as described for a number of infectious organisms, is to gain entry and survive inside host phagocytes, immune cells such as macrophages and dendritic cells. As such, it is very beneficial for any invading organism to avoid the phagocytic response by residing in the very cells meant to destroy them. *F. tularensis*, *Brucella* spp. and *Y. pestis* have all been demonstrated to reside within macrophages (*Y. pestis* does so only transiently) and, by largely unknown mechanisms, replicate in the cytosol as well (13-17, 18, 22). While *Y. pestis* does not replicate intracellulary, the bacteria have been shown to have a considerable intracellular phase, which may serve to prime the bacteria such that they are more resistant to killing by sentinel immune cells. It is apparent that these organisms have evolved to efficiently bypass critical host defenses, although the exact mechanism(s) remains unknown.

Adopting an intracellular lifestyle inside host phagocytes or, in the case of *Y. pestis*, spending a considerable phase of its lifecycle intracellulary, subjects pathogens to a harsh environment. As such, *F. tularensis*, *Brucella* spp. and *Y. pestis* must have a complex array of defenses in order to counteract the phagocyte environment and maintain a hospitable niche in which the bacteria can thrive. Many intracellular pathogens utilize sophisticated protein machines, known as secretion systems, which are capable of injecting bacterial effector proteins directly into host cells. Many of these effector proteins have been well described with functions including neutralizing the low pH characteristic of the macrophage compartment, phosphatases which effectively "turn off"

host proteins involved in immune signaling, etc. Such systems have been well described in *Y. pestis*, and to a lesser extent in *Brucella abortus* (13-15). Both of these pathogens express distinct secretion systems designed to overcome the phagocytic response via protein-protein interactions between host and pathogen, thereby maintaining an intracellular environment favorable to their survival. In contrast, our first analysis of the genome sequence of *F. tularensis* failed to identify such classic secretion systems in this organism (23). This is striking given the ability of *F. tularensis* to persist and replicate within the hostile environment of the macrophage. Whole genome comparisons and comparisons of transcriptional response between organisms of varying degrees of virulence may shed insight into how *F. tularensis* interacts with and evades the host phagocytic response and what genes or genomic regions may be involved.

Given the lack of licensed vaccines for these three pathogens to prevent human disease, it is critical to identify and understand the genetic basis of virulence (for molecular signature development) as well as the mechanism(s) used by these organisms to perpetuate human disease. This knowledge will help us understand the natural prevalence of these infectious agents and aid in the development of effective countermeasures in both human and animal populations.

Research Activities

The focus of this research has been to compare host responses to infection with pathogenic and nonpathogenic strains of *Brucella* and *Francisella* in order to gain insight into how these organisms cause human disease. For these studies, human THP-1 monocytes were infected with various strains of *Brucella* and *F. tularensis* (Table 1) and the cellular response assessed via cytokine and cytotoxicity analyses. The strains used in these studies were selected in order to maximize diversity in terms of virulence, geographic location and host specificity. Comparisons were made between 1) human monocytes infected with pathogenic non-pathogenic *Francisella* strains, 2) human monocytes infected with pathogenic and non-pathogenic *Brucella* strains, 3) human monocytes infected with pathogenic non-pathogenic *Yersinia* strains as well as 4) comparing the host response between infections with pathogenic *Francisella*, *Brucella and Yersinia*. Our goal was to identify commonalities and unique attributes in the host response to both intraspecies as well as interspecies infections.

