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REVIEW

Pathogenesis and Immunobiology of Brucellosis

Review of Brucella*—Host Interactions*

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This review of *Brucella*—host interactions and immunobiology discusses recent discoveries as the basis for pathogenesis-informed rationales to prevent or treat brucellosis. *Brucella* spp., as animal pathogens, cause human brucellosis, a zoonosis that results in worldwide economic losses, human morbidity, and poverty. Although *Brucella* spp. infect humans as an incidental host, 500,000 new human infections occur annually, and no patient-friendly treatments or approved human vaccines are reported. Brucellae display strong tissue tropism for lymphoreticular and reproductive systems with an intracellular lifestyle that limits exposure to innate and adaptive immune responses, sequesters the organism from the effects of antibiotics, and drives clinical disease manifestations and pathology. Stealthy brucellae exploit strategies to establish infection, including i) evasion of intracellular destruction by restricting fusion of type IV secretion system-dependent *Brucella*-containing vacuoles with lysosomal compartments, ii) inhibition of apoptosis of infected mononuclear cells, and iii) prevention of dendritic cell maturation, antigen presentation, and activation of naive T cells, pathogenesis lessons that may be informative for other intracellular pathogens. Data sets of next-generation sequences of *Brucella* and host time-series global expression fused with proteomics and metabolomics data from *in vitro* and *in vivo* experiments now inform interactive cellular pathways and gene regulatory networks enabling full-scale systems biology analysis. The newly identified effector proteins of *Brucella* may represent targets for improved, safer brucellosis vaccines and therapeutics. *(Am J Pathol 2015, 185: 1505–1517; http://dx.doi.org/10.1016/j.ajpath.2015.03.003)*

It is noteworthy that long ago in his publication *Epidemics*, Hippocrates described brucellosis-type syndromes in humans living in the Mediterranean littoral. Many centuries later, British physician, David Bruce, and Greek physician, Themistokles Zammit, in 1886 would discover the causative agent, *Micrococcus melitensis,* of brucellosis and would identify milk products of goats as the source of infection for military troops on the island of Malta. Even after more than a century of extensive research, *Brucella* spp. are still serious animal pathogens that cause brucellosis, a zoonosis that results in substantial economic losses, human morbidity, and perpetuates poverty worldwide.[1] These Gram-negative bacteria infect a diverse array of land and aquatic mammals, including swine, cattle, goat, sheep, dogs, dolphins, whales, seals, and desert wood rats. Traditionally, the genus *Brucella* consisted of six recognized species, grouped according to their primary host preferences, that is, *B. abortus*, cattle; *B. melitensis*, sheep and goats; *B. suis*, pigs; *B. ovis*, sheep; *B. canis*, dogs; and *B. neotomae*, wood desert rats. Recent

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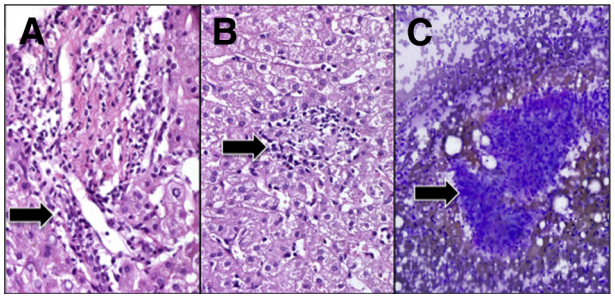

Figure 1 Hepatic and vertebral histopathology of human brucellosis caused by *Brucella melitensis*. **A:** Percutaneous liver biopsy. Mild nonspecific lymphocytic periportal hepatitis (**arrow**); stained with H&E. **B:** Percutaneous liver biopsy, culture positive for *Brucella melitensis*. Early-stage hepatic microgranuloma formation (**arrow**); stained with H&E. **C:** Guided needle core biopsy of vertebral body and epidural abscess, culture positive for *Brucella melitensis*. Lymphohistiocytic discitis osteomyelitis with dense cellular aggregates (**arrow**); stained with Diff-quik. Panels **A** and **B** are reproduced from Young et al[2] with permission from Elsevier. Panel **C** was provided by Drs. Supriya Narasimhan and Michael L. Deftos (Santa Clara Valley Medical Center, San Jose, CA). Original magnification, ×40. H&E, hematoxylin and eosin.

isolates from human (*B. inopinata*), aquatic mammals (*B. pinnipedialis* and *B. ceti*), and a common vole (*B. microti*) are recognized as new species, bringing the current number to 10 species in the genus. The basis for host preference remains an open question, although there may be a role for pseudogenes that influence host adaptation. The global disease burden in livestock is enormous. Conservative estimates are that >300 million of the 1.4 billion worldwide cattle population is infected with the pathogen. Brucellosis in animals results in abortion and other disease manifestations.

Brucella spp. infects humans as an incidental host. Human infection usually results from direct contact with tissues or blood from infected animals or by consumption of contaminated animal products, including unpasteurized milk and cheeses. In fact, >500,000 new human infections are estimated to occur annually. Brucellosis in humans typically presents with high, undulating fever. However, chronic brucellosis may affect many host organs, leading to arthritis, orchitis, hepatitis, encephalomyelitis, and endocarditis[2,3] (Figure 1). Arthritis represents the most common complication. The diverse manifestations of the disease complicate diagnosis. Brucellosis has eluded systematic attempts at eradication for more than a century, even in most developed countries, and no approved human vaccine is available. The low number of virulent organisms required for infection combined with the capacity for aerosolization renders *Brucella* spp. as category B pathogens and potential agents for bioterrorism. With an infectious dose of 10 to 100 organisms, the calculated financial risk of such an attack is second only to anthrax and tularemia. In addition, the threat of deliberate release poses a direct risk to public health in an urban population that cannot be mitigated through the normal approach of animal vaccination. Brucellosis in humans and livestock are relatively uncommon in industrialized nations because of routine screening of domestic livestock and animal vaccination. However, brucellosis is endemic in many developing regions of the globe, including the Middle East, Asia, Africa, and South America, and in the United States where foci of disease remain because of persistent infection in wildlife species. This review of *Brucella*–host interactions and *Brucella* immunobiology is intended to present recent pathogenetic discoveries as the basis for pathogenesis-informed rationales to prevent and treat brucellosis.

