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MACROPHAGE SELENOPROTEINS RESTRICT INTRACELLULAR REPLICATION OF *FRANCISELLA TULARENSIS*

A Dissertation in

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by

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Abstract

The micronutrient selenium (Se) has been suggested as a promising adjuvant for pharmaconutrient intervention in clinical applications. Use of Se in cancer research and for viral infections has been well documented. It has been less well defined in bacterial infections. The application of the micronutrient as a novel therapeutic is rooted in the fact that adequate and supplemental levels of Se are required to maintain optimal expression of selenoproteins. Selenoproteins are a particular class of proteins which contain Se in the form of the amino acid, selenocysteine. It has been well established that deficiency of Se is deleterious to human health, and in the last two decades a large body of work has been published correlating deficiency with individuals suffering from chronic and in a small number of cases acute bacterial infections. Moreover, the increased interest of pharmaconutrient intervention using Se supplementation to improve patient outcome has been supported by evidence that selenoproteins are important mediators of inflammation. Of the 25 selenoproteins expressed in humans and 24 expressed in mice over half perform antioxidant or redox function. In addition to maintaining redox homeostasis, selenoproteins have also been attributed to a wide array of other bimolecular functions vital to immune response. Some that have been more clearly defined then others.

I was particularly interested in addressing the role of selenoproteins during acute infection, specifically understanding how these proteins influenced macrophages a subset cells that are part of the innate immune response. Macrophages are important mediators of immune defenses against bacteria and have the capacity for bactericidal activity. Therefore, I investigated the effect of Se and selenoproteins on macrophages function during an acute intracellular bacterial infection. *Francisella tularensis*, the causative agent of tularemia, is an intracellular bacterium that preferentially infects phagocytes. I have determined that *F. tularensis* lacks the metabolic machinery to specifically incorporate Se into its proteome and the inability to accumulate elemental selenium in its biomass, which indicates the bacteria, is unable to utilize Se. Furthermore, empirical data I have collected shows that Se supplementation does not affect the physiology of the bacteria. For these reasons, *F. tularensis is* ideally suited to investigate the effects of Se on macrophages during acute bacterial infection. Upon entry into a host cell, *F. tularensis* is able to escape the phagosomal compartment, and initiate replication, multiplying approximately 50 to 100 fold within 24 hours.

To determine the effect of Se on *F. tularensis* infection, groups of mice were maintained on Se-deficient (<0.01 ppm of Na_2SO_3) or Se-supplemented (0.4 ppm Na2SO3) defined diets. While 50% of Se-supplemented mice died following infection with *F. tularensis*, 100% of Se-deficient mice succumbed suggesting that Se plays a significant role in protection from infection. Since, F. *tularensis* infects and replicates primarily in macrophages, I investigated the bacterial replication in Se-deficient (treated with 0 nM of Na_2SO_3) and Se-supplemented (200 nM of Na_2SO_3) macrophages. Interestingly, Se-supplemented macrophages restricted bacterial growth by about 20 fold compared to Se-deficient cells. Next, the role of selenoproteins in restricting bacterial growth was tested by using the macrophages derived from $Trsp^M$ mice, which are unable to synthesize selenoproteins in macrophages. $Trsp^M$ macrophages were unable to control the replication of *F. tularensis* compared to wild-type (WT) macrophages. Consistent with these results, $Trsp^M$ mice were significantly more susceptible to *F. tularensis* infection than WT mice. Furthermore, bacterial burden in the livers and spleens of $Trsp^M$ mice were significantly higher than WT mice indicating that macrophage selenoproteins are essential for restricting bacterial replication and promoting host survival.

Given that *F. tularensis* temporally activates the host's autophagy pathway to meet its nutrients and energy requirements during the proliferative phase, and aid in host immune evasion. I examined the expression of autophagy markers in the Se-deficient and Se-supplemented macrophages. The data indicated that Se supplementation suppressed autophagy in a selenoprotein dependent manner. I hypothesize that Se via specific selenoprotein(s) suppresses autophagy limiting available nutrients thereby restricting bacterial replication. This restriction in bacterial replication may lead to a less sever systemic infection and reduces *Ft.* pathogenesis. These date provide insight into the influence of micronutrients on infectious disease pathogenesis and suggest novel strategies to manage infectious diseases

The work presented here focuses on 4 goals: 1.) To assess the potential for Se adjuvant intervention as a therapeutic approach in the treatment of acute bacterial infections. 2.) To validate the *F. tularensis* pathogen model. 3.) To address the role of selenium via selenoproteins on the restriction of intracellular *F. tularensis* replication. 4.) To examine the underlying mechanisms driving the phenotypic restriction of bacterial replication that we have observed under selenium supplemented conditions.

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Abbreviations:

Autophagy related	……..	**ATG**
Bone marrow derived macrophages	……..	BMDM
cluster of differentiation, cluster of designation or classification determinant	……..	CD
Copper	……..	Cu
Francisella tularensis	……..	*F. tularensis*
Glutathione	……..	GHS
Glutathione Peroxidase 1	……..	GPX1
Glutathione Peroxidase 2	……..	GPX2
Glutathione Peroxidase 3	……..	GPX3
Glutathione Peroxidase 4	……..	GPX4
Glutathione Peroxidase 6	……..	GPX6
Iodothyronine Deiodinase	……..	DIO1
Iodothyronine Deiodinase	……..	DIO2
Iodothyronine Deiodinase	……..	DIO3
Iron	……..	Fe
Live Vaccine Strain	……..	LVS
Mechanistic target of rapamycin	……..	mTOR
Methylseleninic acid	……..	MSA
Methionine-R-sulfoxide	……..	MSRB1
Manganese	……..	Mn
prostaglandin E_2	……..	PGE2
Reactive Oxygen Species	……..	ROS
Reactive Nitrogen Species	……..	RNS
Selenium	……..	Se
Selenocysteine	……..	Sec
Selenocysteine Insertion Sequence	……..	SECIS
Selenium nanoparticles	……..	SeNP
Selenomethionine	……..	SeMet
Selenophosphate synthetase 2	……..	SEPHS2
Selenoprotein F	……..	SELENOF
Selenoprotein H	……..	SELENOH
Selenoprotein I	……..	SELENOI
Selenoprotein K	……..	SELENOK
Selenoprotein M	……..	SELENOM
Selenoprotein N	……..	SELENON
Selenoprotein O	……..	SELENOO
Selenoprotein P	……..	SELENOP

Selenoprotein S	……..	SELENOS
Selenoprotein T	……..	SELENOT
Selenoprotein V	……..	SELENOV
Selenoprotein W	……..	SELENOW
Sodium Selenite	……..	Na_2SeO_3
Thioredoxin reductase 1	……..	TXNRD1
Mitochondrial Thioredoxin reductase	……..	TXNRD2
Testis Thioredoxin-glutathione reductase	……..	TXNRD3
Zinc	……..	Zn

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Chapter 1: (Review) The role of selenoproteins during acute bacterial infection

1.1 Introduction:

The CDC's 2013 report claimed that over 2 million individuals in the United States would become ill with antibiotic resistant infections, and approximately 23,000 of those cases would result in death (1). In 2002 there were 1.7 million healthcare acquired infections in the United States (2). Roughly 30% of the healthcare acquired infections were reported to be caused by gram-negative bacterial infections (2). A high percent of healthcare acquired infections are antibiotic resistant strains of bacteria, spurring a concerted effort by the CDC and other agencies to advocate antibiotic stewardship to the medical community. The 2014 CDC report highlights that gram-negative bacteria associated with healthcare acquired infections are of particular concern including: *Clostridium difficile* and carbapenem-resistant *Enterobacteriaceae* (1). These particular bacteria meet the criteria for elevated risk including: availability of effective antibiotics, clinical impact, transmissibility and economic impact to name a few (1). The economist Douglas Scott's report furnished by CDC translates to billions of dollars in medical and incidental costs (3). This cost will only rise if antibiotic efficacy declines and therefore additional therapeutic measures must also be explored.

There has been a growing appreciation for the influence of dietary nutrients on the microbiome as well as host response to infectious disease. The micronutrient selenium (Se) is known to regulate immune functions via selenoproteins, a class of proteins that contain the 21st amino acid selenocysteine. However, the mechanisms by which selenoproteins regulate immune functions during an acute infection are not clear. The need to find novel therapeutic strategies has become urgent as development of new antibiotics has decreased while the emergence and spread of resistant microbes has increased. Perhaps through additional understanding and optimization of endogenous systems which aid the immune response, like that of selenium via selenoproteins, we can extend the life or increase the efficacy of our current antimicrobial portfolio. It also may be the case that we learn valuable information about selenoproteins which lead to novel therapeutic strategies for treating bacterial infections.

1.2 Historical Perspective:

This year marks the 200th anniversary since Jacob Berzelius in 1817 discovered selenium (4). Selenium (Se) was an accidental discovery while working with ore from a particular mine in Falun; his discovery was originally mistaken for another metalloid tellurium[1]. However, it was quickly resolved to be an independent element, though Se does share chemical similarities with tellurium and sulfur (4). The Se field was riddled with controversy over the next century, as the hormic effect of Se was slow to be characterized. It was noted in the 1950's that livestock grazing on grass rich in the micronutrient suffered hair loss and malformations of the hoof (5). It was later noted that population which lived in areas with low selenium content had increased incidence of cardiomyopathy (6). The latter was attributed to the population being unable to mount an effective immune response to coxsackievirus B3 due to Se deficiency (6). The boundaries of beneficial verses toxic effects of Se are impossible to determine as a standard for all, because living systems have pleiotropic effects from the large amount of variables influencing Se status. Additional studies have shown that many of the beneficial effects of Se can be attributed to specific incorporation of selenocysteine into proteins; whereas the toxic effects are mainly attributed to nonspecific incorporation in place of sulfur in some residues (5).

1.3 Selenium in eukaryotic systems:

The micronutrient Se is critical to survival of many organisms. Often referred to as the 21st amino acid, selenocysteine (Sec) is incorporated into the proteome in a specific manner. It is thought that the genes required for biosynthesis and incorporation of Sec are ancient and remarkably conserved, with selenoprotein producing organisms in all three domains of life (7, 8).

In order for an organism to specifically incorporate selenocysteine the organism must have access to a selenium source. The estimated average requirement for a human is 55-70 mcg/day provided generally through diet or supplementation (9). An adequate human would have a measurement of roughly 100 micrograms per liter of Se in whole blood tissue (10). The source and amount of selenium will determine its availability in the hydrogen selenide pool as well as influence the rate at which methylation and demethylation occur to preserve the equilibration with the methylselenol pool (11). Contribution of hydrogen selenide to the

methylselenol pool is important because the methylselenol can be further methylated for excretion. The dimethylselenol is excreted via respiration or trimethlyselenonium and selenosugers are excreted through urination (11). Selenate and its reduced form of selenite are inorganic compounds directly contributing to the hydrogen selenide pool via reduction which is facilitated by glutathione (GSH) (11). Selenoproteins, selenomethione, selenocysteine, and methylselenocysteine can be utilized only after they have been processed by a lyase (11). In the case of methylselenocysteine, and in some cases selenomethionine, methylselenol is then formed which must be demethylated to enter the selenide pool Figure 1.1.

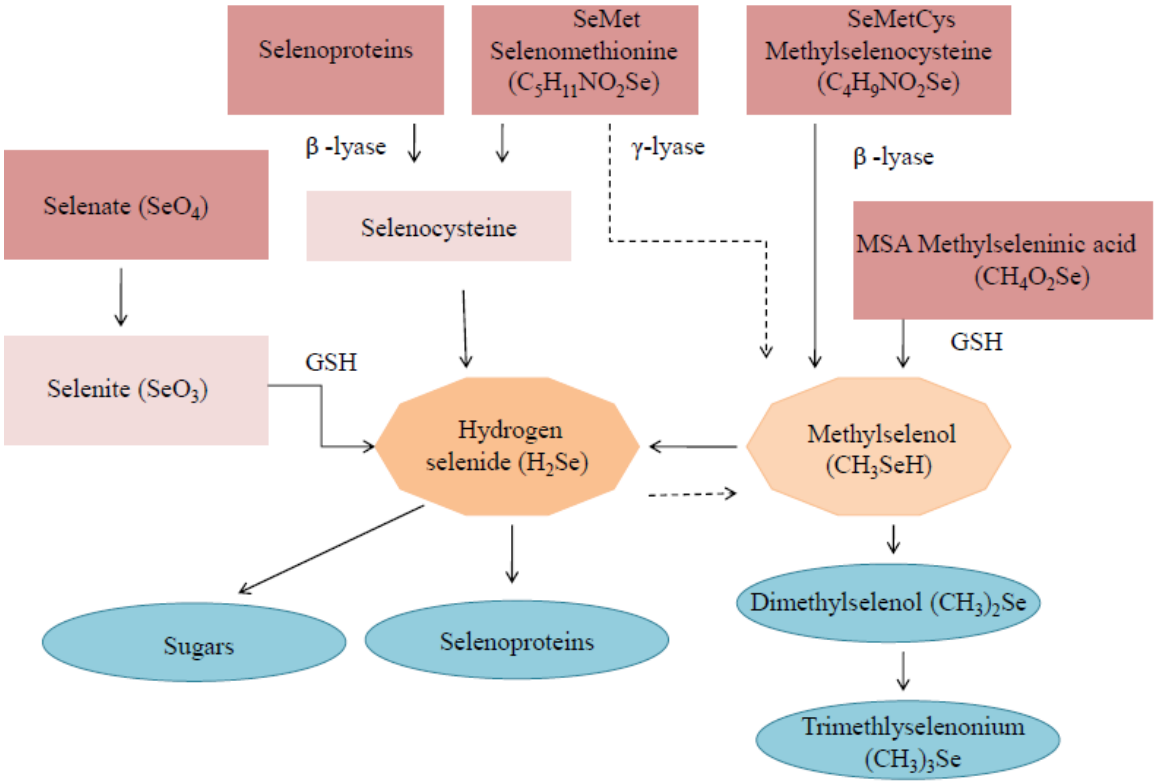

Figure 1.1 Selenium metabolism adapted from Suzuki et al. 2007.

1.4 Specific incorporation of selenocysteine into selenoproteins

Selenoprotein expression is a highly controlled process, beyond available hydrogen selenide, specific incorporation can only occur if the unique transfer ribonucleic acid ($tRNA^{[sec]}$),

an elongation factor, cofactors and key enzymes, selenophosphate synthetase and selenocysteine synthase are present(12). The process begins with serylation of $tRNA^{[sec]}$ followed by phosphorylation, each step requires ATP input into the system (13). In parallel selenide is acted upon by selenophosphate synthestase requiring ATP. The last step in the selenocysteine biosynthesis pathway is the phosphoseryl tRNA conversion (12). This step is made possible by the selenocysteine synthase which utilizes the phosphorylated selenide and ultimately yields selenocysteyl $tRNA^{[ser]sec}$ (12, 13). It should be noted that if selenide is not available an interconversion can occur using sulfur resulting in a cysteine charged $tRNA^{[ser]sec}$ (12, 13). The consequences of cysteine incorporation rather than selenocysteine in an enzyme would influence the enzymatic properties. It would reduce the efficiency which is the case when comparing for example mammalian and bacterial thioredoxin reductase (14). One could speculate that the improved enzymatic activity may influence the positive selective pressure in certain organisms to preserve selenoproteins within their genome. However, there are additional features required for the newly synthesized selenocysteine to be incorporated into the nascent polypeptide chain.

The selenocysteyl $tRNA^{[ser]sec}$ is the limiting factor in cotranslational incorporation of selenocysteine. Messenger RNA with a SElenoCysteine Insertion Sequence (SECIS) element is the feature recognized by the SECIS binding protein 2 (SBP2) (12). This translational cofactor is recruited along with L30 a subunit of the large ribosomal unit, nucleolin and the selenium sensitive eukaryotic initiation factor 4A-III among others (12, 15). When the UGA, typically a stop codon, is reached Sec is added to the growing polypeptide chain (12). Translation of selenoproteins has been determined by studies using deficient animals and cultured cells to be hierarchical (16). Certain selenoproteins are given preference under conditions of low selenide levels. Nucleolin has been described as playing a role in these “essential” selenoproteins (17). GPX1 is a good example of an “essential” selenoprotein which is highly expressed under deficient conditions; and therefore in certain cells like macrophages, GPX can be used as a reliable read out of selenium status.

1.5 Selenoproteins

There are 25 selenoproteins in humans and 24 in mice. Many of the selenoproteins that are well characterized play a role in antioxidant or redox function. Other selenoproteins function in transport, protein folding, selenocysteine biosynthesis and thyroid function. There is a small group of selenoproteins for which a function has not been clearly elucidated and therefore there have been continued efforts to research their function and effects in living systems.

Many of the therapeutic qualities of selenium are attributed to the functions performed by selenoproteins. When considering the role of selenium in bacterial infections the literature describes selenoproteins that maintain normal oxidative tone as the primary mechanism of action. Table 1.1 organizes the selenoproteins by function and details any reports connecting a given selenoprotein to a bacterial disease.

Glutathione peroxidase 3 (GPX3) for example, has been reported as a biomarker for systemic inflammation (18). Unlike GPX3, SELENOP is a selenoprotein in which its main function is to shuttle Se to various tissues, but like GPX3 it has also been described as a biomarker for sepsis (19). Interestingly, a study in germ free mice challenged with nonpathogenic bacteria suggested that SELENOP expression decreased in the gastrointestinal tissue this suggests that bacterial presence alone may decrease expression levels (20). Many of the associations made between selenoproteins and bacterial infections are based on changes of selenoprotein expression levels such as those with the GPX proteins in addition to SELENOP, SELENOK and SELENOS.

Functional tests of individual selenoprotein knockout models have demonstrated that in some instances inflammatory response is escalated during bacterial infection. This is illustrated in the spontaneous colitis model generated using GPX1 and GPX 2 KO mice in combination with *S. enterica* challenge (21). In addition to GPX1 and 2, GPX4 has also been reported to be important during *S. enterica* infection. It has been described that GPX4 affects neutrophil migration during *S. enterica* challenge (22). This phenotype is attributed to its role in the reduction of phospholipid hydroperoxides such as 12-lipoxygenase, an important upstream mediator eicosanoid metabolism during inflammation (22). One of the most interesting selenoproteins to be involved with bacterial infection is DIO3. Typically Iodothyronine

Deiodinase is associated with the thyroid, but DIO3 KO mice had increased bacterial burdens of *S. pneumonia* relative to their wild-type controls after challenge (23). Boelen also reported that DIO3 was particularly upregulated in infiltrating neutrophilic granulocytes after challenge with *S. pneumonia* and *E. coli* (24). Finally, though it was not highlighted on Table 1.1 because it was not reported in a bacterial model system, DIO2 expression levels were also found to be differentially regulated via partial participation of NF-κB pathway after bacterial lipopolysaccharide challenge (25).

Table 1.1 Selenoproteins and their role in bacterial infections

Function	**Selenoprotein**	Abbreviations (2016)	Abbreviation (prior 2016)	Associated with bacterial infection
Antioxidant	Glutathione Peroxidase 1	GPX1	GSHPX1 (Classical or cytosolic GPX)	*S. aureus(26)* *S. enterica(27)*
	Glutathione Peroxidase 2	GPX2	GSHPX-GI (Gastrointestinal GPX)	*S. enterica(21)*
	Glutathione Peroxidase 3	GPX3	Plasma GPX	Sepsis(18)
	Glutathione Peroxidase 4	GPX4	PHGPX (Phospholipid hydroperoxide GPX)	*S. enteric(29)* *D. nodosus(30)*
	Glutathione Peroxidase 6	GPX6	GPX6	N/A
	Methionine-R-sulfoxide	MSRB1	SelR, SelX	N/A
	Selenoprotein W	SELENOW	SelW, SEPW1	N/A
Redox Signaling	Thioredoxin reductase 1	TXNRD1	TrxR1, TR1	N/A
	Mitochondrial Thioredoxin reductase	TXNRD2	TrxR2, TR3	N/A
	Testis Thioredoxin-glutathione reductase	TXNRD3	TrxR3, TR2, TGR	N/A
Thyroid Hormone Metabolism	Iodothyronine Deiodinase	DIO1		N/A
	Iodothyronine Deiodinase	DIO2		N/A
	Iodothyronine Deiodinase	DIO3		*S. pneumonia(24)* *E. coli(25)*
Transport	Selenoprotein P	SELENOP	SEPP1	Sepsis(19)and Non-pathogenic bacterial infection(20)
Protein Folding	Selenoprotein K	SELENOK	SelK	*S. pneumonia(31)*
	Selenoprotein M	SELENOM	SelM	N/A
	Selenoprotein N	SELENON	SelN, SEPN1	N/A
	Selenoprotein S	SELENOS	SelS, VIMP, SEPS1	*H. pylori (32)* *D. nodosus*
	Selenoprotein F	SELENOF	Sep15, (15kDa Selenoprotein)	N/A
Unknown Function	Selenoprotein I	SELENOI	SelI, EPT1	N/A
	Selenoprotein H	SELENOH	SelH, C11 orf31	N/A
	Selenoprotein O	SELENOO	SelO	N/A
	Selenoprotein T	SELENOT	SelT	N/A
	Selenoprotein V	SELENOV	SelV	N/A
Sec Biosynthesis	Selenophosphate synthetase 2	SEPHS2	SPS2	

*Table adapted from Gladyshev et al. 2016 with added information for bacterial infection

Not available (N/A)

While there are some functional studies which elaborate on the intricate mechanisms of selenoprotein involvement with bacterial pathogenesis, it remains that there are large voids in our understanding of selenoprotein functions under homeostatic conditions let alone during periods of pathogenic infection. These proteins are multiple faceted and have numerous interactions with effectors of the immune system compounded by the fact that there are a number of redundancies in their functions. One vital tool used to parse out the individual role of given selenoproteins either globally or in restricted tissues has been the use of murine knockout models.

1.6 Models to understand the contribution of selenoproteins to human health

Selenoproteins are understood to play a large role in the development and homeostasis of organisms. The generation of specific selenoprotein knock out models has been useful in to characterize and functionally annotate individual selenoproteins. A number of selenoprotein such as GPX4, thioredoxin reductases (TXNRD) TXNRD1 and TXNRD3 proved to be absolutely required for development, as knockout models were embryonic lethal (33). Conversely, other selenoproteins knockout models such as GPX1 or GPX2 develop normally, but are less viable after challenge with certain pathogens. Still other knock out models such as Deiodinase (DIO), DIO1, DIO2, DIO3 and selenoprotein P knockout models have varied degree of neurological or tissue dysfunction. Models have also been generated to study the effects of selenoprotein knockout in various tissues. These models were created to mimic existing disease models. For example mice lacking *Trsp* in myocytes will suffer cardiac arrest within weeks of birth (16). This model in combination with the GPX1 knockout and TXNR3 were useful to discover the underlying mechanism of Keshan disease, a congestive cardiomyopathy occurring in a population of selenium deficient individuals infected with coxsackievirus B3 in China (33). Keshan disease may have provided the first evidence that Se, and more specifically selenoproteins, are important factors influencing disease outcome. Keshan disease highlights an important link between sever pathology resulting from selenoprotein deficiencies during acute infection.

1.7 Selenium status and bacterial diseases in humans

Given that a clear link has been established between pathogen induced pathology and selenium status it is reasonable to hypothesize that optimizing nutritional status with positively influence disease outcome. Nevertheless, there has been conflicting clinical evidence that pharmaconutrient therapy is efficacious. Still, what is far less disputed and in some cases, explicitly stated by the authors of extensive metanalyses is that during periods of disease patients have lower levels of serum Se or selenoproteins (34, 35). It has been reported that the lowest levels of Se were observed during acute onset and that in some instances, Se levels increased during the recovery and convalescent phase (36). When considering the body of literature of human trials for patients with bacterial infection, tuberculosis seems to dominate the field. In most cases clinicians measured multiple nutrients, for which Se was often included. Patients suffering from mixed infections such as tuberculosis and HIV or with additional complications such as anemia had some of the lowest Se levels reported (37, 38). It is often the case that only a handful of studies report measurements of additional immune response markers such as cytokine levels or C-reactive protein levels, which in their respective reports were not directly correlated to patient Se levels (38, 39). There are however singular studies that highlight marginal improvement in CD3+/CD4+ cell counts, decreased ulcer formation and reduction in deaths for patients with tuberculosis which were supplemented with a cocktail containing a number of micronutrients including Se (40). Se status may an indicator of risk for a particular patient which could be useful in determining a course of treatment (41). Inherently some of the issues facing the researchers grappling with this data may be the heterogeneity of the subjects, adherence to medication and supplement regimen as well as their status before illness. Often large cohorts of patients suffering from tuberculosis infections are from countries where they may not have had access to adequate nutrition or health care prior to their contracted illness. In some instances a mixed model infection may lead to nutrient absorption issues which can affect the outcome of supplementation.