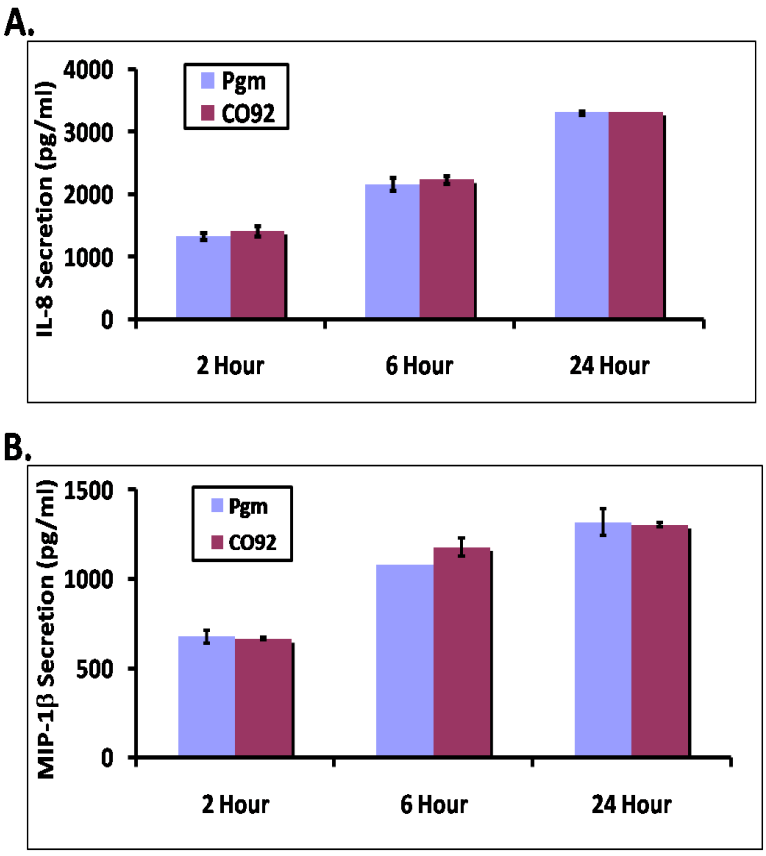
Strain	Species	Phenotype
16M	*Brucella melitensis*	Virulent, field isolated
TAP1	*Brucella melitensis*	16M-derived VirB mutant, attenuated in mice
2308	*Brucella abortus*	Virulent, field isolated
ADH3	*Brucella abortus*	2308-derived VirB mutant, attenuated in mice
1330	*Brucella suis*	Virulent, field isolated
DB2	*Brucella suis*	1330-derived VirB mutant, attenuated in mice
LVS	*Francisella tularensis* subspecies *holarctica*	Avirulent for humans, lab passaged
U112	*Francisella tularensis* subspecies *novicida*	Considered non-pathogenic for humans
Schu S4	*Francisella tularensis* subspecies *tularensis*	Virulent for humans, clinical isolate
CO92	*Yersinia pestis*	virulent for humans

Table 1. Description of *Francisella* and *Brucella* strains used in these studies.

The strains used in these studies are described in Table 1 and will be referred to in the remainder of the text by strain name (1st column, Table 1). Briefly, we performed experiments using both pathogenic (Schu S4, U112, 16M, 2308, 1330, CO92) and non-pathogenic (LVS, TAP1, ADH3, DB2, Pgm) strains of *F. tularensis*, *Brucella* and *Y. pestis*, respectively.

Results/Technical Outcome

THP-1 monocytes infected with disparate *Y. pestis* strains secrete marked levels of the proinflammatory chemokines Interleukin-8 (IL-8), Macrophage Inflammatory protein-beta (MIP-1β) and Regulated upon Activation, Normal T-cell Expressed (RANTES/CCL5) as early as 2 hours post-infection, with levels increasing dramatically over time (Figure 1A-C). Interestingly, this secretion did not depend on the virulence properties of the two strains as both were able to induce comparable levels of chemokine secretion after 2, 6 and 24 hours post-infection. The ability of *Y. pestis* to cause human disease is due, in part, on the ability of the bacteria to disseminate systemically. The upregulation of host chemokines by both pathogenic and non-pathogenic strains may provide an explanation as to how the bacteria are able to move from local lymph nodes to other organs, presumable by being carried to these sites within the hosts own immune cells. Chemokines are potent attractants of circulating immune cells and serve to focus the inflammatory response. As such, by inducing chemokine secretion early on, the bacteria may enable their own dissemination via recruiting host immune cells to the initial site of infection.



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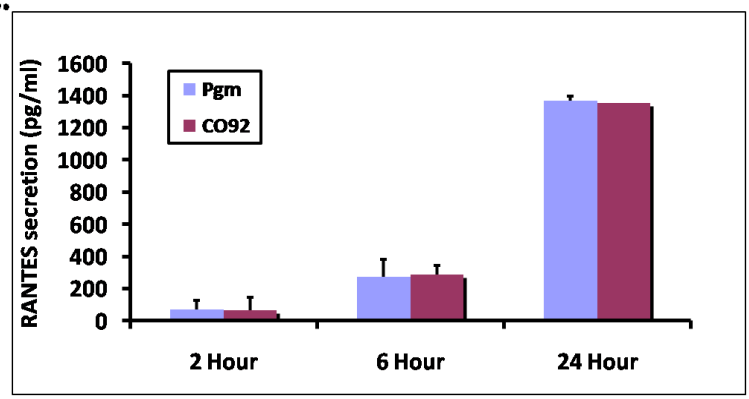


