Host-Adaptation of *Francisella tularensis* Alters the Bacterium’s Surface-Carbohydrates to Hinder Effectors of Innate and Adaptive Immunity

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### Abstract

**Background:** The gram-negative bacterium *Francisella tularensis* survives in arthropods, fresh water amoeba, and mammals with both intracellular and extracellular phases and could reasonably be expected to express distinct phenotypes in these environments. The presence of a capsule on this bacterium has been controversial with some groups finding such a structure while other groups report that no capsule could be identified. Previously we reported *in vitro* culture conditions for this bacterium which, in contrast to typical methods, yielded a bacterial phenotype that mimics that of the bacterium’s mammalian, extracellular phase.

**Methods/Findings:** SDS-PAGE and carbohydrate analysis of differentially-cultivated *F. tularensis* LVS revealed that bacteria displaying the host-adapted phenotype produce both longer polymers of LPS O-antigen (OAg) and additional HMW carbohydrates/glycoproteins that are reduced/absent in non-host-adapted bacteria. Analysis of wildtype and OAg-mutant bacteria indicated that the induced changes in surface carbohydrates involved both OAg and non-OAg species. To assess the impact of these HMW carbohydrates on the access of outer membrane constituents to antibody we used differentially-cultivated bacteria to immunoprecipitate antibodies directed against outer membrane moieties. We observed that the surface-carbohydrates induced during host-adaptation shield many outer membrane antigens from binding by antibody. Similar assays with normal mouse serum indicate that the induced HMW carbohydrates also impede complement deposition. Using an *in vitro* macrophage infection assay, we find that the bacterial HMW carbohydrate impedes TLR2-dependent, pro-inflammatory cytokine production by macrophages. Lastly we show that upon host-adaptation, the human-virulent strain, *F. tularensis* SchuS4 also induces capsule production with the effect of reducing macrophage-activation and accelerating tularemia pathogenesis in mice.

**Conclusion:** *F. tularensis* undergoes host-adaptation which includes production of multiple capsular materials. These capsules impede recognition of bacterial outer membrane constituents by antibody, complement, and Toll-Like Receptor 2. These changes in the host-pathogen interface have profound implications for pathogenesis and vaccine development.

### Citation

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### Introduction

*Francisella tularensis* is an extremely infectious gram-negative bacterium which is readily aerosolized. Inhalation of this Category A select agent can lead to pulmonary tularemia which has a mortality rate of ~35% in the absence of treatment. Reportedly, antibiotic-resistant strains of this bacterium were developed by at least one nation’s biological weapons program [1]. The specter of such an agent being maliciously employed, in concert with the current lack of a licensed tularemia vaccine, has evoked a ground swell of interest in *F. tularensis*.

In addition to being a potentially fearsome biological weapon, *F. tularensis* also is a naturally-occurring zoonotic bacterium found in strikingly diverse environments including an array of warm- and cold-blooded hosts (mammals, insects, arthropods, fresh water protozoans) [1,2]. The *tularensis* subspecies (ssp., comprised of type A strains and found in North America) is maintained primarily in a terrestrial cycle involving lagomorphs and rodents with transmission via ticks and tabanid (deer, horse, etc) flies. In contrast, the *holartica* ssp. (type B strains which are distributed throughout the northern hemisphere) has a stronger association with water-borne disease and, perhaps consequently, with transmission by mosquitoes (as well as ticks and biting flies). In addition to the broad range of hosts, it has recently become appreciated that within both mammalian and protozoan infection models, the bacterium has both intracellular (replicative) and extracellular (transmissive)
phases [3–7] suggesting additional environments to which the bacterium must adapt. Furthermore, a growing body of evidence indicates that as F. tularensis progresses through the intracellular cycle (phagocytosis and phagosomal escape, cytoplasmic replication, induction of autophagic vacuoles and cellular escape) the bacterium adapts to distinct micro-environments by modulating expression of many genes [8–10].

The mutability of this bacterium, which endows such a broad host range, also poses a challenge to vaccine development as the nature of the inoculum (with potentially distinct antigenic compositions) can vary widely. Upon entering the mammalian environment the bacterium will presumably adapt regardless of its immediate history. However, it must be considered that the time required for complete bacterial adaptation of a F. tularensis inoculum is currently unknown - as is the impact that this adaption might have on tularemia vaccination strategies. A salient example of how bacterial adaptation can affect vaccination against vector-borne pathogens is provided by the now defunct LYMErix vaccine that induced antibody (Ab) against OspA of Borrelia burgdorferi, the tick-borne Lyme disease spirochete. This vaccine was developed largely based on work with non-host-adapted bacteria in which OspA was characterized as an abundant, surface-exposed outer membrane protein which could serve as a protective antigen in syringe-inoculation models of murine borreliosis. After many years of development and commitment of financial resources, it was subsequently revealed that, to be effective, the host’s bactericidal α-OspA Ab had to enter the feeding tick early in the blood meal to kill the tick-adapted spirochete. This was necessary because, in preparation for exiting the tick and entering the mammalian host, the bacterium dramatically down-regulates expression of OspA. Consequently, any host-adapted (OspA negative) spirochetes that entered the host were “immune” to the host’s vaccine-induced immunity [11], an observation which correlated well with the vaccine’s sub-optimal efficacy. Production of LYMErix was halted in 2002. The desire to work with host-adapted F. tularensis as a platform for vaccine-discovery prompted our previous efforts to recapitulate the bacterium’s mammal-adapted phenotype during in vitro growth [12].

As F. tularensis replicates within host cells, cell-mediated immunity has long been considered paramount to the control of disease. However, the growing recognition that the bacterium has a significant extracellular phase [4,5,7] provides a mechanistic explanation for the early observation that adoptively-transferred immune serum provides a degree of protection [13]. This original report has been confirmed and extended under more defined experimental conditions/infection models using both the attenuated type B, live vaccine strain (LVS) [14–19] and the human-virulent, Type A strain SchuS4 [20,21] as challenge inoculums. It should be noted, however, that none of these more recent challenges were initiated with host-adapted bacteria. Where therapeutic administration of either immune serum or Ag-specific Ab has been examined, the serum/Ab’s full therapeutic potential was realized by administration beginning no later than 24 hr post-infection (PI) [16,20,22]; further delays in Ab-therapy (potentially allowing for further bacterial adaptation) result in dramatic reductions and/or loss of transferrable protection [16]. Consistent with this susceptibility of non-host-adapted bacteria to Ab, it has recently been shown that animals receiving multiple transfers of immune serum (on days −1, +3, +6, +9, and +12 relative to challenge on day zero) are no more protected from challenge than animals that receive a single equal dose of immune serum one day before infection [21].

In addition prophylactic and/or therapeutic approaches involving immunization with attenuated/inactivated bacteria and/or adaptive transfer of serum/Ab, several purified or semi-purified antigens have been assessed as sub-unit vaccine candidates. Perhaps the most well-characterized is lipopolysaccharide (LPS) which in gram-negative bacterium is readily antibody-accessible – provided the bacterium is not heavily encapsulated. Preparations of F. tularensis LPS (a poor TLR4 agonist) have been shown to be protective immunogens by multiple groups [23–28]. However, as we and others [25,29] have observed, the most widely-cited method for preparing LPS from this bacterium (hot-pheno1 extraction) also co-purifies a significant amount of HMW carbohydrate/putative capsule; this material can amount to 40% of the total “LPS” preparation [25]. This raises the specter that some of the protection ascribed to LPS immunization may, in part, represent anti-capsule responses. Indeed a recent report showed that active immunization with OAg capsule or passive immunization with a mAb directed against the capsular OAg was protective [30].

Our long term interests include the identification of bacterial surface proteins which are both i) accessible to host antibodies and/or cellular receptors and, importantly, ii) are expressed during mammalian infection. In support of this goal we previously identified in vitro culture conditions that recapitulate the bacterium’s mammal-adapted, extracellular phenotype. Here we describe the further pursuit of our objective which has led us to the discovery that host-adaptation includes multiple changes to the bacterium’s surface carbohydrates (LPS and capsules) which cumulatively hinder recognition of OM constituents by Ab, complement, and TLR2.

Results

Francisella tularensis grown in brain-heart infusion broth display the bacterium’s extra cellular, host-adapted phenotype

Host-adapted F. tularensis (those replicating in macrophages or in the tissues of mice) display a phenotype distinct from that of bacteria cultivated in vitro using standard media such as in Mueller Hinton broth (MB) or Chamberlain’s defined media (CDM) [31,32]. The phenotypic differences include expression of a distinct bacterial proteome as well as the elicitation of a different reaction from naïve macrophages (MΦ) responding to the bacteria [33]. Recently we reported that growth of F. tularensis in BHI induces a phenotype that is indistinguishable from that of bacteria which have emerged from infected MΦ (i.e. extracellular, host-adapted F. tularensis) [12]. This work was undertaken in support of our longer-term goal: the direct biochemical identification of surface-exposed, outer membrane (OM) proteins (OMPs) which are expressed during mammalian infection. As candidates for a sub-unit vaccine, such proteins could serve as targets for antibody (Ab) during the bacterium’s extra-cellular phase [5,7].

Here, prior to commencing our quest for such OMPs, we first sought to confirm and extend the notion that growth of F. tularensis in BHI induces the bacterium’s host-adapted phenotype. To this end we used western blot analysis of several differentially-expressed proteins to gauge the similarity between MBH-, BHI-, and extracellular, MΦ-grown F. tularensis. To extend our understanding of the regulatory genes and environmental cues which govern host-adaptation we also probed BHI-grown, mglA and pncA strains along with wildtype (WT) F. tularensis grown in the presence of high concentrations of free amino acids [12] (in the form of casamino acids - CA) or spermine [34]. As shown in Fig 1, F. tularensis that have emerged from infected MΦ and those which were grown in BHI contain similarly elevated levels of the Intracellular Growth Locus (Igl) proteins B and C, catalase (KatG),
and PmrA - a response-regulator protein [35,36]. These proteins were minimally-expressed by *F. tularensis* grown in MHB (which contains 17.5 g/l casamino acids) or in BHI supplemented with casamino acids to 17.5 g/l (BCA). These protein changes during host-adaptation were entirely dependent on the transcriptional regulator MglA [37] which responds to amino-acid deprivation [39]. MglA activates transcription of the Iglg and represses that of KatG [39]. PmrA had a detectable, but less pronounced, contribution to these regulatory changes. Supplementation of MHB with spermine (MS), which induces multiple transcriptional changes in *F. tularensis* [34], had minimal impact on the abundance of these proteins.