A few studies have investigated Se levels and single pathogen infections, for example Group A streptococcal infections. Group A strep is the causative agent of strep throat and scarlet fever. Group A streptococcus can also have deleterious effects on cardiac tissue. A study of patients with Group A Strep and heart disease describes a correlation with lower Se and Zn

levels in patients with reoccurring rheumatic disease (42). Conversely, a later study found children with acute rheumatic fever had only slightly lower Se levels before treatment as compared to healthy controls, and this data was deemed not statistically significant (43). It can be surmised that the sample collection time is an important in determinant if a true correlation exists between bacterial infection and Se status in non-systemic infections.

For pathogenic infections that are limited to a given tissue measuring the serum selenium levels may not be useful, which is the case with *Helicobacter pylori* infections. Se as measured in the serum is equivalent to controls in acute cases, but gastric tissue levels are elevated in the samples of infected patients (44). Additional studies have suggested that serum selenium levels will decrease in chronic infections with precancerous lesions (45). Interestingly, data published on a study looking at polymorphisms in the Selenoprotein S (SELENOS) promoter in conjunction with incidences of *H. pylori* infection show an increased onset of gastric cancer (32). It has been established that SELENOS expression levels are inversely tied to proinflammatory cytokines such as 1L-1β, TNF-α and IL-6 (46). The authors speculated that due to the increased pro-oxidative response triggered by *H. pylori,* compounded by inadequate SELENOS response individuals with certain polymorphisms may have a higher risk factor for gastric cancer (32). This data further emphasizes that selenium supplementation and moreover, the increase of selenoproteins, may impact immune response in patients with certain forms of bacterial infections. This study also highlights the idea that the mechanism may extend beyond the simple measurements of antioxidant selenoproteins such GPX 1 or GPX3. To truly understand how Se is utilized and affects cells of the immune system we must better understand the functions of all the selenoproteins, irrespective of their position on the hierarchal selenotranscriptome. Data compiled from profiling experiments suggests that selenoprotein expression levels are highly variable depending on tissue specificities and conditions under which the samples were obtained as illustrated in Figure 1.1 (16).

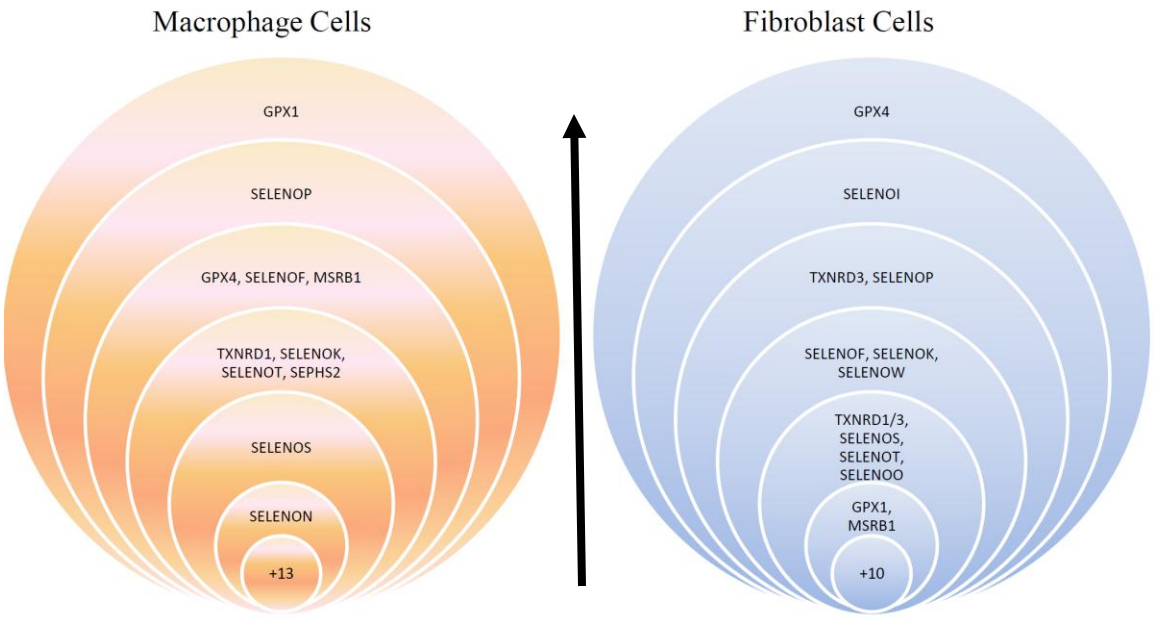

Figure 1.2: Depiction of selenoprotein expression adapted from Carlson et al. 2009. Macrophage BMDMs and NIH3T3 embryonic fibroblasts were analyzed by real-time qPCR to determine relative selenoprotein expression levels.

1.8 Selenoproteins and immune response

Given that it has been established selenium affects immune function in humans and mice and that this is mediated primarily through selenoproteins. The absence of selenoproteins will profoundly hinder both the innate and adaptive arms of the immune system. Carlson et al. have described that macrophages unable to synthesize selenoproteins have higher levels of reactive oxygen species (ROS under homeostatic conditions, additionally these macrophages have reduced abilities to migrate (16). Se status also profoundly affects the infiltration and activity of natural killer cells during bacterial infection (47). The Prabhu lab has shown that arachidonic acid metabolism is sensitive to selenium status and that these effects are mediated by selenoproteins with pleiotropic affects from inhibition of histone acetylation, to down regulating inflammatory cytokine expression, and initiation of apoptosis of leukemia cells (48, 49 and 50).

Selenium has been reported to play a role in T cell activation, proliferation and differentiation (51). Unpublished data collected in the Kirimanjeswara lab provides evidence that B cell functions such as endocytosis is greatly impaired under Se deficient conditions. Furthermore, there have been reports of Se supplementation increasing antibody titers in animal models after bacterial challenge (52, 53). On the other hand this is a controversial conclusion given that there are also published reports that dispute antibody titers change in response to an animal's Se status (54). In light of the debate of phenotypic differences, Se and selenoproteins are crucial factors determining response to acute bacterial infections and it can be suggested that optimizing selenium status, or rather a select selenoprotein status has the capacity to facilitate a more highly tuned immune response thereby limiting pathogenicity during acute infection.

In an effort to more completely present immune function as it relates to selenoproteins it is important to understand how selenoproteins are functioning in these different cellular compartments of the immune system. Here I will highlight a few examples of mechanisms by which selenoproteins regulate immune response.

SELENOK is an important selenoenzyme located in the endoplasmic reticulum. Its general function has been described to aids in protein folding. Surprisingly, SELENOK also moonlights in a number of other functional roles. Those roles become exceedingly important in the context of immune function. Hoffmann's group have described that SELENOK participates in palmitoylation (55). SELENOK palmitoylation of protein ASAP2 allows for a downstream cleavage event by calpain-2 resulting in ability for Fc_{γ} receptor enclosed endosomes to undergo maturation (56). During bacterial infections antibody coated microbes will be targeted by phagocytic cells for opsonization via the Fcγ receptor. The participation of SELENOK in facilitating the upstream events leading endosomal maturation of the Fcγ receptor compartment is an important immuomodulatory function.

TXNRDs are likely one of the most well known selenoproteins, due in part to their roles as antioxidants and in maintenance of redox homeostasis. It is the antioxidant and redox related proteins like GPX and TXNRDs which allow for recoil of innate cellular defenses of reactive oxygen nitrogen species (RONS). Case and point, hypothiocyanous acid is a ROS initiated as part of an innate cellular defense; TXNDR1 is able to reduce hypothiocyanous acid in a NADPH dependent manner at physiological levels thereby protecting the host cells from

continued damage (14). Additionally, TXNDR1 was also found to be upregulated with TXNRD2 in T cells stimulated with IL-15, which is thought to be important for establishment of memory in CD8+ cells (57). Antioxidant profiles in addition to their protective role during cellular stress may also play an important function in tuning immune response by calibrating chemical signals to enhance phenotypic response in a discrete fashion.

1.9 Selenium as adjuvant treatment for bacterial diseases in humans

GPX proteins have long been recognized as important mediators to alleviate oxidative damage. Their antioxidant functions and resulting depletion during periods of excessive inflammation are the basis for their use as biomarkers. A number of clinicians such as Forceville and Manzanares preempted their large clinical trials of Se adjuvant therapy with prospective studies to measure patient Se levels, and in fact, Manzanares utilizes the selenoprotein GPX3 as a biomarker of sepsis in the 2009 trial (58, 18). The conclusion that critically ill or septic patients have lower serum Se levels during discrete periods of infection is generally accepted, but going forward, statements about the efficacy of pharmaconutrients and Se adjuvant therapies are highly disputed even now. As researchers continue to annotate and reveal clear functions for selenoproteins during periods of acute infection, clinicians are eager to implement therapies using the researchers' finds as evidence that Se supplementation will improve patient outcomes. Unfortunately, the prospective randomized trials available for examination are riddled with inconclusive results. Table 1.2 represents a selection of studies that involve critically ill patients which have undergone Se adjuvant therapy with the main form of supplementation sodium selenite (pentahydrate). Additional studies were rejected if they did not involve pathogenic infection, were supplemented with other forms of Se or if the primary literature was not available in English. Utilizing a primary endpoint of mortality it is clear that 78% of the studies found no significant differences between Se supplemented and control, or placebo groups. The Se adjuvant studies that reported statistically significant differences between groups that received intervention comprise just 22% of Table 1.2, and these studies were conducted by one group, Angstwurm et al 1999 and 2007. In the majority of the studies where Se levels or GPX levels were measured, patients who received Se intervention had an increase in Se and/or GPX3 measurements as compared to baseline measurements, and this level of increase in Se or GPX3 trended above the groups which did not receive Se intervention (59-65). Furthermore, in all but one study there were multiple other endpoints measured such as C-reactive protein levels, sepsis related organ failure scores and ventilator or hospital acquired pneumonia, which were for the most part were negatively correlated with Se or GPX levels (62-64, 66). Lastly, added emphasis must be placed on clinicians who performed due diligence in completion of post hoc testing on their data set to determine if there is an interaction between the dependent and independent variables, or if the data is influenced by other parameters. An example would be the Forceville

study, which fails to separate septic patients that undergo emergency surgery, and in this particular study, the supplemented cohort has undergone emergency surgery 5X more than the control cohort (58). Patients in the Janka study were divided into four groups, intervention verses control, and those groups were then subdivided based on surgical intervention (65). The influence of surgery on data collected may be substantial leading to misinterpretation of the results. It is clear with such a mix response how clinicians have struggled to determine if there is sufficient evidence to introduce interventions such as Se adjuvant therapy as a standard of care.

Table 1.2 Human trials of selenium adjuvant therapy

Study	Year	Patients	Selenium	Dose	Levels of Se/GPX	Mortality
Angstwurm(59)	1999	42	Selenite	535 µg/24hr/3d to 285 µg /24hr/3d to 155 µg/24hr/3d then maintained as control group on 35 µg/24hr/d	Increased	Total p=0.13 Post hoc p=0.0278
Berger(60)	2006	41	Selenite	copper 2.5 -3.1 mg/d selenium 315-380µg/d zinc 26.2 - 31.4 mg/d	Increased	p=0.57
Angstwurm(61)	2007	249 (238, 189)	Selenite	1,000 µg/bolus followed by 1,000 µg/24hr/14d or placebo	Increased	p=.109 p=0.049
Forceville(58)	2007	60	Selenite	4,000 µg/24hr/1d to 1000 µg /24hr/9d or placebo	N/A*	p=0.691
Mishra(62)	2007	40	Selenite	474 µg/24hr/3d to 316 µg /24hr/3d to 158 µg/24hr/3d then maintained as control group on 31.6 µg/24hr/d	Increased	p=0.94
Andrews(66)	2011	502	Selenite	Parenteral glutamine 20.2 g/24hr/7d or selenium 500 µg/24hr/7d	N/A*	p=0.54
Manzanares(63)	2011	35	Selenite	1,000 µg/bolus followed by 1,000 µg/24hr/14d or placebo	Increased	p=0.55 p=0.95
Valenta(64)	2011	150	Selenite	1000µg/24hr/1d to 500µg/24hr/13d then maintained as controls group on 75µg/24hr/14d	Increased	p=0.367
Janka(65)	2013	72	Selenite	750 µg/24 h/6d or placebo	Increased	p=0.159

***N/A signifies information that is not available.**

To normalize and increase the data pool, multiple groups have performed metanalyses on the prospective studies available in the body of literature. Unfortunately, these metanalyses also lead to more questions than answers. Table 1.3 below describes four analyses, which utilize some or all of the prospective studies from Table 1.2.

Table 1.3 Metanalyses of human trials of selenium adjuvant therapy

Study	Year	# Studies	Major finding
Vincent (67)	2008	4	Not enough studies have provided conclusive results and toxicity of high bolus doses is understudied
Manzanares (68)	2012	20	Dose and dosage regimen have a significant effect on outcome including mortality
Alhazzani (69)	2013	9	In sepsis mortality is reduced when patients receive supraphysiological doses
Allingstrup (70)	2015	16	Study found a lack of evidence to support Selenium or ebselen affected endpoints considered

The data pooled from the metanalyses shows an even split among groups that found supporting evidence for Se adjuvant therapy and those which did not conclude there was evidence to support Se adjuvant therapy. The Vincent study in 2008 was considered neutral because it speculated that there was not enough data to provide conclusive results at the time of the analysis. An inference was made by a number of authors that supraphysioligical doses are required to observe a measurable effect of Se intervention on prescribed endpoints, and Manzanares points out that dose and administration of doses may be factors as well (63, 67). It should be noted that with the inclusion of the Manzanares metanalysis some of the data reported includes studies which are related to severe inflammatory reaction caused by autoimmunity or other underlying causes of inflammation and this may skew the data in favor of Se intervention, so caution must be exercised in determining overarching conclusions from this group of analyses.

The Manzanares metanalysis remained in Table1.3, because it has included subsections sections of analyses focusing on sepsis populated with relevant studies.

The Allingstrup metanalysis can be interpreted as the most critical opponent of Se or Ebselen (an organic compound containing Se used therapeutically) intervention. Allingstrup reported the highest risk of study bias were due to randomization and selective reporting. This can be clearly illustrated when considering overall mortality, the studies considered in this metanalysis reported on varied intervals, and have confounding attrition rates of the subjects included (70). In total, it seems perhaps the heterogeneity, background and inevitability of study bias that exist in human trials is a limitation, which thwarts efforts to fully realizing the potential of Se adjuvant therapy. An alternative that may be more plausible in studying an overall effect of selenium, and more specifically selenoproteins during acute bacterial disease may be to look for patterns in other mammalian models.

1.10 Se and selenoproteins during bacterial infections in animal models

Laboratory and agricultural animal models of disease have numerous benefits. Though their physiology is not identical to humans there are similarities which can be applied to human medicine. Some of the benefits of using animal models are that the researcher can gain control over conditions of the experiment, such as environment, food, water and medication adherence. Furthermore, using animal models expedites the timeline of infection and recovery to provide a more full picture of the effects Se supplementation or deficiency can have under given circumstances. Finally, the ability to select for homogeneity in the genetics of a study cohort or specifically construct genetic model to investigate the role of a specific selenoprotein is unequivocally the single most useful tool in a researcher's arsenal. This section will highlight some of the animal models used to collect evidence for the role of Se and selenoproteins in heterogeneous as well as homogeneous genetic populations of a variety of mammalian species during bacterial infections.

Odocoileus (deer): Wildlife ecologists have collected data for the Se status of deer in the United States. In two of these studies the authors noted that populations sampled in the study were Se deficient (71, 72). In the case of the Sleeman study, deer were found to succumb to

Mycobacterium avium subsp. Paratuberculosis, a common pathogen normally afflicting agricultural ruminants, but not typically known to cause disease in wild ruminant populations. The authors attributed the increased incidence of *M. avium* pathogenic infection in this population to low Se levels as measured in the hepatic tissues (72). Similarly, a mule deer population surveyed by Myers et al. provided data that lead the authors to conclude the populations tested were Se deficient, and it was further speculated that the deficiency in combination with the pathogens detected may increase the risk of population decline (71). Conversely, Wolf et al. noted that data collected on the deer populations in the Dakotas, did not suggest Se status was a risk factor influencing pathogen prevalence in the sampled populations (73). It could be noted that the Dakotas are reported to have high Se content in their soil and at those levels, the animals in this study were readily sufficient which may have contributed to the lack of evidence correlating Se status to pathogen burden. The wildlife ecology studies show a similar pattern in disease susceptibility to deficient populations much like what was observed in a clinical setting.

Bos taurus(cow): Supporting the observations from the wildlife studies, bovine case studies have also been reported in which cattle have increased susceptibility to infection of *Yersinia pseudotuberculosis, Clostridium botulinum* and *Escherichia coli* infection under Se deficient conditions (74, 75 and 76). The McLennan study is an interesting example of a farm in Australia whereby the soil was known to be deficient in Se and Cu. The farm reported that it stopped supplementation of sodium selenite drenching after toxicity was reported (74). The author reported an inverse relationship between the cows GPX (Se levels) and the pathology of *Y. pseudotuberculosis* resulting in increased mortality within the herd (74). Conversely, Downs reported that there was no risk associated with Se status as measured by GPX levels in the serum of cattle in relationship to bovine tuberculosis, but that Cu levels may have conferred susceptibility to the pathogen (77).

Bovine models have also been used to complete controlled studies often testing Se status as it relates to mastitis. Mastitis is a condition of mammillary gland inflammation which can be attributed to infection with one or a few of the following pathogens: *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus uuberis,* and/or *Escherichia coli.* The six studies reported here differed greatly in their Se intervention which included yeast derived organic Se,

generically labeled Se dry matter or undisclosed vitamin mixtures containing Se, though most were administered in diet. Another aspect of these studies that varied greatly was the technique used to induce mastitis of which their pathogen was the most obvious difference. Surprisingly, despite the variation in protocols between the studies they all reported that their endpoints, which also varied, were influenced by Se status. In two reports the bacterial shedding was higher in Se deficient animals as compared to supplemented, which resulted in a decrease of milk production in the deficient animals (76, 78). Moreover, a study reported that Se deficient animals had higher levels of prostaglandin E_2 (PGE_2) relative to Se supplemented with four animals in the deficient cohort which required euthanasia, suggesting a more pronounced inflammatory response was produced in the deficient animals (79). Two groups looked at polymorphonuclear leukocytes and phagocytes cells and found there was a decrease in apoptosis and infiltration in the supplemented groups (80, 81). The latest report considered here described data demonstrating a negative correlation between supplementation of the heifers and mastitis (82). The bovine model corroborates the findings that deficiency in Se status may increase risk of pathogen associated disease. In addition, this model begins to show how Se supplementation leads to improved immune response through recruitment of viable immune cells and regulation of immune modulating factors like PGE_2.

Ovis (Sheep): Perhaps when considering the conflict between the reports of Angstwurm and Forceville which differ in their administration of a bolus followed by continuous Se treatment, it is useful to consider the Ovis model presented here. Wang et al. induced sepsis using a peritonitis model in ewes and reported that a sodium selenite bolus (2mg) followed by continuous injections (6μg/kg) prolonged time to death in supplemented animals as compared to control without Se intervention (83). This model as in the case of Forceville did not have a direct outcome on overall mortality, but does suggest there is inherently regulation of immune function resulting in a beneficial outcome. Unfortunately, this study did not probe the molecular mechanism leading to prolonged survival. What has been reported using a *Dichelobacter nodosus* (foot rot) model in sheep is that gene expression levels of L-selectin, interleukin-8 receptor (IL-8R) and Toll-like receptor 4 (TRL4) were increased in supplemented animals, suggesting supplementation is important for expression of genes associated with innate immune function (84). Unfortunately, Se supplementation did not decrease the incidence, severity or length of foot rot disease in the sheep which were tested (84, 85).

Gallus (chicken): Studies selected from the chicken model further illustrate the effects of selenium on the immune system. In an enteric infection model induced through a co-infection of the Gram-positive bacteria *Clostridium perfringens* and the protist *Eimeria maxima,* Se supplementation (via sodium selenite or organic yeast based diet) has been observed to increase cytokine levels, antimicrobial effectors and reduce lesions; supporting the idea that Se supplementation is protective against necrotic enteritis (86, 87, 88). The Lee report provided evidence of increased expression levels of IL-1β, IL-6, IL-8 and inducible nitric oxide (iNOS), in addition to higher antibody titers in the Se supplemented group, while the Se deficient cohort exhibited higher levels of GPX7(88). This evidence is further validated by the observation that supplemented animals have a lower mortality rate due to Enteropathogenic *E. coli* then control animals (89). The thymus of chicks supplemented with Se was larger than that of the control group after challenged with *Salmonella typhimurium,* this group also described a better antibody response in the supplemented group compared to the controls (90). If however, chicks are over supplemented with Se to toxic levels of 20ppm they have been found to be more susceptible to *Salmonella gallinarum*, emphasizing how targeting the dose of Se is a key component to optimizing immune response (91).

Rodentia (mouse, rat, and guinea pig): By far one of the most useful animal modeling systems used to study Se and bacterial pathogenesis is *Rodentia*. While most studies are conducted in mice specific pathogen infections are better reflected in other species. This is the case with *H. pylori* infections where guinea pigs (Table1.4), but not mice develop gastritis similar to that observed in human patients (92). Given that evidence from human polymorphisms in SELENOS have correlated to increased risk of gastric cancer for individuals who also suffer from *H. pylori* infections, and that Se status correlates to expression levels of antioxidant selenoproteins during active *H. pylori* infection the use of a guinea pig model is appealing to understand the interaction between these two phenotypes. In this study Se supplementation increased antioxidant levels protecting animals from gastritis, hence it is also conceivable that patients with H. *pylori* infections may benefit from Se adjuvant therapy.

The other more obvious pattern, which immerges from the rodentia Se models is that animals maintained under deficient conditions suffer increased proinflammatory immune response often accompanied by increased pathogenesis Table1.4. Se deficient mice infected with

Citrobacter rodentium or *Staphylococcus aureus* were reported to have elevated levels of pro-inflammatory cytokines, increased activation of Toll-like receptor response and an inability to control bacterial burden resulting in more tissue damage compared groups with access to dietary Se (93-96). Under conditions where lipopolysaccharide from *E. coli* was administered it was noted there was a decrease in GPX expression in deficient animals, which further supports the role of selenoproteins during infection (97). Data reported by Wang using a *Listeria monocytogenes* infection model confirms these findings, and adds that the activity of GPX, superoxide dismutase and catalase were also found to be lower under conditions of deficiency (98). Moreover, bacterial burden of *L. monocytogenes* was higher in deficient animals as compared to sufficient animals, with Altimira et al reporting increase in the number of lesions found in the central nervous tissue (98, 99). Conversely, decreases in bacterial burden were reported in a study of pathogenic *E. coli* where the authors found a synergistic effect between use of Se and the antibiotic ciprofloxacin, which given the growing emergence of drug resistant microbes is promising (100). In opposition, data reported by Boyne et al. described no effects of Se deficiency on the immune response or ability to control bacterial burden in rats infected with *S. typhimurium* or *S. aureus* (101). It is possible that the dose of Se Boyne et al administered to the animals was too low to observe a phenotype, or that the rats were maintained for only 8 weeks (101). Smith et al. noted that in mice, the phenotypes observed with the Se deficient mice were influenced by the length of time the diet was administered (94).

Table 1.4 Se and selenoproteins during bacterial infections in animal models

Study	Year	Model	Pathogen	Selenium*	Dose & Route**	Major Findings
Gao(93)	2016	Mouse	*S. aureus*	NaSe	0.03, 0.13, 1.5mg/kg diet	Deficiency lead to higher levels of proinflammatory cytokines, MPO activity, TLR2 signaling and NF-κB activation.
Smith(94)	2011	Mouse	*C. rodentium*	NaSe	0 or 0.2µg/g diet	Deficiency lead to increased cytokine levels, pathology and colonic hyperplasia. Maintenance of diet for 20 weeks verses 5 weeks exacerbated phenotype.
Smith(95)	2011	Mouse	*C. rodentium*	NaSe (Vit E)	0 or 0.2µg/g diet	Vitamin E and Se deficiency lead to increased bacterial burden reduced response and increased pathology.
Wang(98)	2009	Mouse	*L. monocytogenes*	Se	0.005 or 0.2mg/kg diet	Deficiency lead to increased bacterial burden, less infiltrating immune cells in the spleen and NK cells had lower activity.
Berg(97)	2005	Mouse	*E. coli LPS*	NaSe	0.05, 0.15, 2g/kg diet	Deficiency was marked by reduced levels of GPX in a dose dependent manner.
Altimira (99)	2000	Mouse	*L. monocytogenes*	Se	350 or <8µg/g diet	Deficiency leads to greater damage in CNS tissue.
Liu(96)	2016	Rat	*S. aureus*	MCS/MSA	1.5mg/kg Se i.p.	Deficiency leads to increased TRL-2 activation increasing caspase cleavage and apoptosis.
Kim(100)	2012	Rat	*E. coli*	Se	12µg/g water	Se with ciprofloxacin decreased bacterial burden.
Boyne(101)	1986	Rat	*S. typhimurium* *S. aureus*	Se	0.01 or 0.1 mg/kg	Deficiency had no effect on response to infection.
Sjunnesson (92)	2001	Guinea Pig	*H. pylori*	Se (Vit: A,C, E)	0.15 or 1mg/kg diet	Dietary antioxidant levels increased with supplementation this protected against type-B gastritis. Data suggest there may be a correlation between bacterial load and gastric scores.