Figure 1. Secretion of inflammatory chemokines by human THP-1 monocytes infected with *Y. pestis* strains. Cultured THP-1 monocytes were infected with pathogenic *Y. pestis* CO92 and a nonpathogenic *Y. pestis* Pgm- strain for 1 hour. Panels A-C: Culture supernatants were harvested 2, 6, and 24 hours post infection and specific capture ELISAs were performed to quantify A) IL-8 secretion, B) MIP-1β secretion and C) RANTES secretion. Supernatants from uninfected THP-1 cells did not contain measurable levels of IL-8, RANTES, or MIP-1β. All results are presented as the mean of three separate experiments ± SEM.

In contrast, THP-1 monocytes infected with disparate *Brucella spp*. do not secrete marked levels of 30 known proinflammatory mediators (Figure 2A, B) as analyzed using Panomics cytokine protein arrays, compared with uninfected control samples after 24 hours post-infection. These results are consistent with previous reports describing the "stealth" nature of *Brucella* in that the bacteria appear to evade recognition by host immune cells. These observations may account for the ability of *Brucella spp*. to invade, replicate and survive in host macrophages over long periods of time.

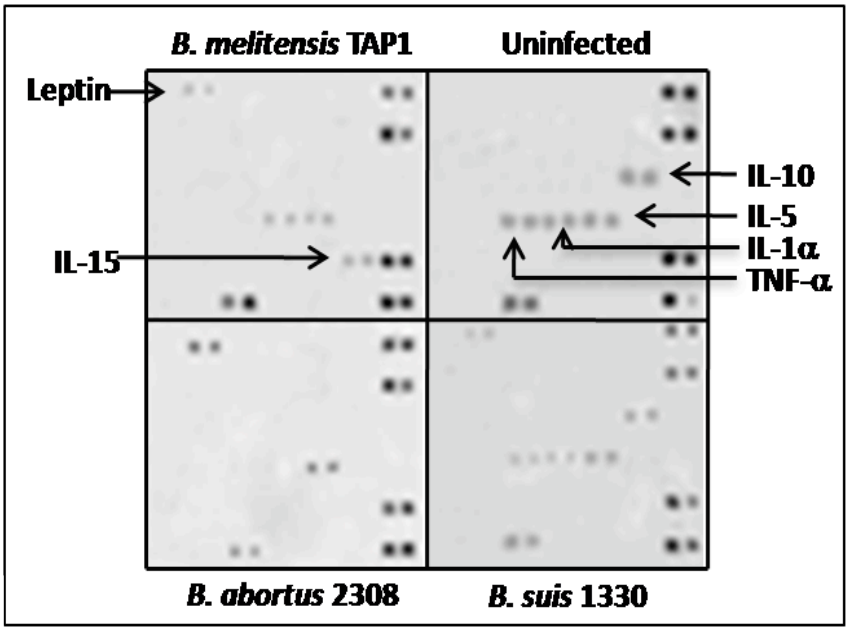
THP-1 monocytes infected with disparate *Francisella spp*. secrete marked levels of known inflammatory chemokines (Figure 3) as analyzed using Panomics cytokine protein arrays, compared with uninfected control samples after 24 hours post-infection. Interestingly, pathogenic strains appear to elicit more robust levels of secretion of IL-8 RANTES and MIP-1β compared with the non-pathogenic LVS strain. These results indicate that much like *Y. pestis*, *F. tularensis* may induce host chemokine secretion in order to facilitate its own dissemination to peripheral organs. Such findings might help explain, in part, the inability of LVS to cause human disease.

Cytotoxicity analyses of *Brucella* and *F. tularensis* infected THP-1 cells reveal low levels of cytotoxicity (Figure 4A, B) after 24 and 48 hours post-infection (14% and 25%, respectively). These data indicate that the bacteria are not likely replicating to high levels within the host cells in order to prevent cell lysis. Rather, it appears that there is a balance between bacterial replication and cell lysis such that a level of replication is maintained that minimizes host cell lysis and ensures bacterial survival.