Host Interactions

Pathology of Brucellosis

Brucellae display strong tissue tropism and replicate within vacuoles of macrophages, dendritic cells (DCs), and placental trophoblasts. However, the pathogen has the ability to replicate in a wide variety of mammalian cell types, including microglia, fibroblasts, epithelial cells, and endothelial cells. The intracellular lifestyle of *Brucella* limits exposure to the host innate and adaptive immune responses,[4] sequesters the organism from the effects of some antibiotics, and drives the unique features of pathology in infected hosts, which is typically divided into three distinct phases: the incubation phase before clinical symptoms are evident, the acute phase during which time the pathogen invades and disseminates in host tissue, and the chronic phase that can eventually result in severe organ damage and death of the host organism. Nonspecific influenza-like symptoms observed in humans include pyrexia, diaphoresis, fatigue, anorexia, myalgia, and arthralgia. Furthermore, increasing evidence from endemic regions suggests that an elevated risk of human abortion is associated with exposure.[5] Chronic infection results from the ability of the organism to persist in the cells of the host in which brucellae are distributed by way of the lymphoreticular system to eventually cause cardiovascular, hepatic, lymphoreticular, neurologic, and osteoarticular disease (Figure 1). Measurable splenomegaly is associated with increased lymphohistiocytic cells in the spleen, slightly reduced percentage of splenic $CD4^+$ and $CD8^+$ T cells, and major increases in the percentage of splenic macrophages.

Biology of *Brucella*

Brucellae quickly translocate across the mucosal epithelium layer[6] *in vivo* and are endocytosed by mucosal macrophages and DCs. *Brucella* survive and replicate inside professional phagocytic cells, evade and modulate the host immune response, and disseminate to preferred tissues through cellular tropism, for example, placental trophoblasts in pregnant females, fetal lung, reticuloendothelial system, and reproductive tract.[7] *In vitro* studies were used as models to understand adhesion, internalization, intracellular trafficking, survival, and replication of *Brucella* in susceptible hosts. Thus, after attachment to the surface of mucosal epithelial cells, *Brucella* induces a zipper-like mechanism for internalization.[8] As yet incompletely defined binding molecule(s) are activated before and/or

on contact with epithelial cells, *Brucella* bind to epithelial cell surface receptors that contain sialic acid and sulfated residues.[9] Binding promotes activation of small GTPases that trigger a signaling cascade that reorganizes the actin cytoskeleton to induce a host cell membrane rearrangement along the surface of the pathogen that enhances invasion. Entry occurs within a few minutes after interaction which requires full activation of a mitogen-activated protein kinase signaling pathway.[8] *Brucella* survive and replicate inside nonprofessional phagocytic cells up to 72 hours *in vitro* and move across the epithelium *in vivo* by subverting the mucosal epithelial barrier function to facilitate *Brucella* transepithelial migration.[6] Simultaneously, this interaction initiates a minimal innate immune response with weak proinflammatory activity.[8,10] Once translocated through the epithelium, *Brucella* are engulfed by mucosal phagocytic cells in which $<10\%$ of phagocytized bacteria survive an adaptation period. To delay being recognized by the immune system and initiating an immune response, *Brucella* reduce, modify, or cloak their pathogen-associated molecular patterns[10]; however, some Toll-like receptors (TLRs; mainly TLR2, TLR4, and TLR9) initiate limited intracellular signaling that activates the transcription factor NF-κB to control expression of inflammatory cytokine genes,[11] although at a level that is 10-fold less than enterobacteria.

Inside mononuclear phagocytic cells, *Brucella* reside in a special vacuole (*Brucella*-containing vacuole, BCV), modify intracellular trafficking, and transform the vacuole into a replicative compartment or *brucellosome*.[12] Experimental evidence indicates that the microenvironment inside the BCV is one of limited nutrient availability[12] to which *Brucella* adapts soon after invasion. Initially, the pathogen undergoes quantitatively reduced gene expression and protein synthesis involved in anabolic metabolism while increasing amino acid catabolism, switching to alternative energy sources, and altering respiration to adapt to low oxygen tension.[13] In an *in vitro* brucellosis infection model, expression of a type IV secretion system (T4SS) early after infection is essential for intracellular survival and multiplication inside mammalian cells. Yet, *in vivo* studies found that the T4SS is not necessary for invasion, systemic dissemination, or establishment of initial infection, but it is essential for prolonged persistence[14] (C.A. Rossetti, K.L. Drake, S.D. Lawhon, J. Nunes, T. Gull, S. Khare, L.G. Adams, unpublished data) in which expression of T4SS stimulates an inflammatory reaction that was proposed as a mechanism to recruit cells that contribute to persistence.

Over the course of infection, invading brucellae surviving the adaptation period gradually recover the expression of key metabolic process-encoded genes. Transporters, iron metabolism, and cell membranes are primary targets for this transcription–translation reactivation.[13] *Brucella* initiate replication concurrently with the resumption of expression of necessary functions, including virulence genes that in some cases are also tightly regulated by quorum-sensing molecules.[15,16] Infected mononuclear phagocytic cells trigger extensive transcriptional changes in response to infection during the adaptation stage and return to normal