***Forms of Se: sodium selenite (NaSe), unknown or labeled as selenium (Se), selenium nanoparticles (SeNP), methylselenocysteine (MCS), methylseleninic acid (MSA)**

**** Intraperitoneal injection (i.p.)**

1.11 Toxicity associated with the micronutrient selenium

While the nutritional properties of selenium have been the focus of this review the toxicity associated with selenium, selenosis has been well documented. The man who discovered Se, Jacob Berzelius was thought to suffer from selenosis based on the accounts of his housekeeper (102). Hallmarks of selenosis are garlic breath, loss of hair, deformities of nails or hoofs, (in the case of ungulates) neurological dysfunction ataxia, lethargy, skin lesions and granulomas in the lung tissues (when selenium is aerosolized). Exposure can be environmental such as in the cases of China and North Dakota where soil selenium levels are particularly rich and dietary sources exceed recommended values (103, 104). Environmental exposures can occur due to water runoff in areas where mining or agricultural pollution has occurred. Occupational exposures have been documented in copper refineries and other industries that utilize Se in their processes such as semiconductor (105, 106). Often occupational exposures result in inhalation of high levels of selenium resulting in "rose cold" (105). By far the most common form of selenosis comes from accidental overdose. The FDA documented incidences of manufacturers' mislabeling the contents of supplements resulting in isolated cases of selenosis in 1983(107). This manufacturing oversight resulted in a major recall, but as of 2008 the FDA reported that there have been over 201 cases of selenium overdose associated with mislabeling (107). While fatalities due to accidental overdose are rare, a percentage of patients who suffered overdose reported unresolved symptoms even after 2.5 years (106). In the case of accidental overdose it was reported that betaine treatment reversed some of the effects on liver enzymes and may be a viable therapeutic option in some instances (108). Earlier studies completed on buffalo calves suggested administration of glutathione as a substrate to reduce the toxicity in cases of selenosis (109).

Selenium taken in high doses elevates the level of enzyme activity of glutathione peroxidase (GPX) and superoxide dismutase, which may seem beneficial (108). However, GPX in high amounts will utilize glutathione GSH as a substrate and deplete GSH levels in the tissue (109). To compound this increase in GPX, levels of glutathione reductase, glucose-6-phosphate dehydrogenase, catalase, and paraoxonase-1 are reported to be reduced during selenosis (Harisa et al. 2013). Additionally, excess levels of selenium intake can nonspecifically be incorporated

into select residues in keratin, can cause adducts on DNA and interact with thiols in oxidative reactions (110, 111, 5).

It is difficult to pinpoint the exact optimal consumption of selenium for every individual at any given time because of variables such as genetics, health and normal dietary composition. However, it has been generally accepted that selenosis is measured at 480-400 micrograms per liter in whole blood (10). In addition selenosis is also dependent upon the form of selenium, for example selenate and methylselenocysteine (the form often associated with agricultural selenosis) will be absorbed and excreted at different rates, thus the differences in pharmacokinetics of selenocompounds also determines the levels required to cause selenosis (110). In stark contrast to the defined average estimated recommendations for Se levels, sodium selenite in high doses has been proposed for use as a chemopreventative and as adjuvant therapy in patients receiving with certain forms of cancer. In a recent study by Brodin they tested for toxicities and found that chemo patients could tolerate up to 10 mg/m^2 of sodium selenite, though the efficacy of this treatment will be addressed in future trials, this is a promising advance (111). The results of this trial also encourage researchers to find alternative forms of selenium that can increase dose with reduced toxicity. Where ebselen failed, selenium nanoparticals may fill this void.

Nanoparticles have become the new buzz in therapeutic applications and selenium biology is no different. Selenium nanoparticles (SeNP) have been shown to reduce cytotoxicity, prevent microbial biofilm formation and have antimicrobial properties at physiologically sustainable levels (112, 113). SeNPs can be produced either synthetically or with the use of microorganisms such as: *Stenotrophomonas maltophilia, Bacillus mycoides* and *Bacillus licheniformis JS2* (113, 114). SeNPs purified from *Bacillus licheniformis JS2* for example, were capable of preventing proliferation of prostate adenocarcinoma cells, and moreover the SeNPs induced the cell to express essential enzymes required for programmed cell death of the cancer cells, without affects observed in other healthy cells (113). There have been reports that nanoparticles may reduce barrier function in lung during respiratory exposure, which can cause susceptibility to certain pathogens such as *Pseudomonas aeruginosa*, a causative agent of pneumonia, though SeNPs have not yet been shown to cause this issue in host pathogen models (115).

1.12 Some prokaryotic organisms utilize selenium

Toxic doses of selenium may actually increase the level of susceptibility to certain pathogenic bacteria, as in the case of *Salmonella Gallinarum* infected chicks fed 20ppm selenium (91). Conversely, in some instances selenium even at physiological levels may prove advantageous to some pathogenic microorganisms. A good example is blackleg, a disease in cattle caused by *Clostridium chauvoei*. Farmers have been reported to stop selenium drenching, even in areas of low soil selenium, due to increased incidences of the disease (74). Some prokaryotes have the ability to utilize Se either as selenoproteins or as cofactors. In anaerobic bacteria, selenium improves the bioenergetics of the stickland reaction, whereby amino acids are utilized in a fermentation process to produce energy when other nutritional sources are unavailable (116). *Treponema denticola, Clostridium difficile* and *Clostridium botulinum* are three examples of anaerobic bacteria that undergo stickland reactions (117). It could be speculated that a patient presenting symptoms of sepsis may have a *C. difficile* infection and would not necessarily benefit from selenium adjuvant therapy; for that reason, in cases of bacterial infection the pathogen must be considered before therapeutic intervention with selenium is recommended.

1.13 Concluding remarks

At present the evidence supports the claim that the micronutrient Se when administered at optimal doses can be beneficial in some clinical situations. The major hurdle clinicians must face is the lack of clear evidence about what is the optimal dose and how it should be administered based on the individual and the disease. The data that has been collected suggests that selenoproteins may be the predominant mediators of immune response to Se during bacterial infection. Therefore, it becomes paramount to elucidate the function and targets of selenoproteins thereby allowing for develop of novel therapeutic strategies to implement for acute bacterial infections. The importance of this endeavor becomes even greater with the emergence of drug resistant bacteria and their increasing spread throughout the undeveloped as well as the developed world.

Key Concepts:

- Se incorporation into the proteome of humans and mice is a highly specialized and strictly regulated process
- Selenoproteins are thought to be the principal regulators in the immune modulation observed under Se supplementation
- Use of Se adjuvant therapy has mixed successes, due in part to the limited understanding of the multifaceted roles that selenoproteins play in immune regulation and basic physiology
- Type, dose and timing of Se administration is a key aspect of optimizing Se adjuvant therapy for the regulation of immune response
- Se utility in therapeutic applications during pathogenic infection must be carefully considered given that some prokaryotes utilize this micronutrient as well

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Chapter 2: Restriction of intracellular replication of *Francisella tularensis* by selenoproteins

Abstract

Selenium has begun to gain momentum as a micronutrient used for pharmaconutrient intervention. While the levels of serum selenium have been reported to be decreased during acute infections, treatment with Se has been shown to be beneficial to patients with sepsis underscoring the importance of this micronutrient. Selenium is thought to regulate the immune functions via selenoproteins, a class of proteins that contain the 21st amino acid selenocysteine. However, the mechanisms by which Se regulates immune functions during an acute infection are not clear. Moreover, Se supplementation to bolster immune response to bacterial infections has had limited success, partially due to the fact that certain bacteria also utilize Se. I was interested in better understanding the role of Se in the immune response of macrophage cells to the bacterial pathogen *Francisella*. However, it was not clear from the literature if the pathogen *Francisella* utilized Se. Selenium can be utilized in all three domains of life, which speaks to its significance as a nutrient and its conservation through speciation events. Se can be utilized by prokaryotes in one of three ways. The first is through specific incorporation of selenocysteine (Sec), the 21st amino acid, into the organism's proteome. This requires a number of special enzymes, cofactors, an elongation factor and most significantly a highly unique tRNA encoded by the *sel*C gene. The second fashion a prokaryote may incorporate Se is through the modified base 2-selenouridine, which like the selenocysteine biosynthetic pathway requires the selenophosphate synthetase *sel*D gene product. Finally, prokaryotes can utilize Se as a cofactor either bound or unbound to a variety of molybdenum hydroxylases enzymes. All three methods of Se utilization confer a selective advantage to the microorganisms. And while this advantage maybe good for the microorganism; if that microbe is a pathogen, it may not be beneficial to the host.

To determine if *Francisella* was able to utilize Se as either a selenoprotein or modified base the genomes of *Francisella tularensis* was interrogated for evidence of the required genes in the selenocysteine biosynthetic pathway using reference genomes from known selenocysteine incorporating bacteria *Escherichia coli* and *Desulfovibrio vulgaris.* The analysis failed to identify the required genes necessary to incorporate Se into proteins or modified bases. Additionally, to rule out that *Francisella* utilized Se as cofactor an atomic absorption spectrometry analysis was performed on bacterial lysates of *Francisell*a and *E. coli.* While the

analysis showed an increase in the level of measured Se in the *E. coli* biomass it failed to show an increase of Se in the *Francisella* biomass above the measured Se of the culture media. Finally, elemental Se was tested to determine if it influenced growth, virulence or pathogenicity of *Francisella* through a series of *in vitro* and *in vivo* assays. These studies did not demonstrate differences in the growth, virulence or pathogenicity of *Francisella* that was cultured in the presence or absence of Se. This led to the conclusion that *Francisella* neither utilizes nor is influenced by the presence of elemental Se in supraphysiological levels up to 200nM. Therefore, *Francisella* is an ideal model to study acute bacterial infections in mammalian systems.

To determine the effect of Se on *F. tularensis* infection, groups of mice were maintained on Se-deficient (<0.01 ppm of Na_2SO_3) or Se-supplemented (0.4 ppm Na_2SO_3) defined diets. While 50%of Se-supplemented mice died following infection with *F. tularensis,* 100% of Se-deficient mice succumbed suggesting that Se plays a significant role in protection from infection. Since, F. *tularensis* infects and replicates primarily in macrophages, I investigated the bacterial replication in Se-deficient (treated with 0 nM of Na_2SO_3) and Se-supplemented (200 nM of Na_2SO_3) macrophages. Interestingly, Se-supplemented macrophages restricted bacterial growth by about >20 fold compared to Se-deficient cells. Next, the role of selenoproteins in restricting bacterial growth was tested by using the macrophages derived from $Trsp^M$ mice, which are unable to synthesize selenoproteins in macrophages. $Trsp^M$ macrophages were unable to control the replication of *F. tularensis* compared to wild-type (WT) macrophages. Consistent with these results, $Trsp^M$ mice were significantly more susceptible to *F. tularensis* infection than WT mice. Furthermore, bacterial burden in the livers and spleens of $Trsp^M$ mice were significantly higher than WT mice indicating that macrophage selenoproteins are essential for restricting bacterial replication and promoting host survival.

2.1 Introduction

Selenium (Se) is an essential micronutrient, often sufficiently supplied from dietary sources, it is important for a large repertoire of molecular and immunological functions. The hormetic effect of selenium is dependent on specific incorporation of Se into the proteome of humans, which is tightly regulated under homeostatic conditions, but can be temporally shifted to a deficient state during periods of microbial infection (1). Reports have suggested that the use of Se in therapeutic programs may provide benefits, primarily due to an increase in antioxidant levels which directly corresponds to expression of a select class of Se containing proteins called selenoproteins (2). Selenoproteins have been shown to affect immune functions through multiple interacts, including but not limited to antioxidant and redox function. Multiple human and animal trials have been completed to assess the effects of Se supplementation as a therapeutic adjuvant, specifically in cases of systemic inflammation like that caused by some bacterial infections (3-12). Se in combination with antibiotic treatment has been shown to act synergistically to reduce bacterial burden, though the molecular mechanism was not fully characterized (13). It has also been reported that Se supplementation can reduce toxicity in prokaryotes which also utilize Se as cofactors and selenoproteins (14). Additionally, there are a few reports published with evidence that Se supplementation enhances the bioenergetics of stickland reactions in bacteria, a type of fermentation used to extract energy under limited conditions, which have been described to promote survival (15). *Clostridium difficile* is an example of a microorganism which utilizes this process (16). The CDC has classified *Clostridium difficile* as an urgent hazard in its 2014 report based upon a set of predefined criteria (17). One of the major concerns is the emergence of antibiotic resistant strains of gram-negative bacteria, like *C. difficile*. While Se adjuvant intervention may confer benefits in multiple clinical instances of bacterial infection, it may have devastatingly different results for pathogens like *C. difficile.* Clearly, a disconnect lies in our understanding of the pleiotropic effects of Se and selenoproteins on immune function and perhaps through interrogation of those interactions we can more acutely target novel functions of selenoproteins to use as new therapeutic strategies. To address the role of Se and selenoproteins on immune function in the host system alone a suitable bacterial model that does not utilize Se must be selected. To this end we propose using *Francisella*.

Bacteria that incorporate selenocysteine into proteins do so using a highly conserved pathway with many similarities to eukaryotic and archaea systems. This pathway involves the products of the *sel*A, *sel*B, *sel*C and *sel*D genes (18, 19). The pathway is centered around a unique tRNA species specific for the UGA codon $tRNA^{[sec]}$ (the *sel*C gene product) (20). $tRNA^{[sec]}$ is initially charged with serine; the seryl moiety is then converted to a selenocysteyl moiety. Selenocysteine synthase (the *sel*A gene product) catalyzes the serine-to-selenocysteine reaction, which requires selenophosphate as a donor (provided by selenophosphate synthetase, (the *sel*D gene product) (21). Selenoprotein mRNAs feature a stem-loop secondary structure known as a SElenoCysteine Insertion Sequence (SECIS) element; in bacteria this is found immediately downstream of a UGA codon unlike eukaryotes where it is located in the 3'untranslated mRNA (19). The positioning of the UGA codon next to the SECIS element reduces the need for additional cofactors in formation of the biosynthetic complex. For the UGA codon to be translated as selenocysteine, a specialized translational elongation factor (the *sel*B gene product) must interact with the selenocysteyl- $tRNs^{[sec]}$, the SECIS element and GTP at the ribosome (22). Thus, bacteria that translate proteins containing selenocysteine generally require the *sel*A, *sel*B, *sel*C and *sel*D genes and their respective products.

Alternatively, bacteria may utilize Se as a part of a unique 2-selenouridine base in the wobble position of select tRNAs (23). *YbbB* can be synthesized independently of *sel*A, *sel*B, *sel*C, but is thought to require *sel*D (23). Lastly, the final method of Se utilization in prokaryotes is as a cofactor in molybdenum enzymes. In the case of molybdenum hydrolyses it is suggested the Se is not bound, but the crystal structure of Nicotinate dehydrogenase (NDH) from *Eubacterium barkeri* suggests the Se atom is bound (24, 25). Figure 2.1 illustrates the prokaryotic utilization of Se.

Understanding the evolution of Se utilization has been made possible through the robust sequencing efforts of the last decade. Large metagenomic analyses have provided rich data sets allowing researchers to understand how Se is utilized in all branches of life, as well as trace its utilization back to earlier ancestors supporting the hypothesis that selenoproteins are a highly conserved and ancient (23, 26). The universal last common ancestor, while not containing selenoproteins was thought to rely on Se as a cofactor (27). Even though selenoproteins, modern lineage-specific or original (ancient) like Formate dehydrogenase are common among

prokaryotes, not all prokaryotes utilize elemental Se (26). Strong relationships have been established between prokaryotes utilizing Se and characteristics of the prokaryotes' biological requirements such as oxygen and temperature, for which anaerobes in physiologically relevant temperatures are most likely to utilize Se (28). Others have looked at patterns emerging form ecological niches that suggest bacteria found in a host have a higher probability of utilizing Se (29). Ultimately, the determining factor for retention or loss of the selenoprotein biosynthetic genes in an organism's genome may be tied to the organism's need for enhancement of catalytic function which elemental Se offers (28). Romero et al. and Zhang et al. have published large scale metanalyses of bacterial utilization of Se (23, 28) Romero et al. published an extensive list of bacteria containing genes for the biosynthetic pathway of selenocysteine for which Gammaproteobacteria like *Yersinia pestis,* an intracellular, gram-negative highly pathogenic coccobacillus bacteria is listed (23). Similarly, the Zhang study published a report that of the 79 Gammaproteobacteria sampled 38 species had been found to utilize Se (28). *Francisella,* was not found to be reported by either Romero or Zhang, but it is Gammaproteobacteria with some of the characteristics associated with Se utilization.

Francisella tularensis is a facultative intracellular gram-negative, non-motile, aerobic bacterium that causes the acute disease tularemia in humans and animals. *F. tularensis* has a broad host range and versatile tropism (30, 31). *F. tularensis* infections can occur from ingestion, inhalation, and direct physical contact or from arthropod vectors. Clinical manifestations of tularemia are as an ulceroglandular, glandular, oculoglandular oropharyngeal, typhoidal or pneumonic infection (32). The type of infection depends heavily on the route of infection. The symptoms of tularemia are similar to that of the flu, but unlike the flu *F. tularensis* can be treated with antibiotics. Cases of tularemia in humans are rare and amount to approximately 300 cases annually in the US, though strains of *F. tularensis* are found in many places around the world and incidences of tularemia vary greatly by location (33). There are four subspecies of *F. tularensis,* which include *tularensis, holartica, novicida* and *mediasiatica*. Of the subspecies *Francisella tularensis* subsp. *tularensis* is considered the most virulent to humans. Due to a lack of an approved vaccine, virulence, severity of pulmonary infection, ease of aerosolization and its historical use as a bioweapon *F. tularensis tularensis* ssp. *tularensis* SCHU4 is categorized as a class A select agent (34). A *F. tularensis* live vaccine strain (LVS) has been produced from *F. tularensis holartica*, but LVS does not confer suitable protection, means of attenuation are

unknown, which has resulted in failure to gain approval from regulatory agencies. LVS as it will be referenced henceforth is however a useful model system that can be used in a laboratory setting with a reduced biosafety level of 2 compared to SCHU4 which must be handled at a level-3.

F. tularensis preferentially infects phagocytic cells. *F. tularensis* modulates the antimicrobial defenses of macrophages, and utilizes the cytoplasmic environment for replication and evasion of the host immune system (35). Despite the classification of facultative intracellular, *F. tularensis* is auxotropic for 16 amino acids and relies heavily on the cellular compartment to carryout growth (36). *F. tularensis* utilizes numerous cellular receptors to gain entry into a cell. Once inside the cell the *F. tularensis* escapes the endocytic compartment as it matures to the cytoplasm where it undergoes numerous rounds of replication. Typically, in a laboratory *in vitro* assay the bacteria will increase on the order of 1 to 2 logs (36). *F. tularensis* has an arsenal of virulence factors which allow it to evade cellular defense systems, these factors including structural components like its O-antigen and lipid A as well as enzymatic effectors like superoxide dismutase, catalases and other proteins like *Igl*C which has been linked to phagosomal escape and the type 6 secretion system (36, 37). In approximately 24 hours the bacteria has entered the cell, completed replication, and begins to egresses as the apoptotic cell ruptures. Conversely, macrophages are one of the few immune cells capable of killing the intracellular bacteria with costimulatory signaling from interferon gamma (IFN-γ) (38). However, *Francisella* utilizes a number of virulence factors that allow it to evade detection such as its unique O-antigen and lipid A as well as other effector molecules which suppress RONS once inside the host cell (35, 39).

When macrophages are activated and mount an ROS/RNS response to microbial infection there are cellular mechanisms that help to dissipate these radicals to prevent damage to the host. The 21st amino acid, selenocysteine is an integral residue of selenoproteins which performs a wide variety of functions including maintaining redox, antioxidant as well as protein folding and possibly many other functions that have yet to be determined. Models have been generated in animals that use Cre/loxP technology to disrupt their selenocysteinyl tRNA$^{[Sec]}$ (Trsp$^{fl/fl}$) (2). These models revealed that broadly impairing selenocysteine incorporation resulted in an embryonic lethal phenotype (2). However, mutant animals with the Trsp$^{fl/fl}$ gene in

conjunction with a cell specific Cre expression patterns are viable, and have been used to elucidate functional roles for selenoproteins in immune response. Specifically, macrophage and T cell functions have been shown to be profoundly impaired from Se deficiency (40). Se supplementation has been reported to modulate macrophage response and plasticity (42). Up-regulation of key factors involved in phagocytosis, cell migration and cellular metabolism has been attributed to selenoprotein activity (2, 40, 43). It remains to be fully elucidated if the functional shift in the macrophages' characteristics provides an advantage to innate immune response during acute bacterial infection. It is also unclear if the mechanism of action in our model is due to a specific selenoprotein(s) or if it is the result of another Se-containing molecule.

Since *F. tularensis* contain genes that encode several enzymes involved in selenocompound metabolism such as methionyl-tRNA synthase and thioredoxin reductase, it was suggested that *F. tularensis* may need selenium for its intracellular growth or survival (44). In support of this prediction, microarray data from the same group indicated that *F. tularensis* activated the genes for selenium utilization in the host cell. Therefore, I sought to determine the contribution of selenium in *F. tularensis* pathogenesis. Our data indicate that *F. tularensis* does not contain specific genes critical for selenocysteine or 2-selenouridine synthesis. Additionally, the presence or absence of selenium in growth medium does not influence the bacterial replication or virulence. In contrast, selenium was found to be critical for limiting the bacterial growth in macrophages. Furthermore, our data indicate that the effects of selenium was primarily mediated via selenoproteins. Therefore, I speculate that the upregulation of host genes for selenium utilization may be a host defense mechanism against F. tularensis.

2.2 Methods

Bacterial stocks were generated through by expansion of 1 colony forming unit of *F. tularensis* LVS in Chamberlains defined media (CDM) pH adjusted to 6.2 prepared as described (45). The stocks were cultured and serially passaged at least 4 times in CDM under deficient 0nM Na_2SeO_3, adequate 50nM Na_2SeO_3 or supplemented 200nM Na_2SeO_3 conditions. Cultures were grown in a shaker incubator maintained at 37ºC, with a speed of 175 rpm until they reached an optical density measured at 600nM (OD_{600nM}) of 0.5OD. Cultures were then snap frozen in CDM with a measured viability of $1.2x10^9$ colony forming units (CFU) per mL.

Bacterial growth assays were carried out in 96 well flat bottom plates (Costar) with the *F. tularensis* LVS starting at 0.002OD in CDM with Na_2SeO_3 adjusted to a final concentration of 0nM, 50nM or 200nM. OD_{600nM} measurements were taken every 30 min for a period of 25 hours using a Spectromax spectrophotometer (Molecular Devices). To determine the viability of the bacteria in culture, samples were diluted in phosphate buffered solution (PBS) (Hyclone) and plated every 8 hours on chocolate agar plates prepared from Mueller-Hinton agar (Becton, Dickinson and Company) supplemented with 1% (w/v) bovine hemoglobin (Remel) and 0.5% (v/v) IsoVitaleX™ (Becton, Dickinson and Company). Plates are incubated at 37°C in a 5% CO2 for 72 hrs.

Bacterial virulence gene expression was measured using isolated RNA from pelleted bacteria grown to log phase in CDM with or without Se. The RNA was extracted using TRIzol™ reagent (Invitrogen) and purified using a RNA isolation kit (Amigen). cDNA was prepared and Real-Time PCR was carried out with SYBR™ Green (ThermoFisher) reaction mix and measured using the CFX96 Touch™ Real-Time PCR Detection System (BioRad). Fold change values were calculated by comparing the CT values of *fop*A(FTL _1328), *igl*C(FTL_1159 and *tul*4(FTL _0421) to the internal control of *pol*A (FTL _1666) (primers listed in Table 2.1).