The quest for outer membrane proteins in host-adapted *F. tularensis* reveals putative capsular material(s)

To aid our search for differentially-expressed OMPs we fractionated MHB- and BHI-grown *F. tularensis* using Triton X-114 (Tx) phase-partitioning [40,41,42,43] combined with differential membrane solubilization of the Triton-insoluble material. To validate this approach, the resulting fractions along with the parental whole cell lysates (WC) were probed with antibodies specific for proteins of known or predicted location. Consistent with previous reports of IgA, IgIB, IgIC, and GroEL (a chaperone) and KatG being soluble cytoplasmic and periplasmic proteins respectively [31,44–46], we found that these species quantitatively partition into the Tx-aqueous (A) phases (Fig 2A). Curiously, KatG and GroEL along with SodB (superoxide dismutase) and Bir (a bacterioferritin homolog) have also been found in the culture supernatants of non-host-adapted *F. tularensis* [47,48]. Active secretion of the bacterial antioxidant enzymes into host cells could limit cellular activation by altering redox-dependent host cell signaling [49]. Given the alterations of KatG and SodB expression we observed in host-adapted *F. tularensis* (Fig 1 and reference [12]), we quantitatively assessed the distribution of KatG, GroEL, SodB, and Bir in MHB- and BHI-grown cells and cell-free supernatants. To aid the interpretation of our findings, we similarly analyzed the distribution of the *E. coli* α-hemolysin (HlyA), a secreted pore-forming protein [50,51] whose expression is increased during mammalian infection [52,53]. As expected for a secreted protein, HlyA was readily detected (96–97% of the total) in the culture supernatants of *E. coli* with only scant levels observed within the bacterial cells (Fig. S1). In stark contrast, the *F. tularensis* proteins were readily detected in bacterial cells with only scant amounts (~1% of the total) detected in the supernatants and only those of MHB-grown *F. tularensis*. Based on these findings, we conclude that these *F. tularensis* proteins are not actively secreted - consistent with the findings of Hager et. al. for LVS [54]. A similar controversy over the active secretion of catalase, chaperones, superoxide dismutase, peroxiredoxins, and others played out in the *Mycobacterium tuberculosis* field in the 1990s including notions of how these bacterial enzymes could modify host cell behavior [55–59]. Ultimately, active secretion of these Mtb proteins (and presumably their effector functions within host cells) was unambiguously disproven by Tullius et. al. who used approaches similar to those used here to show that the presence of these proteins in cell-free culture supernatants resulted, not from active secretion, but instead from bacterial cell lysis in concert with the high abundance and extra-cellular stability of these proteins [60].

Of the membrane proteins, FsaP contains a single internal, transmembrane domain [61] which would place it in the cytoplasmic membrane [62,63], a location consistent with both our detection of FsaP in the Tx-detergent phases (which lack the OM markers FopA, TolC, and LPS OAg, Fig 2A) and the inability of polyclonal FsaP-specific Ab to label the surfaces of either intact *F. tularensis* or recombinant *E. coli* expressing FsaP [61]. While the quest for outer membrane proteins in host-adapted *F. tularensis* reveals putative capsular material(s)

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Figure 1. *F. tularensis* grown in MΦ or in BHI are nearly indistinguishable. *F. tularensis* LVS strains were harvested from the culture supernatants of infected macrophages (MΦ) or grown in vitro as indicated: MHB (M), BHI (B), MHB supplemented with spermine to 200 μM (MS), BHI supplemented with casamino acids to 17.5 g/l (BCA). Ten micrograms of protein from these bacteria were resolved by SDS-PAGE and transferred to membranes for western blot analysis with antibodies specific to the indicated proteins.

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Figure 2. Fractionation of *F. tularensis* reveals the presence of an inducible HMW carbohydrate in host-adapted bacteria.

Whole cells (WC) of MHB- (M) and BHI-(B) grown *F. tularensis* LVS were Tx114 phase-partitioned into aqueous (A), detergent (D), and Tx-insoluble phases. The insoluble material was treated with 0.2% sarkosyl to generate soluble (SS) and insoluble (SI) fractions. A) Intact fractions derived from 10 μg of WC protein were probed by western blot for the indicated proteins. Predicted locations were derived from the LVS protein sequence with the aid of PSORT (http://psort.hgc.jp/form.html). B) Carbohydrates present in proteinase K-treated fractions derived from 10 μg of WC protein were visualized by Emerald Green staining of resolved samples.

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contrary to the interpretations of Mellio et al. [61], our assignment of FsaP to the inner membrane is also consistent with the protein’s lack of heat-modifiability — a characteristic property of membrane-spanning OM proteins [62] — displayed by FopA but not FsaP (data not shown). Pal (peptidoglycan-associated lipoprotein) and Tul4 (as known as LpnA) are predicted and confirmed lipoproteins respectively [45,64]. As the rules for lipoprotein sorting in F. tularensis have not been established [45], it is not yet possible to predict with confidence the location of these molecules which could be lipid anchored to i) the outer leaflet of the inner membrane, ii) the inner leaflet of the outer membrane, or iii) the outer leaflet of the outer membrane [65]. Pal homologues in gram-negative bacteria are sub-surface moieties anchored by their N-terminal lipids to the inner leaflet of the OM allowing the protein to bind the periplasmic peptidoglycan [65], Tul4 is a potent TLR2 agonist [66,67] which is reportedly surface-exposed in capsule-deficient F. tularensis but not in the WT bacteria [68]. These cellular locations are consistent with our detection of the lipoproteins primarily in the Tx detergent (D) phases and, to a lesser extent, the Tx-insoluble, sarkysol-soluble (SS) phases (Fig 2A). ToLC-B and FopA are well characterized membrane-spanning OMPs [69] and as expected, we detected these proteins in the sarkysol-insoluble (SI) fraction (Fig 2A).

As a final means of validating our approach, we analyzed these same fractions (after proteinase-K treatment) by SDS-PAGE followed by periodic acid oxidation and fluorescent staining to visualize carbohydrates such as those in LPS - a known OM constituent. As expected, the typical ladder pattern of repeating LPS O-antigen (OAg) units was observed in the WC and SI fractions but not in the A, D, or SS phases (Fig 2B). However, this carbohydrate staining also revealed unexpected and striking differences between MHB and BHI-grown F. tularensis.

First, despite equivalent amounts of lipid A core, the “short” LPS (containing one to three OAg repeats) was more abundant in MHB-grown bacteria whereas the LPS from BHI-grown F. tularensis was more heavily populated by “long” LPS (see Fig. 2B and Fig. S2 for long and short exposures of the same gel). The increased abundance of “short” LPS in MHB-grown F. tularensis was confirmed by western blots (data not shown) with mAb FB11 which is specific for the bacterium’s LPS OAg [30]. Another significant difference was apparent in the high-molecular weight (HMW) carbohydrates of MHB- and BHI-grown F. tularensis. The MHB WC samples contained a diffuse region of staining extending roughly from ~100 kDa to slightly above 225 kDa (Fig 2B). In the BHI-grown samples, this region of staining extended well above the 225 kDa marker to nearly the bottom of the loading wells. Upon phase-partitioning, this BHI-specific ≥225 kDa carbohydrate was found in the aqueous phase along with a BHI-specific, protease-resistant, ~200 kDa putative glycoprotein that is labeled by both carbohydrate- and protein-specific stains (Fig S3). The phase-partitioning behavior of the HMW carbohydrate was similar to that of the F. tularensis OAg capsule recently described by Apicella et al. [30]. As both capsules and extended-length LPS molecules can markedly impact the accessibility of OMPs [70,71], we next sought to determine if host-adaptation of F. tularensis alters the production of OAg capsule and/or the accessibility of Ab to OM constituents.

Host-adapted F. tularensis produce more OAg capsule and bind less Ab directed against OM constituents

To probe the surface of F. tularensis for OAg capsule and OM accessibility we incubated intact bacteria with various Ab and probed the washed bacteria for bound immunoglobulin heavy chain (HC) by western blot. Following development of the Ig HC signals, we re-probed the membranes for total FopA and quantified the data as surface Ab/total FopA and normalized the ratios to the corresponding MHB result. As shown in Fig. 3A and B, when F. tularensis was incubated with immune mouse serum (IMS) the amount of IgG bound to the surface of BHI-grown, WT F. tularensis was significantly reduced relative to that of its MHB-grown counterpart. The amount of IgG bound to the surface of MHB-grown, WT F. tularensis and the BHI-grown, OAg mutant was not significantly different (Fig 3B) and were no independent experiments are indicated along with standard deviations in parentheses.

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**Figure 3.** Host-adapted F. tularensis produce more OAg capsule and bind less Ab directed against OM constituents. A) Intact bacteria (WT and wbtA), grown in MHB (M) or BHI (B), were incubated with the indicated sera/Ab, washed and probed for IgG heavy chain by western blot. The probed membranes were stripped and re-probed for total signals for the indicated proteins. B) Surface-bound Ab-to-FopA ratios from 5–8 independent experiments were quantified and expressed relative to that of the MHB-grown WT with same Ab. Asterisks indicate values that differed significantly (p ≤0.05, T-test with Bonferroni correction). C) MHB- and MHB-grown F. tularensis were incubated with Ab specific for the OAg-capsule and Tul4 and analyzed as in A above. Mean FopA-normalized, surface Ab values from 3 independent experiments are indicated along with standard deviations in parentheses.
significant differences in the amount of IgM bound to the surfaces of these three bacteria (Fig 3B).