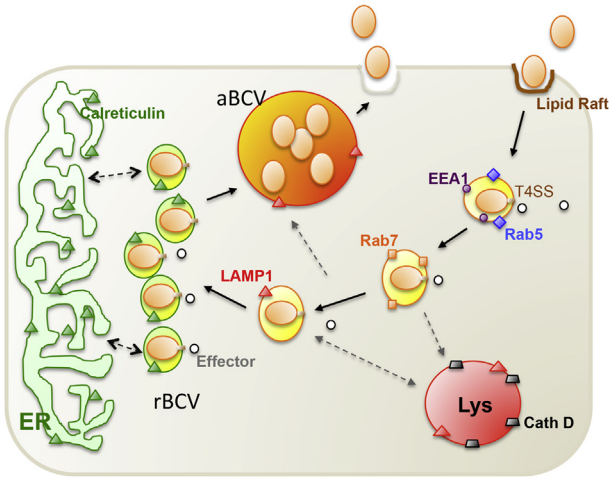

Figure 2 Working model of *Brucella* intracellular trafficking in macrophage cells. Plasma membrane-associated lipid rafts mediate the internalization of smooth *Brucella* into macrophage cells. As the BCV matures, it sequentially associates with markers for early (EEA1, **purple circle**; Rab5, **blue diamond**) and late (Rab7, **orange square**) endosomes. The biogenesis and trafficking of BCVs is regulated by bacterial effector proteins (**white circles**), which are secreted through the *Brucella* T4SS. BCVs that contain virulent organisms do not fuse with lysosomes (cathepsin D, **gray trapezoid**), although transient association with LAMP1-positive membranes (**orange triangles**) is observed. The pathogen replicates in tight rBCVs that are decorated with calreticulin (**green triangle**), a marker for the ER. At a later point after infection (48 to 72 hours), the pathogen is observed in LAMP1-positive aBCVs that also contain LAMP1. The biogenesis of aBCVs depends on the activities of a subset of autophagy proteins, including ULK1 (not shown) and Beclin1 (not shown). Finally, the pathogen is released from the cell through lytic or nonlytic (shown) mechanisms. aBCV, autophagic *Brucella*-containing vacuole; BCV, *Brucella*-containing vacuole; Beclin1, coiled-coil myosin-like BCL2-interacting protein; EEA1, early endosome antigen 1; ER, endoplasmic reticulum; LAMP1, lysosome-associated membrane protein 1; rBCV, replicative *Brucella*-containing vacuole; T4SS, type IV secretion system; ULK1, Unc-51-like kinase 1.

levels after 12 hours, a time corresponding to the initiation of *Brucella* replication. Among the early transcription changes that contribute to adaptation, *Brucella* has several clever strategies to establish and maintain a chronic infection, including inhibition of apoptosis of infected mononuclear cells, prevention of DC maturation, reduced antigen presentation, and reduced activation of naive T cells.[17] Once adapted to the intramacrophage environment, *Brucella* extends its intracellular persistence indefinitely, which contributes to systemic metastasis and infection of preferred targeted cells or tissues, such as placental trophoblasts, fetal lung, male genitalia, skeletal tissues, reticuloendothelial system, and endothelium. Currently, there is minimal information available to describe the interaction of *Brucella* with these target cells and tissue[18,19] to provide a more holistic systems biology analysis of the pathogenesis of brucellosis at the level of the whole host organism.

Intracellular Trafficking

Brucellae evade intracellular destruction by restricting fusion of the BCV with the lysosomal compartment. Some

Table 1 Pathogenesis-related *Brucella* spp.—host molecular interacting elements

Brucella element	Host factor	Reference
Adhesion and internalization		
SP41	Sialic acid residues	9
Hsp60	PrP^c	26
LPS	Scavenger receptor SR-A	27
Intracellular trafficking		
RicA	GTPase Rab2	25
Intracellular survival		
Cyclic B1,2-glycan	Cholesterol	28
Evasion of immunity		
BtpA/Btp1/TcpB	MyD88	29,30
	MAL	
BtpB	MyD88	24
Proinflammatory reaction		
VceC	BiP/Grp78	31

Hsp60, heat shock protein 60; LPS, lipopolysaccharide; MAL, MyD88-adaptor like; MyD88, myeloid differentiation response gene 88; PrP^c, cellular prion protein; SP41, 41-kDa surface protein.

BCVs that harbor internalized *Brucella* traffic from endocytic compartments to a replicative niche within BCVs that contain markers of the endoplasmic reticulum (ER). BCV seizure of ER membranes and components is accompanied by structural characteristics and functional restructuring of the ER.[20] BCVs later accumulate autophagic features and exhibit lysosome-associated membrane protein-1 positivity, constituting a distinctive aspect of the intracellular *Brucella* lifestyle (Figure 2). The VirB T4SS regulates *Brucella* intracellular trafficking,[21,22] and organisms that lack this system fail to establish an intracellular replicative niche *in vitro*. The T4SS of *Brucella* is thought to secrete effector molecules that control the intracellular and stealthy lifestyle of the pathogen.[23–25] Table 1 summarizes several of the established interactive host and pathogen elements in the pathogenesis of brucellosis.

Two-Component Regulatory Systems

Two-component regulatory systems also play an important role in the stealth program. Among the best studied are the BvrR/BvrS two-component regulatory system and the LuxR-like transcriptional regulator VjbR.[16,32] The absence of the BvrR/BvrS sensory-regulatory system results in major changes in the bacterial outer membrane that alters cellular uptake of the organism.[33] The BvrR/BvrS regulon also includes carbon and nitrogen metabolic functions and the expression of additional transcriptional regulators among 127 differentially regulated genes.[32] In the absence of a functioning BvrR/BvrS, the organism fails to replicate intracellularly and is avirulent in the mouse model. Among the genes regulated by BvrR/BvrS, there are 10 transcriptional regulators, including *vjbR*. The protein-encoded *vjbR* was shown to regulate expression of the VirB locus that encodes the T4SS necessary to prevent phagosome—lysosome fusion.[21] The absence of VirB alone may explain the attenuated virulence of *bvrR/bvrS* mutants.

Identification of T4SS-Secreted Substrates

Identification of *Brucella* virulence factors that facilitate invasion and infection was restricted to the surface O-polysaccharide until the late 1990s.[34] At that time, several research groups used multiple approaches to genetically inactivate target genes. These reductionist approaches led to the conclusion that *Brucella* persists in nutrient-poor BCVs within the host,[35] in which the organism replicates and from which infection spreads with minimal activation of the host cell.[36] In contrast to the numerous metabolic functions shown to be necessary for intracellular replication, the T4SS stood out as a notable target for further investigation of virulence potential.[37,38] Yet despite a decade of research, the complete mechanism of action remains undefined. Although shown to be required to prevent trafficking to the lysosome, the mechanistic steps involved, including interacting partners, enzymatic reactions, protein modifications, and detailed intracellular trafficking, are only now being described after a series of experiments in which *Brucella* gene reporter fusions were found to be secreted in a T4SS-dependent manner.[23,25,39]

Putative effector candidates were identified *in silico* on the basis of several criteria, including shared features with effectors expressed by other bacteria, eukaryotic motifs, GC content, and limited distribution across bacterial genera. Candidate effectors were shown to target secretory pathway compartments when expressed ectopically and impaired host protein secretion.[31,39] Genetic studies have found a redundancy of function among effector candidates, consistent with the failure to identify effectors by using early genetic screens. Although several factors are now identified, the list is far from complete, and the mechanism by which replication is enhanced and lysosomal fusion is prevented remains undefined. Effector enzymatic activity, protein—protein interactions, and the identity of effector targets all remain to be identified.

