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The intracellular lifecycle of *Brucella* spp.

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Abstract

Bacteria of the genus *Brucella* colonize a wide variety of mammalian hosts, in which their infectious cycle and ability to cause disease predominantly relies on an intracellular lifestyle within phagocytes. Upon entry into host cells, *Brucella* undergo a complex, multistage intracellular cycle in which they sequentially traffic through, and exploit functions of, the endocytic, secretory and autophagic compartments, via Type IV secretion (T4SS)-mediated delivery of bacterial effectors. These effectors modulate an array of host functions and machineries to first promote conversion of the initial endosome-like *Brucella*-containing vacuole (eBCV) into a replication-permissive organelle derived from the host endoplasmic reticulum (rBCV), then to an autophagy-related vacuole (aBCV) that mediates bacterial egress. Here we detail and discuss our current knowledge of cellular and molecular events of the *Brucella* intracellular cycle. We discuss the importance of the endosomal stage in determining T4SS competency, the roles of autophagy in rBCV biogenesis and aBCV formation, and T4SS-driven mechanisms of modulation of host secretory traffic in rBCV biogenesis and bacterial egress.

THE *BRUCELLA* INTRACELLULAR CYCLE

Brucella Pathogenesis

Bacteria of the genus *Brucella* belong to the α2-proteobacteria, a phylogenetic subgroup which includes a variety of bacteria that are either animal or plant pathogens or symbionts. As such, these bacteria have experienced a long-standing co-evolution with eukaryotic hosts that has likely shaped their biology. The genus *Brucella* is composed of an increasing number of species that infect a wide variety of mammals as primary hosts, such as bovine (*B. abortus*), caprine (*B. melitensis*), swine (*B. suis*), ovine (*B. ovis*), camels, elk, bison (*B. abortus*), canine (*B.* canis), rodents (*B. neotomae*, *B. microti*), monkeys (*B. papionis*), as well as marine mammals such as seals, purpoises, dolphins and whales (*B. pinnipidialis* and *B. ceti*), and also amphibians (*B. inopinata*) (1). Most species cause in their hosts a disease named brucellosis, which manifests as abortion, sterility and lameness in animals, and which can also be transmitted to humans via inhalation of aerosolized bacteria, ingestion of, or contact with, contaminated tissues or derived products, classically by the most pathogenic species *B. melitensis*, *B. suis* and *B. abortus*, with additional cases due to *B. canis* and *B. neotomae* (2–4). Human brucellosis is characterized by non-descript flu-like symptoms during an early acute phase, which is followed by a chronic infection with debilitating

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consequences, including recurrent fever, osteomyelitis, arthritis, neurological symptoms and endocarditis, if not treated with antibiotic therapy in a timely manner (4). Both animal and human brucellosis share common pathophysiological features at the cellular level, where bacteria undergo an intracellular cycle that ensures their survival, proliferation and persistence within phagocytic cells of various tissues, including macrophages and dendritic cells (4, 5). Initially described in placental tissues of infected animals (6), the ability of *B. abortus* to extensively proliferate in mammalian cells was reproduced in a variety of tissue culture models of epithelial and phagocytic cells that have been instrumental in defining the main features of the bacterium's intracellular cycle (7–12).

A Multi-Stage Intracellular Cycle.

Upon entry into either phagocytic or non-phagocytic cells, *Brucella* are enclosed within a membrane-bound compartment that has been named the *Brucella*-containing vacuole (BCV) (8, 13) (Fig. 1), based on the concept that the original phagosome is functionally modified by the bacterium into an idiosyncratic membrane-bound vacuole. Early microscopy-based trafficking studies have established that the initial BCV normally traffics along the endocytic pathway, sequentially acquiring early then late endosomal membrane markers (7, 8), and becomes acidified, reaching a pH of 4.5 (14–16), consistent with a complete phagosomal maturation process. Based on the endosomal nature of the bacterial vacuole at this stage of the intracellular cycle (between 0 and 8 h post infection), it was named eBCV (for endosomal BCV; Fig. 1 and 2A) (17).

Subsequent to eBCV interactions with the endocytic compartment, the vacuole progressively loses endosomal markers (between 8 and 12 h post infection), concomitant with sustained interactions with endoplasmic reticulum (ER) structures, and eventually acquires ER membrane-associated markers such as Calreticulin, Calnexin and Sec61β (7, 8, 13), indicating a turnover of eBCV membranes and accretion of ER-derived membranes. These vacuoles also gain structural and functional features of the ER (7, 8), further indicating a conversion of the eBCV into an ER-derived organelle. These structural and functional changes correlate with the onset of bacterial replication, suggesting that these organelles provide intracellular conditions that promote bacterial growth and replication. Based on these considerations, these vacuoles were named rBCVs (for replicative BCVs; Fig. 1 and 2B) (17). Original ultrastructural characterizations of rBCVs suggested these vacuoles were individual and rarely connected with ER cisternae in either macrophage or HeLa cells (7, 8) (Fig. 1D), while other studies had observed bacteria within the ER lumen of trophoblasts (6), leaving the actual organization of rBCVs uncertain. Recent electron tomography 3-dimensional reconstruction of rBCVs clearly determined that these vacuoles are intricately connected with the ER and also seem to interact with vesicular traffic between the ER and the Golgi apparatus (18), demonstrating intimate interactions with the host secretory pathway. The rBCV stage is tightly associated with bacterial proliferation (between 12 and 48 h post infection) and causes a dramatic reorganization of the host cell ER into the rBCV network (8).