Analyses of intracellular survival in human monocytes 2, 6 and 24 hours post-infection indicate that virulent strains of *Y. pestis*, *F. tularensis* and *Brucella* survive better than their avirulent counterparts (Figure 5). These data may underlie, in part, the

inability of avirulent strains to cause disease in immunocompetent humans. Interestingly, we have observed that mouse macrophages are permissive for the replication and survival of all *F. tularensis* strains we have tested (10 fully virulent clinical isolates, LVS, Novicida and Schu S4) to date, including LVS, a strain not considered to be able to cause human disease (data not shown). These observations may suggest that for *F. tularensis* infections, the mouse model may not be the ideal model to study human disease early during the infectious process. Studies to compare trafficking of *F. tularensis* in both human and mouse macrophages are currently ongoing in our laboratory.

A



B

Human Cytokine Array 3.0

	1 2	3 4	5 6	7 8	9 10	11 12	13 14
A	Apol/Fas	Leptin	Rantes	ICAM-1	IL-2	IL-7	POS
B	CTLA	MIP1α	TGF-β	VCAM-1	IL-3	IL-8	POS
C	Eotaxin	MIP1β	IFN-γ	VEGF	IL-4	IL-10	NEG
D	GM-CSF	MIP4	TNF-α	IL-1α	IL-5	IL-12(p40)	NEG
E	EGF	MIP-5	TNFRI	IL-1β	IL-6	IL-15	POS
F	IP-10	MMP3	TNFRII	IL-1ra	IL-6R	IL-17	POS

Figure 2. Brucella-infected THP-1 human monocytes do not secrete appreciable levels of inflammatory mediators as determined by antibody-based protein arrays which utilize the sandwich ELISA method for detecting protein. A. THP-1 monocytes were uninfected or infected with various strains of *Brucella* at an MOI of 10 for 1 hour followed by Gentamicin treatment for 2 hours. Culture supernatants were harvested 24 hours post-infection and assayed for the presence of 36 cytokines and chemokines using the Panomics TranSignal Human Cytokine Antibody Array 3.0. B. Key of layout of the Human Cytokine Array 3.0. The relative expression levels of cytokines (dots) were determined by comparing signal intensities.

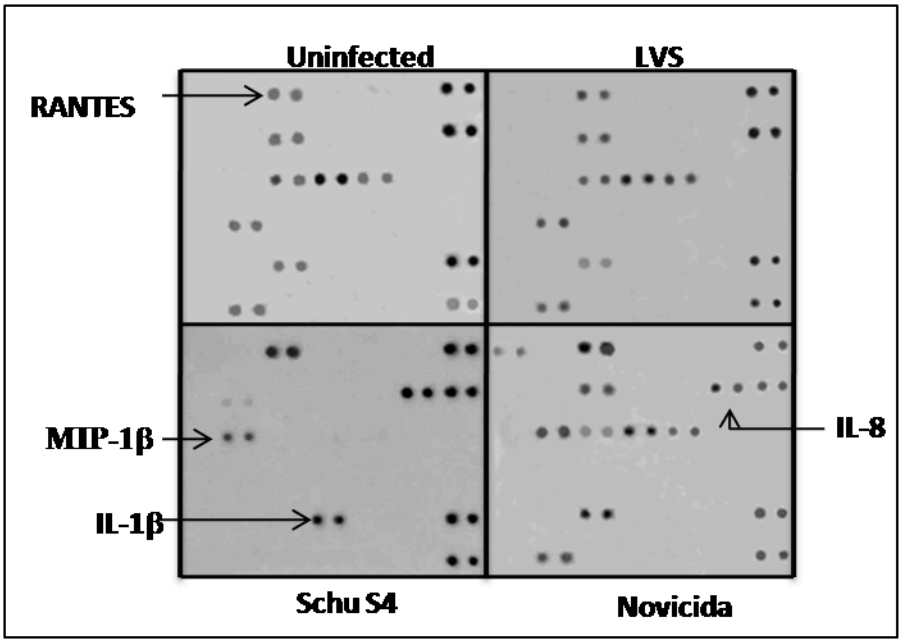
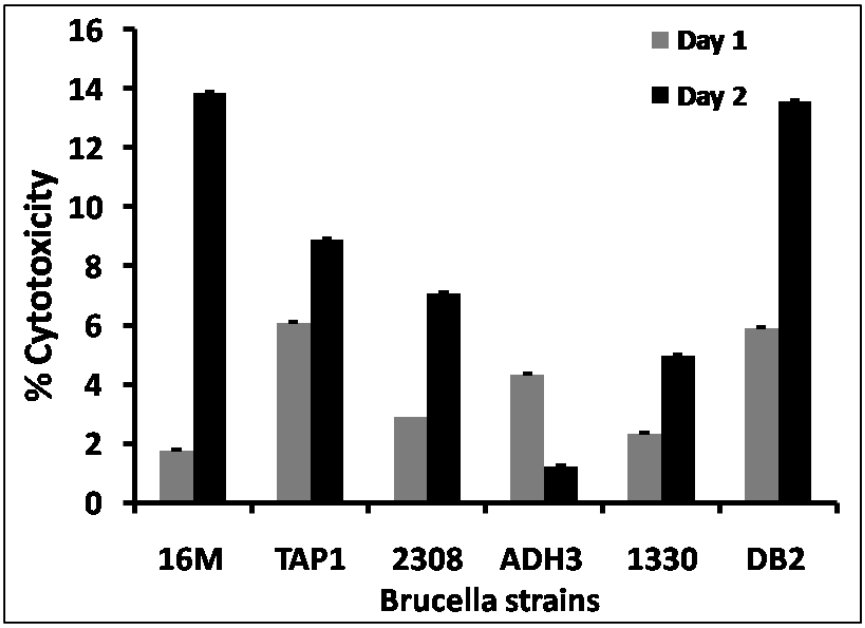