Gentamicin protection assays were carried out using bone marrow derived macrophages suspended in DMEM 5% fetal bovine sera (Hyclone, Lot AYC60564) 2mM L-glutamine (Corning), 1.5mM HEPES(Hyclone), 1mM sodium Pyruvate (Hyclone) and 1X Non-Essential Amino Acids (Hyclone) referred to as DMEM complete media. BMDMs were prepared as previously described in deficient conditions and seeded at 3-5x105 cells/well in a 24 well tissue culture treated plate (Cellstar) (46). *F. tularensis* LVS in deficient CDM was revived and grown in a shaker incubator maintained at 37ºC, with a speed of 175 rpm until it reached 0.05 to 0.1 OD. *F. tularensis* LVS was then diluted in DMEM complete media to a final targeted concentration of 2.5 $X10^7$-5 $X\ 10^7$ CFU/mL. Macrophages were infected at an MOI of 1:50 to 1:100 CFU. Cells were centrifuged at a speed of 300 x g, 25°C for 10 min. Cells were then incubated at 37°C, 5% CO2 for 20 min. Media containing bacteria was aspirated and the wells were supplied fresh DMEM complete media containing 100µg/mL of gentamicin (Gibco) and incubated at 37°C, 5% CO2 for 1 hour to remove extracellular bacteria. Three PBS washes were

completed for all wells; some wells were resuspended in DMEM complete media under deficient conditions, while others were lysed for CFU enumeration at 2hrs post inoculation. Cells were lysed with a solution of 0.1% deoxycholate in PBS solution for 2-5 min at RT. Cell lysates were then diluted in PBS and plated as described.

F. tularensis and *E. coli* were grown in CDM or Brain Heart Infusion broth (Becton, Dickinson and Company) culture pH 6.2 with Na_2SeO_3 added to a final concentration of 0nM, 50nM or 200nM. Cultures were incubated in a shaker and agitated at 175 rpm, 37°C overnight. Cultures were centrifuged at3222xg, 20 min, 4°C. Pellets were washed with PBS and centrifuged for a total of 3 washes. Pellets were then resuspended in 2ml of MS grade water (Sigma) and pulsed using a digital sonicator (Branson, Digital Sonifier) for 3 cycles of 30 secs at a power of 20. Bacterial lysates or control media was then filtered through a 0.45μM filter and analyzed by The Water Quality Laboratory, Penn State Institute of Energy and the Environment (PSIEE).

In silico data analysis was completed with the data sets described in Table 2.1. Data was retrieved from the REFSEQ genomic and protein databases in FASTA file format (Table 2.2). A blastp 2.5.0+ function using the default settings was performed on known selenoprotein sequences using the reference sequences from *E. coli* (NC_000913.3) and *D. vulargis* (NC_002937.3) against *F. tularensis* (NC_006570.2, NC_007880.1) sequences, and this was followed by a reciprocal function (47, 48). *F. tularensis* genomes were interrogated for $tRNA^{[Sec]}$, using the tRNADB-CE6 and GtRNAdb7 databases (49, 50). *E. coli* K12 was used a reference using the Conserved Domain Architecture Retrieval Tool (CDART) (51). Ontology was assessed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (52).

C57Bl/6 mice were placed on specialized Harlan Teklad diets (Harlan Laboratories, TD.92163, TD.96363 and TD.07326). Se deficient Teklad diet is estimated to contain less than 0.01 ppm of background Se. A Se adequate diet (control) has 0.1ppm (0.1mg/kg) Se from the Na_2SeO_3 additive. The Se supplemented diet has 0.4ppm (0.4mg/kg) Se from the Na_2SeO_3 additive. Mice were maintained on these diets for greater than 12weeks as recapitulated from other Se diet studies (42).

$Trsp^{M}$ animals lacking macrophage specific selenoproteins were a kind gift from the Hatfield lab and maintained in the PSU animal resource program (40). The $Trsp^{M}$ animals are $Trsp^{fl/fl}$ that also carry either heterozygous or homozygous for cre-recombinase under the lysozyme M promoter as described (40). The control "wild type" (WT) animals have the following genotypes: $Trsp^{fl/fl}$ $LysM^{wt/wt}$, $Trsp^{fl/wt}$ $LysM^{cre/wt}$ or $Trsp^{fl/wt}$ $LysM^{cre/cre}$. $Trsp^{M}$ and WT animals were greater than 6 weeks old and maintained standard chow diets. All mouse husbandry and experimentation was conducted in accordance with guidelines and approved IACUC protocols.

Genotyping was performed on transgenic animals to verify successful deletion of the Trsp gene as previously described (53). Briefly, ear tissue samples were taken from all mice and placed in 200µl tail lysis buffer (50mM Tris-HCL, 25mM EDTA, 50mM KCL and 0.4%(v/v) NP40 Tween-20) with added in a 1:50 (v/v) OB Protease Solution (Omega) and incubated at 55ºC overnight. Lysates are vortexed then heated to 95ºC for 5 min. The lysates are then spun at 10,000xg for 2 mins at RT. The supernatant is removed and used for PCR. The PCR is prepared using molecular grade water(Hyclone) with 1:5(v/v) of 5X GoTaq Buffer (Promega), 1µM Magnesium chloride (New England BioLab), 200µM dNTPs (New England BioLab) 0.20µM of each primer 1U of Go Taq DNA polymerase, and 1µl of lysates containing DNA (Nelson et al. 2011).The PCR amplification is completed using the following program 95ºC/5min (1 cycle), 95ºC/0.5min, 59 ºC for Trsp or 54 ºC for LysM/1min, 72 ºC/1min (40 cycles) followed by a final 72 ºC/3min (1cycle). Products were run on 1.8% Agaros gels and imaged using the Gel doc (Bio-Rad, Universal hood II) and Image Lab software (Bio-Rad, version 5 build18). The primers used to detect regions of interest and product sizes are listed in Table 2.5.

In vivo studies were completed through inoculation of mice with *F. tularensis* (LVS) CDM frozen stocks diluted to inoculums which ranged from 15-35 CFU/µl. Mice were anesthetized using isofluorene gas (Piramal Healthcare) 400ppb for 30 secs. Mice were then inoculated by placing 2-5droplets of PBS comprising 50µl of LVS culture over the nostrils. Mice were observed daily and body mass measurements were taken twice daily.

For bacterial enumeration organs were harvested at the described endpoint or when greater than 20% body mass had been loss, which ever occurred first. Organs (lung and spleen) were homogenized in PBS with 1.0mm Zirconi/silicon beads (RPI Research products) in a Bead

Blaster™24 (Bench Mark D2400) using 6 cycles of 1 min with a 30sec break at a power level of 7. Whole livers were placed in whirl bags and crushed in 1mL of PBS. Homogenates were diluted and plated on chocolate agar or GC (Becton, Dickinson and Company) plates to enumerate CFU.

Bone marrow derived macrophages (BMDMs) were prepared as described previously (46). In brief, the femur and tibia of euthanized mice were crushed through a 70μm strainer in Complete DMEM (Hyclone, SH3008102), 20% (v/v), 5% (v/v) fetal bovine serum (Hyclone, Lot# AWG18462), 2mM L-Glutamine (Gemini 400-106), 1.5mM HEPES (Corning), 1mM sodium pyruvate (Hyclone) and 1X nonessential amino acids (Hyclone) here after referred to as DMEM complete media. Bone marrow was then centrifuged at 400 x g, 10 min at 25°C. The supernatant was decanted and the pellet was resuspended in Complete DMEM media with 20% (v/v) L929 conditioned media (containing granulocyte colony stimulating factor) supplemented. Na_2SeO_3 (Sigma) was added to culture of adequate (50nM) or supplemented (200nM) cells. Cells were plated at approximately 5 x 105 cells/dish in $100cm^2$ petri dishes (VWR) and allowed to rest for 48 hours. On days 3 thru 5 cells were feed fresh Complete DMEM media supplemented with 20% L929 with or without Na_2SeO_3.

Western blot analyses were completed by first lysing cells with RIPA buffer plus 1X HALT™ Protease Inhibitor cocktail- 1X EDTA solution (ThermoFisher) for 10 min followed by dilution with 2X laemmli buffer (Biorad). MiniProtean®TGX™ precast gels (Biorad) were used to separate proteins. Proteins were then transferred to PVDF membranes using the Biorad Turbo Blotsystem and Trans-Blot®Turbo™ transfer packs (Biorad). Blots were blocked in 5% fetal bovine albumin (Sigma) and probed using primary antibodies: 1:1000 dilutions of rabbit anti-mouse GPX-1 (Abcam) and 1:100,000 rabbit anti-mouse β-actin. This was washed and probed with a goat anti-rabbit H&L chain secondary conjugated to horseradish peroxidase enzyme (HRP) at a dilution of 1:7500. The blots were developed with HRP substrate (Millipore). The blots were imaged and analyzed using a Gel doc (Bio-Rad, Universal hood II) and Image Lab software (Bio-Rad, version 5 build18).

Statistical analyses were performed using unpaired t-test with Welch's correction for two samples, and analysis of variance (ANOVAs) were used for analyses comparing three or more groups followed by a Bonferroni's Multiple Comparison Test. Log-Rank Mantle-Cox tests were

used to determine significance for survival studies. All statistical analyses were performed using the GraphPad software Prism for Mac OS X (version 5).

2.3 Results

***In Silico* Analyses reveals that *F. tularensis* lacks the machinery for selenocysteine incorporation into proteins**. To determine if *F. tularensis* can incorporate selenocysteine into proteins, I interrogated *F. tularensis* genomes using a range of bioinformatics tools and databases (Table 2.3). Firstly, we ran blast to determine if there were any selenoproteins predicted in the *F. tularensis* proteome using a blastp 2.5.0+ tool. Using protein sequences allowed us to overcome inherent codon usage bias between our reference organisms and *F. tularensis*. The protein blastp function was enhanced by the curated domain (CD) search tool, but resulted in hits with extremely low scores and high E-values (54). Upon closer examination of the hits it was clear that there was a very low identity between resulting hits, which strongly suggests that the matched hits are highly conserved among organisms including *F. tularensis* and therefore retain important domains of essential genes such as those for tRNA (*sel*C). It was known that *F. tularensis* did carry a copy of formate dehydrogenase subunit (*fdh*), and of course this was also a hit using the *E. coli* reference genome. However, as with the other hits the identity was exceedingly low.

Next, it was assessed whether *F. tularensis* encodes $tRNA^{[sec]}$, using the tRNADB-CE6 and GtRNAdb7 databases. Both sources confirmed the presence of $tRNA^{[Sec]}$ in known selenocysteine incorporating bacteria (*E. coli* and *D. vulagaris*), but failed to identify the $tRNA^{[sec]}$ in any published *F. tularensis* genomes that were analyzed, including the LVS and SchuS4 strains. The lack of a $tRNA^{[sec]}$ with the appropriate anticodon strongly suggests *F. tularensis* is unable to incorporate selenocysteine into proteins.

Nonetheless, to determine whether any of the other components of the selenocysteine incorporation machinery are present in *F. tularensis*, the CDART and KEGG database tools were utilized. Additionally, using the amino acid sequences of *E.coli sel*A and *sel*B gene products as templates, CDART identified architectures comprised of two and five conserved domains, respectively. Filtering by the NCBI taxonomy tree, these domain architectures were found in the

genomes of other known selenocysteine incorporating bacteria (e.g. *D. vulgaris*), but were absent in all *F. tularensis* genomes. Furthermore, individual domains specific to selenometabolism (Selenocysteine synthase N-terminal domain in *sel*A, *sel*B-winged helix domain in *sel*B) were completely absent in *F. tularensis* which strongly suggests functional copies of these genes are not present. Consistent with the above findings, KEGG resources demonstrate that in all available *F. tularensis* genomes, *sel*B is not among the translation elongation factors, and *sel*A, *sel*D and SeU (*Ybb*B) are absent in the KEGG selenocompound metabolism pathway – while all are present for *E. coli* and *D. vulgaris* (excluding *Ybb*B). Collectively, bioinformatics analysis of *F. tularensis* genomes suggests these bacteria lack all four canonical factors and SeU that are required for Se incorporation into proteins. Our data strongly supports our conclusion that *F. tularensis* does not incorporate Se.

Another concern was that *F. tularensis* may indiscriminately acquire Se from the host cell because it posses the enzyme selenocysteine lyase/cysteine desulfurase (CsdB, FTL_1333). The enzyme is capable of two reactions: 1) L-cysteine + acceptor = L-alanine + S-sulfanyl-acceptor 2) L-selenocysteine + reduced acceptor = selenide + L-alanine + acceptor. Attempts to use molecular techniques to disrupt or eliminate this gene were unsuccessful suggesting it is an essential gene (data not shown).

Atomic absorption spectrometry measurements failed to detect accumulation of elemental Se in bacterial lysates from *Francisella.* Using a Database of Trace Element Utilization (dbTEU) (55) it was noted that *Francisella* utilizes primarily copper and zinc. Unlike other prokaryotes which encode Se-dependent molybdenum hydroxylases (SDMH), *F. tularensis* does not use Se as cofactor. Still, *F. tularensis* and *E. coli* lysates were empirically tested for detectible levels of Se over the defined or undefined culture broth. The data collected suggested that elemental Se was either not detected or present in the relative concentration of the culture media in LVS (Figure 2.2). By contrast, the reference organism *E. coli*, had appreciable levels of Se when cultured in supplemented CDM as well as detectable levels in neat BHI with increased levels measured in Se supplemented BHI. This data provides convincing evidence that *Francisella* does not utilize Se as a selenoprotein, modified base or as a cofactor.

The presence of Se in bacterial culture broth does not affect growth kinetics or virulence of *Francisella.* In order to establish that Se is affecting macrophage physiology

exclusively it was important to rule out adverse effects Na_2SeO_3 may have on bacterial physiology. To test this *F. tularensis* was cultured in CDM adjusted with Na_2SeO_3 to final concentrations of 0nM, 50nM or 200nM for 25 hours. The changes observed in OD over time were similar among all Se conditions tested (Figure 2.3a). Using a linear regression model the slopes were analyzed during log phase growth between 10 and 20 hours and the slopes were found to be similar without significant difference (data not shown). Furthermore we plated the liquid growth cultures at 0, 8, 16and 24 hours and recovered similar numbers of CFU among all Se conditions (Figure2.3b). Given, that we were unable to detect significant differences in the growth or viability of the bacteria among all Se conditions it can be concluded that Se does not influence *F. tularensis* growth in liquid culture.

We next performed RT- PCR to assess changes in the expression of LVS genes associated with virulence: OmpA family protein (FTL_1328, *fop*A), Intracellular growth locus, subunit C (FTL _1159, iglC) and 17kDa major membrane protein (FTL _0421, *tul*4) (references for these genes). DNA polymerase (FTL _1666, polA) gene expression was measured as the internal control. There were no observed differences in the expression levels of genes associated with virulence (Table 2.4).

To empirically determine if Na_2SeO_3 in the bacteria culture could influence the ability of the bacteria to enter or replicate in a Se deficient macrophage a gentamicin protection assay was performed. The bacterial entry (Figure 2.4a) and bacterial burden 24 hours post inoculation (Figure 2.4b) were similar between Se conditions. This data provided additional evidence that the presence of Na_2SeO_3 at concentrations below or equal to 200nM had no effect on the bacterial physiology or its ability to infect. Finally, an *in vivo* assay was completed to determine if *F. tularensis* supplemented with Na_2SeO_3 in liquid culture would have an effect on the ability of the bacteria to colonize and replicate in mouse lung tissue. Similarly, bacterial burden at 6, 12 or 24hours post inoculation was comparable between Se conditions (Figure 2.5). Taken together, this data indicates the presence or absence of Na_2SeO_3 in liquid bacterial culture has no effect on the physiology or infectivity of *F. tularensis.*

Se deficiency increased susceptibility of the mice to pulmonary tularemia. I tested if mice maintained on a specific Se diet had similar response to *F. tularensis* infection. Animals were feed special diets of <0.01ppm of Na_2SeO_3 (deficient), 0.1ppm of Na_2SeO_3 (adequate), or

0.4ppm of Na_2SeO_3 (supplemented) for greater than 12 weeks. Intranasal inoculated was then performed with *F. tularensis* LVS at 1500CFU/mouse for all mice. All animals feed a deficient diet of <0.01ppm succumb to infection with the lethal dose (LD_{100}) equal to 1500 CFU (Figure 2.6a). By contrast, mice with Na_2SeO_3 added into a defined diet had increased rates of survival and loss less body weight with a LD_{50} of 1500 CFU (Figure 2.6b) suggesting that selenium plays an important role in the host-pathogen interaction during *F. tularensis* LVS infection. Due to the small sample size of 4 animals per group the power of the experiment did not yield significant results in mortality at day 14 using a Log-Rank Mantle-Cox test. Still, a strong trend existed among groups. This suggested that deficiency created a loss of immune protection during pulmonary infection and to this end I wanted to understand if the decreased survival was due to an inability to control the bacterial infection. Since macrophage cells have the ability to kill intracellular *Francisella*, and it has been well documented macrophage function can be affected by Se I wanted to test if this compartment of the immune response was compromised during deficiency leading to an inability to control the bacterial replication (56).

To measure the potential for intracellular growth of *F. tularensis* LVS, a gentamicin protection assay was performed using BMDMs cultured from diet mice and maintained *ex vivo* in Na_2SeO_3 under deficient (0nM), adequate (50nM) or supplemented (200nM) conditions. The data I collected suggested cells deficient in selenium had the highest bacterial burden 24 hours after inoculation as compared to the Se conditions (Figure 2.7a). The bacterial numbers recovered from deficient BMDMs agrees with other reports that have shown approximately a 50 to 100-fold increase in *F. tularensis* over 24 hours (46). Interestingly, Se supplementation seemed to limit bacterial growth in a dose-dependent manner as less growth was observed in adequate and supplemented groups (Figure 2.7b). The data indicates that the presence of Se is restricting *F. tularensis* LVS intracellular growth, and moreover suggests this may be a factor in the conference of protection during pulmonary challenge.

It is well documented that Se supplementation increase selenoprotein levels in cells and tissues. It has also been established that the increases in expression are hierarchical and that protein levels of certain selenoproteins are more dramatically correlated with Se status then others, for example macrophage Glutathione peroxidase-1 (GPX1) (40) In our hands, BMDMs GPX1 protein expression was also increases in a dose dependent manner through Se

supplementation (Figure 2.8a). Se supplemented BMDMs had 12X the relative expression of GPX1 as compared to the deficient cells (Figure 2.8b). Paradoxically, expression of GPX1, an antioxidant would reduce intracellular ROS/RNS. ROS/RNS are cellular defense mechanisms used by macrophages to eliminate intracellular bacteria. I next needed to determine if selenoprotein expression was related to the intracellular bacterial replication restriction observed. I first wanted to determine if BMDMs supplemented with alternative forms of selenocompounds would have similar expression patterns in selenoprotein GPX-1 levels and if these compounds would also limit intracellular bacterial growth. To this end BMDMs supplemented with 200nM of methylseleninic acid (MSA), selenomethionine (SeMet), Na_2SeO_3, and those maintained under deficient conditions were tested. A reduced intracellular growth was observed in BMDMs supplemented with MSA and Na_2SeO_3 as compared to BMDMs maintained in deficient media or supplemented with SeMet (Figure 2.9a). Furthermore, GPX-1 protein expression levels for BMDMs cultured *ex vivo* in MSA and Na_2SeO_3 were higher than for BMDMs maintained at deficient conditions or supplemented with SeMet (Figure 2.9b). This data further supported the conclusion that the bacterial replication restriction was influenced by the expression levels of selenoproteins. I speculate that the supplementation of SeMet in BMDMs did not result in an increase of selenoproteins due to low expression levels of γ-lyase, a required enzyme for the utilization of SeMet in the methylselenol pool (57). I next wanted to understand if lack of macrophage selenoproteins were contributing to a reduction in survival during pulmonary tularemia.

Macrophage selenoproteins are required for control of *F. tularensis* during pulmonary infection. Provided that Se was found to be important for the BMDM specific bacterial replication restriction of *F. tularensis* LVS, and that restriction was observed only with selenocompounds which enhanced selenoprotein expression as measured by GPX1. I next wanted to investigate if macrophage selenoproteins were important for survival of tularemia. Using a conditional selenoprotein knock out model ($Trsp^M$) the role of macrophage selenoproteins was examined during an active LVS infection (40). Mice underwent an intranasal inoculation of 750, 1500 or 1750 CFU of *F. tularensis* LVS and their body mass was monitored over 14 days (Figure 2.10). These studies demonstrated that $Trsp^M$ animals were more susceptible to pulmonary infection, with a statistically significant higher mortality rate compared to the WT cohort (Figure 2.10 a, b). This data suggested that selenoproteins were required for

macrophage response to *F. tularensis* LVS infection. Though, it should be recognized that the presence of macrophage selenoproteins are not enough to improve the survival rate animals inoculated beyond the LD_{100} (Figure 2.10c).

Next bacterial burden was assessed in the lung, liver, spleen and blood of animals 5 and 7 days after intranasal inoculation with 750 CFU. By day 5 the liver of $Trsp^{M}$ animals harbored statistically significant higher bacterial numbers as compared to the WT controls, with this difference becoming more pronounced by day 7 (Figure 2.11b). Differences observed in bacterial burden between $Trsp^{M}$ and WT animals were statistically significant on day 7 in the blood and spleen as well (Figure 2.11 c, d). Moreover, the trend of increased bacterial burden was observed in all the organs of $Trsp^{M}$ animals as compared to WT. This data indicates $Trsp^{M}$ animals with macrophages lacking selenoproteins were less able to control *F. tularensis* LVS infection as compared to the WT controls. This data suggests that animals lacking macrophage selenoproteins are unable to control the bacterial infection and that this influenced the decreased survival rate we observed in these studies.

Selenoproteins restrict *F. tularensis* replication in macrophages independent of Se supplementation. *In vivo* studies had provided evidence that macrophage selenoproteins were important for controlling *F. tularensis* LVS infection. Thus, it was important to establish if the bacterial replication restriction observed in the diet model (Figure 2.7b) was mediated in a selenoprotein specific manner. To our knowledge selenoproteins have not been reported to participate in bacterial replication restriction directly. Indeed, using a gentamicin protection assay it was observed that $Trsp^{M}$ macrophage cells had higher bacterial numbers 24 hours post inoculation (Figure 2.12a). The increased bacterial replication of *F. tularensis* LVS in the $Trsp^{M}$ macrophages could not be reduced through the addition of Na_2SeO_3 (Figure 2.12b), indicating that the bacterial replication restriction observed was a result of a selenoprotein mediated mechanism. This data supported the conclusion that macrophage specific selenoproteins are required for control of *F. tularensis* LVS replication both *in vivo* and *in vitro*.

2.4 Discussion

The data presented provides evidence that *F. tularensis* is an excellent model to study the effects of the micronutrient Se on the host system during acute bacterial infection. I have demonstrated through the *in silico* analysis that *F. tularensis* does not possess the required machinery for selenocysteine biosynthesis or specific incorporation. Furthermore, I have shown through atomic absorption spectrometry that Se is not accumulated in the biomass of the bacteria when cultured in supraphysiological levels. Finally, I have shown that Se at or below 200nM do not influence growth, virulence or the bacteria's ability to colonize murine tissues. Going forward tularemia is an excellent disease model to better understand how Se status influences molecular mechanisms of the host immune response. Particularly, how macrophages, given that they are among few cells that can directly kill *Francisella* will respond under varied Se status.

Se deficiency has been shown to have detrimental effects from dysfunction of neutrophil and natural killer cell recruitment to altered cytokine expression (58, 59). The obvious and most recognized result of Se deficiency is down regulation of selenoprotein expression, most notably of selenoproteins involved in redox homeostasis (60, 61). Consistent with findings in other bacterial infection models, Se deficient mice had a more pronounced phenotype, and an inability to survive pulmonary challenge with *F. tularensis* LVS. Other studies have focused on a reduction in polymorphonuclear leukocytes recruitment and only speculated that the PMN's have an inability to control infection were as the data described here demonstrated Se deficient macrophages have an inability to control intracellular bacterial infection (62).

Supraphysiological levels of Se supplementation decreased the intracellular *F. tularensis* LVS replication in macrophages. To our knowledge this is the first report of bacterial control of *F. tularensis* LVS through Se supplementation. Additionally, multiple mechanisms of the bioactivity of Se can be attributed to selenoproteins, and macrophages lacking selenoproteins have higher levels of bacterial burden implicating their direct role in bacterial control. This was corroborated by *in vivo* studies where $Trsp^{M}$ animals demonstrated higher levels of mortality after pulmonary challenge. This increased mortality demonstrates the critical role macrophage selenoproteins play during tularemia.