The trend of Ab binding significantly better to the surfaces of MHB-grown F. tularensis also was observed with Ab specific for Tul4A, FopA, and LPS OAg (Figs 3A and 3B). This trend, however, was reversed when we probed MHB- and BHI-grown F. tularensis with Ab specific for the OAg capsule. BHI-grown F. tularensis bound 3-fold more α-OAg capsule Ab than did the same bacterium grown in MHB. MΦ-grown F. tularensis also displayed increased production of OAg capsule and reduced OMP (Tul4) accessibility compared to the non-host-adapted bacteria (Fig 3C). As with the BHI-grown bacteria, the reduced Ab binding was not explained by a reduction in Ab expression.

While the increased levels of OAg capsule and reduced accessibility of Ab to OM constituents noted in Fig 3 were internally-consistent, the ~3-fold increase in OAg capsule observed with BHI-grown F. tularensis seemed disproportionate to the 60–95% reduction in binding of α-OMP Ab by the same cells. Additionally, removal of the OAg capsule (in BHI-grown wbtA) did not, in all cases, fully restore Ab binding to the levels observed with MHB-grown bacteria.

For these two reasons, we postulated that host-adapted F. tularensis might produce an additional capsule-like material.

Host-adapted F. tularensis produce an additional high molecular weight carbohydrate that also correlates with reduced accessibility of Ab to OMPs

To determine if growth in BHI induced production of a HMW carbohydrate in addition to OAg capsule we examined the carbohydrate composition of MHB- and BHI-grown wbtA F. tularensis. As shown in Fig 4A, the BHI-grown, wbtA strain produced far more HMW carbohydrate than its MHB-grown counterpart. This HMW carbohydrate was similar in size (>225 kDa) to the material present in WT, BHI-grown F. tularensis. Upon phase-partitioning this material, along with the ~200 kDa putative glycoprotein, was found in the aqueous phase (Fig S3) similar to what was observed for WT BHI-grown F. tularensis (Fig 2B). The aqueous-phase, HMW carbohydrate material derived from BHI-grown, WT F. tularensis was slightly more abundant than that of the BHI-grown, wbtA1 mutant (Fig S3) likely indicating that the WT HMW carbohydrate material contains both OAg capsule and the non-OAg HMW carbohydrate.

To determine if the presence of this non-OAg, BHI-specific material correlated with reduced binding of α-OMP Ab, we used MHB- and BHI-grown WT and wbtA F. tularensis to immuno-precipitate α-Tul4A and α-FopA Ab as was done for Fig 3. As expected, the wbtA strain bound more α-OMP Ab than the similarly grown WT strain. However even in the absence of OAg, BHI-grown wbtA bound significantly less surface Ab than its MHB-grown, wbtA counterpart (Fig 4B and 4C). This diminished binding could not be explained by differences in loading or Ag expression (data not shown), but could be explained by the BHI-specific, HMW carbohydrate shielding the OMPs from Ab.

Host-adaptation reduces interaction with effectors of innate immunity

Since the access of many OM constituents to Ab was reduced in host-adapted F. tularensis, we postulated that OM constituents might also be shielded from elements of the innate immune system. We tested this notion in vitro by examining the ability of differentially-grown bacteria to promote i) complement deposition and ii) TLR2-dependent cellular activation of naive MΦs.

As a respiratory and blood-borne pathogen, F. tularensis would be expected to complement during the bacterium’s extra-cellular phase within mammalian hosts [72]. Since LPS- and/or capsule-mutants of F. tularensis fix complement more readily than WT bacteria [73–77], we hypothesized that the regulated LPS and capsule changes that are commensurate with host-adaptation would diminish complement deposition. As shown in Fig 5, MHB-grown F. tularensis exposed to complement-active normal mouse serum rapidly accumulated a ~53 kDa C3 fragment (consistent with the size of C3d) that failed to accumulate on BHI-grown WT bacteria. Fragments consistent with the sizes of the ζ-chain in C3b (~100 kDa), iC3b and iC3b (~68 and ~62 kDa) followed a similar pattern of deposition, albeit with different kinetics (Fig S4). Detection of these fragments is consistent with the reported ability of F. tularensis to bind factor H [78] which, in conjunction with Factor I, cleaves C3b [79]. As expected [73], deposition of complement was accelerated by the absence of OAg (wbtA1 strains).

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Host-adaptation reduces interaction with effectors of innate immunity

Since the access of many OM constituents to Ab was reduced in host-adapted F. tularensis, we postulated that OM constituents might also be shielded from elements of the innate immune system. We tested this notion in vitro by examining the ability of differentially-grown bacteria to promote i) complement deposition and ii) TLR2-dependent cellular activation of naive MΦs.
Host-adaptation accelerates tularemia pathogenesis

Previously we reported that mice challenged with BHI-grown F. tularensis LVS reproducibly succumb to infection ~1 day sooner than mice challenged with an equivalent dose of MHB-grown bacteria [12]. At the time, we assumed that this difference was largely an indication that the host-adapted bacteria were better prepared for the metabolic/nutritional environment of the mammalian host. In light of the LPS and capsule changes noted above, it is likely that the differences in the survival also reflected the heightened susceptibility of non-adapted bacteria to host innate immune effectors that could target the bacterium for clearance before it could host-adapt. Here we sought to extend our findings with LVS to the human-virulent strain F. tularensis SchuS4.

We have previously found protein expression by differentially-grown SchuS4 to follow a pattern similar to that of differentially-grown LVS [12]. Accordingly, here we examined the carbohydrate composition of differentially-grown SchuS4. As shown in Fig. 7A, two features of BHI-grown LVS are conserved in BHI-grown SchuS4 – i) an increase in the proportion of LPS molecules bearing a large number of OAg-repeating units, and ii) an increase in the size of the HMW carbohydrate. Interestingly, SchuS4 appears to produce more HMW carbohydrate than LVS. Next we examined TNF-α and IL-1β production by BMDM responding to differentially-grown SchuS4. As shown in Fig 7B and similar to our observations with LVS, BHI-grown SchuS4 elicited significantly lower levels of these pro-inflammatory cytokines from murine BMDMs and human U937 cells (data not shown) compared with that provoked by MHB-grown SchuS4 (p<0.001 for both TNF-α and IL-1β from BMDMs).

Finally, we examined survival of C57BL/6 mice challenged with MHB- or BHI-grown, F. tularensis SchuS4. In our experience, SchuS4 grows slightly faster than LVS suggesting that these bacteria might host-adapt more quickly than LVS and...
that differences in survival of SchuS4-infected animals might be less than was previously observed with LVS [12]. Consistent with the above predictions and the presumably heightened susceptibility of non-adapted bacteria to the restrictions present in the host environment (nutritional, innate immune, etc.), we observed (in 3 independent experiments initiated with distinct preparations of bacteria) that lethality among mice challenged with MHB-grown SchuS4 began later and took longer to complete than in mice challenged with BHI-grown bacteria (data not shown). For the mice infected with BHI-grown SchuS4, mortality began 5 days (120 hrs) post-infection (PI) with a median survival time of 132 hrs. By day 6 (144 hrs) PI, all mice had succumbed to infection with BHI-grown bacteria. For the mice challenged with MHB-grown SchuS4, mortality began at 126 hrs (median survival time = 144 hrs) but was not complete until day 7.25 (174 hrs) PI. The differences in survival of mice infected with MHB-grown and BHI-grown F. tularensis SchuS4 were highly-significant (p<0.001).

**Discussion**

We began these studies with the goal of identifying Ab-accessible OMPs expressed by host-adapted F. tularensis; these proteins could then be evaluated as subunit vaccine candidates. Pursuit of this goal led us to the discovery that host adaptation includes multiple changes to the bacterium’s surface carbohydrates (LPS and capsules) which cumulatively hinder Ab, complement, and TLR2-mediated recognition of OM constituents.

Within the mammalian host, F. tularensis undergoes intracellular replication yet has a significant extracellular phase [5,7] which likely facilitates dissemination. During this latter phase F. tularensis is presumably exposed to a variety of anti-bacterial factors such as complement, antibody, and phagocytes - which if activated – can hinder the bacterium’s progress [85]. For many pathogens, significant protection is afforded by a large polymeric capsule that surrounds the bacterium and limits access of immune effectors to the bacterium’s limiting membrane [86–88]. In this regard, it is not surprising that F. tularensis also would utilize this highly successful strategy. However, the existence and/or composition of F. tularensis capsule(s) have remained a subject of controversy.