Central Role for the Host UPR

The unfolded protein response (UPR) is an evolutionarily conserved pathway that mediates cellular adaptation to protein-folding stress in the ER. The stress sensors activating transcription factor 6, protein kinase RNA-like ER kinase, and inositol-requiring enzyme 1 α (IRE1α), which are located in the ER membrane, trigger UPR when unfolded proteins accumulate in the lumen of the ER. Activating transcription factor 6 regulates the expression of chaperones that facilitate protein folding. Protein kinase RNA-like ER kinase mediates transient translational attenuation and promotes apoptosis in unresolved stress. IRE1α plays a central role in initiating the UPR by catalyzing the splicing of X box binding protein I (XBP1) mRNA when

unfolded proteins accumulate in the ER. The spliced message is then translated to generate active XBP1 transcription factor.[40] XBP1 controls the expression of UPR genes that encode ER chaperones, proteins involved in ER-associated degradation, and other proteins that mitigate the harmful consequences of unfolded protein accumulation.[41]

Subversion of the UPR is critical to the intracellular lifestyle of *Brucella*.[22,42] Murine embryonic fibroblasts that harbor deletions in IRE1α were used to demonstrate that this protein supports intracellular replication of *Brucella in vitro*. Host phosphatidylinositol 3-kinase (PI3K) activity was confirmed to mediate *Brucella* uptake; however, replication after uptake was unaffected.[42] Celli and colleagues[43] showed that *Brucella* replication occurs in an ER-derived replicative BCVs preceding the acquisition of autophagic markers. The gradual appearance of autophagic BCVs involves the acquisition of several autophagy proteins, including Unc-51-like kinase 1, Beclin 1. The formation of autophagic BCVs is independent of autophagy-elongation proteins, including ATG5 and LC3b,[43] and gives rise to lysosome-associated membrane protein-1–positive, calreticulin-negative, pathogen-containing compartments. Importantly, autophagic BCVs were shown to play an important role in cell-to-cell spread of the pathogen.[43] Finally, *Brucella* infection induces XBP1 splicing and UPR gene expression in host macrophages, both *in vitro* and *in vivo*.[20] Moreover, treatment of host cells with tauroursodeoxycholic acid, a pharmacologic chaperone that ameliorates the UPR, impairs *Brucella* replication.[20] Taken together, these data suggest that *Brucella* subverts host IRE1α signaling cascades to secure an intracellular niche that supports nutrient acquisition, pathogen replication, or pathogen cell-to-cell spread.

Host PI3K Activity and *Brucella* TIRAP-Containing Proteins

The role of PI3K in *Brucella* uptake is especially intriguing. Instrumental in the formation of phosphatidylinositol 3,4,5 trisphosphate, PI3K promotes binding of Toll–IL-1 receptor (TIR)-containing proteins on the plasma membrane and subsequent TLR signaling. *Brucella* prevents this by expressing at least two TIR-containing proteins to ultimately restrict the proinflammatory response and DC maturation.[20,29,30,44,45] Details of the mechanism remain to be established, but evidence suggests that the presence of TIR-containing protein TcpB or BtpA and/or BtpB compete with myeloid differentiation response gene 88 (MyD88) for TIR domain-containing adaptor protein (TIRAP) binding which results in enhanced degradation of TIRAP by and interference with TLR4/TLR2 signaling.[30] The presence of phosphoinositol phosphate binding sites in TcpB/BtpA and BtpB is consistent with binding at the plasma membrane and inhibition of downstream events that prevent activation of NF-κB–mediated transcription and development of an effective proinflammatory response.

Systems Biology and Omics Analysis of *Brucella* and Hosts

The expansion of genomics, next-generation sequencing, and omics technologies has enabled in-depth analysis of the pathogenesis of brucellosis. Large-scale simultaneous *Brucella* and host global expression data sets can now be combined with proteomics and metabolomics data from *in vitro* and *in vivo* experiments in target species and nonhuman primates to generate cellular pathway and gene regulatory networks[46–48] that enable full-scale systems biology analysis[49] and improved whole system understanding of *Brucella* pathogenesis. Around the turn of the century, the community of *Brucella* investigators generated genomes and data sets.[7,50,51] *Brucella melitensis* strain 16M was the first *Brucella* genome sequenced and published.[52] More than 18 complete and 415 whole genome shotgun *Brucella* genomes were sequenced, published in peer-reviewed journals, and are available online for analysis (Broad Institute, *http://www.broadinstitute.org/annotation/genome/brucella_group/Downloads.html*; Pathosystems Resource Integration Center, *http://patricbrc.org/portal/portal/patric/Taxon?cType=taxon&cId=234*; and National Center for Biotechnology Information, *http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=224914*; all last accessed February 10, 2015). These data provide baselines for the analysis of comparative gene structure and homologies, conservation and variability, and gene expression, regulatory networks, and protein synthesis, interaction, and metabolic pathways. DNA sequence availability of several genomes of host species, including domestic animals and humans, together with experimental omics technologies and software tools for data analysis will increasingly facilitate construction of *in silico* interactome models of the infection biology of *Brucella*–host relations.