It could be speculated the inability of $Trsp^M$ mice to control bacterial burden in the liver tissue may have been due to a selenoprotein dependent reduction in interferon-γ (IFN-γ) and IL-6 cytokine levels (63). It will be important in future studies to target cytokine expression levels in an effort to better understand the mechanism contributing increased mortality of the $Trsp^M$ animals. This is especially true, given that using an LD_{100} I observed similar levels of pathology in the lung, liver and spleen (data not shown). It has been well documented that a primary component of macrophage bactericidal activity against *F. tularensis* is mediated through iNOS expression after IFN-γ signaling (38). However, in the $Trsp^M$ animal studies, excluding granulocytes expressing lysozyme M, all other cells have intact selenoprotein synthesis and therefore given the redundancy observed in an immune response it is likely that other cells would contribute to cytokine expression *in vivo.* It could also be speculated that without the presence of selenoprotein K, FCγR phagocytosis of macrophages is compromised reducing the ability to take up opsonized bacteria (43). This too may be unlikely a factor because, it has been reported that naive serum provides little protection, and actually may overwhelm phagocytic response providing inadequate bacterial control during *F. tularensis* infection (64). While it could be argued that these larger systemic effects may play a role in the mouse model, the contributions of these mechanisms *in vitro* would be irrelevant. The bacterial replication restriction observed in the $Trsp^M$ macrophages is independent of IFN-γ and antibody involvement and to that end I am interested in studying the intracellular mechanism for bacterial replication restriction.

Unlike studies completed in peritoneal macrophages challenged with *S. aureus* bactericidal activity in BMDMs under selenium supplementation was not observed (65). I speculate that one factor may be the supplementation of Se reported in the Aribi study that elicited the bactericidal effects was 10X higher at 2000nM, compared to the more physiologically relevant concentration of 200nM we utilized in these studies (65). However, irrespective of the differences between the studies determining intracellular RNS/ROS and arginase levels must be further scrutinized. Future studies will also address levels of iNOS expression, which if found to be increased may warrant further biochemical examination of proteins to determine if nitrosative stress has occurred. Monitoring levels of ROS/RNS and arginase are important to fully understand the underlying mechanisms contributing to bacterial replication restriction.

Key Concepts:

- *F. tularensis* lacks the required genes to biosynthesize or specifically incorporate selenocysteine.
- *F. tularensis* does not accumulate Se in its biomass, therefore it is unlikely it utilizes Se as a cofactor.
- *F. tularensis* is not physiologically affected by the presence of elemental selenium at supraphysiological levels up to 200nM.
- Se deficiency increased susceptibility of the mice to pulmonary tularemia
- Macrophage selenoproteins are required for control of *F. tularensis* during pulmonary infection
- Selenoproteins restrict *F. tularensis* replication in macrophages independent of Se supplementation

2.5 Tables and Figures:

Table 2.1: Primers used for RT-PCR

Gene	Forward	Reverse
FTL _1666, *Pol*A	5'-TACACGACCATTTGCGTCCA-3'	5'-CAGCAAAGGGTCAAGTGTCG-3'
FTL _0421, *Tul*4	5'-GTGCCATGATACAAGCTTCC-3'	5'-GCTGTCCACTTACCGCTTCA-3' 5'-GCTGTCCACTTACCGCTACA-3'
FTL _1159, *Igl*C	5'-CCAGGCTCTATAAATCCAACAATA-3'	5'-TTTCATATCTGTAGCACTTGCTTG-3'
FTL _1328, *Fop*A	5'-AGGATCTGTTCAAGGTGCTT-3'	5'-GCATTAGGCTGGTTCATTCC-3'

Table 2.2: Data used to in the *in silico* analyses

Organism	REFSEQ: (accession)	Assembly:
Escherichia coli	>NC_000913.3 Escherichia coli str. K-12 substr. MG1655, complete genome	EColiK12MG1655_Full_GCF_000005845.2_ ASM584v2_protein
Desulfovibrio vulagaris	>NC_002937.3 Desulfovibrio vulgaris str. Hildenborough chromosome, complete genome	Desculfovibrio vulgaris str. Hildenborough_ full_GCF_000195755.1_ ASM19575v1_protein
***Francisella tularensis ssp. tularensis* SCHU4**	>NC_006570.2 Francisella tularensis subsp. tularensis SCHU S4 chromosome, complete genome	*F. tularensis*ularensis SCHUS4_Full_GCF_000008985.1_ ASM898v1_protein
***Francisella tularensis ssp. holartica* Live Vaccine Strain**	>NC_007880.1 Francisella tularensis subsp. holarctica LVS complete genome	*F. tularensis*ularensis holarctica LVS_Full_GCF_000009245.1_ ASM924v1_protein

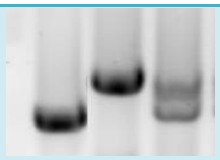
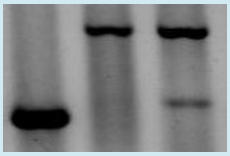
Table 2.3: Genes required for synthesis and incorporation of selenocysteine

Gene	Product	Function	*E. coli*	*D. vulgaris*	*F. tularensis Live Vaccine Strain*	*F. tularensis tularensis* Schu S4
selA	selenocysteine synthase	generates selenocysteine from serine + selenophosphate	Present	Present	Absent	Absent
selB	SelB	specialized elongation factor for selenocysteine	Present	Present	Absent	Absent
selC	$tRNA^{Sec}$	SeCys tRNA, specific for UGA codons	Present	Present	Absent	Absent
selD	selenophosphate synthetase	generates selenophosphate, the Se donor required by selenocysteine synthase	Present	Present	Absent	Absent
ybbB	tRNA 2-selenouridine synthase	Catalyzes 2-thiouridine to 2-selenouridine from selenophosphate donor	Present	Absent	Absent	Absent

Table 2.4: Relative gene expression levels measured by Real-Time PCR

Virulence Gene	$2^{-\Delta\Delta CT}$	SD	Significance
FopA	1.11	0.11	ns
IglC	0.91	0.51	ns
Tul4	0.62	0.36	ns
Control Gene			
DNApol	1		

Table 2.5: Genotyping primers and products

Description	Sequence	Product	
Trsp CKNO2	5'-GCAACGGCAGGTGTCGCTCTGCG-3'	**$Trsp^{wt/wt}$ = 900 bp $Trsp^{fl/fl}$= 1100 bp $Trsp^{wt/fl}$ = 900bp &1100bp**	
Trsp CKN08RP	5'-CGTGCTCTCTCCAGTGGCTA-3'		
MLys1	5'-CTTGGGCTGCCAGATTTCT-3'	**$LysM^{wt/wt}$ = 350bp $LysM^{Cre/Cre}$ = 700 bp $LysM^{wt/cre}$=700 bp &350bp**	
MLys2	5'-TTACAGTCGGCCAGGCTGAC-3'		
LysM Cre8	5'-CCCAGAAATGCCAGATTACG-3'		

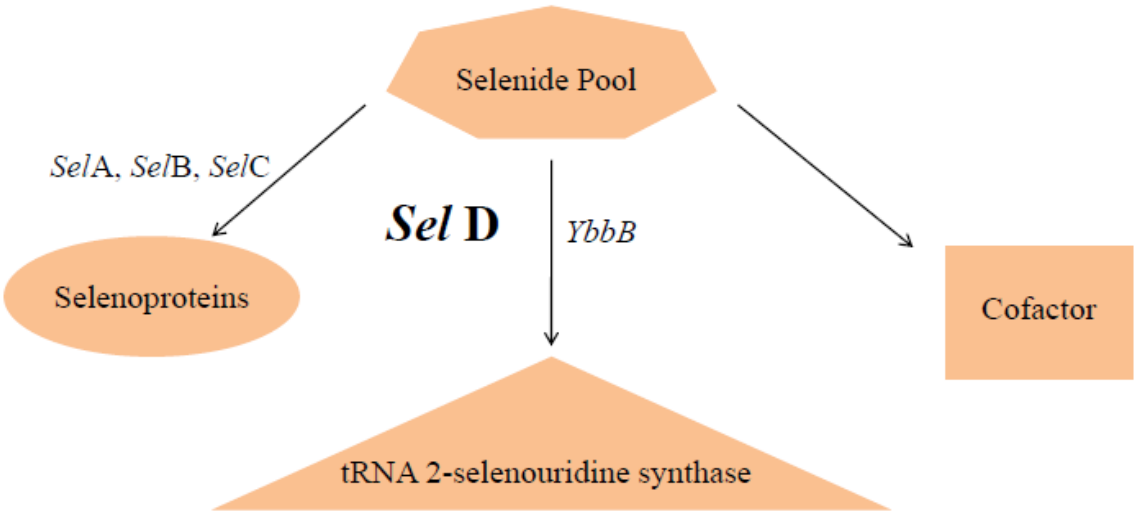


Figure 2.1: Prokaryotic utilization of Se. Figure adapted from Self, WT presentation at University Park 2016.

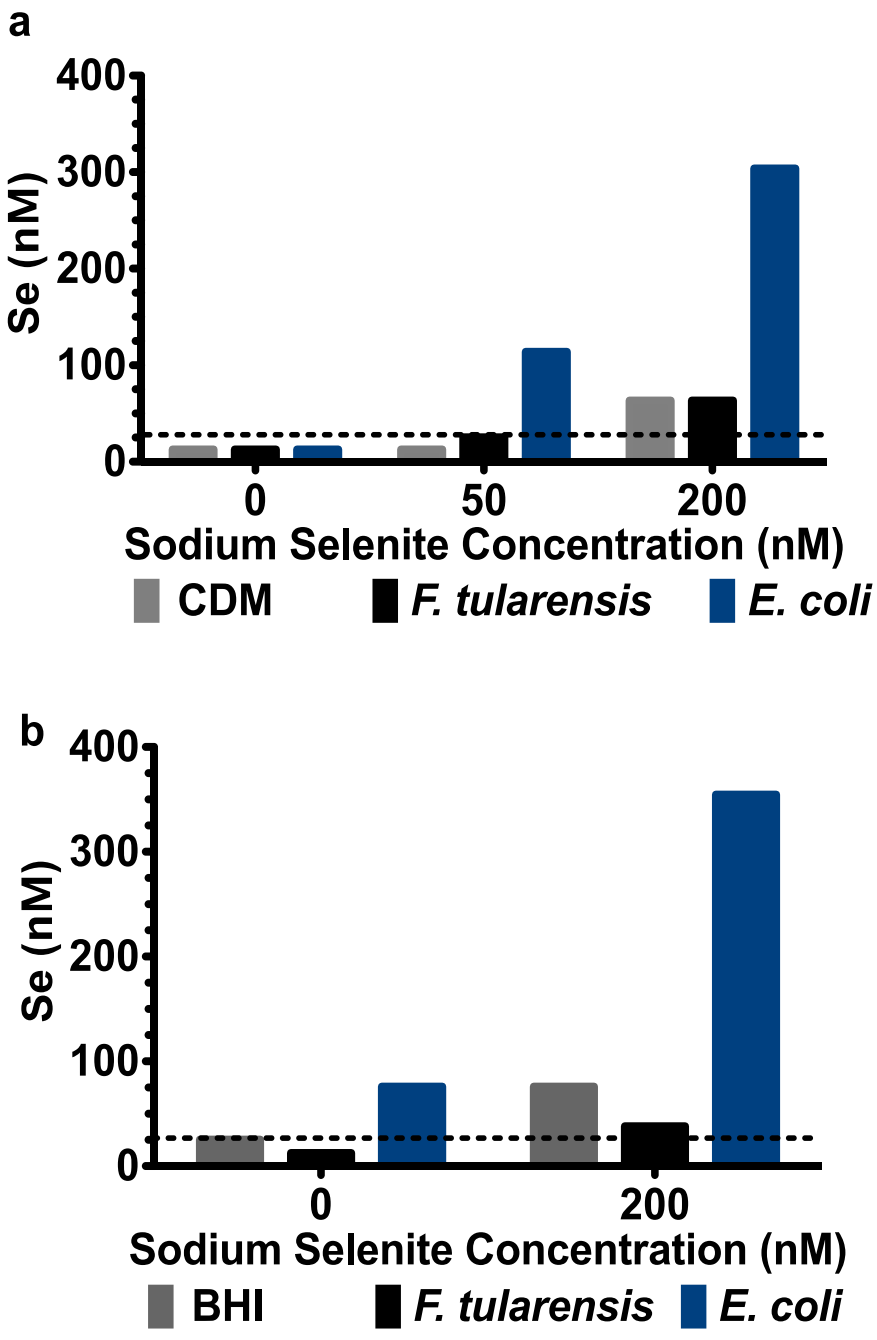


Figure 2.2: *F. tularensis* LVS does not incorporate Se. *F. tularensis* or *E. coli* K12 was grown in (a) CDM or (b) undefined BHI broth to saturation equating to approximately 10^{11} CFU/mL. All media was supplemented with Na_2SeO_3 as indicated. Bacteria pellets were washed well and reconstituted in ultrapure water. Concentrations of elemental Se in bacterial lysates were measured using Atomic Absorption Spectrometry.

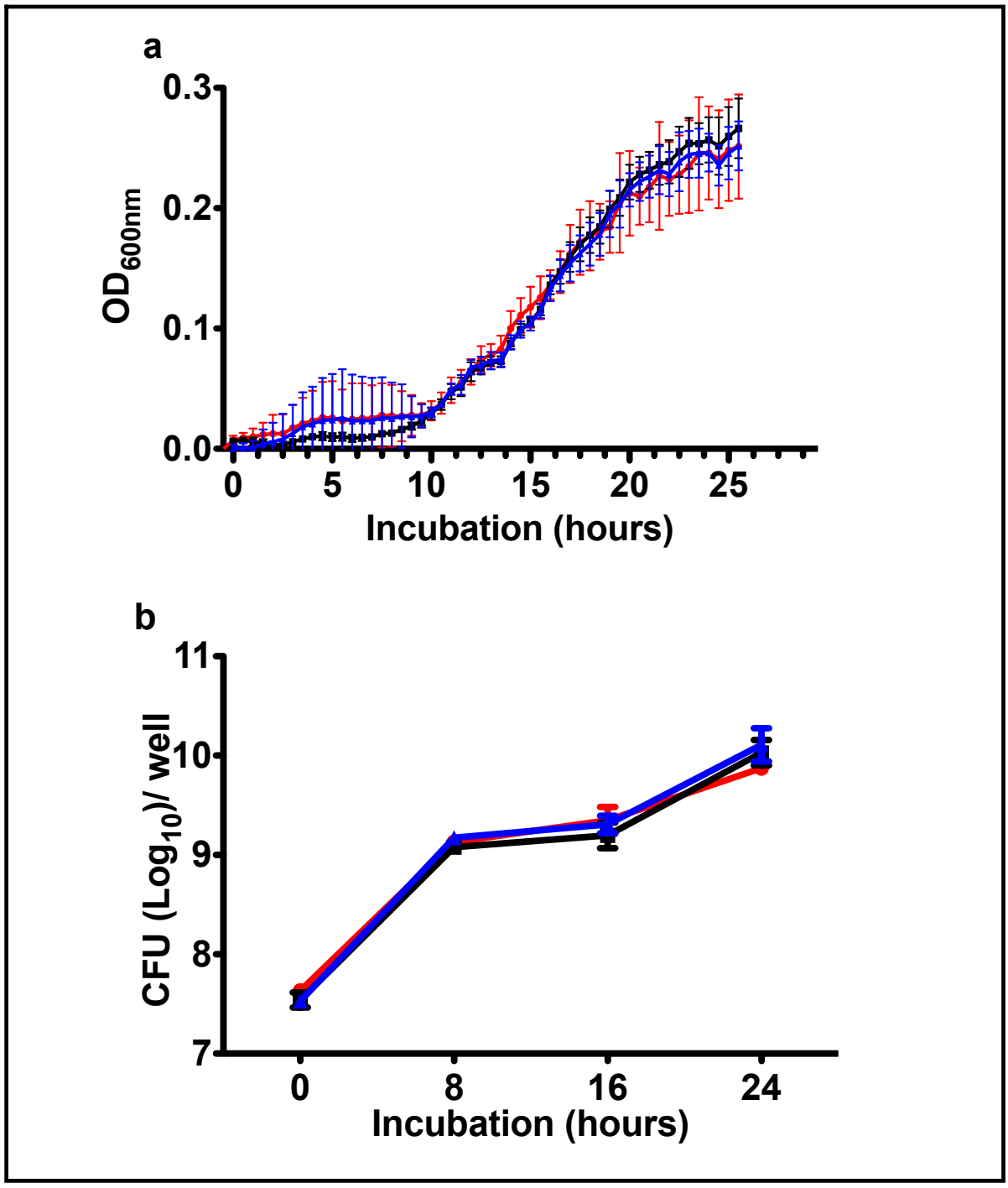


Figure 2.3: Se in liquid culture does not affect the growth of *F. tularensis* LVS.

Growth of *F. tularensis* was monitored over 24 hours in CDM under various Se statuses by measuring the (a) OD_{600nM} and (b) CFU recovery at indicated times.

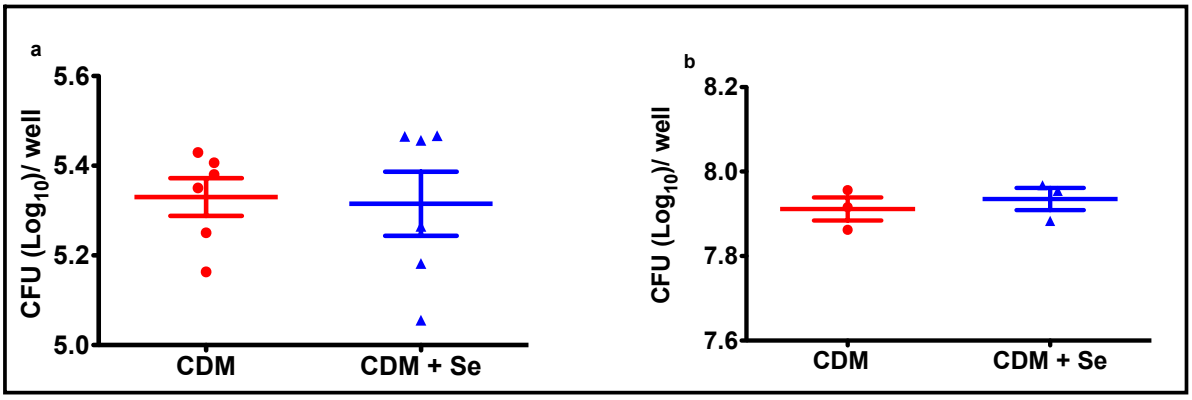


Figure 2.4: Se status of bacterial culture does not affect *in vitro* entry and replication *of F. tularensis* in macrophages. BMDMs were prepared from 6-8 week old C57Bl/6 mice and maintained *ex vivo* under deficient conditions. BMDMs were infected with *F. tularensis* LVS at MOI of 1:100. Entry at 1.5hrs and intracellular growth of bacteria 24 hrs post infection were enumerated. (a) The number of bacteria recovered at 24 hrs represented as the mean of triplicates. (b) Bacterial growth over 24 hrs represented as fold change. Data presented here represents of one of three similar experiments. Statistical significance was assessed using an unpaired t-Test with Welch's correction ($p<0.05$).

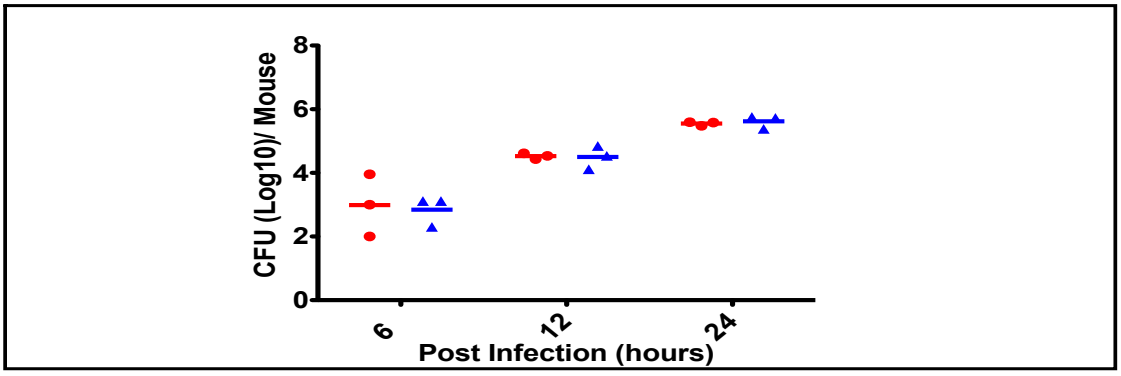


Figure 2.5: Se status in bacterial culture does not affect in vivo replication of *F. tularensis* LVS in the lungs of mice. 6-8 week old C57Bl/6 mice were inoculated with 10,000 cfu of *F. tularensis* LVS by intranasal route. Bacterial burden was measured at 6, 12 and 24 hours post inoculation. A Two-way ANOVA was performed to assess statistical significance ($p>0.05$).

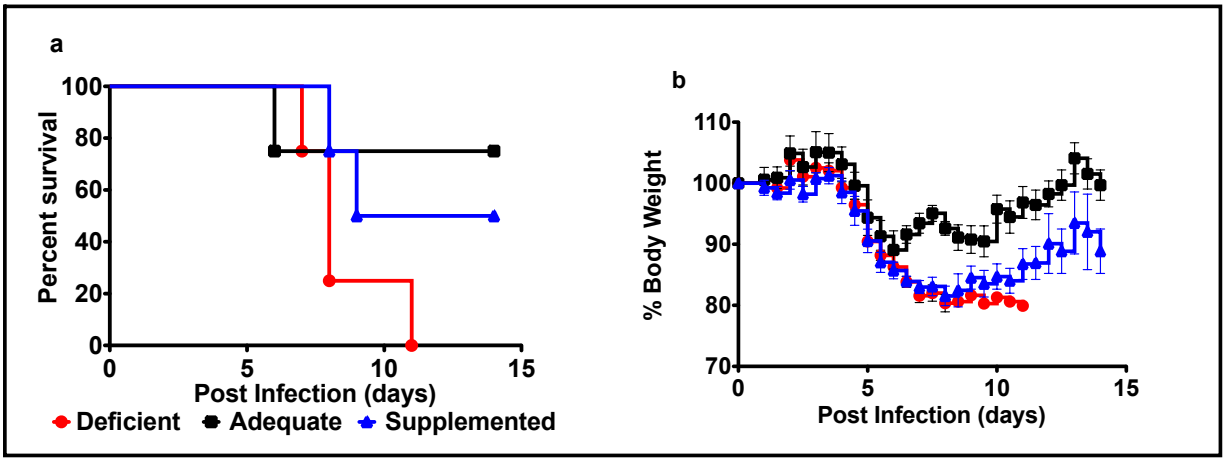


Figure 2.6: Dietary Selenium is required for survival from *F. tularensis* LVS infection. 6-8 week old C57Bl/6 mice were placed on Se deficient, adequate or supplemented diets and maintained for a minimum of 12 weeks (n= 4/group). These mice were inoculated with 1500 cfu of Ft LVS by intranasal route and body weight was monitored daily for 14 days. Mice that lost >20% of their starting body weight were euthanized. (a) Statistical significance of survival was assessed using the Log-rank (Mantel-Cox) test. (b) Daily weights were monitored and represented as the mean % body weight of the surviving animals.

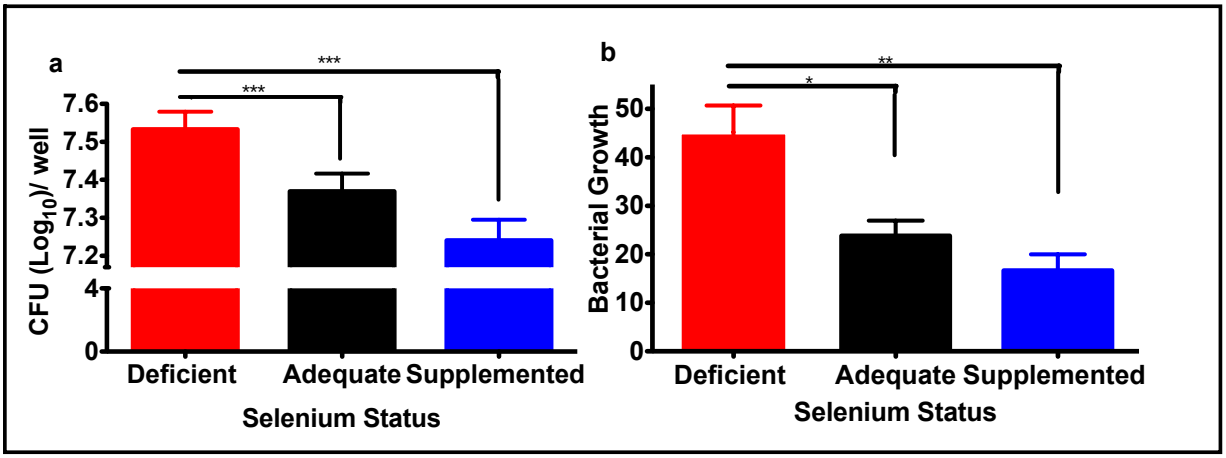


Figure 2.7: Selenium restricts *F. tularensis* LVS replication in macrophages. 6-8 week old C57Bl/6 mice were placed on Se deficient, adequate or supplemented diets and maintained for a minimum of 12 weeks. BMDMs were prepared and maintained *ex vivo* under deficient, adequate or supplemented conditions. BMDMs were infected with *F. tularensis* LVS at MOI of 1:100 and intracellular growth of bacteria 24 hrs post infection were enumerated. Graphs shown represent one of three experiments performed (a) The number of bacteria recovered at 24 hrs represented as the mean and standard deviation of triplicates. (b) Bacterial growth over 24 hrs represented as fold change. Statistical significance was assessed using a One-way ANOVA followed by a Bonferroni's Multiple Comparison Test ($p<0.05$). Data presented here represents one of three similar experiments.