Several electron microscopic (EM) analyses have noted circumferential labeling of bacteria that had been negatively-stained with heavy metals or immuno-labeled with α-F. tularensis sera - thereby providing support for the existence of an F. tularensis capsule [89,90,76]. In contrast, several recent attempts to detect a capsule-structure and/or material have been unsuccessful [73,91,92], prompting speculation that LPS OAg might be the only capsule-like structure produced by the bacterium [92]. Initial clues to the resolution of this conceptual discord were provided by the observation that repeated serial passage of F. tularensis in a chemically-defined, synthetic medium increased the abundance of an EM-visible capsule-like material [93]. This finding suggested that capsule production by F. tularensis might be environmentally regulated, however, it cannot be excluded that extended passage led to accumulation of encapsulated variants. Recently Apicella et al. provided another advancement by generating a capsule-specific reagent (mAb 11B7) and utilizing SDS-PAGE to visualize the

**Figure 6. F. tularensis surface carbohydrates impact the accessibility of TLR2 ligands and the extent of TLR2-dependent macrophage activation.** F. tularensis LVS WT and wbtA strains were grown in MHB (M), BHI (B), MHB supplemented with spermine (MS), or BHI supplemented with casamino acids (BCA). A) Bacterial lysates containing 10 μg of protein were treated with proteinase K prior to SDS-PAGE resolution and Emerald Green staining of carbohydrates. B) Intact bacteria were incubated with anti-Tul4A Ab, washed and probed for IgG heavy chain by western blot to detect surface-bound (S.) α-Tul4A Ab. The probed membranes were stripped and re-probed for the total FopA and total Tul4. C) Viable F. tularensis were incubated with C57BL/6 BMDMs at an MOI of 100 for 24 hrs prior to analysis of secreted cytokine levels shown as means and standard errors. doi:10.1371/journal.pone.0022335.g006
bacterium’s HMW carbohydrates [30]. These tools, along with biophysical and immunological approaches, were employed to determine that \textit{F. tularensis} produces an OAg capsule that is physically distinct from that of the LPS OAg. Here we synthesized these multiple observations and experimental approaches and found that host-adaptation of \textit{F. tularensis} increases production of (i) OAg capsule, (ii) longer polymers of LPS OAg, and (iii) an additional HMW carbohydrate/glycoprotein material(s) with capsule-like functions. We speculate that the putative glycoprotein described here may be related to the capsule-like complex, a putative glycoprotein, recently characterized by Bandara et al [94]. Collectively these changes shield OM constituents from recognition by Ab, complement, and TLR2-dependent macrophage activation – functions of capsules and/or long LPS which have been well-characterized in other pathogens [70,71,87,95–97].

Our working model for the interactions of differentially-grown \textit{F. tularensis} with select immune effectors is presented in Figure 8.

Our results provide both some confirmatory observations and several novel findings. Perhaps most striking is the marked induction of multiple capsular-materials and functions that is commensurate with host-adaptation of \textit{F. tularensis} (observed with bacteria grown in MΦ or BHI). Host-adapted bacteria bound significantly higher levels of the mAb 11B7 (which labels OAg-capsule) and contained additional, non-OAg, HMW carbohydrate(s) and reduced cytokine elaboration. \textit{F. tularensis} LVS (WT and wbtA) and SchuS4 were grown in MHB (M) and BHI (B). A) Proteinase K-treated lysates were resolved through 8-16 % SDS-PAGE Protein II large gels and stained with Emerald green to visualize carbohydrates. B) Viable \textit{F. tularensis} SchuS4, grown as indicated, were co-incubated with C57BL/6 BMDMs at an MOI of 100 for 24 hrs prior to analysis of secreted cytokine levels. Results are means and standard errors from three independent experiments each conducted in duplicate.

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Figure 7. BHI-grown \textit{F. tularensis} SchuS4 also displays increased production of HMW carbohydrate(s) and reduced cytokine elaboration. \textit{F. tularensis} LVS (WT and wbtA) and SchuS4 were grown in MHB (M) and BHI (B). A) Proteinase K-treated lysates were resolved through 8-16 % SDS-PAGE Protein II large gels and stained with Emerald green to visualize carbohydrates. B) Viable \textit{F. tularensis} SchuS4, grown as indicated, were co-incubated with C57BL/6 BMDMs at an MOI of 100 for 24 hrs prior to analysis of secreted cytokine levels. Results are means and standard errors from three independent experiments each conducted in duplicate.

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with LVS followed by SchuS4) as well as pAb and/or mAb specific for the lipoprotein Tul4A, the integral OMP FopA, and the LPS OAg. Interpreting the reduced binding of IMS IgG by host-adapted \textit{F. tularensis} is complex in that this sera has a broad range of specificities that likely include capsule(s) as well as Tul4, FopA, LPS OAg and many others. However, since Tul4, FopA and LPS are immuno-dominant Ags [29,32,64], and capsule is reported to be poorly-immunogenic [90], our observation (that host-adapted [capsule-expressing] bacteria bind less IMS IgG) is consistent with an increased amount of poorly-immunogenic capsule blocking access of Ab to the more highly immunogenic OM moieties. The use of WT and OAg-mutant bacteria allowed us to conclude that both the OAg and non-OAg HMW carbohydrates inhibit Ab binding. However, based on our findings, we can not dissect the relative contributions of (i) the OAg capsule and (ii) the longer LPS OAg polymers to the shielding of OM moieties from Ab.

The results with the mono-specific Abs are most readily interpreted as an indication that elevated capsule production limits access of Ab to Ag. Alternatively, host-adapted bacteria could have increased production (or acquisition) of a protease that cleaves bound Ab. Indeed \textit{F. tularensis} has been reported to bind exogenously-added, active plasmin [98,99] which, in one report, cleaved bound Ab, opsonizing IgG and unbound, non-specific IgG [99]. For several reasons (in addition to the fact that we did not add plasmin) we conclude that proteolysis of Ab is not the mechanism underlying the differences we observed. First, while the amount of bound IgG specific for OM constituents (\(\alpha\)-Tul4, \(\alpha\)-FopA, \(\alpha\)-LPS OAg) was reduced in host-adapted bacteria, the amount of surface-bound IMS-IgM was unchanged and the amount of bound IgG specific for another constituent (\(\alpha\)-OAg capsule) was increased. Secondly, the reduction in binding of \(\alpha\)-OMP Ab (\(\alpha\)-Tul4, \(\alpha\)-FopA) by BHI-grown bacteria was largely negated in bacteria (wbtA) unable to produce intact LPS and OAg capsule. Lastly, the addition of protease inhibitors to BHI-grown bacteria did not increase the amount of bound Ab (data not shown). Accordingly, we conclude that the elevated capsule production by host-adapted \textit{F. tularensis} limits the access of Ab to multiple OM antigens.

While both (i) complement activation (and subsequent deposition), and (ii) TLR2 engagement (and subsequent cytokine production), are more complex processes than Ab binding, we postulate that the increased capsule present on host-adapted \textit{F. tularensis} is also limiting access of these innate immunity molecules to bacterial OM ligands. Activation of C3 leads to deposition of C3b that contains an \(\alpha\)-chain of \(\sim100\) kDa which is covalently-bound to an acceptor molecule on the bacterial surface. The cooperative activities of Factors H and I cleave C3b sequentially to yield \(\alpha\)-chain fragments (iC3b\(1\), iC3b\(2\), and C3dg/C3d) of \(\sim68,\sim62\), and \(\sim40/\sim35\) kDa; these C3b fragments retain the ability to be bound by complement receptors but do not further promote the complement cascade [79,100]. In our assays with normal mouse sera as the source of complement, we observed the above cascades from activation to C3d (or C3dg) production. However, this was only observed with MHB-grown WT bacteria and OAg-deficient \textit{F. tularensis}. In contrast, host-adapted WT bacteria showed no evidence of C3 deposition, and BHI-grown, OAg-deficient bacteria accumulated less C3d/C3dg than their MHB-grown counter parts. The simplest (although not exclusive) interpretation of these findings is that the C3-activating moieties (natural Ab, mannose-binding protein, or targets of the alternative pathway) are shielded from their ligand(s) by capsule similar to what we hypothesize for the reduced binding of OM-specific Ab to host-adapted \textit{F. tularensis}. It is conceivable that the heightened sensitivity of MHB-grown bacteria to complement may have
contributed to the more rapid demise of mice infected with BHI-grown *F. tularensis* that we report here for strain SchuS4 and observed previously with strain LVS [12].

The activation of naïve M\(_{\text{W}}\)s in vitro by MHB-grown bacteria has been shown by multiple groups to be TLR2-dependent and to characteristically culminate in the elaboration of T\(_{\text{H}1}\)-type pro-inflammatory cytokines [80-83]. However, these cytokines are largely undetectable in the lungs of mice during early respiratory tularemia (days 0 to 3) even though this is a time and site of exponential bacterial replication. This apparent discord has prompted the notion that the bacterium is actively suppressing the inflammatory response. The active-suppression hypothesis is consistent with the observations of many that *F. tularensis*-infected cells and/or animals show blunted T\(_{\text{H}1}\)-type pro-inflammatory responses to exogenous TLR agonists [101–104]. Active-suppression of host-cell responses by bacteria is typically mediated by bacterial effector proteins which are delivered to the host cell cytoplasm by either a bacterial syringe-like apparatus (T3SS and T4SS) or as the cargo components of AB-toxins. *F. tularensis* is notable for its non-proinflammatory LPS, its lack of identifiable toxins and its lack of T3SS and T4SS syringe-like apparatuses [105]; a related species *F. novicida* may have a functional T6SS [106].

Our findings - that host-adapted bacteria produce sufficient surface carbohydrates to shield known TLR2 ligands (such as Tul4A) from Ab, and apparently from TLR2, - suggest a distinct mechanism to explain the apparent hypo-responsiveness of M\(_{\text{W}}\)s engaging *F. tularensis*. Specifically we postulate that host-adapted bacteria, through shielding of TLR2 ligands with abundant capsular carbohydrates, either i) simply fail to trigger the TLR2 signaling cascade that results in production of TNF-\(\alpha\) and IL-1\(\beta\), and/or ii) preferentially engage a distinct receptor that provokes an non-T\(_{\text{H}1}\) type response. Along these lines, the mannose-receptor, which has been implicated in the phagocytosis of *F. tularensis* by M\(_{\text{W}}\)s and suggested to be a “safe portal of entry” [107,108], is an attractive candidate as pathogen-mediated ligation of this receptor can blunt TLR-driven T\(_{\text{H}1}\)-type pro-inflammatory responses [109]. We envision that the mannose receptor could be preferentially engaged by the abundant HMW carbohydrate capsule present on host-adapted bacteria. An early analysis of total capsular material (presumably containing both OAg capsule and non-OAg capsule) detected high levels of mannose [90] while a recent analysis of purified OAg capsule revealed an absence of mannose [30]. These observations would be consistent with the non-OAg capsule (enriched on host-adapted bacteria) being the presumptive ligand of the mannose receptor. It is conceivable that earlier and/or more extensive engagement of the mannose receptor, combined with reduced TLR2 engagement, may have contributed to the more rapid demise we observed with mice infected with BHI-grown SchuS4.