In vitro and *in vivo Brucella* and/or host gene expression and proteome data sets were generated by several investigators during the past decade, progressing toward a more comprehensive analysis of the host and pathogen.[6,8,13,16,18,32,53–60] This broad spectrum of data sets was analyzed to identify candidate genes and biomarkers of *Brucella* and hosts,[61] to analyze[62] and predict *Brucella* antigenic proteins, to identify subunit vaccines,[63] to understand gene regulatory networks,[54] and to characterize the stringent *Brucella* stress response[64] and modulation of host responses.[53]

Time-series studies of *in vitro* or *in vivo* experimental infection data were performed with a systems-based perspective of complex biological organizations to categorize the interactions of individual proteins within *Brucella*–host protein–protein networks. Time-series studies of *in vivo* system have

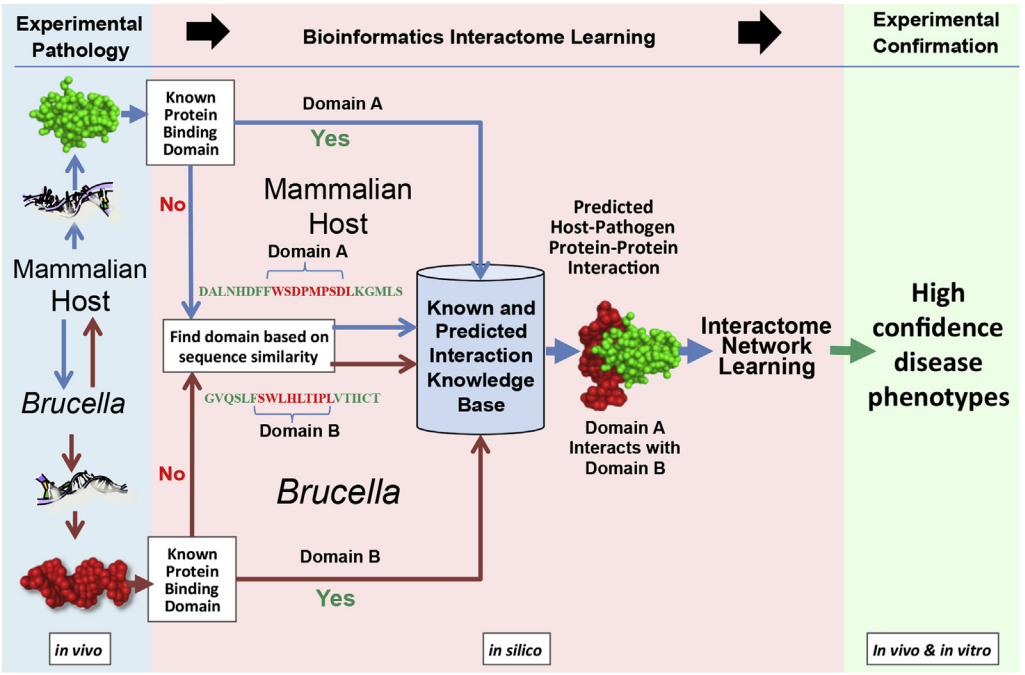

Figure 3 Scheme of *in vivo* and/or *in vitro* systems biology analysis of host and *Brucella* interactive pathogenesis. Experimental pathology includes the collection and omics (transcriptomics, proteomics, metabolomics, etc.) data from both mammalian host and *Brucella* samples from an *in vivo* time series of *Brucella* infection of a target organ (eg, Peyer patch, lung, spleen, liver) in a natural target animal (eg, cattle, sheep, goat, pig, nonhuman primate). The resulting omics (eg, transcriptomics, proteomics, metabolomics) data sets are fused and bioinformatically analyzed for known and computed structural modeling of predicted host–pathogen protein–protein interactions to develop an *in silico* interactome structure learning model. Proteins are inferred from genes if not directly measured. Pairs of predicted candidate *Brucella*–host protein–protein mechanistic genes from interactive pathways are phenotyped *in vitro* in standardized gentamicin killing assays by using specific deletion mutants of *Brucella*, siRNA knockdown of host genes, and confocal microscopy. *Brucella*–host protein–protein pairs with positive *in vitro* phenotypes are phenotyped *in vivo*, and high confidence positive candidate protein pairs undergo pull-down analysis and quantitative selected reaction monitoring mass spectrometry for further confirmation of *Brucella*–host protein–protein interactions in *Brucella* pathogenesis. **Blue lines** and **arrows** indicate flow of *in vivo* data and results from the host, and **brown lines** and **arrows** indicate flow of data and results from *Brucella* into *in silico* analysis to identify and model domain A from a host protein predicted to interact with domain B of *Brucella*.

served as a source of *Brucella* gene expression data interacted computationally with bovine host gene expression data to identify mechanistic genes of interacting cellular pathways as novel biosignatures and potential druggable targets on the basis of predicted protein–protein structural homologies (Figure 3).[61,65] In another time-series study, results of systems biology analysis of *Brucella* and bovine host gene expression data[61,65] were combined with reverse vaccinology[62] to identify effective candidate subunit vaccines protective against virulent challenge in the mouse model.[66] These studies provide provocative examples in support of using systems biology to more effectively integrate and exploit data for model development, for causal discovery, for the prediction of biological activities, for improving the design of *in vitro* and *in vivo* experiments, for finding biomarkers for enhanced brucellosis diagnosis, and for druggable targets for more effective treatment of brucellosis.

Immunobiology of Brucellosis

Central Role for Immunoregulatory Components

The stealthy nature of *Brucella* was attributed in large part to the structure of the smooth lipopolysaccharide (LPS) on the cell surface.[10] The presence of elongated fatty acid molecules on the lipid A portion was shown to reduce the toxicity of *Brucella* LPS and to reduce the immune response by serving as a poor TLR4 agonist, consistent with the capacity of the organism to invade with minimal activation of the host cell. However, rough brucellae, lacking the O-polysaccharide portion of the LPS, are cytotoxic to macrophage cells. Although a comparative analysis of the lipid A from smooth and rough organisms has not been performed, there is no scientific reason to assume that the length of the fatty acids will be altered. Furthermore, the complete eradication of cytotoxic activity in the absence of the T4SS argues against a role for rough LPS in the cytotoxic activity. The simplest explanation at the current time is that *Brucella* LPS is a weak TLR4 agonist, whereas the O-polysaccharide is instrumental to the stealthy behavior of the organism. As an inducer of PI3K activity, weak TLR4 activation may be expected to reduce uptake as observed with smooth *Brucella*, whereas enhanced uptake observed with rough *Brucella* is consistent with an enhanced PI3K activity and a reduction in stealth.[42]