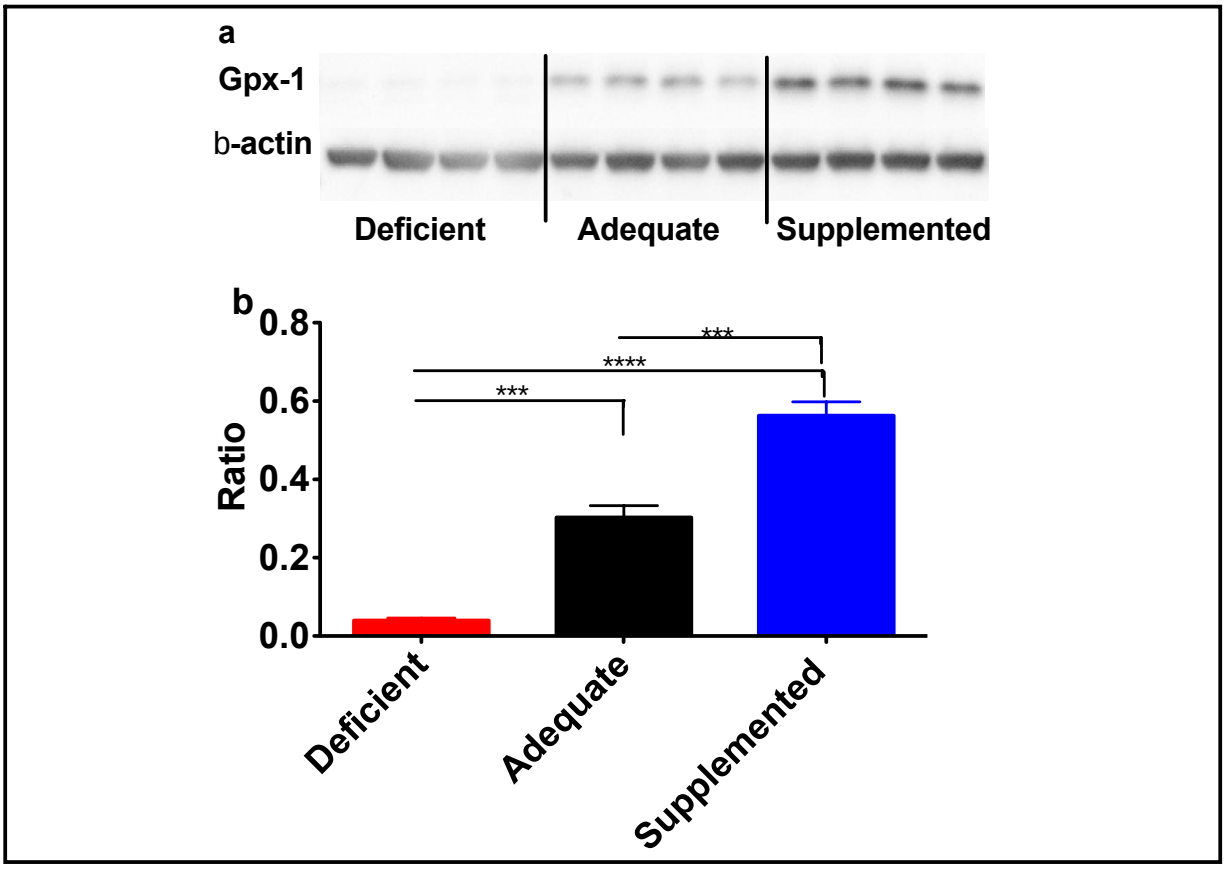


Figure 2.8: Glutathione peroxidase-1 expression in BMDMs under varied Se conditions. 6-8 week-old C57Bl/6 mice were placed on Se deficient, adequate or supplemented diets and maintained for a minimum of 12 weeks. BMDMs were prepared and maintained *ex vivo* under deficient, adequate or supplemented conditions. Lysates were prepared and relative expression of GPX1 by western blot analysis using four technical replicates. The data here represents one of three experiments performed.

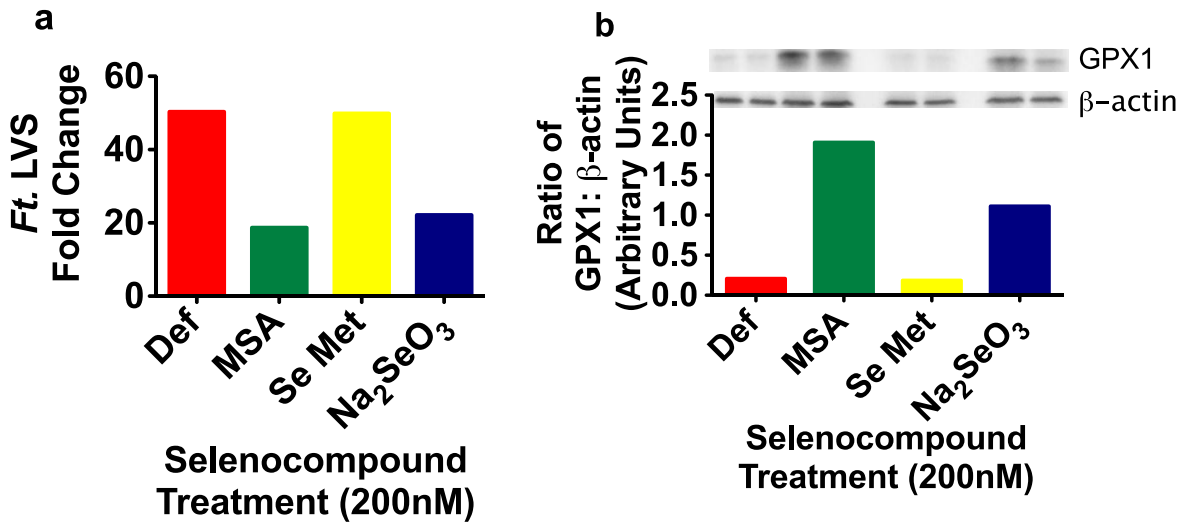


Figure 2.9: Methylseleninic acid and sodium selenite restricts *F. tularensis* LVS replication in BMDMs that may be due to an increase in selenoproteins as measured by glutathione peroxidase-1 expression. 6-8 week-old C57Bl/6 mice were placed on Se deficient or supplemented diets and maintained for a minimum of 12 weeks. BMDMs were prepared and maintained *ex vivo* under deficient or supplemented conditions with MSA, SeMet and Na_2SeO_3. (a) BMDMs were infected with *F. tularensis* LVS at MOI of 1:100 and intracellular growth of bacteria 24 hrs post infection were enumerated. The number of bacteria recovered at 24 hrs represented as the mean of triplicates. (b) Lysates were prepared and relative expression of Gpx-1 by western blot analysis using two replicates.

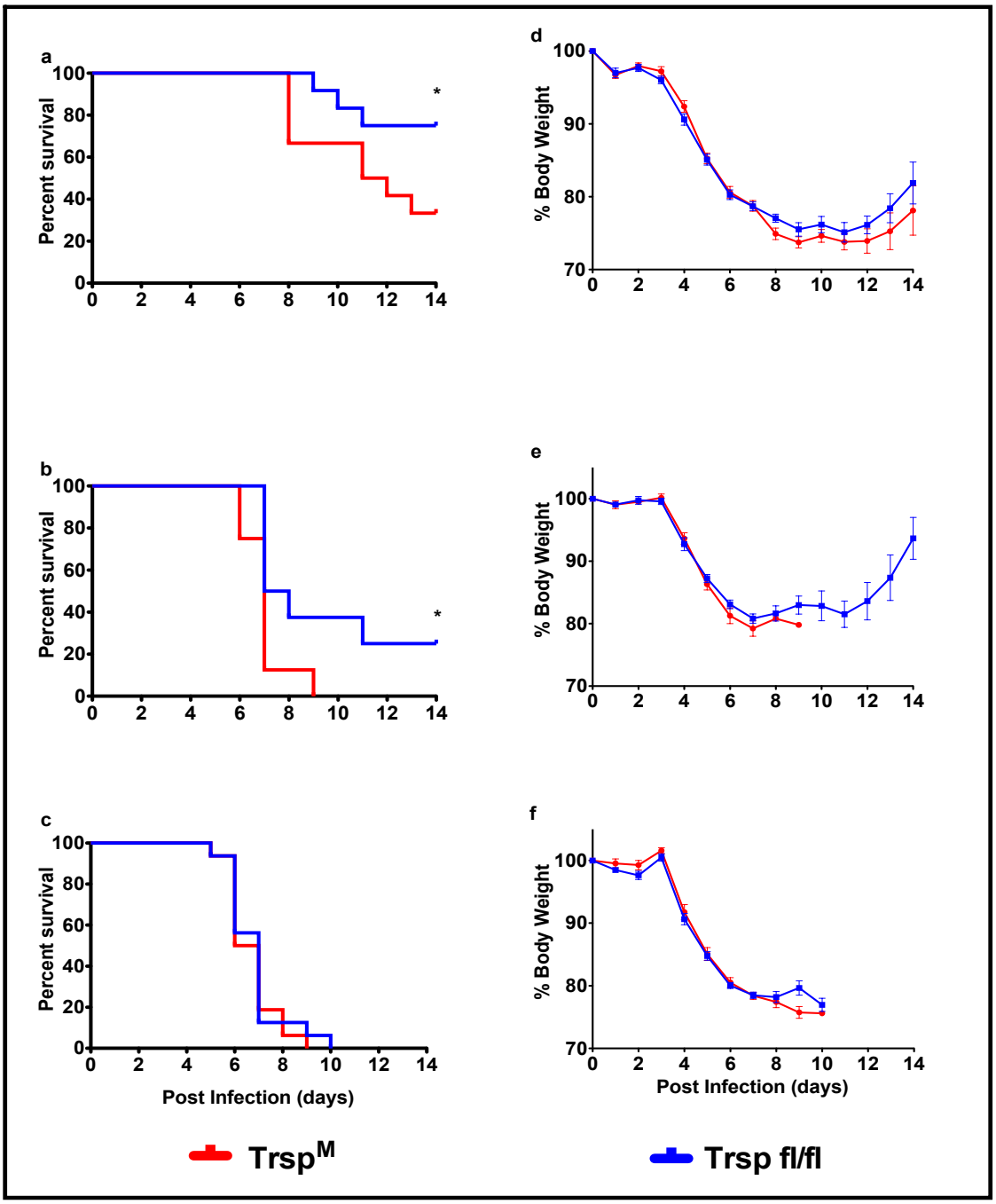


Figure 2.10: Macrophage selenoproteins are required for survival of *F. tularensis* LVS infection. 6-8 week-old Trsp^{M} and WT mice (n= 8-16/group) were inoculated with (a/d) 750, (b/e) 1,500 or (c/f) 1,750cfu of *Ft* LVS by intranasal route and body weight was monitored daily for 14 days. Mice that lost 20% -30% body weight were euthanized. Statistical significance of survival was assessed using the Log-rank (Mantel-Cox) test (a-c). Daily weights were monitored and are represented as the mean % body weight of the surviving animals (d-f).

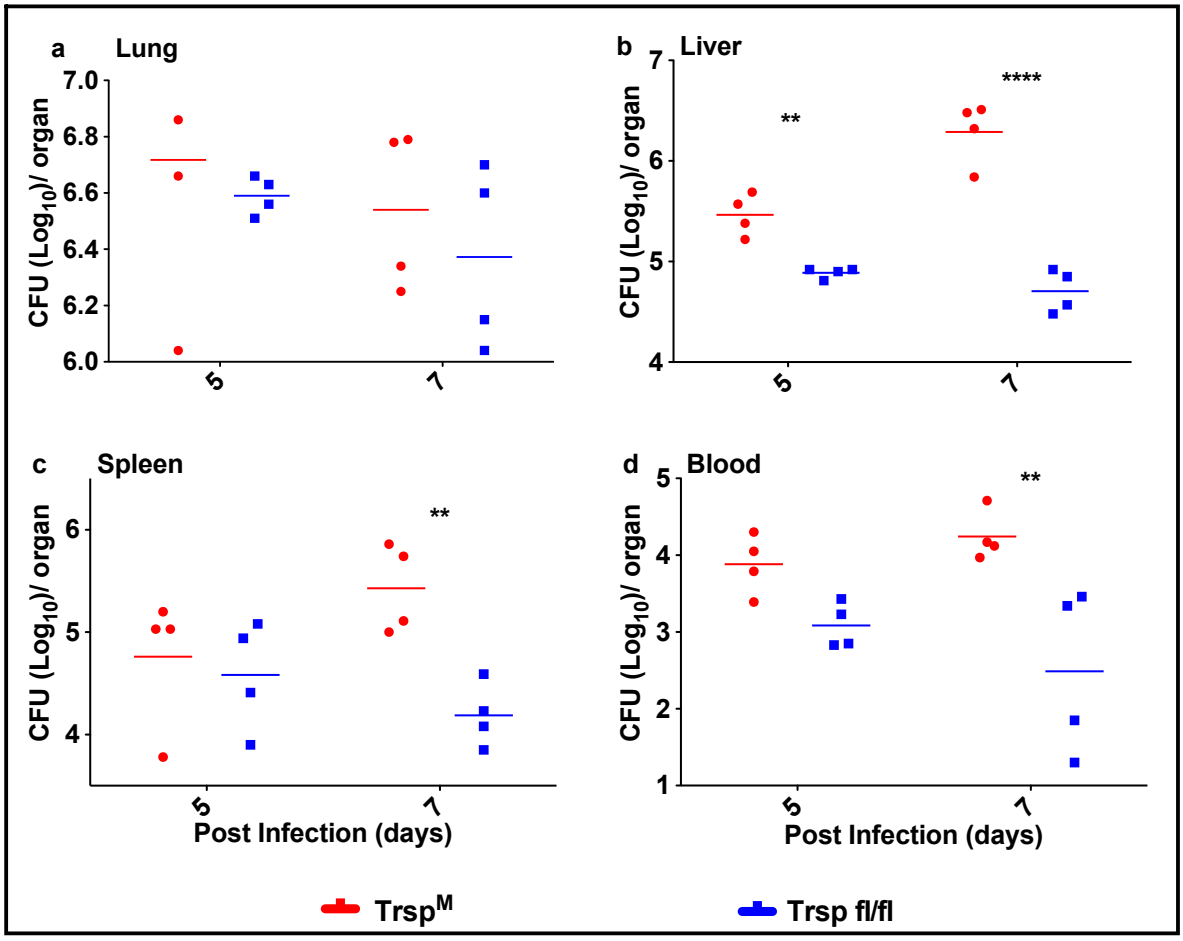


Figure 2.11: Macrophage selenoproteins are required for control of *F. tularensis* LVS infection in mice. 6-8 week-old $Trsp^{M}$ and WT mice (n= 4/group) were inoculated with 750 cfu of Ft LVS by intranasal route and body weight was monitored daily for 5-7 days. Mice were euthanized on day 5 and 7. (a)Lungs, (b) liver, (c) spleen and (d) blood were collected, organs were homogenized and bacteria were enumerated. Statistical significance was assessed using a Two-way ANOVA followed by a Bonferroni's post-test ($p<0.05$). The data presented here represents one of four similar experiments.

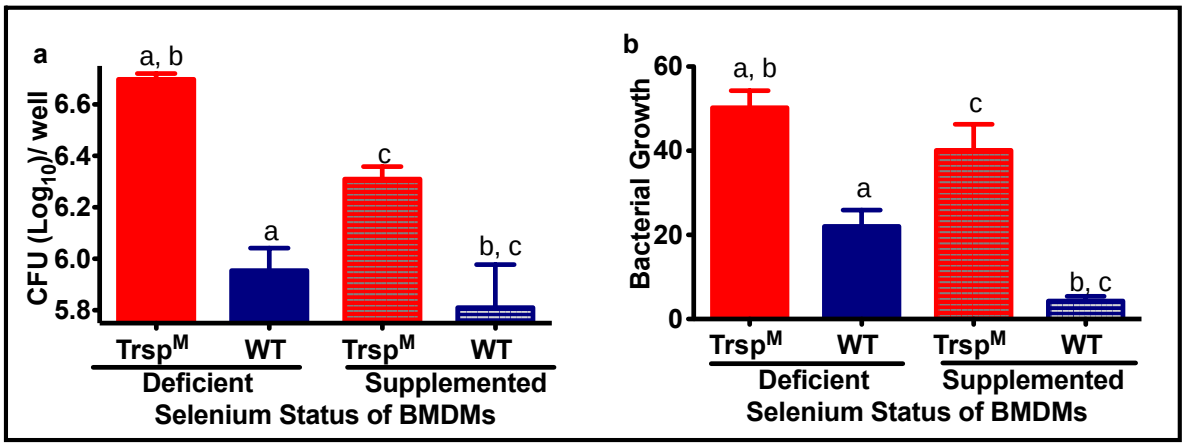


Figure 2.12: Macrophage selenoproteins restrict intracellular replication of *F. tularensis* LVS. Bone marrow was collected from 6-8 week old $Trsp^{M}$ and WT mice. BMDMs were prepared and maintained *ex vivo* under deficient or supplemented conditions. BMDMs were infected with Ft LVS at MOI of 1:50 and intracellular bacteria were enumerated 24 hrs post infection. (a) The number of bacteria recovered at 24 hrs represented as the mean of triplicates. (b) Bacterial growth over 24 hrs represented as fold change. Statistical significance was assessed using a One-way ANOVA followed by a Bonferroni's Multiple Comparison Test ($p<0.05$). The data here represents one of three similar experiments.

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Chapter 3: Insights into the mechanism by which selenoproteins restrict intracellular bacterial replication

Abstract

The micronutrient Se has been shown in our work to limit the intracellular replication of *Francisella tularensis* (*F. tularensis*). I have shown that the replication restriction is dependent on macrophage selenoproteins. Selenoproteins are characterized by their incorporation of the amino acid selenocysteine. Selenocysteine is the 21st amino acid and its incorporation is specific and tightly regulated through a biochemical pathway with specialized enzymes, cofactors, a unique tRNA and the SECIS element, a hairpin structure formed in the 3' untranslated region of the mRNA downstream from the AUG codon. Se status as well as loss of selenoproteins synthesis dramatically influences immune response. Trsp^{M} mice which lack macrophage selenoproteins due to the deletion of the tRNA$^{[Sec]}$ gene, show phenotypic differences in response and plasticity (1,2). This influences their ability to effectively mount a bactericidal defense against pathogens. Since they are one of the few cells capable of killing the intracullar pathogen *F. tularensis*, this dysfunction in response leads to decreased protection for infected Trsp^{M} animals.

Francisella is a gram-negative pathogenic bacterium, which is the causal agent of the acute infection, tularemia. *F. tularensis* has been used as a model system because this bacterium does not incorporate, utilize or display physiological phenotypes when exposed to supraphysiological levels of Se equal to or below a concentration of 200nM. *Francisella tularensis* subsp. *holarctica* live vaccine strain *(*LVS) is less virulent, but has been shown to utilize the intracellular compartment to complete replication through temporal phagosomal escape similar to that of the more virulent Category A agent *F. tularensis* subsp. *tularensis*[7]. Microarray data has suggested that suppression of ROS and expression of extra cellular matrix remodeling proteins occur during an active *F tularensis* infection. In addition, rapid replication of *F tularensis* has been shown to require the induction of autophagy in the macrophage[8].

The cytosolic environment of a macrophage is not an ideal niche for the survival or replication of bacteria. To compensate for a lack of nutrients and to evade host defenses *Ft.* modulates the host cytosolic environment (3). We hypothesize that Se via specific selenoprotein(s) downregulates autophagy limiting available nutrients thereby restricting bacterial replication. This restriction in bacterial replication may lead to a reduction in systemic infection and decrease *F. tularensis* pathogenesis.

3.1 Introduction

Selenium is an essential micronutrient, important in multiple aspects of human health from cancer to thyroid function and immune response. It has been well established that the main mechanism by which Se mediates immune function is through selenoproteins, a special class of proteins which incorporate the amino acid selenocysteine. It has been demonstrated that selenoproteins are crucial to normal immune function and response. Using transgenic mice selenoproteins have been eliminated through removal of the required $tRNA^{[Sec]}$ gene (1). Deletion of selenoproteins globally results in an embryonic lethal phenotype, so conditional knockout models were created to better characterize their functional roles within the select immune compartments (4). This is true for both T cells and macrophage cells (5). Macrophage selenoprotein knock out cells have been shown to have higher basal levels of reactive oxygen species (1). Moreover, it was reported that the cells have a reduced ability to migrate, a fundamental function as part of the innate immune response (1).

Macrophage cells are typically derived from the monocytes lineage. Macrophages are unique in that there are two populations; one which arose from embryogenesis and the other which occurs through hematopoiesis derived from the bone morrow in humans and mice (6). These two populations are dispersed throughout the tissues in the body (6). One set are differentiated and tissue restricted, these cells generally carry out functions to remove dying cells or debris and stand guard. The second are a rapidly generated from monocytes circulating in the blood, their differentiation and homing is triggered by injury or infection (6). To add to this complexity macrophage cells have pleiotropic phenotypes based on signals from their microenvironment. While they can descriptively be classified as either, M1 proinflammatory or M2 anti-inflammatory, they may also display phenotypes which fall somewhere in between (6, 7). It has been shown that macrophage phenotype may also play a role in the level of basal autophagy through activation of mammalian target of rapamycin (mTOR) (8). The versatile physiology of the macrophage allows this cell to perform a wide range of functions such as scavenging, wound healing and act as a defensive mechanism from microbial assault (6). The latter of which will be the focus of this chapter.

Resident macrophage cells within the tissue often are the first cells to “see” the infection. Macrophages are equipped with a number of receptors to sense and respond. Pattern recognition

receptors, for example can detect pathogen associated molecular patterns, like that of lipopolysaccharide derived from *E. coli* (6). Alternatively, if the pathogen has been opsonized this may trigger the FCγ receptor endocytosis (6). In both instances once a receptor has been triggered a signal cascade begins, the result of which may be proinflammatory or anti-inflammatory depending on the signal and the microenvironment of the macrophage.

In the case of professional phagocytes like macrophages they will engulf the receptor-antigen complex which will then undergo endosomal maturation processes. This will result in the degradation of the endosomal contents after lysosomal fusion. The macrophage may also up-regulate genes associated with cellular defense mechanisms. Often this is in the form of enzymes to generate reactive oxygen (ROS) and nitrogen species (RNS); example would be inducible nitric oxide synthetase (9). If bacteria do breach the confines of the confines of the endosomal compartment cells may utilize another approach to eliminate bacteria through autophagy.

Autophagy is an endogenous process by which cells degrade cytoplasmic contents to be used as new substrate for the cell. There are three forms of autophagy, however, macroautophagy would be the form triggered by intracellular pathogens (10). In this process cellular signals such as AMPK or activation of molecular target of rapamycin complex mTORC would be activated leading to protein complex formation and nucleation of a double walled membrane (10, 11). Through a series of enzymatic events, the phagosomal membrane is elongated and loaded with cargo (12). Phagosomes will mature into autophagosomes where lysosomal fusion will take place and the contents will be degraded and provide a nutrient source for the cell (10).