Figure 8. A model of the interaction of MHB-grown and host-adapted *F. tularensis* with select mammalian immune effectors. The cellular locations depicted for FopA, Tul4, Pal, and FsaP are based upon data in figure 2A and the relevant text in which this data is discussed. The depiction of host-adapted bacteria having longer LPS O-Ag carbohydrate chains and elevated levels of capsular material is based upon results in figures 2B, 3, 4, 6A, 7A, 52, and 53. The depiction of antibodies binding differentially to host-adapted and non-adapted bacteria is based on results presented in figures 3, 4, and 6. The differential binding/activation of complement protein C3 is based upon data in figures 5 and S4. Our model of TLR2-dependent production of T\(_{\text{H}1}\)-type pro-inflammatory cytokines by macrophages responding to *F. tularensis* is based upon results presented in figures 6B and 7B.

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While we used host-adapted and non-host-adapted bacteria primarily as tools to explore the impacts of capsule production on immune effector functions, we believe that our results also have implications for understanding the regulation of capsular components during the host-cell infection cycle. Within the cytoplasm of mammalian host cells the concentrations of both free amino acids and spermine are high compared to those of the extracellular environment. We have shown previously [12], and confirmed here, that BHI-grown *F. tularensis* and extracellular, host-adapted bacteria (those which have emerged from infected Mφ) are phenotypically indistinguishable; much of this MgL-dependent phenotype is repressible by high concentrations of free amino acids. Using microarrays, RT-PCR, and transcriptional reporters to compare *F. tularensis* grown in MHB and MS or harvested from the cytoplasm of infected cells, Carlson et al [34] found that MS-grown and intracellular bacteria are highly similar; spermine activates promoters within multiple insertion sequences including one upstream of wbtA – thought to be involved with the production of OAg. Here we found that, compared to bacteria grown in MHB, MS-grown *F. tularensis* have increased binding of mAb specific for the OAg-capsule (shown in Fig 6). We also found that production of OAg capsule was also stimulated by high concentrations of free amino acids. The responsiveness of OAg capsule to spermine and amino acids suggests that the OAg capsule is important, not only for extracellular survival, but also for intracellular growth. Indeed Lindeleman et. al. have recently shown that mutants with defects in production and/or export of OAg capsule, in addition to being complement sensitive, have impaired cytosolic replication despite showing normal phagosome-escape [75].

In contrast to the OAg capsule, we believe that one-or-more of the non-OAg capsular materials is repressed by high concentrations of free amino acids possibly suggesting a primarily extracellular role for this material. We found that addition of casamino acids to BHI decreased the production of HMW carbohydrate by *F. tularensis* and that this decrease was not attributable to changes in OAg (shown in Fig 6). We speculate that this amino-acid inhibitable, extracellular HMW carbohydrate(s) that we observed maybe similar to the putative capsule noted by Golovliov et al. [89]. This material was abundant on extracellular bacteria yet markedly reduced/absent on intracellular bacteria. Down-modulation of this non-OAg capsular component once inside the host cell could facilitate more rapid host-pathogen interactions such as nutrient acquisition. In the extracellular milieu this capsular component could impede recognition by complement, Ab, and TLR2. We postulate that the higher level of capsule (OAg and non-OAg) present on BHI-grown *F. tularensis* may have contributed to the more rapid demise we observed of mice infected with BHI-grown SchuS4 (this work) and LVS [12].

The survival times we observed for G57Bl/6 mice challenged with ~20 CFU of MHB-grown SchuS4 (median survival time - 6 days) are highly similar that those recently reported by Ireland et al in which Balb/c mice were challenged with 25 CFU of MHB-grown SchuS4 (mean time-to-death of 5.8 and 3.4 days in separate experiments) [110]. On the other hand, the statistically significant differences we observed between mice challenged with MHB- and BHI-grown SchuS4 (median survival time - 12 hr difference, time-to-complete lethality - 30 hr difference) are similar to differences recently reported by groups examining the virulence of genetically distinct *F. tularensis* type A strains. Molins et al monitored survival of mice challenged with different clades of type A strains and observed mean time-to-death differences of 9 hr (between A1a strains and A2 strains) and 13.5 hrs (between A1a strains and A1b strains) [111]. Other groups have examined survival of mice challenged with WT type A strains and isogenic mutants (pilOQ, tolC, np, hfp, and FTF6069) and found differences in time-to-complete lethality of 24 hrs [112,113] – similar to the 30 hr difference we report here for host-adapted vs non adapted SchuS4. Thus the impact of host-adaptation on murine lethality experiments is similar in magnitude to the bacterial genetic differences mentioned above.

In addition to the above experiments with mice lacking pre-existing specific immunity; we predict that the use of host-adapted or non-adapted challenge inoculums could also impact the outcome of vaccine efficacy trials. This could be especially true in the case of OMP-based, sub-unit vaccines - where the accessibility of the OMPs to Ab differs depending on how the challenge inoculum was prepared (consider Fig 8). In this case, vaccine-induced γ-OMP Ab (through recruitment of phagocytes and/or complement) could clear a portion of the challenge inoculum before the bacteria had host-adapted; after host-adaptation these Ab would have reduced/no efficacy. In total, such an experiment could report the vaccine to be “partially protective” when in fact this might be true only for a MHB-grown challenge dose. Infection with *F. tularensis* emanating from a tick, tabanid fly, mosquito, aerosolized logomorph, or the “lab” of a terrorist that does not use MHB could proceed unhindered in the face of such vaccine-induced immunity. Accordingly, we suggest that vaccine discovery and testing should incorporate the use of host-adapted *F. tularensis* to avoid re-trekking the OSpA path.

### Materials and Methods

#### Bacteria and Media

Wild-type (WT) *F. tularensis* LVS and the isogenic mutant strains mglA, pmrA, and wbtA have been described previously [12,36,114]. *F. tularensis* SchuS4, originally isolated from a human case of tularemia, was obtained from the U.S. Army Medical Research Institute for Infectious Diseases (Frederick, MD). All experiments using SchuS4 were conducted within the Albany Medical College ABSL-3/BSL-3 facility which has been certified by the Center for Disease Control. SchuS4 lysates for protein analysis to be performed outside of this facility were generated by boiling harvested bacteria in 50 mM Tris, pH 8.0 containing 1 % SDS followed by sterility testing. *Escherichia coli* WAM 1824, which secretes γ-hemolysin (HylA), has been described [50].

Routine culturing of *F. tularensis* involved streaking aliquots of frozen bacterial glycerol stocks onto Mueller Hinton Chocolate agar plates [Becton, Dickinson and Company (BD), Sparks, MD] followed by 2-3 days of growth in a humidified chamber maintained at 37°C. Starter cultures were generated by resuspending several isolated colonies in ~100 μl of BHI from which ~50 μl was immediately used to inoculate 3–5 ml of BHI and MHB for ~18 hrs of growth on an orbital shaker operating at 200 rpm and 37°C. Mature starter cultures were used at a 1:100 dilution to inoculate larger volumes (10–50 ml within 125–250 ml Erlenmeyer flasks) of fresh media appropriate for each experiment. Unless stated otherwise, all bacterial cultures used in this work were in mid-log phase (OD<sub>600</sub> = 0.4–0.6). Preparation of MHB and BHI media as well as supplementation of BHI with casamino acids has been described previously [12]. Supplementation of MHB with spermine to 200 μM was performed as described [34].

#### Tissue Culture

Unless stated otherwise, tissue culture was performed using high-glucose DMEM (HyClone, Logan, Utah) containing 10% fetal bovine serum (Pel-Freez. Rogers, AK), 100 U/ml penicillin/100 μg/ml streptomycin (Gibco/Invitrogen. Grand Island, NY),
2 mM GlutaMAX (Gibco), 10 mM HEPES (Mediatech, Inc. Herdon VA), 0.075 % sodium bicarbonate (Mediatech), and 50 μM β-mercaptoethanol (Sigma). Bone-marrow-derived macrophages [BMDM] were harvested from C57/BL6 mice (Taconic Farms, NY); the murine macrophage-like cell line RAW 264.7 was cultured as described [33]. Murine hybridomas producing mAb directed against IgB and IgC were graciously provided by Francis Nano (U. of Victoria).

**Generation of macrophage-grown bacteria**

To generate macrophage (MΦ)-grown *F. tularensis* LVS, adherent RAW cells at ~75 % confluence within four-to-six T-75 flasks were rinsed and replenished with antibiotic-free DMEM media. MΦ-grown bacteria were harvested by centrifugation (4000 x g, 10 min) and resuspended in 1 ml of antibiotic-free DMEM media. MΦ infections were initiated at an MOI of 500 and allowed to proceed for 8 hrs within a tissue-culture incubator. Monolayers were rinsed and incubated for 1 hr with DMEM containing 50 μg/ml gentamicin. Following five further rinses, the cultures were replenished with fresh antibiotic-free DMEM prior to further incubation. At 36 hrs post-infection, the extracellular bacteria present in the tissue-culture supernatant were subject to 2–4 low-speed (500 x g, 10 min) spins to remove any MΦ; supernatants from each spin were examined by darkfield microscopy for contaminating eukaryotic cells. The bacteria present in the MΦ-free supernatants were harvested by centrifugation (7000 x g, 10 min) and gently resuspended to ~5×10^9/ml in antibiotic-free DMEM.