Figure 3. *Francisella*-infected THP-1 human monocytes secrete inflammatory cytokines and chemokines as determined by antibody-based protein arrays. THP-1 monocytes were uninfected or infected with various strains of *Francisella* at an MOI of 10 for 1 hour followed by Gentamicin treatment for 2 hours. Culture supernatants were harvested 24 hours post-infection and assayed for the presence of 36 cytokines and chemokines using the Panomics TranSignal Human Cytokine Antibody Array 3.0. The relative expression levels of cytokines (dots) were determined by comparing signal intensities. The cytokine antibody array key is shown in Figure 2B above.

A



B

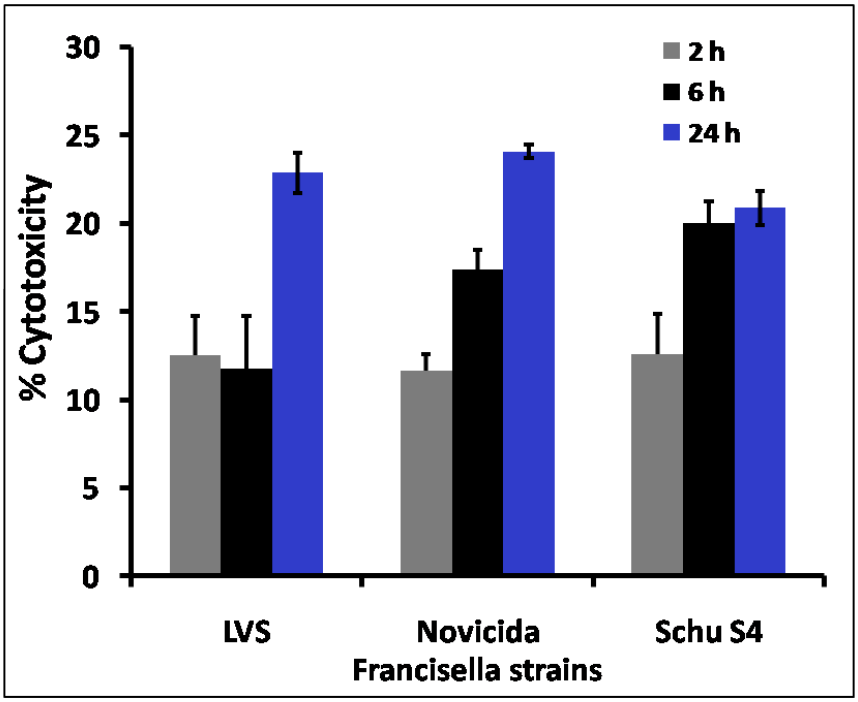


Figure 4. Cytotoxicity analyses of human monocytes infected with disparate strains of *Brucella* and *Francisella*. Human monocytes were infected with various Brucella and Francisella strains at an MOI of 10 for 1 hour, followed by a 2 hour Gentamicin treatment. A. Culture supernatants from THP-1 cells infected with *Brucella spp*. were harvested 24 and 48 hours post-infection and assayed for the presence of lactate dehydrogenase. B. Culture supernatants from THP-1 cells infected *Francisella spp*. were harvested 2, 6 and 24 hours post-infection and assayed for the presence of lactate dehydrogenase.