In addition to the weak agonist activity of *Brucella* LPS, the organism expresses novel immunoregulatory

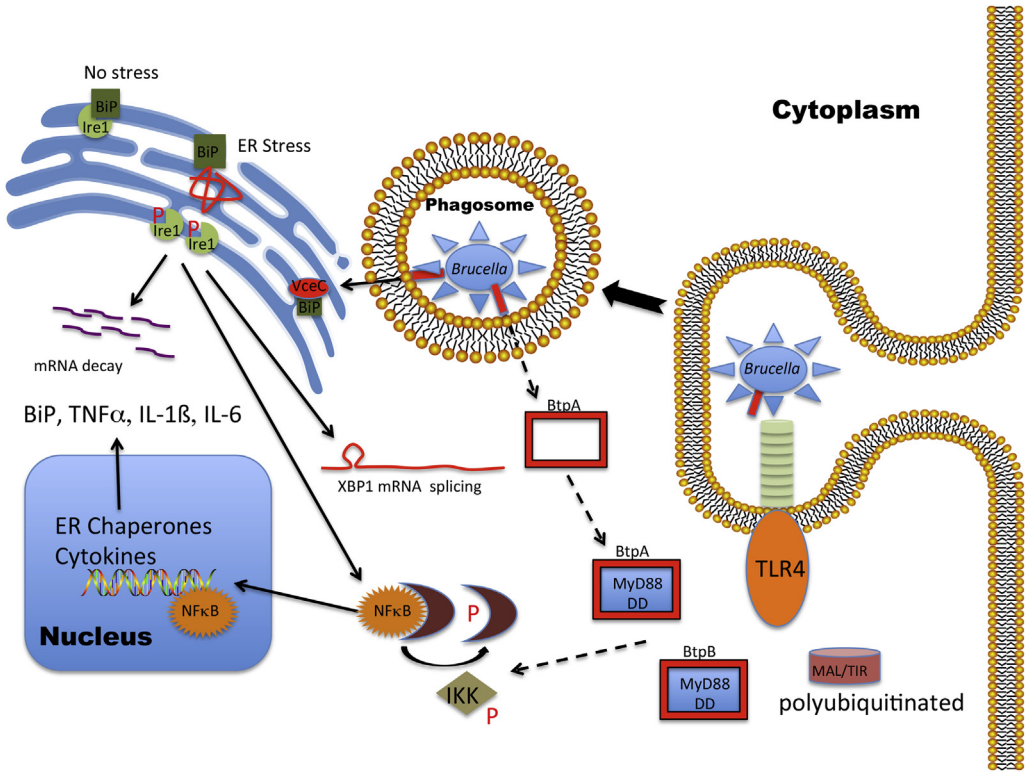

Figure 4 *Brucella* is bipolar. *Brucella* both inhibits and promotes a proinflammatory immune response. TLR4 signaling during infection is restricted by the presence of elongated fatty acid chains that reduce the toxicity of the LPS (**blue studs** on *Brucella* surface) and by blocking downstream IKK phosphorylation via MyD88 binding (**blue squares**) with *Brucella* TIR-containing proteins, BtpA and BtpB (**red squares**), leading to enhanced polyubiquitination and degradation of MAL. However, the T4SS (VjbR-controlled expression) effector VceC (**red oval**) stimulates an innate immune response via interaction with BiP, an ER molecular chaperone (**green squares**) to release and phosphorylate IRE1 to promote mRNA splicing of XBP1 and activation of UPR. IRE1 phosphorylation also promotes the proinflammatory response via the release of NF-κB from the complex with IκBα (**maroon crescent**). The critical distinction between these two pathways may reside in the timing of activation. Inhibiting the host early or MyD88-mediated response may promote acquisition of a replicative niche, whereas the delayed T4SS-mediated VceC effector (VjbR-controlled expression) response may enhance the spread of the organism. **Solid arrows** indicate *Brucella*-mediated activation, whereas **dotted arrows** indicate *Brucella*-mediated inhibition. ER, endoplasmic reticulum; IκBα, inhibitor of κB protein α; Iκκ, IκB kinase; IRE1, inositol-requiring enzyme 1; LPS, lipopolysaccharide; MAL, MyD88-adaptor like; MYd88, myeloid differentiation response gene 88; T4SS, type IV secretion system; TIRAP, Toll—IL-1 receptor domain-containing adaptor protein; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor α; UPR, unfolded protein response; XBP1, X-box binding protein 1.

factors that suppress the innate immune response (Figure 4). The mechanism has been elaborated for some of these proteins, such as the TIR-containing protein or *Brucella* TIR protein, TcpB/BtpA.[20] Similar to TLRs, TcpB contains a TIR domain through which it interacts with cytoplasmic MyD88 adaptor like/TIRAP. One study concluded that TcpB binding prevents MyD88 binding to MyD88 adaptor like/TIRAP, thereby accelerating MyD88 adaptor like/TIRAP degradation and subverting TLR signaling and proinflammatory cytokine production.[30,45] In another study, TcpB was shown to interact with MyD88 to prevent downstream interactions and short-circuiting TLR-mediated activation.[29] Although Δ*tcp*B knockout mutants exhibit minimal change in survival in the wild-type mouse model, replication in macrophages is reduced at late time points (≥48 hours) after infection.[20] In addition, current evidence suggests that the absence of *tcpB* expression results in an elevated state of immune activation that reduces overall organism survival. One implication of this hypothesis is that TcpB may act through protein kinase B to relieve tuberous sclerosis complex 1/2-mediated inhibition of target of rapamycin activity to ultimately suppress the NF-κB—mediated proinflammatory response and induce IL-10 production. To do so, TcpB must enhance the production or stability of phosphatidylinositol-3,4,5 triphosphate, which would deplete the levels of phosphatidylinositol 4,5-bisphosphate and diminish plasma membrane interactions to support TLR signaling.