**SDS-PAGE and western blot analysis**

Samples of *Francisella* [10 μg (~1×10^8 cells)] were mixed with Laemelli sample buffer and boiled for 10 min prior to resolution through 4–12 % gradient SDS-PAGE pre-cast gels (Invitrogen). The running buffer was NuPAGE MES SDS buffer from Invitrogen; gels were variously run at 90–160 V. Resolved gels were stained with either coomassie blue (BioRad) or transferred to nitrocellulose membranes. Coomassie-stained gels were scanned into Adobe Photoshop using an HP 2820. Membranes to be probed with polyclonal sera against the secreted protein HlyA was purchased from Abcam. Biotinylated goat α-Ig heavy (γ) chains of mouse, rat, and rabbit IgG or IgM were from SouthernBiotech (Birmingham Al) and used in western blots O/N at a dilution of 1:1000; these primary Ab were detected using strepavidin-conjugated HRP. FITC conjugated Goat α-mouse C3 was purchased from Immunology Consultants Laboratory. By comparing the reactivity of this Ab in western blots against whole NMS prepared in the presence and absence of β-ME, we found that this Ab to be selective for the β-chain of C3.

**Fractionation of Francisella**

Mid-log phase *F. tularensis* was harvested by centrifugation (8000 x g, 15 min, 20°C). Supernatants were decanted and sterile (0.22 μm)-filtered for subsequent analysis; the bacterial pellet was resuspended in fresh growth media (MHB or BHI), transferred to pre-weighed Eppendorf tubes, pelleted as above after which the transfer supernatant was aspirated. Pellet wet weights were determined and used to estimate cell numbers based upon the estimate of 1 mg of wet weight = 5×10^8 bacterial cells. Cells were resuspended to 2.5×10^7/ml (higher bacterial concentrations (DC protein assay, BioRad, Hercules, CA), equilibrated bacterial suspensions were supplemented with EDTA to 5 mM, lysozyme and RNAse to 100 μg/ml each, Benzonase (1 μ/ml, Sigma), and Triton X-114 (Acros, NJ) to 1% from a 10% stock in PBS. Following a 1 hr incubation at room temperature with periodic gentle agitation, a 1/20 vol. aliquot was saved as the whole-cell (WC) fraction; the WC and remaining sample were stored at ~20°C overnight. The samples were thawed and incubated at RT until further viscosity reduction abated. Following 15 min incubation on ice, the samples were centrifuged (20 min, 16,500 x g, 4°C) to yield Triton X-114 soluble (TxS) and insoluble (TxI) fractions. The TxS fractions were transferred to fresh tubes and centrifuged again to remove any TxI carry-over; the TxI fractions were washed twice with cold PBS (1 vol). The TxS fractions were incubated in a 32°C water bath for ~10 min followed by centrifugation (7000 x g, RT, 15 min) to effect phase partitioning [40–43,64] into aqueous (top) and detergent (bottom) phases (any protein precipitation at this step rendered the samples useless, such experiments were discarded). Recovered phases were washed by supplementation (to 1% Triton X-114 - A or 10 vol PBS - D) followed by phase separation; three washes per recovered phase were performed. The washed Triton insoluble (TxI) phases were resuspended in PBS containing 0.2% sarcosyl (Fisher Scientific, Fair Lawn, NJ) and incubated for 20 min with gentle agitation followed by centrifugation (30 min, 4°C, 16,500 x g). The sarcosyl-soluble (SS) fraction was recovered and centrifuged a second time to remove any carryover of insoluble material. The sarcosyl-insoluble (SI) material was washed once with PBS, 0.2% sarcosyl and dissolved in 10 mM Tris, pH 8.0 containing 1 % SDS. WC, A, D, SS, and SI fractions were resolved by SDS-PAGE.
Emerald Green staining of SDS-PAGE gels

Boiled samples of *F. tularensis* in Laemelli sample buffer (1 ug/ul protein) were de-proteinated with proteinase K (0.4 ug/ul final) at 60°C for 1 hour and re-boiled prior to resolution through 1-12 % gradient SDS-PAGE pre-cast gels (Invitrogen). Each lane was loaded with the de-proteinated material generated from *F. tularensis* lysate containing 10 ug of protein. Carbohydrates were visualized in situ using the Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit (Invitrogen) as instructed. Briefly, resolved gels were fixed over-night in 5 % acetic acid, 50 % methanol. Following two 20 minute washes in 3 % acetic acid, the gels were incubated for 30 minutes in oxidizing solution, washed 3 times in 3 % acetic acid prior to fluorescent staining of oxidized carbohydrates. After two 20 minute washes in 3 % acetic acid, the gels were visualized using a SYBR-green filter and an Alpha Innotech imaging system in movie mode. Following acquisition of the emerald-green signal, gels were de-stained overnight in 3 % acetic acid and subsequently stained with SYPRO Ruby fluorescent protein stain (Invitrogen) and visualized using an ethidium bromide filter as above.

Antibody- and complement-binding assays

To assess binding of Ab or complement to the surface of *F. tularensis*, 5 x 10^6 bacteria in 25 ul were mixed with 25 ul of sterile, pre-cleared (10,000 x g for 10 min) 0.5 X PBS containing 0.1 % glucose and either i) 1-5 µl (empirically-determined) of the test Ab/sera (sera were heat-inactivated for Ab-binding assays or ii) 12.5 µl of intact or heat-inactivated normal mouse sera (for complement-binding assays). To assess the impact of protease inhibitors on Ab-binding, a protease inhibitor cocktail (Sigma, p2714 – which includes aprotinin, a plasmin inhibitor) was included in the above Ab mixture. Samples were incubated at 37°C for 1.5 hrs (for Ab-binding assays) or for various times (10 min – 1 hr) for complement-binding assays. Following incubation, bacteria were pelleted by centrifugation (10,000 x g for 10 min) and 45 µl of supernatant was removed. The bacteria were gently resuspended in 1 ml of 0.5 X PBS containing 0.1 % glucose and pelleted by centrifugation (10,000 x g for 10 min) after which the supernatants were aspirated. Following one additional wash, the cells were resuspended to 25 µl in 50 mM Tris pH 8.0 containing protease inhibitors followed by the addition of 25 µl of sample buffer prior to boiling and SDS-PAGE. 1 x 10^6 bacteria per lane were resolved by SDS-PAGE and probed for bound immunoglobulin (IgG or IgM) heavy chain (HC) by western blot. Following development of the Ig HC signals, we re-probed the membranes for total FopA and quantified the data as surface Ab/total FopA ratios to the corresponding MHB membranes for total FopA and normalized the ratios to the corresponding MHB.

Macrophase infections for cytokine analysis

BMDM seeded in 24 or 48 well plates were co-incubated at an MOI of 100 with *F. tularensis* that had been grown in vitro (MHB, BHI, MS, BCA) or harvested from previously-infected BMDM. After 24 hrs the media was analyzed by ELISA (eBiosciences) to measure TNF-α and Cytometric Bead Array (BD Pharmingen) to detect IL-1β. Statistical analysis was performed using the 2-tailed, T-test with Bonferroni corrections when appropriate and significance set at p<0.05.

**Ethics Statement**

All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all animal work was approved by the Albany Medical College Animal Care and Use committee (Approval # 901398). Guidelines provided by the NIH were followed in all experimentation.

**Murine infections**

We generated two independent sets of matched challenge stocks (MHB- and BHI-grown *F. tularensis* SchuS4). Each matched set was generated from a common pool of single isolated colonies, picked from a single chocolate agar plate and used to inoculate both MHB and BHI broth; each broth culture was expanded side-by-side and harvested at O.D.₆₅₀₀ = 0.2. In three independent experiments totaling 26–28 mice per media type, groups consisting of 6–10 C57BL/6 mice, 8–12 wks of age, were anesthetized by intra-peritoneal injection of 100 µl of xylazine (20 mg/ml) and ketamine (1 mg/ml) and challenged by intranasal instillation of ~20 CFU of MHB- or BHI-grown *F. tularensis* SchuS4 in 20 µl of PBS. Exact CFUs/inoculum were determined by duplicate plating of the inoculums at the time of challenge. Mice were monitored once-to-three daily until all mice succumbed to infection (here less than 8 days). Two of the three experiments used one matched set of challenge stocks; the other experiment used the other set of matched challenge stocks. Analysis of the survival data by the Cox proportional hazard model determined that i) there were no significant differences among the three experiments (p>0.79) and ii) the small variation in inoculum size (21–26 CFU) had no significant impact (p>0.68). This analysis did however find that inoculum type (MHB- or BHI-grown *F. tularensis* SchuS4) had a significant (p=0.0046) impact on survival with a hazard ratio for BHI-grown bacteria of 2.38 (95% C.I. of 1.31–4.43). This indicates that at any point in the experiment, a mouse infected with BHI-grown SchuS4 was 2.4 times more likely to die as a mouse infected with MHB-grown SchuS4. The observation that the three experiments were not significantly different also allowed for analysis of the combined survival data from all 54 mice by the Kaplan-Meier log-rank test.

**Supporting Information**

**Figure S1** The cellular distribution of the *F. tularensis* KatG, GroEL, SodB, and Bfr proteins is markedly distinct from that of a secreted protein (*E. coli* HlyA). *F. tularensis* LVS and *E. coli* WAM 1824 cultures grown in MHB (M) or BHI (B) to mid-log were quantified and harvested by centrifugation. Sterile-filtered, cell-free supernatants from 5 x 10⁸ bacteria were precipitated with 10 volumes of acetone, washed twice with 70 % ethanol and resuspended in 1 % SDS. Cells and supernatants from the indicated number of bacteria were resolved by SDS-PAGE and either stained with coomassie blue (top panels) or analyzed by western blot (lower panels) for the indicated proteins. Note that the immunoblot signal intensity for MHB-grown *F. tularensis* is similar in 10⁷ cells and 10⁸ supernatant equivalents indicating that ~1 % of each protein is found in the MHB supernatant. Expression of HlyA is environmentally-regulated and, in uropathogenic *E. coli*, is increased during mammalian infection. (TIF)

**Figure S2** Fractionation of *F. tularensis* reveals the presence of an inducible HMW carbohydrate in host-adapted bacteria. Whole cells (WC) of *F. tularensis* LVS grown in MHB (M) or BHI (B) were Tx114 phase-partitioned into
aqueous (A), detergent (D), and insoluble fractions (TxI). The TxI material was treated with 0.2% sarkosyl (S) resulting in soluble (S) and insoluble (SI) fractions. Following proteinase-K treatment and SDS-PAGE resolution, the samples were stained for carbohydrates. Different exposures (top panel-short exposure, bottom panel-longer exposure) of the same gel are shown here and in Figure 2B. Western blots with mAb FB11 (specific for LPS OAg, data not shown) confirmed the identification of the band labeled “LPS core + 1 OAg unit”.