Not all pathogens are susceptible to intracellular defenses. In fact some pathogens such as *Francisella tularensis* can avert responses through expression of virulence factors (13). *Francisella tularensis* is a gram negative coccobacillus and the causative agent of tularemia. *F. tularensis* while facultative is auxotrophic for 16 amino acids (3). *F. tularensis* preferentially infects phagocytic cells gaining entry through a number of receptors including the mannose receptor and scavenger receptors among others (14). *Francisella* does not elicit a robust signaling response from toll-like receptors due to the structure of its O-antigen and lipid-A (15). Once inside the cell, *F. tularensis* escapes the endosomal compartment prior to lysosomal fusion via a series of virulence factors including Intracellular growth locus, subunit C (*Igl*C) (16). The

macrophage cytosol is not a hospitable environment, but *F. tularensis* produces additional virulence factors to suppress ROS/RNS responses (17, 18). Once in the cytosol *F. tularensis* undergoes multiple rounds of replication depleting the cell of nutrients. To overcome nutrient limitations, *F. tularensis* will induce autophagy within the macrophage to gain additional nutrients required to sustain its robust replicative burst (3). Though, it is not clear at this time the mechanism by which *F. tularensis* initiates autophagy, it is thought that bacteria avoids xenogaphy through additional virulence factors(14). The use of pharmacological inhibition of autophagy by others has been reported to cause a replication deficient phenotype, we observed similar results through Se supplementation of macrophage cells (3). Therefore, I hypothesized that Se via selenoproteins is suppressing autophagy leading to a restriction in *F. tularensis* bacterial replication.

The data collected and presented here will support the conclusion that bacterial replication restriction occurs at later time points during the *F. tularensis* infection. Furthermore, I will provide evidence that shows Se via selenoproteins is able to reduce induction of LC3 processing during stress induced autophagy. The selenoprotein dependent regulation of LC3 processing during stress induced autophagy is similar to the observed phenotype during *F. tularensis* infection, whereby Se supplementation dampens autophagic LC3 processing as compared to Se deficient cells. This data supports the idea that inhibition of *F. tularensis* induced autophagy at later time points during the infection can impede replication of the bacteria through nutrient deprivation.

3.2 Methods

C57Bl/6 mice were placed on specialized Harlan Teklad diets (Harlan Laboratories, TD.92163, TD.96363 and TD.07326). Se deficient Teklad diet is estimated to contain less than 0.01 ppm of background Se. A Se adequate diet (control) has 0.1ppm (0.1mg/kg) Se from the Na_2SeO_3 additive. The Se supplemented diet has 0.4ppm (0.4mg/kg) Se from the Na_2SeO_3 additive. Mice were maintained on these diets for greater than 12weeks as recapitulated from other Se diet studies (7).

$Trsp^M$ animals lacking macrophage specific selenoproteins were a kind gift from the Hatfield lab and maintained in the PSU animal resource program (1). The $Trsp^M$ animals are

$Trsp^{fl/fl}$ that also carry either heterozygous or homozygous for cre-recombinase under the lysozyme M promoter as described (1, 4). The control "wild type" (WT) animals have the following genotypes: $Trsp^{fl/fl}$ $LysM^{wt/wt}$, $Trsp^{fl/wt}$ $LysM^{cre/wt}$ or $Trsp^{fl/wt}$ $LysM^{cre/cre}$. $Trsp^{M}$ and WT animals were greater than 6 weeks old and maintained standard chow diets. All mouse husbandry and experimentation was conducted in accordance with guidelines and approved IACUC protocols.

Genotyping was performed on transgenic animals to verify successful deletion of the Trsp gene as previously described (2). Briefly, ear tissue samples were taken from all mice and placed in 200µl tail lysis buffer (50mM Tris-HCL, 25mM EDTA, 50mM KCL and 0.4 %(v/v) NP40 Tween-20) with added in a 1:50 (v/v) OB Protease Solution (Omega) and incubated at 55ºC overnight. Lysates are vortexed then heated to 95ºC for 5 min. The lysates are then spun at 10,000xg for 2 mins at RT. The supernatant is removed and used for PCR. The PCR is prepared using molecular grade water(Hyclone) with 1:5(v/v) of 5X GoTaq Buffer (Promega), 1µM Magnesium chloride (New England BioLab), 200µM dNTPs (New England BioLab) 0.20µM of each primer 1U of Go Taq DNA polymerase, and 1µl of lysates containing DNA (Nelson et al. 2011).The PCR amplification is completed using the following program 95ºC/5min (1 cycle), 95ºC/0.5min, 59 ºC for Trsp or 54 ºC for LysM /1min, 72 ºC/1min (40 cycles) followed by a final 72 ºC/3min (1cycle). Products were run on 1.8% Agaros gels and imaged using the Gel doc (Bio-Rad, Universal hood II) and Image Lab software (Bio-Rad, version 5 build18). The primers used to detect regions of interest and product sizes are listed in Table 3.1.

Bone marrow derived macrophages (BMDMs) were prepared as described previously (19). In brief, the femur and tibia of euthanized mice were crushed through a 70µm strainer in Complete DMEM (Hyclone, SH3008102), 20% (v/v), 5% (v/v) fetal bovine serum (Hyclone, Lot# AWG18462), 2mM L-Glutamine (Gemini 400-106), 1.5mM HEPES (Corning), 1mM sodium pyruvate (Hyclone) and 1X nonessential amino acids (Hyclone) here after referred to as DMEM complete media. Bone marrow was then centrifuged at 400 x g, 10 min at 25°C. The supernatant was decanted and the pellet was resuspended in Complete DMEM media with 20% (v/v) L929 conditioned media (containing granulocyte colony stimulating factor) supplemented. Na_2SeO_3 was added to culture of adequate (50nM) or supplemented (200nM) cells. Cells were plated at ~5 x 10^5 cells/dish in 100cm^2 petri dishes and allowed to rest for 48

hours. On days 3 thru 5 cells were feed fresh Complete DMEM media supplemented with 20% L929 with or without Na_2SeO_3.

Griess Assay was performed as reported elsewhere (20). Briefly, supernatants were collected and spun at 4ºC to remove cellular material. Supernatants were added in equal parts to Griess reagents 1:1 (A/B) 1% sulfanilamide-HCl (Sigma) and 0.1% NED (Sigma), 6% phosphoric acid with the addition and colorimetric analysis was performed in a 96 well plate(costar) at 540nM using the Spectromax spectrophotometer (Molecular Devices). Data values were calculated based on a standard curve using known amounts of nitrogen dioxide NO_2 (sigma) serially diluted from 400µM to 0.

Western blot analyses were completed by first lysing cells with RIPA buffer plus 1X HALT™ Protease Inhibitor cocktail- 1X EDTA solution (ThermoFisher) for 10 min followed by dilution with 2X laemmli buffer (Biorad). MiniProtean®TGX™ precast gels (Biorad) were used to separate proteins. Proteins were then transferred to PVDF membranes using the Biorad Turbo Blotsystem and Trans-Blot®Turbo™ transfer packs (Biorad). Blots were blocked in 5% fetal bovine albumin (Sigma) and probed using primary antibodies: 1:1000 dilution of the following rabbit anti-mouse antibodies were used anti-LC3 (Cell Signaling and MBL), anti-GPX-1 (Abcam) and 1:100,000 rabbit anti-mouse β-actin. This was washed and probed with a goat anti-rabbit H&L chain secondary conjugated to horseradish peroxidase enzyme (HRP) (Cell Signaling) at a dilution of 1:7500 to 1:2000. The blots were developed with HRP substrate (Millipore). The blots were imaged and analyzed using a Gel doc (Bio-Rad, Universal hood II) and Image Lab software (Bio-Rad, version 5 build18).

Statistical analyses were performed using unpaired t-test with Welch's correction for two samples, and analysis of variance ANOVAs were used for analyses comparing three or more groups followed by Bonferroni's Multiple Comparison Test. All statistical analyses were performed using the GraphPad software Prism for Mac OS X (version 5).

3.3 Results

Macrophage selenoproteins restrict intracellular replication of *F. tularensis* after 12 hours of infection. Data collected from gentamicin protection assays comparing the intracellular bacterial growth between $Trsp^M$ and WT BMDMs indicated that the restriction was observed at

later time points during the infection (Figure 3.1). I wanted to first assess if there were cellular defense mechanisms that might suppress the bacteria in the WT BMDMs. Since it has been reported that basal levels of ROS are higher in $Trsp^{M}$ macrophages as compared to WT it was doubtful that this was the mechanism influencing the bacterial replication restriction (1). However, macrophages are known to participate in bactericidal activity via generation of RONS and Se has been shown to improve receptor response in other cell types. Therefore, I wanted to investigate if it was plausible that selenoproteins participated in sensitizing the macrophage to the bacterial presence and amplifying the response to generate higher levels of RONS. To test this in a dose dependent fashion I utilized our diet model which yields deficient, adequate and supplemented BMDMs.

Se status of BMDMs does not influence nitric oxide production in response to *F. tularensis* infections. BMDMs were prepared from diet feed mice and cultured *ex vivo* under deficient, adequate or supplemented Se conditions. Rested BMDMs were then infected with *F. tularensis* with a multiplicity of infection (MOI) ranging from 10 to 1000 in a gentamicin protection assay. Supernatants were collected at 24 hours post infection and prepared for analysis using Griess Reagent. The data collected suggested that Se status of BMDMs did not influence nitric oxide levels in cells when infected with *F. tularensis* (Figure 3.2a). This data is in agreement with other reports of the poor immunogenicity of *F. tularensis* (14). Additionally, I measured ROS levels using a CellROX™ flow cytometry based assay and were unable to detect increased levels of ROS in LVS infected cells (data not shown). I was interested if Se would influence the levels of nitrite produced after stimulation. To address this question, I measured the nitrite levels of deficient, adequate and supplemented BMDMs after stimulation with interferon gamma (IFN-γ) and *Escherichia coli lipopolysaccharide* (LPS), known activators of inducible nitric oxide synthetase (9). The data collected indicated that the highest levels of nitrite were observed in the Se supplemented group as compared to the deficient or adequate group and that this increase was statistically significant (Figure 3.2b). This suggested that Se supplementation did not inhibit production of nitrite when two stimulus signals were provided to activate BMDMs. It also suggested that the bacterial replication restriction I was investigating was not likely due to changes in ROS or RNS within the cell.

Given that ROS and RNS were not leading to the bacterial replication restriction I next turned our attention to autophagy. *F. tularensis* is well adapted for life in the cytosol and as such this pathogen is known to evade sequestration into autophagosomes via virulence factors or may down regulate autophagy (14). Conversely, it has been shown that *F. tularensis* directly or indirectly activates the autophagy pathway utilizing it as a means to source additional nutrients that become depleted during replicative burst (3). Therefore, it was our goal to assess if macrophage selenoproteins could influence autophagy. I first tested this by eliminating *F. tularensis* from our model system. *F. tularensis* at any given time period of the infection may have the capacity to up or down-regulate the autophagy pathway and choose a starvation model to activate autophagy.

Macrophage selenoproteins suppress autophagy during periods of starvation as measured through microtubulin-associated protein 1A/1B light-chain 3 (LC3) processing. The LC3 protein is an important part of autophagosomal formation. In order to be incorporated proLC3 must first be cleaved by **autophagy**-related (ATG) 4. This cleavage event is followed by a lipidation event in which ATG3 and ATG7 (ligases) facilitate the conjugation of phosphatidylethanolamine (PE) to the now cleaved LC3I (21). The PE conjugated form of LC3 is known as LC3II. These steps are integral for phagosome formation. I measured the ratio of LC3II protein levels to LC3I protein levels in order to indirectly measure autophagy. A clear pattern emerged whereby $Trsp^{M}$ BMDMS had consistently higher ratios of LC3II/LCI as compared to WT controls (Figure 3.3). While, using LC3 expression alone is not sufficient to claim increased autophagy is occurring in the $Trsp^{M}$ macrophages this data is strongly suggestive of selenoprotein involvement in suppression of autophagy.

To further support the LC3 processing data I have begun to compile samples and measure the levels of other autophagy related proteins. This includes Beclin1 an important for induction of autophagy, ATG7 an E1 ligase, ATG5 an E3 ligase and part of the ATG12/ATG16L1 complex (10). I utilized our starvation model testing TrspM deficient cells and WT Se supplemented cells. I validated our TrspM samples through assessment of the increased ratio of LC3 cleavage over WT supplemented cells (Figure 3.4 a). Next I checked that the GPX1 protein, a useful read out of selenium status in WT cells, was expressed at 0 and 6hrs after starvation (Figure 3.4b). It has been reported that mTOR an upstream activator of autophagy can

increase GPX1 expression, but I did not observe large increases in GPX1 protein expression of our WT Se supplemented cells (22). Finally, I measured relative levels the autophagy related proteins (Figure 3.4 c/d). The relative levels of expression of these genes at this point seem to be comparable, which is contrary to findings that Se deficiency increases transcript levels of these proteins (23). However, the data represented here is one biological replicate and should be considered preliminary in nature. Additionally, measurements of ATG5 may misrepresent actual levels given that ATG5 also participates in the ATG5/ATG12 complex with ATG16L1. I am in the process of obtaining these measurements and they will be used to then draw conclusions regarding the relative expression between $Trsp^{M}$ and WT.

Finally, I wanted to understand if there was a difference in the ratio of LC3II and LC3I expression as measured over the course of the infection. I observed that there was a large difference in the change of the LC3 II to LC3I ratio measured in Se deficient samples as compared to the WT from 1.5hrs to 24 hrs post infection (Figure 3.5). The data comparing deficient BMDMs to supplemented BMDMs during infection is one of two biological replicates I have measured. Indirectly, this suggests that the supplemented BMDMs have a greater potential to control autophagic flux initiated by *F. tularensis* in a selenoprotein dependent manner. Our working model is that by suppressing the activation of autophagy the Se supplemented BMDMs are able to limit the available intracellular nutrients thereby restricting the replication of *F. tularensis*.

3.4 Discussion

Previous work has demonstrated that Se supplementation of BMDMs restricts intracellular replication of *F. tularensis*. I have shown that this mechanism is mediated through selenoproteins by using a transgenic model in which the TrspM BMDMs could not synthesize selenoproteins and therefore demonstrated a permissive phenotype by which *F. tularensis* growth exceeded the WT by at least 20% depending on the Se status of the cells. The goal now is to determine the mechanism by which selenoproteins are mediating the replication restriction.

The data I have collected measuring levels of intracellular RNS and ROS suggest that *F. tularensis* does not elicit a robust immune response to stimulate the macrophage to synthesize those effectors in resting macrophage cells. Furthermore, *F. tularensis* produces a number of

virulence factors that inhibit NAPDH oxidase, as well as superoxide dismutase and catalase to cope with ROS/RNS that may be present (14, 17). However, it may be important to look redox in the context of autophagy. It has been demonstrated that ablating the activities of thioredoxin reductase TXNRD1 and 2 can leads to redox dysfunction and activation of serine-threonine kinase (AKT) as well as AMP activated protein kinase (AMPK) (24, 25). This is an area that requires further investigation given $Trsp^{M}$ animals would have lost TXNRD function and may be exhibiting related pathways contributing to increased levels of autophagy.

I have compiled strong evidence that autophagy is suppressed by Se supplementation in a selenoprotein dependent manner. However, a limitation of the methods employed here are that LC3 protein expression alone does not describe if the pathway is intact. For example if Bafilomycin A1 is added to the system the ratio of LC3II may in fact be elevated, but given that Balfilomycin A1 inhibits autophagosomal-lysosomal fusion the pathway is rendered incomplete. Great efforts will be taken to validate our findings through alternative methods. Measuring autophagic flux will be another important aspect of understanding if Se is uniformly suppressing autophagy or if a low endogenous level is observed with a dampening of acute fluxes.

The preliminary findings on expression levels of beclin and ATG 5 do not match published reports whereby basal expression levels were higher in chickens feed a deficient diet as compared to controls (23). It will be important to complete additional studies with an increased sample size to draw meaningful conclusions from this our data. Unlike other reports that have observed increases in GPX1 protein expression, I did not observe this phenomena with our WT Se supplemented cells after starvation (22). It may be that I have to target mTOR directly and reanalyze expression levels. If in fact selenoprotein expression levels are increased by autophagy, it may demonstrate a negative feedback loop by which the bacteria will initiate autophagy thereby expressing higher levels of selenoproteins in the cell, which then act to dampen the autophagic flux. In this case $Trsp^{M}$ macrophages, which lack selenoproteins, would have an inability to utilize this method of autophagic suppression.

Finally I have shown that there is a pattern of increased LC3 cleavage of deficient cells over the course of a *F. tularensis* infection. This data would suggest that deficient cells are more permissive or more sensitive to microbial dependent induction of autophagy required for F. tularensis replicative burst (3). This piece of data suggests there is a link between the replication

restriction I have observed and the decreased levels of induced autophagy in the WT supplemented macrophage cells. This may be a form of "nutritional immunity," though that term is generally used to describe host sequestration of metals (Fe, Cu, Mn, and Zn) in order to prevent pathogen acquisition (26). It maybe that "nutritional immunity" extends beyond metals to more broadly describe the host's innate abilities to also limit carbon and energy sources from pathogens as a means to control infection.

In conclusion, the studies describe here point to the importance of selenoproteins capacity to suppress autophagy and thereby limit available nutrients and decrease the capacity for F. *tularensis* replication. The studies described here need to be more thoroughly vetted through alternative methods of measuring autophagic flux, by microscopy, for example. Additionally, a clear correlation must be made between *F. tularensis* replication restriction, selenoprotein presence and autophagy. This is currently underway with functional assays using pharmacological inhibition of autophagy and exogenous supplementation to rescue bacterial replication. Finally future work must identify the selenoproteins which are directly or indirectly contributing to the suppression of autophagy. Through identification and validation of selenoproteins responsible for the activation of autophagy a targeted approach can be developed and applied to other bacterial models. This would also suggest a possible biomarker to determine the ability of bacterial control during infection.

Key Concepts:

- ***F. tularensis* activates autophagy during infection to acquire nutrients**
- **The bacteria replication restriction occurs after multiple rounds of replication at a time when intracellular nutrients are likely depleted**
- **Selenium suppresses autophagic related protein expression of LC3**
- **Selenoproteins are mediating the decrease in LC3 processing**

3.5 Tables and Figures

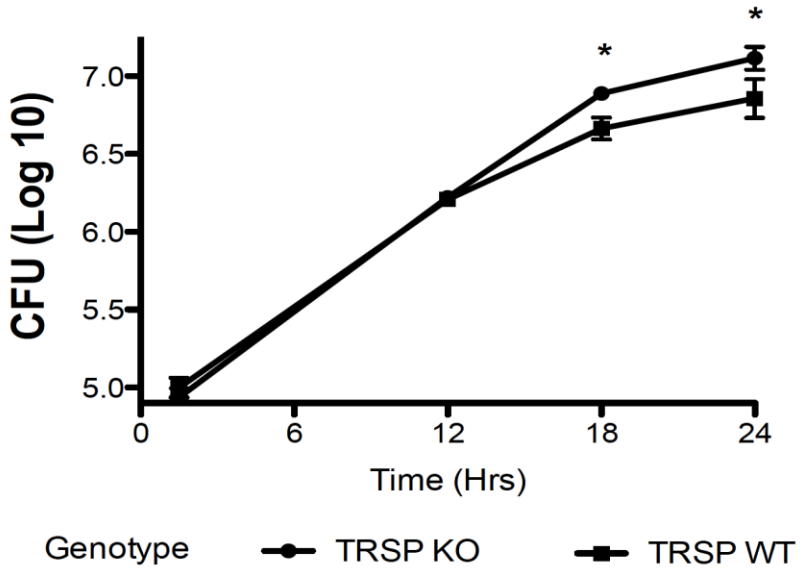

Figure 3.1: Macrophage selenoproteins restrict intracellular replication of *F. tularensis* after 12hrs post infection. Bone marrow was collected from 6-8 week old $Trsp^{M}$ and WT mice. BMDMs were prepared and maintained *ex vivo* under deficient or supplemented conditions respectively. BMDMs were infected with Ft LVS at MOI of 1:50 and intracellular bacteria were enumerated at 2, 12, 18 and 24 hours. Statistical significance was assessed using a Student's t-Test on individual time points.

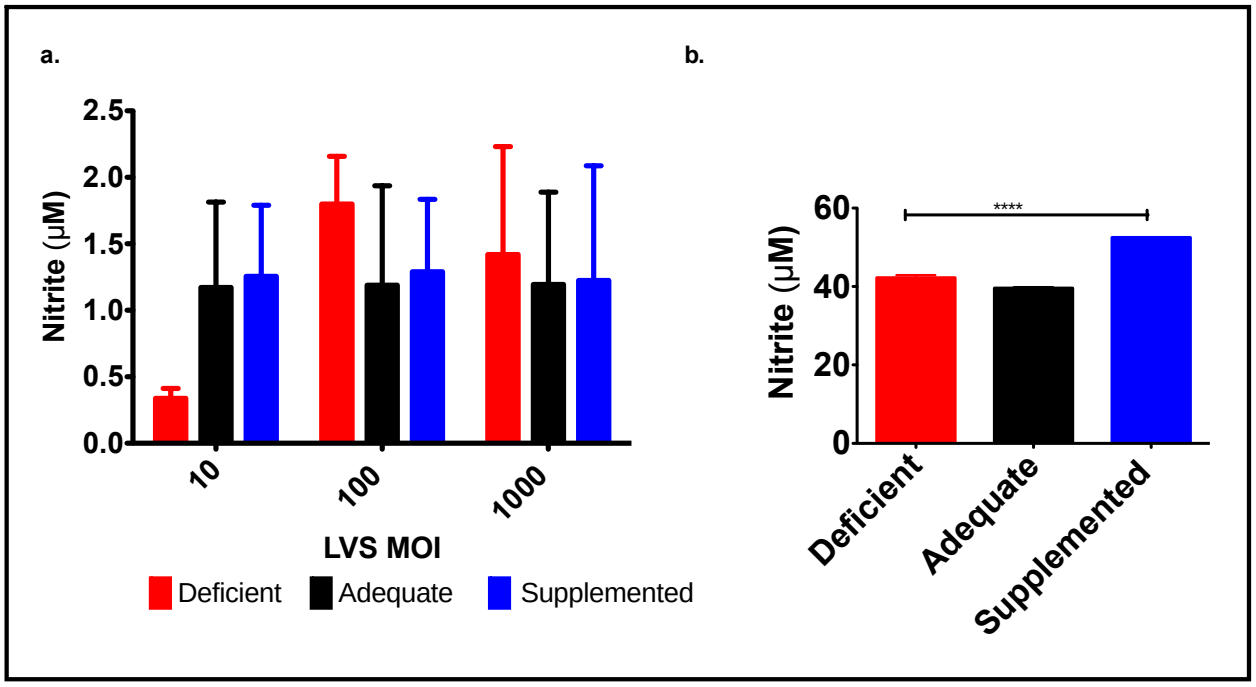

Figure 3.2: Se status does not influence nitric oxide production during F. *tularensis* infection. 6-8 week old C57Bl/6 mice were placed on Se deficient, adequate or supplemented diets and maintained for a minimum of 12 weeks. BMDMs were prepared and maintained *ex vivo* under deficient, adequate or supplemented conditions. (a) BMDMs were infected with *F. tularensis* LVS at MOI of 1:10 to 1:1000 and intracellular growth of bacteria was allowed to progress for 24 hrs. Lysates were removed and tested for nitric oxide. Data represented as the mean and standard deviation of triplicates. Statistical significance was assessed using a Two-way ANOVA followed by a Bonferroni's Multiple Comparison Test (ns). (b) BMDMs were treated with and 100nM of IFN-γ for 24 hrs and 1μg/mL of LPS for 16hrs. Supernatants were collected and assayed for Nitrite. Data represented as the mean and standard deviation of triplicates. Statistical significance was assessed using a One-way ANOVA followed by a Bonferroni's Multiple Comparison Test ($p<0.001$).