Recently, a second protein BtpB, containing TIR structural domains, was reported to interfere with TLR signaling through MyD88 to prevent DC maturation.[24] Redundancy of TcpB/BtpA and BtpB function may explain the failure to identify these immunoregulatory genes by using simple transposon screens. Interaction between BtpA/BtpB and MyD88 may also explain the substantial increase in survival of *Brucella* in MyD88$^{-/-}$ knockout mice in contrast to TLR4$^{-/-}$/TLR2$^{-/-}$ knockout mice in which no substantial increase over wild-type was observed.[67] The absence of MyD88 may adversely affect the proinflammatory response

that results from a reduction of both ER stress and TLR-induced innate immune responses.[31]

Protective Immunity against Stealthy *Brucella*

Knowledge of protection against infection is derived from a number of disparate animal models, including mice, guinea pigs, ruminants, nonhuman primates, and humans. The importance of a T helper cell type 1 (Th1) response against *Brucella* is supported by numerous studies and is summarized elsewhere,[51,68,69] particularly the roles of $CD4^+$ and $CD8^+$ T cells, although these results were contradictory at times. Natural killer cells play an important role in some hosts, but their role was shown to be minimal in the mouse model.[70] Passive transfer experiments suggest that antibody to LPS (O-polysaccharide) may contribute to protection,[71–75] yet the effectiveness of the T helper cell type 2 (Th2) humoral immune response remains unclear,[76] and the efficacy of rough *Brucella* vaccines contradicts the role of anti-LPS antibodies in protective immunity.

Cytokines are key players in protection against brucellosis, mediating both innate and adaptive immune responses. IL-12 produced by B cells and macrophages leads to a Th1 response and induction of interferon-γ, which activates macrophages. The activity of is interferon-γ is maximized by tumor necrosis factor-α produced by macrophages and natural killer cells. Reports also indicate that IL-1–dependent induction of colony-stimulating factor increases neutrophil and macrophage infiltration into the spleen.[77] This phenomenon may also explain a role for IL-6 produced by T cells. Splenocytes of infected hosts express higher levels of mRNA for IL-2, interferon-γ, and IL-10 and reduced levels of mRNA for IL-4, consistent with a Th1 response.[78,79] Increased IL-10 observed later in infection may support the capacity of *Brucella* to avoid immune surveillance, resulting from repression of a protective Th1 response.[80] Interestingly, cellular and humoral immune responses against similar *Brucella* strains vary significantly among susceptible hosts. This confounding aspect of *Brucella* immunobiology has presented important challenges in the identification of reliable correlates of immune protection in tractable model animal systems.

Resistance to other innate immune system components (eg, complement, opsonins, phagocytic cells, innate lymphocytes, cytokines, and other barriers) was in most cases suspected of being inherent to *Brucella* and provides passive resistance to intracellular killing mechanisms. However, on the basis of the importance of the T4SS to the long-term success of infection, it is becoming clearer that resistance mechanisms alone are not sufficient for the success of infection. Brucellae, and other intracellular pathogens, alter the innate immune response with the immediate aim of establishing a replicative niche and long-term persistence. To restrict long-term protective immunity, the organism first avoids the innate immune response by stealthy entry into host cells. From there, the organism controls aspects of protein secretion, intracellular trafficking, and bacterial replication,[31,39] ultimately altering the course of the innate and adaptive immune responses.[81]

Failure of long-term protection against *Brucella* infection is the result of a weakened adaptive immune response controlled in part by the attenuated innate immune response.[71,82–84] As a stealth invader, *Brucella* enters the host cell without apparent activation of the innate immune response through TLR ligand interaction. Knockout mice and animals deficient in either or both TLR2 and TLR4 exhibit little relevant change in the ability to control infection. In contrast, cells deficient in MyD88 sustain a two-log increase in bacterial infection.[85] This finding may be best explained by a redundancy in host functions. However, it may also reflect that the primary goal is prevention of long-term adaptive immune response rather than rescue at early stages of infection. Evasion of the host-induced innate immune response may allow the organism to gain a foothold, whereas stimulation at later times aids the spread of infection. Manipulation of the innate immune response was found for at least three factors TcpB/BtpA, BtpB, and VceC (Figure 4). Although numerous other effectors were identified,[23–25,31,39] their contribution to pathogen survival remains to be demonstrated. However, it seems clear that the wild-type *Brucella* has at its disposal a complete battery of effectors and that any delay in the innate immune response induced by these proteins could potentially be manipulated so as to improve the potential for more protective and safer vaccines.

Pathogenesis-Informed Approaches to Therapeutics

Human *Brucella* infection is typically treated with combination antibiotic therapy. But therapeutic relief from infection is neither certain nor rapid, because the intracellular location reduces antibiotic efficacy. Treatment can be prolonged and associated with undesirable side effects. Moreover, relapse after antibiotic therapy constitutes a substantial risk to the treated patient. As such, the recommended treatment usually entails a combination of drugs for at least 30 days,[86] each of which falls into Food and Drug Administration pregnancy categories C (rifampin) or D (tetracycline/doxycycline) that are poor to unsuitable for use in gravid women.

To address these issues, development of novel therapeutic strategies to address brucellosis has been pursued. Recent advances in our understanding of the molecular mechanisms that mediate interactions between *Brucella* and host cells have facilitated this pursuit. For example, Baron et al[87] have spearheaded the development of novel antivirulence compounds that target virulence functions while leaving essential cell functions unharmed. One advantage of the antivirulence approach is that molecules that do not target essential

functions will fail to induce selective pressure for antibiotic resistance. To identify novel compounds that inhibit the virulence of *Brucella*, a high throughput small molecule screen was performed for compounds that inactivated the function of VirB8,[88] a central component of the bacterial T4SS, which is essential for virulence and pathogenicity of *Brucella,*[89] and a wide variety of other animal and plant pathogens. This endeavor identified several potent and specific inhibitors of T4SS function. X-ray crystallography and docking studies were used to demonstrate that VirB8 inhibitors bind to a surface groove opposite to the dimerization interface,[88,90] thereby providing insight into the mechanism of action of this potential therapeutic.

Pathogenesis-Informed Approaches to Vaccines

Nonviable *Brucella* vaccines historically have a poor record of success that started with heat-killed *Brucella*, crude extracts, and then subunit and DNA vaccines. This poor track record is primarily explained by a failure to induce an effective Th1 response comparable with live, attenuated vaccines (LAVs).[91] In contrast, LAVs have a long history of successful use against brucellosis and other intracellular pathogens in laboratory animal models and in target species, such as ruminants. However, given the safety concerns associated with human use, including the threat of persistent infection or reversion to virulence extreme, caution must be used in LAV development.