**Figure S3** *F. tularensis* grown in BHI produces a both an OAg and non-OAg HMW carbohydrate and a ~200 kDa putative glycoprotein that partition into the Tx-114 aqueous phase. Tx-114 aqueous phases from MHB (M)- and BHI (B)-grown *F. tularensis* WT and wbtA were treated with proteinase K (PK) and resolved by SDS-PAGE. The resolved samples were sequentially stained to visualize carbohydrates (left panel) followed by visualization of protein (right panel). Note that the bottoms of the loading wells are visible.

**(TIF)**

**Figure S4** Host-adaptation of *F. tularensis* reduces complement activation. MHB- or BHI-grown bacteria were incubated for 10 min or 1 hr with 25% normal mouse serum (NMS), heat-inactivated (hi) NMS, or in the absence of NMS. Washed bacteria were probed by western blot with a polyclonal Ab directed against mouse complement protein C3; the C3 Ab was found to be selective for the α-chain of C3. Results are representative of three independent experiments.

**(TIF)**

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**Author Contributions**

Conceived and designed the experiments: KROH. Performed the experiments: TMZ AS CB TR KROH. Analyzed the data: PJF KROH. Contributed reagents/materials/analysis tools: BS EJG TJS. Wrote the paper: KROH.

**References**

1. Oytosny PC, Stojched A, Tithall RW (2004) Tularemia: bioterrorism defense renws interest in Franciella tularensis. Nat Rev Microbiol 2: 967–978.

2. Petersen JM, Mead PS, Schriefer ME (2009) Franciella tularensis: an arthropodborne pathogen. Vet Res 40: 7–.

3. Abd H, Johansson T, Golovlev I, Sandstrom G, Forman M (2003) Survival and growth of *Franciella tularensis* in *Acanthamoeba castellanii*. Appl Environ Microbiol 69: 600–606.

4. Bar-Haim E, Gat O, Markel G, Cohen H, Shafferman A, et al. (2008) Interrelationships between dendritic cell trafficking and *Franciella tularensis* dissemination following airway infection. PLoS Pathog 4: e1000211-.

5. Forestal CA, Malik M, Catlett SV, Savitt AG, Benach JL, et al. (2007) *Franciella tularensis* LVS has a significant extracellular phase in infected mice. J Infect Dis 196: 134–137.

6. Thelaus J, Anderson A, Mathiesen P, Forslund AL, Noppa L, et al. (2009) Influence of nutrient status and grazing pressure on the fate of *Franciella tularensis* in lake water. FEMS Microbiol Ecol 67: 69–80.

7. Yu JJ, Randle JK, Morris AK, Guerrier MN, Klose KE, et al. (2007) The presence of infectious extracellular *Franciella tularensis* subsp. novicida in murine plasma after pulmonary challenge. J Eur J Clin Microbiol Infect Dis 26: 927–935.

8. Cheng A, Wehrly TD, Nair V, Fischer ER, Barker JR, et al. (2008) The early phagosomal stage of *Franciella tularensis* determines optimal phagocytic escape and Franciella pathogenicity island protein expression. Infect Immun 76: 5485–5499.

9. De Bruin OM, Ludus JS, Nano FE (2007) The Franciella pathogenicity island protein Ig9α localizes to the bacterial cytosol and is needed for intracellular growth. BMC Microbiol 7: 1–10.

10. Wehrly TD, Chong A, Virtaneva K, Sturdevant DE, Child R, et al. (2009) Intracellular biology and virulence determinants of *Franciella tularensis* revealed by transcriptional profiling inside macrophages. Cell Microbiol 11: 1126–1130.

11. Barthold SW, Fikrig E, Bockerstedt LK, Persing DH (1995) Circumvention of outer surface protein A immunity by host-adapted *Borrelia burgdorferi*. Infect Immun 63: 2255–2261.

12. Hazlett KRO, Caudin SD, McArthur DG, Cirillo KA, Kirimanevorsa GS, et al. (2008) Adaptation of *Franciella tularensis* to the mammalian environment is governed by cues which can be mimicked in vitro. Infect Immun 76: 4095–4102.

13. Fosha Y (1949) Tularemia: a summary of certain aspects of disease including methods for early detection and the results of serum treatment in 600 patients. Medicine 19: 1–41.

14. Drabick JJ, Narayanan RB, Williams JM, Lede JW, Nacy CA (1994) Passive protection of mice against lethal *Franciella tularensis* (live tularemia vaccine strain) infection by the sera of human recipients of the live tularemia vaccine strain. Am J Med Sci 308: 83–87.

15. Fortier AH, Slayter MV, Ziemba RH, Mezler MS, Nacy CA (1991) Live vaccine strain of *Franciella tularensis*: infection and immunity in mice. Infect Immun 59: 2922–2928.

16. Kirimanjeswara GS, Golden JM, Bakshi CS, Metzger DW (2007) Phagolytic and therapeutic use of antibodies for protection against respiratory infection with *Franciella tularensis*. J Immunol 171: 532–539.

17. Lavine CL, Clinton SR, nugroho-Fischer I, Marion TN, Bina XR et al. (2007) Immunization with heat-killed *Franciella tularensis* LVS elicits protective antibody-mediated immunity. Eur J Immunol 37: 3007–3020.

18. Rhinehart-Jones TR, Fortier AH, Elkins KL (1994) Transfer of immunity against lethal murine Franciella infection by specific antibody depends on host gamma interferon and T cells. Infect Immun 62: 3129–3137.

19. Stemmark S, Lindgren H, Tarwak A, Stojched A (2003) Specific antibodies contribute to the host protection against strains of *Franciella tularensis* subsp. holarctica. Microb Pathog 35: 73–80.

20. Kimpel GR, Eaves-Pyles T, Moen ST, Taormina J, Peterson JW, et al. (2006) Levofloxacin rescues mice from lethal intra-nasal infections with virulent *Franciella tularensis* and induces immunity and production of protective antibody. Vaccine 26: 6874–6882.

21. Mara-Koosham G, Hurt JA, Lyons CR, Wu TH (2011) Antibodies contribute to Effective Vaccination Against Respiratory Infection by Type A *Franciella tularensis*. Infect Immun.

22. Savitt AG, Men-Tahoupa P, Mensale G, Benach JL (2009) *Franciella tularensis* infection-derived monoclonal antibodies provide detection, protection and therapy. Clin Vaccine Immunol.

23. Cole LE, Yang Y, Elkins KL, Fernandez ET, Qureshi N, et al. (2009) Antigen-specific B-1a antibodies induced by *Franciella tularensis* LPS provide long-term protection against F. tularensis LVS challenge. Proc Natl Acad Sci U S A 106: 4343–4348.

24. Crowl JW, Shen H, Webb A, Perry MB (2002) Mice vaccinated with the O-antigen of *Franciella tularensis* LVS lipopolysaccharide conjugated to bovine serum albumin develop varying degrees of protective immunity against systemic or aerosol challenge with virulent type A and type B strains of the pathogen. Vaccine 20: 3463–3471.

25. Crowl JW, Vinogradov E, Monteiro MA, Perry MB (2003) Mice intradermally-inoculated with the intact lipopolysaccharide, but not the lipid A or O-chain, from *Franciella tularensis* LVS rapidly acquire varying degrees of enhanced resistance against systemic or aerosol challenge with virulent strains of the pathogen. Microb Pathog 34: 39–45.

26. Dreisbach VC, Cowley S, Elkins KL (2000) Purified lipopolysaccharide from *Franciella tularensis* live vaccine strain (LVS) induces protective immunity against LVS infection that requires B cells and gamma interferon. Infect Immun 68: 1988–1996.

27. Fulop M, Manche R, Tithall R (1995) Role of lipopolysaccharide and a major outer membrane protein from *Franciella tularensis* in the induction of immunity against *Franciella tularensis*. J Immunol 155: 1250–1255.

28. Fulop M, Manche R, Tithall R (1996) Role of two outer membrane antigens in the induction of protective immunity against *Franciella tularensis* strains of different virulence. FEBS Immunol Med Microbiol 13: 243–247.

29. Fulop M, Webber T, Manche RJ, Kelly DG (1991) Production and characterization of monoclonal antibodies directed against the lipopolysaccharide of *Franciella tularensis*. J Clin Microbiol 29: 1407–1412.

30. Apicella MA, Post DM, Fowler AC, Jones BD, Rasmussen JA, et al. (2010) Identification, characterization and immunogenicity of an O-antigenic capsular polysaccharide of *Franciella tularensis*. PLoS ONE 5: e11060-.

31. Golovilov I, Ericsson M, Sandstrom G, Tarwak A, Stojched A (1997) Identification of proteins of *Franciella tularensis* induced during growth in macrophages and cloning of the gene encoding a prominently induced 23-kilodalton protein. Infect Immun 65: 2183–2189.

32. Twin SM, Mykytczuk NG, Petit MD, Shen H, Stojched A et al. (2006) In vivo proteomic analysis of the intracellular bacterial pathogen, *Franciella tularensis*, isolated from mouse spleen. Biochem Biophys Res Commun 345: 1621–1633.
33. Loegering DJ, Drake JR, Banas JA, McNealy TL, Mr. Arthur DG, et al. (2006) Francisella tularensis LVS grown in macrophages has reduced ability to stimulate the secretion of inflammatory cytokines by macrophages in vitro. Microb Pathog 41: 218-225.