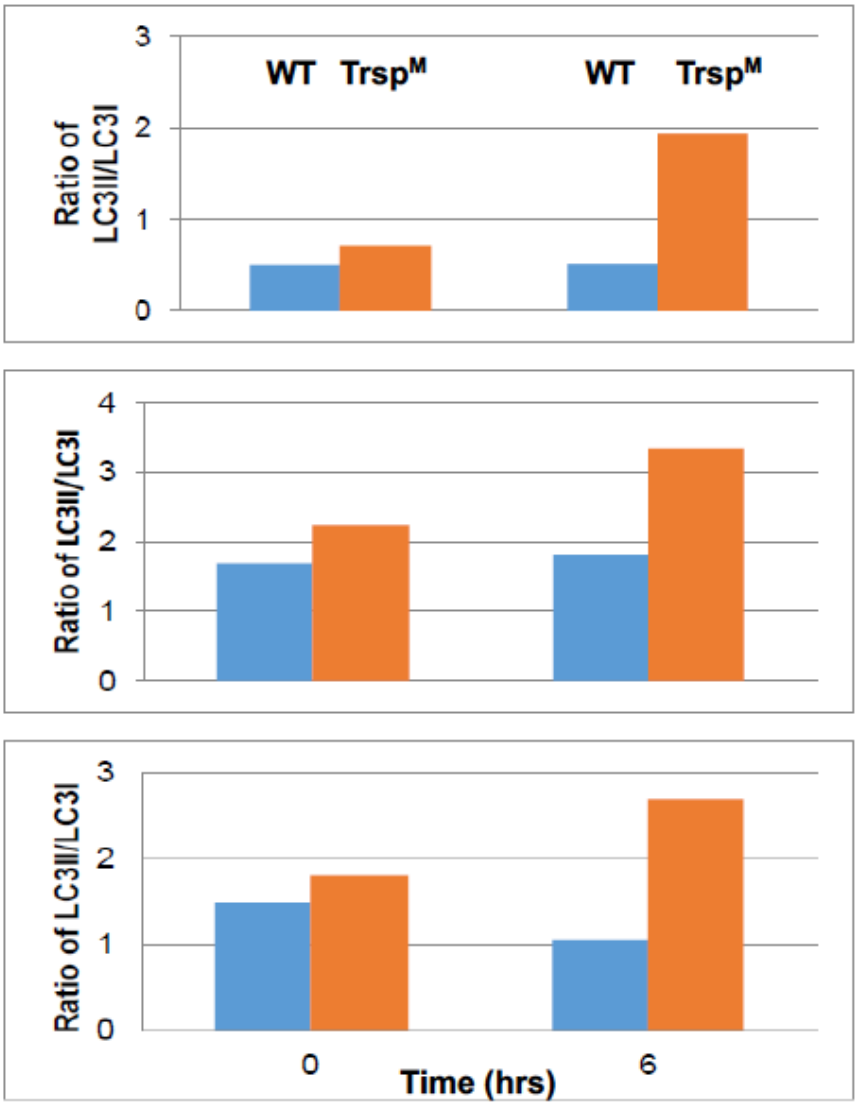

Figure 3.3: Macrophage selenoproteins influence the autophagy as measured by LC3 cleavage. Bone marrow was collected from 6-8 week old Trsp^{M} and WT mice. BMDMs were prepared and maintained *ex vivo* under deficient or supplemented conditions respectively. Lysates were prepared and relative expression of LC3I and LC3II by western blot analysis. Data represents 3 biological replicates.

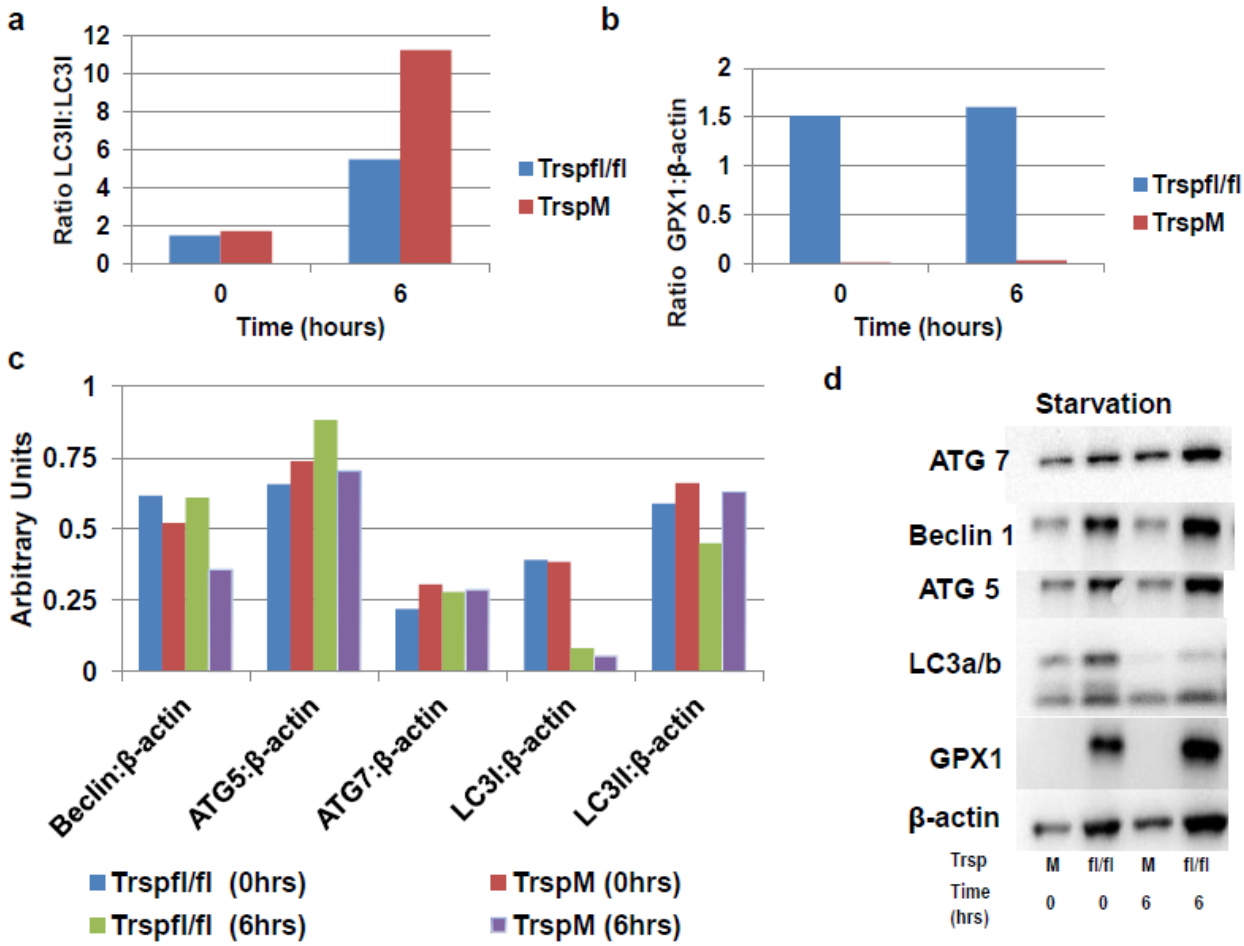

Figure 3.4: Macrophage selenoproteins influence the autophagy related protein expression. Bone marrow was collected from 6-8 week old Trsp^{M} and WT mice. BMDMs were prepared and maintained *ex vivo* under deficient or supplemented conditions respectively. Lysates were prepared and western blot analyses were performed to determine the (a) ratio of LC3II to LC3I (b/d) GPX1 expression level (c/d) relative expression of ATG7, ATG5, Beclin 1, LC3I and LC3II as compared to β-actin. Data represents 1 of 3 biological replicates tested.

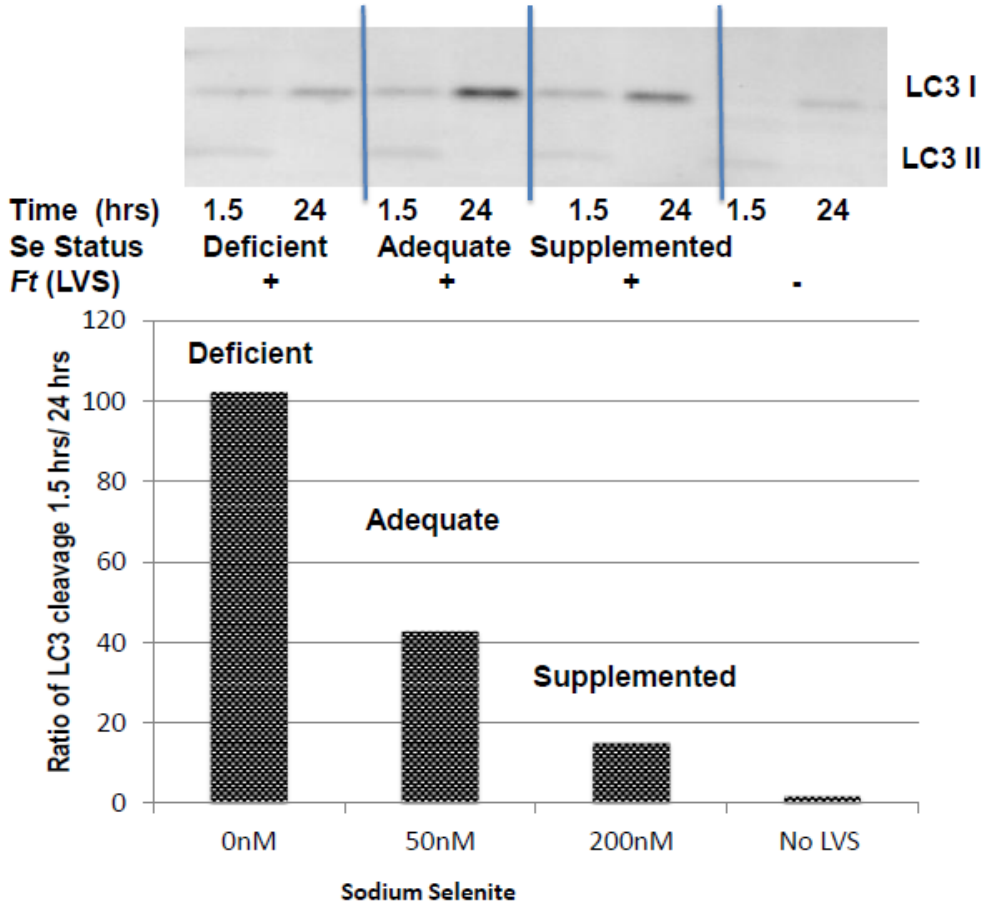

Figure 3.5: Se supplementation decreases the relative change in autophagy related protein LC3 cleavage during F. *tularensis* infection. 6-8 week old C57Bl/6 mice were placed on Se deficient, adequate or supplemented diets and maintained for a minimum of 12 weeks. BMDMs were prepared and maintained *ex vivo* under deficient, adequate or supplemented conditions. BMDMs were infected with *F. tularensis* LVS at MOI of 1:100 and intracellular growth of bacteria was allowed to progress for 24 hrs. Lysates were prepared at 1.5 and 24 hrs post infection in order to perform western blot analysis. Data represents one of two biological replicates.

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Chapter 4: Summary, Conclusions and Future Directions

The focus of this work has been to tackle four main goals. The first of which is to address the use of Se adjuvant intervention as a plausible therapy for acute bacterial infections. The information in both human and animal studies is fairly limited. The objective of chapter 1 was to provide a foundation for the use of Se as a treatment in acute bacterial infections and highlight some of critical components in the success or failure of these studies.

The human trials I included had numerous limitations and most likely the largest factor was the inconsistency in the data. This primarily was attributed to heterogeneity of the genetics in the human population and study design (1).It was agreed that the Se status of the patient had a correlation with risk assessment and outcome (2). It was also established that in systemic infections selenoproteins, such as GPX3, are excellent biomarkers (3). The biosynthesis and incorporation of selenocysteine is a tightly controlled and specific process, it is reasonable that polymorphisms in any number of genes involved in the process, or in specific selenoproteins would have a variety of effects. This is exemplified with the increased risk of gastric cancer in patients with select SELENOS gene polymorphisms and *H. pylori* infections (4). The most important finding from the collection of data pertaining to human trials is that the type of Se, the dose of Se and the dosage regimen are critical in the design and likely have the most influence on the endpoints measured. It seems that supplementation with Se in the form of sodium selenite may be most favorable. However, new therapies utilizing Se nanoparticles are still being established for proof of principal (5, 6). It is reasonable Se nanoparticles may provide better results with less associated risk of cytotoxicity, though they have not been considered in the context of our studies (7, 8).

Animal studies provided more conclusive results and also detailed some of the immunological mechanism by which Se was influencing immune function. Like the human trials sodium selenite was consistently used in many of the studies. Though, like the human trials experimental design in the dosage and dosage regimen varied greatly. It was also clear looking at the diet models that the length of time used to induce deficiency in animals may influence the pathology (9). Surprising, Se supplementation may increase the incidence of mortality in certain types of bacterial infections, such as *Clostridium chauvoei* (10). Additionally, the pathogen *Legionella pneumophila* was reportedly enhanced with Se supplementation in liquid culture (11) This highlights a very important consideration that the utilization and specific incorporation of

Se is a very ancient, and a highly conserved function in multiple domains of life, including prokaryotes. Bacteria, especially anaerobic microbes which undergo stickland fermentation reactions may benefit from Se supplementation (12). Extreme caution must be observed when considering Se supplementation for therapeutics against *Clostridium difficile* infections (13). Therefore the influence of Se on the pathogen must be considered in efforts to examine the role of Se and selenoproteins on host immune function.

Chapter 2 described the available literature on prokaryotic utilization of Se either through specific incorporation of selenoproteins, as a modified base, or as a cofactor. A number of massive bioinformatics analyses on hundreds of sequenced prokaryotic genomes had been completed prior to our study (14). Unfortunately, some of these analyses were going to press around the same time the *F. tularensis* genome sequences were being deposited in NCBI's databases. An *in silico* analysis on the *F. tularensis* genome using E. *coli,* a bacteria which is known to synthesize selenoproteins was completed. This analysis failed to identify any the required genes for selenocysteine synthesis or incorporation. With the exception of a subunit of the formate dehydrogenase gene this analysis also failed to detect known prokaryotic selenoproteins. The default settings were applied for the Blastp searches. The loose constraints of this approach yielded a few false positives which were then sorted through manually for elimination. The false positive hits had extraordinarily high E values. Additionally, few of the false positive hits had identities which were higher than ~30%. These thresholds were similar to those used by others in more recent reports, confirming the validity of our approach (15). Additionally, other bioinformatics tools and databases were used to thoroughly interrogate the *F. tularensis* genome for the presence of selenoproteins or selenoprotein related genes (Table 2.3). The utilization of Se was ruled out empirically by testing bacterial lysates from *F. tularensis* and *E. coli* and measuring elemental Se via atomic absorption spectrometry (AAS). AAS has a sensitivity of 25nM, far below the threshold to be considered adequate. The analysis failed to detect accumulation of elemental Se in *F. tularensis* lysates (Figure 2.2). However, microarray data from F. *tularensis* infected alveolar epithelial cells yielded a hit which related to the activation of (L)-selenoaminoacids incorporation in proteins during translation and it was speculated by the authors that this may have biological relevance to *F. tularensis* infection (16). There were no differences found in the growth, virulence or pathogenicity of bacteria cultured in 200nM of Na_2SeO_3 (Table 2.4, Figures 2.3-2.5). One of the key findings from Chapter 2 was that

F. tularensis did not utilize, nor was it physiologically influenced by Se. This allowed us to ask questions related to the role of Se in the host.

I go on in Chapter 2 to focused on the role Se and selenoproteins play in the innate immune response to *F. tularensis*. I found that animals supplemented with Se in their diet are protected from infection (Figure 2.6). Moreover, macrophages that were Se supplemented displayed a bacterial replication restriction phenotype (Figure 2.7). A number of selenocompounds were tested, including selenomethione and methylseleninic acid. When performing these assays the bacterial replication restriction phenotype could only be recapitulated using selenocompounds which increased GPX1 levels (Figure 2.8-2.9). This data suggested that selenoproteins were important in mediating the bacterial replication restriction. I hypothesized that selenomethione supplementation did not increase GPX1 levels due to low expression of γ-lyase, which is required for the catabolism of (SeMet). However, this should be verified. I was able to test whether macrophage selenoproteins were protecting animals during pulmonary infection. The data from our diet studies was indeed corroborated by the $Trsp^{M}$ studies, showing that TrspM animals had an increased mortality rate compared to WT animals in sub-lethal challenges (Figure 2.10).

Since macrophage selenoproteins conferred protection to WT mice during pulmonary infection it was possible that there was a reduction in pathology. Unfortunately, in a systemic infection at LD_{100} I was unable to detect any differences in the pathology of the lung, liver or spleens (data not shown). I am in the process of repeating this experiment by inoculating at an LD_{50} of *F. tularensis* to examine pathology in a sub-lethal infection. This may provide more promising results given that I have observed increased bacterial numbers in the liver, spleen and blood on day seven using a sub-lethal dose (Figure 2.11). Currently, I am analyzing cytokine levels in addition to measuring lymphocyte infiltration in the spleen and liver five days post infection. I speculate that I will see increased levels of proinflammatory cytokines in $Trsp^{M}$ mice, but at this time that is not congruent with our current pathology findings. However, the complexity of the *in vivo* model should not overshadow the most important observation made in chapter 2; that irrespective of selenium status, macrophages, which lacked selenoproteins facilitated higher levels of *F. tularensis* growth (Figure 2.12). The evidence I have collected conclusively implicates selenoproteins as important mediators in the *F. tularensis* replication

restriction. This satisfied our goal 3 to determine the role of selenoproteins in the bacterial replication restriction. This also raised the question of how selenoproteins were regulating this response.

In chapter 3 I address our final goal in examination of the underlying mechanism of selenoprotein mediated *F. tularensis* replication restriction. I speculate that the answer may be in the relationship between selenoproteins and autophagy. It has been shown that Se status of cells influences either activation or suppression of autophagy related protein and gene expression (17, 18).

I have utilized the $Trsp^{M}$ BMDMs to show that loss of selenoproteins increases levels of the cleaved and lipidated LC3 protein, a marker for autophagy (Figure 3.3). The diet model was used to demonstrate that there is an increased level of LC3 processing during *F. tularensis* infections (Figure 3.5). Future studies will focus on functionally demonstrating that $Trsp^{M}$ BMDMs, unable to restrict bacterial replication, can be pharmacologically treated with Balfilomycin, and inhibitor of autophagy, to decrease bacterial burden. Similarly, ablation of the selenoprotein dependent bacterial replication restriction through amino acid supplementation in WT Se supplemented BMDMs. Additional studies will also address the measurement of autophagic flux through the use of microscopy.

Finally, it has been reported that macrophage polarization may influence the level of basal autophagy (19). IL-4 expression has been reported during *Francisella* infection; therefore it would be prudent to assess levels of this cytokine in our *in vivo* analysis (20). If IL-4 levels are increased in our Se adequate WT mice, it would stand to reason that our WT macrophage cells may polarized to more of an M2 phenotype (21). M2 macrophages have been reported to express lower levels of mTOR activation (19). mTOR is a kinase, which is responsible for upstream activation of canonical autophagy. Therefore, the decreased bacterial burden in the liver of the WT mice compared to the $Trsp^{M}$ animals (Figure 2.11) may be attributed to lower levels of autophagy. Future work can address this through isolation and characterization of Kupffer cells from *F. tularensis* infected $Trsp^{M}$ and WT mice. The Kupffer cells are a subset of liver macrophages, and given that there was a dramatic loss of bacterial control in the liver of *F. tularensis* infected mice, these cells may have an exacerbated phenotype in the regulation of autophagy.

It is clear that there is a gap in our understanding of how selenoproteins regulate autophagy, despite a few reports which identify a relationship. Se adjuvant therapy like most broad interventions may have unintended consequences when used to treat pathogens that utilize Se. Our efforts to more fully elucidate the antimicrobial effects of Se supplementation with the *F. tularensis* model may identify specific selenoprotein targets. This would be useful to circumvent supplementing the pathogen while trying to optimize the immune response of the host through selenoprotein therapy. This highlights why elucidating the specific selenoprotein(s) responsible for driving the observed bacterial replication restrictions is critical to understanding and applying the information I have learned from our studies understanding how macrophage selenoproteins mediate bacterial infection. Aside from the use of selenium as adjuvant therapy there have also been attempts to implement mimetics of selenoenzymes, like Ebselen described in chapter 1 (22, 23). Like selenium supplementation, memetics of selenium have been reported to have limited therapeutic capacity, which may be because a more dramatic focus to the individual role of selenoenzymes must be initiated (23). A better approach may be to target selenoproteins that participate in autophagy regulation, which could then be applied to many aspects of human health. Understanding the relationship and molecular interactions between the selenoprotein of interest and the autophagy machinery will allow for design of novel compounds that contain similar structure to recapitulate the catalytic activity of the target selenoprotein(s) allowing for manipulation of autophagy.

The work described here also raises interesting questions. For example, which selenoproteins are important in the regulation of autophagy? Is there a direct or indirect regulation mechanism, and at what point in the pathway are selenoproteins participating in the regulation? Additionally, given that GPX1 and GPX4 expression maybe linked to mTOR activation, is it possible that a negative feedback loop exists by which autophagy leads to increased selenoprotein expression thereby down-regulating the pathway itself (24)? Given that autophagy participates in multiple aspects of cellular response it is likely that I will observe similar patterns in other cells of the immune system.

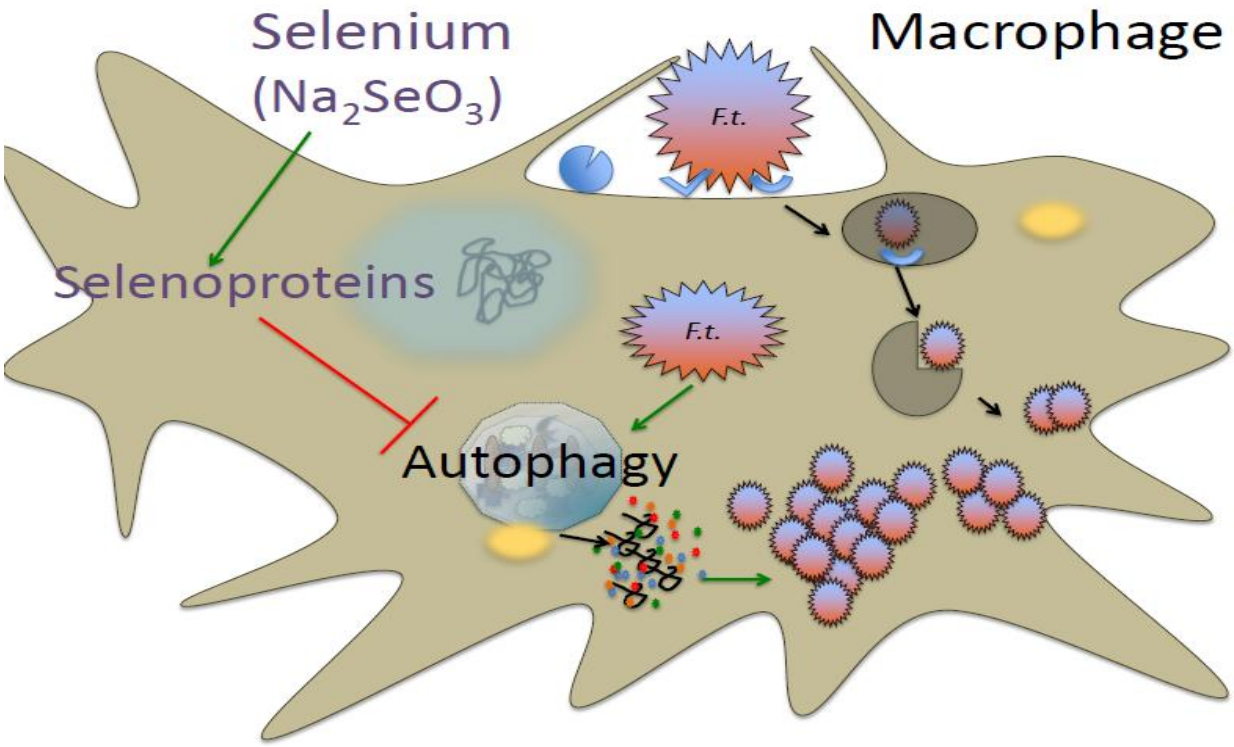

Figure 4.1: Proposed mechanism by which Se via specific selenoprotein(s) suppresses autophagy limiting available nutrients, and thereby restricting bacterial replication.

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Vita

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Statement of Focus

My current research focus is to understand the influence of nutrition on host-pathogen interactions. The objective of my doctoral thesis is to determine the effect of the trace element selenium on the outcome of acute bacterial infections, and establish the underlining mechanism. Specifically, I have established that selenoproteins restrict the intracellular replication of the gram-negative bacteria *Francisella tularensis* by suppression of autophagy in macrophage cells.

Education

2011-Present	PhD Candidate, Immunology & Infectious Disease (IID) The Pennsylvania State University, University Park, PA Thesis: "Restriction of intracellular replication of *Francisella tularensis* by selenoproteins" Advisor: Girish Kirimanjeswara Expected Graduation: Spring, 2017
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2000-2004	B.S. Biology Gettysburg College, Gettysburg, PA Dual Major: Visual Arts Minor: Philosophy Thesis: "Allelopathic Effects of *Helianthus annuus* on *Hordeum vulgare*"

Work Experience

2011- Present	Graduate Assistant, Pennsylvania State University, University Park, PA Supervisor: Girish Kirimanjeswara
2010 – 2011	Cooperative learning Experience, Merck & Co. (Vaccines Basic Research), West Point, PA Supervisor: Eberhard Durr