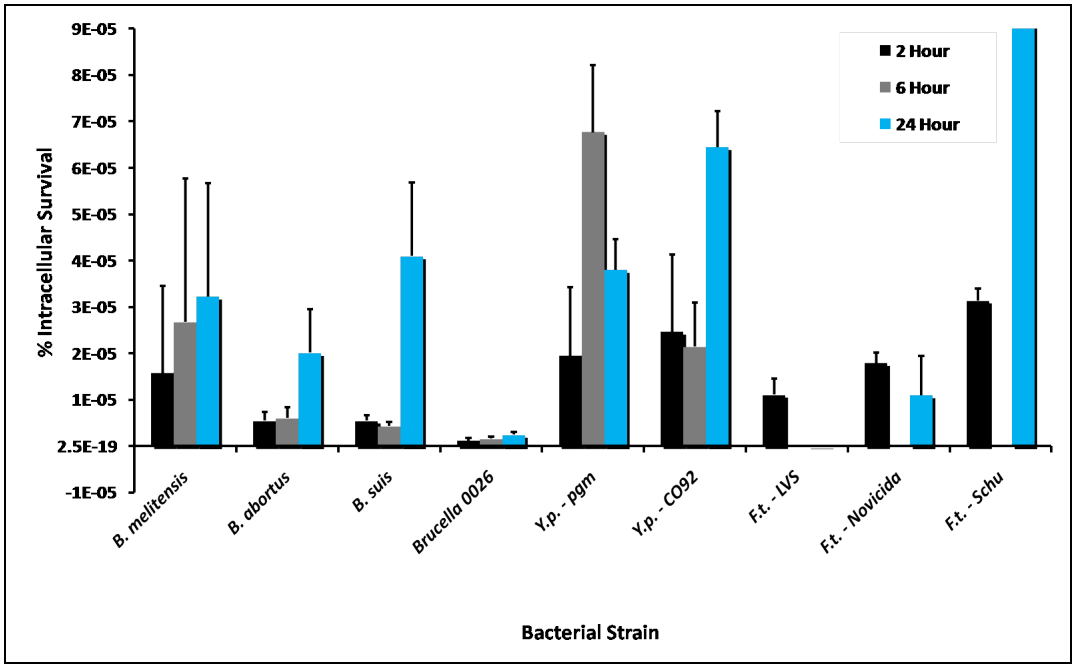


Figure 5. Comparison of survival in human monocytes of various *Brucella*, *Francisella* and *Yersinia* strains. Human monocytes were infected at an MOI of 10 for 1 hour, followed by a 2 hour Gentamicin treatment. Cells were lysed at 2, 6 and 24 hours post-infection using 1% saponin and plated to enumerate CFU.

Summary

Taken together, our preliminary results demonstrate that the host response to infection with three disparate biothreat pathogens is distinguishable, to some extent, based on host cytokine profiles. Pathogenic *F. tularensis* and *Y. pestis* strains induced marked secretion of known host chemokines, and may highlight a strategy whereby pathogenic isolates recruit host immune cells to the site of infection in order to aid their own dissemination to peripheral organs. Interestingly, Brucella infection resulted in minimal secretion of a number of known host inflammatory mediators and may help explain in part, the ability of pathogenic Brucella strains to survive in host macrophages for extended periods of time.

All three pathogens demonstrate a marked ability to survive intracellulary after 24 hours post-infection compared with their non-pathogenic counterparts, an observation that may account, in part, for the inability of avirulent strains to cause human disease. Given the ability of Brucella species to survive intracellulary in host macrophages for long periods of time, future studies should include analyses of host innate immune responses with a focus on Toll-like receptor signaling pathways and oxidative response pathways.

Exit Plan

The data presented in this report laid the foundation, in part, for a Strategic Initiative at LLNL aimed at rapid countermeasure development for Select Agent pathogens (Jane Bearinger, PI). The goal of this effort is to rapidly identify and validate targets in both the pathogen and host that can be exploited for downstream countermeasure development. Further, experiments aimed at characterizing *F. tularensis* interactions with host cells will provide the basis for an NIH proposal to be submitted in FY10. Efforts to identify metabolic targets for countermeasure development in *F. tularensis* has been submitted to DTRA for funding consideration.

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