34. Carlson PE, Jr., Horwitz MA, J. Dey DM, Robinson CM, Neophytou P, et al. (2009) Global transcriptional response to spermine, a component of the intramacrophage environment, reveals regulation of Francisella gene expression through insertion sequence elements. J Bacteriol 191: 6853-6864.

35. Mohapatra NP, Soui S, Bell BL, Warren R, Ernst RK, et al. (2007) Identification of an opsonic response regulator required for the virulence of Francisella spp. and transcription of pathogenicity island genes. Infect Immun 75: 3305-3314.

36. Samuelson-Jackson WL, McClelland K, Maner-Cirioni JN, Menzer DW, Bakshi GS, et al. (2006) Generation and characterization of an attenuated mutant in a response regulator gene of Francisella tularensis live vaccine strain (LVS). DNA Cell Biol 25: 387-403.

37. Baron GS, Nano FE (1998) MglA and MglB are required for the intramacrophage environment, reveals regulation of Francisella gene expression through insertion sequence elements. J Bacteriol 191: 6853-6864.

38. Moayeri M, Welch RA (1997) Prelytic and lytic conformations of erythrocyte-enzyme activities potentially involved in the pathogenicity of Mycobacterium tuberculosis. Microbiol 62: 227-237.

39. Williams MD, Ouyang TX, Fleckinger MC (1994) Starvation-induced expression of SpaA and SpaG: the effects of a null mutation in spaA on Escherichia coli protein synthesis and survival during growth and delayed starvation. Mol Microbiol 11: 1029-1043.

40. Charney JC, Costante-Hammon MM, Balon EL, Boyd DH, Rubin EJ, et al. (2007) T3N-yRNA polymerase-associated proteins control virulence gene expression in Francisella tularensis. PLoS Pathog 3: e94.

41. Border C (1981) Phase separation of integral membrane proteins in Triton X-114 solution. J Biol Chem 256: 1641-1649.

42. Hazlett KRO, Seliassi T, Nguyen TQ, Cox DL, Clawson MI, et al. (2001) The TprK protein of Toponyma pallidum is periplasmic and is not a target of opsonic antibody or protective immunity. J Exp Med 195: 1015-1026.

43. Hazlett KRO, Seliassi T, Funderburke M, Bennett MP, Davisers DC, et al. (2005) TP0435, a concealed outer membrane protein of Toponyma pallidum, enhances membrane permeability. J Bacteriol 187: 6699-6698.

44. Radolf JD, Chamberlain NR, Clawson MI, Norgard MV (1989) Identification of an O-antigen polymerase (Wzy) of Francisella tularensis. Infect Immun 60: 4781-4792.

45. Kovacs-Simon A, Titball RW (2010) Lipoproteins of bacterial pathogens. Infect Immun.

46. Thakran S, Li H, Levine CL, Miller MA, Bina JE, et al. (2006) Identification of Francisella tularensis Lipid That Stimulates the Toll-like Receptor (TLR) 2/TLR1 Heterodimer. J Biol Chem 283: 3751-3760.

47. Forestal CA, Gil H, Monlett M, Noah CE, Platz GJ, et al. (2008) A conserved and immunodominant lipoprotein of Francisella tularensis is a prominalyn for but not essential for virulence in murine models. Microbiol 154: 512-522.

48. Sandstrom G, Tarnvik A, Sandstrom G (1991) The T-cell-stimulating 17-kilodalton protein of Francisella tularensis LVS is a lipoprotein. Infect Immun 59: 656-663.

49. Sjostedt A, Tarnvik A, Sandstrom G (1991) The T-cell-stimulating 17-kilodalton protein of Francisella tularensis LVS is a lipoprotein. Infect Immun 59: 656-663.

50. Russo TA, Beanan JM, Olson R, MacDonald U, Cope J (2009) Capsular polysaccharide and the O-specific antigen impede antibody binding: a potential obstacle for the successful development of an extraintestinal pathogenic Escherichia coli vaccine. Vaccine 27: 388-395.

51. Strunk RC, Eidlen DM, Mason R (1988) Pulmonary alveolar type II epithelial cells synthesize and secrete proteins of the classical and alternative complement pathways. J Clin Invest 81: 1419-1426.

52. Clay GD, Suss S, Gunn JS, Schlaifer LS (2008) Ossation of complement-mediated lysis and complement C3 deposition are regulated by Francisella tularensis lipopolysaccharide O antigen. J Immunol 181: 5568-5578.

53. Li J, Ryder C, Mandal M, Ahmed F, Azadi P, et al. (2007) Attenuation and protective efficacy of an O-antigen-deficient mutant of Francisella tularensis LVS. Microbiology 153: 3141-3153.

54. Lindemann SR, Peng K, Long ME, Hunt JR, Apicella MA, et al. (2010) F. tularensis Schu S4 mutants in O-antigen and capsular biosynthesis genes delay early cell death in macrophages. Infect Immun 78: 4479-4488.

55. Sandstrom G, Logven T, Tarnvik A (1989) A capsule-deficient mutant of Francisella tularensis LVS exhibits enhanced sensitivity to killing by serum but diminished sensitivity to killing by polymorphonuclear leukocytes. Infect Immun 56: 1194-1202.

56. Sebastian S, Dillon ST, Lynch JG, Balon E, et al. (2007) A defined O-antigen polysaccharide mutant of Francisella tularensis live vaccine strain has attenuated virulence while retaining its protective capacity. Infect Immun 75: 2561-2602.

57. Ben NA, Klempel GR (2008) Subversion of complement activation at the bacterial surface promotes serum resistance and opsonophagocytosis of Francisella tularensis. J Leukoc Biol.

58. Sahu A, Lambird JD (2001) Structure and biology of complement protein C3, a connecting link between innate and acquired immunity. Immunol Rev 180: 35-48.

59. Cole LE, Shirley BA, Barry E, Santiago A, Balabhadhuri P, et al. (2005) Toll-like receptor 2-mediated signaling requirements for Francisella tularensis live vaccine strain infection of murine macrophages. Infect Immun 73: 4127-4137.

60. Li H, Nookala S, Bina XR, Bina JE, Re F (2006) Innate immune response to Francisella is mediated by TLR2 and caspase-1 activation. J Leukoc Biol 80: 766-773.

61. Malik M, Bakshi CS, Sahu B, Shah A, Lotz SA, et al. (2006) Toll-like receptor 2 is required for control of pulmonary infection with Francisella tularensis. Infect Immun 74: 3657-3662.

62. Martinoon F, Chen X, Lee AH, Glumcher LH (2010) TLR activation of the transcription factor XBP1 regulates innate immune responses in macrophages. Nat Immunol 11: 411-418.

63. Parra MC, Shaffer SA, Hajar AM, Gallis BM, Hager A, et al. (2010) Identification, cloning, expression, and purification of Francisella lipop: an immunogenic lipoprotein. Med Microbiol Res 165: 311-345.

64. Edwards JA, Rocke-Brouwer D, Nair V, Celi J (2010) Restricted cytosolic growth of Francisella tularensis subsp. tularensis by IFN-gamma activation of macrophages. Microbiology 156: 327-339.
100. Comstock LE, Kasper DL (2006) Bacterial glycans: key mediators of diverse host immune responses. Cell 126: 847–850.

101. Schneider MC, Edey RM, Ram S, Sim RB, Tang CM (2007) Interactions between Neisseria meningitidis and the complement system. Trends Microbiol 15: 233–240.

102. Stollerman GH, Dale JB (2008) The importance of the group a streptococcus capsule in the pathogenesis of human infections: a historical perspective. Clin Infect Dis 46: 1038–1045.

103. Goleviev I, Baranov V, Krocova Z, Kovarova A, Stojchedt A (2003) An attenuated strain of the facultative intracellular bacterium Francisella tularensis can escape the phagosome of monocytic cells. Infect Immun 71: 5940–5950.

104. McManus PR, Wara WM (2000) Virulence of Francisella tularensis. J Hig (Lond) 75: 47–60.

105. Cherwonogrodzky JW, Knodel MH, Spence MR (1994) Increased encapsulation of Enterococcus faecalis by leukocytes. J Immunol 153: 93–99.

106. Bandara AB, Champion AE, Wang X, Berg G, Apicella MA, et al. (2011) Roles of capsule and lipopolysaccharide O antigen in interactions of Francisella tularensis with human immune cells in vitro and pulmonary cells in vivo. Infect Immun 78: 154–167.

107. Telepnev M, Golovliov I, Grundstrom T, Tarnvik A, Stojchedt A (2003) Francisella tularensis inhibits Toll-like receptor-mediated activation of intracellular signaling and secretion of TNF-alpha and IL-1 from murine macrophages. Cell Microbiol 5: 41–51.

108. Lin T, McNamara J, Neil P, Leake M, Moss J, et al. (2007) Francisella tularensis LVS initially activates but subsequently down-regulates intracellular signaling and cytokine secretion in mouse monocytic and human peripheral blood mononuclear cells. Microb Pathog 38: 239–247.

109. Ireland R, Olivares-Zavaleta N, Warawa JM, Gherardini FC, Jarrett C, et al. (2010) Effect of the type IV pilin, PilA, on survival of Francisella tularensis subspecies tularensis SCHU S4 in a mouse intradermal infection model. PLoS ONE 4: e5463-.

110. Chase JC, Celli J, Bosio CM (2009) Direct and indirect impairment of human dendritic cell function by virulent Francisella tularensis Schu S4. Infect Immun 77: 180–193.

111. Ireland R, Oliva Z, Cana GA, Danna-Dey H, Cordero OY, et al. (2010) Identification of genes contributing to the virulence of Francisella tularensis SCHU S4 in a mouse intradermal infection model. PLoS ONE 4: e5463.-

112. Su J, Yang J, Zhao D, Kawula TH, Banaz JA, et al. (2007) Genome-wide identification of Francisella tularensis virulence determinants. Infect Immun 75: 3089–3101.