Prevention of animal disease was found to provide substantial protection against human disease. Historically, the focus of immune protection against *Brucella* infection in animals has been the use of spontaneously attenuated strains.[92,93] Their stable and effective use in animals over decades was used to justify support for human trials.[94] Three vaccines are used extensively in animals to provide immune protection: *B. abortus* S19 and RB51 and *B. melitensis* Rev.1.[95–97] S19 and Rev.1 are fortuitously attenuated isolates. One strain was obtained accidentally, and the second was obtained after a stepwise process to identify streptomycin-dependent and then streptomycin-independent isolates. The two strains are considered to be smooth (expressing intact LPS with O-polysaccharide) which distinguishes them from RB51, a rough strain lacking the O-polysaccharide. S19 and Rev.1 provide superior protection but exhibit substantial human virulence and cannot be used in gravid female animals. RB51 is considered to be safe for use in gravid females and does not induce O-polysaccharide antibodies that can be used to distinguish field strain-infected from vaccinated animals. Despite well-defined differences in immune potential among these vaccines, no marker or correlate has been identified that can be used to predict immune protection.

Attenuated virulence may be derived from simple point mutations or from genetic rearrangements, including gene deletion. Although the potential for reversion of a mutant that bears a complete gene deletion is already small, the potential for reversion to virulence can be made infinitesimal by the introduction of a secondary mutation. Optimally, the additional mutation would affect the same pathway as the primary mutation, serving only as a backup to prevent reversion, but inducing no additional reduction in virulence or negative effect on protective immunity.

Obviously, care must be taken so that a balance is struck that supports survival sufficiently to enhance immune protection without posing a risk of inducing disease. One approach to this task is encapsulation of the attenuated vaccine strain to release the organism over time and to provide the added advantage of providing a natural booster response.[68,98] This approach uses a vaccine depot from which the attenuated *Brucella* is gradually released over a 30-day period and significantly enhances immune protection by using highly attenuated LAVs to improve both efficacy and safety.[99] In addition, recent cell biology findings have revealed the dependence of *Brucella* infection on the UPR, specifically IRE1α.[20,42] This dependence may be exploited in an effort to provide LAVs that provide enhanced immune protection. ER stress and TLR signaling provide a synergistic stimulation of the proinflammatory response.[100] The key to an optimal LAV development strategy is to identify vaccine candidates that fail to restrict the innate immune response and as a result induce an effective adaptive immune response without safety or reversion concerns.

Perspectives

Although *Brucella* spp. infect humans as an incidental host, 500,000 new human infections occur annually, yet no human vaccine or patient-friendly treatments exist. In addition, the threat of deliberate release poses a direct risk to public health that cannot be mitigated through the usual approach of animal vaccination to protect the public. Nonspecific influenza-like symptoms observed in humans include pyrexia, diaphoresis, fatigue, anorexia, myalgia, and arthralgia. Chronic infection results from the ability of the organism to persist in the cells of the host in which *Brucella* are distributed through the lymphoreticular system to eventually cause cardiovascular, hepatic, lymphoreticular, neurologic, and osteoarticular disease.

Brucellae quickly translocate across the mucosal epithelium layer and are endocytosed by mucosal macrophages and DCs. Brucellae display strong tissue tropism for the lymphoreticular system and an intracellular lifestyle that limits exposure to the host innate and adaptive immune responses, sequesters the organism from the effects of antibiotics, and drives the unique features of the clinical disease manifestations and pathology. *Brucella* uses several clever strategies to establish and maintain infection, such as evading intracellular destruction by restricting fusion of the type IV secretion system-dependent BCVs

with the lysosome compartment, inhibition of apoptosis of infected mononuclear cells, prevention of DC maturation, inhibition of antigen presentation, and activation of naive T cells. Once adapted to the intramacrophage residence, *Brucella* prolongs its intracellular lifestyle indefinitely yet may systemically metastasize to infect other preferred targeted cells or tissues, such as placental trophoblasts, fetal lung, male genitalia, skeletal tissues, reticuloendothelial system, and endothelium. The mechanistic steps involved in interacting partners, enzymatic reactions, protein modifications, and detailed process of inhibiting intracellular trafficking to the lysosome are only now being described. Subversion of the host UPR proteins was found to be critical to the intracellular lifestyle of *Brucella*.[101] Recent data suggest that *Brucella* subverts host IRE1α signaling cascades to secure an intracellular niche that supports nutrient acquisition, pathogen replication, or pathogen cell-to-cell spread.

By strategically converging on the entire range or holistic systems biology with traditional reductionist approaches, remarkable progress is being made toward improved design of brucellosis vaccines, enhanced biomarker diagnostics, and identifying specific druggable targets while avoiding the threat of antimicrobial resistance. Current reductionist approaches have generated extensive information about pathogen and host genes and pathways, yet clearer understanding of the interactions of these genes at the level of the whole host system has yet to emerge.

Despite well-defined differences in immune potential among existing vaccines, no marker or correlate has been identified that can be used to predict immune protection in livestock. LAVs have a long history of successful use against brucellosis and other intracellular pathogens in laboratory animal models and in target species. The key to an optimal LAV development strategy for humans is to identify vaccine candidates that fail to restrict the innate immune response and as a result induce an effective adaptive immune response without safety or reversion concerns. *Brucella* has a complete battery of effector proteins that delay the innate immune response which could be favorably manipulated to provide safer, more protective brucellosis vaccines and therapeutics.

Summary

After more than a century of research human and animal brucellosis still remains an important challenge to health and well-being. Moreover, no currently available safe, efficacious vaccines or patient-friendly treatments are available for human brucellosis, a disease that also needs low-cost bedside differential diagnostics. However, with the emergence of clearer understandings of the pathology and molecular pathogenesis of brucellosis, opportunities are substantially enhanced to identify and evaluate new druggable therapeutic targets and to apply reverse vaccinology and other postgenomic tools to develop safe, effective subunit or human LAVs.

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