Following extensive bacterial replication, an additional stage in the *Brucella* intracellular cycle consists of the progressive capture of rBCVs by crescent like membrane structures,

leading to the formation of multi-membrane vacuoles structurally reminiscent of autophagosomes that contain small to large groups of bacteria and acquire late endosomal/lysosomal features (17). These remodeled rBCVs, which are called aBCVs (for autophagic BCVs; Fig. 1 and 2C) form between 48 and 72 h post infection and are associated with bacterial release from infected cells and new infection events, indicating that they facilitate completion of the *Brucella* intracellular cycle (17).

By their functional and spatial diversity, the sequential eBCV, rBCV and aBCV stages of the *Brucella* intracellular cycle point towards their necessity to the bacterium's infectious cycle. Hence, significant efforts have been put in the last two decades to understand the underlying mechanisms that define these stages and how the bacterium exploits the corresponding cellular pathways to complete its intracellular cycle.

The VirB Type IV Secretion System

A hallmark of *Brucella* spp. virulence is their expression of a VirB family Type IV-A secretion system (T4SS), which was identified by homology to the VirB T4SS of the plant pathogen *Agrobacterium tumefaciens* and shown to be required for virulence in a murine model of chronic brucellosis and intracellular replication of *B. abortus*, *B. melitensis* and *B. suis* in various host cell models (19–21). Indeed, deletions of various genes within the *virB* operon, or transposon insertions in different *virB* genes, rendered *Brucella* unable to convert eBCVs into rBCVs and replicate intracellularly (8, 13, 22), and unable to establish a chronic infection in mice (23, 24), emphasizing major roles of this T4SS in rBCV biogenesis and *Brucella* intracellular replication. Based on the ability of various T4SS to deliver effector proteins or nucleoproteins complexes across biological membranes, and by analogy to those that contribute to bacterial virulence (25, 26), the VirB T4SS of *Brucella* was presumed to deliver effector proteins into host cells across the eBCV membrane to modulate specific cellular functions and mediate rBCV biogenesis. This assumption was confirmed by the discoveries of an array of *Brucella* effector proteins translocated into host cells in a T4SS-dependent manner (27–32), which opened avenues to understand at the molecular level the underlying mechanisms of the *Brucella* intracellular cycle.

THE ENDOSOMAL BCV

Early BCV Maturation

Upon uptake by phagocytes or entry into non-phagocytic cells, *Brucella* initially resides in a phagosome that rapidly acquires markers of the early endocytic compartment, including the small GTPase Rab5 that controls early endosomal maturation, and the early endosomal antigen EEA-1 (8, 13, 33). Since these markers are acquired with kinetics similar to those of classical phagosome, it is likely that the early BCV undergoes an unaltered maturation process.

Consistently, the vacuole then acquires markers of late endosomes, such as the lysosomal-associated membrane proteins LAMP1 (Fig. 2A) and LAMP2, CD63 and the small GTPase Rab7 (7, 8, 13, 16), which controls fusion with late endocytic compartments and lysosomes, further indicating a normal vacuolar maturation process within the first hours post-infection.

Importantly, the BCV also rapidly becomes acidified to a pH range of 4.5–5.0 (14), further supporting a maturation process into phagolysosomes. However, early attempts at detecting delivery of lysosomal luminal content to the BCV as evidence for fusion with lysosomes failed to demonstrate accumulation of Cathepsin D by immunostaining (7, 13, 34). This led to the proposal that the BCV avoids fusion with terminal degradative lysosomes, despite advanced maturation events, thus preventing bacterial killing and ensuring intracellular survival.

The eBCV, A Necessary Evil?

Several studies monitoring bacterial viability within eBCVs have revealed that a large proportion of intracellular bacteria (up to 90% in primary bone marrow-derived macrophages) are killed (8, 16), arguing for bactericidal conditions within eBCVs. Using live cell imaging of fluorescent fluid phase markers that were pre-chased to terminal lysosomes, as a method that precludes any issue with detection of soluble lysosomal antigens, Starr *et al* showed that eBCVs in epithelial HeLa cells undergo fusion with terminal lysosomes, although to a restricted extent (16), directly demonstrating that the eBCV matures into a compartment resembling a phagolysosome. While these findings are consistent with the loss of bacterial viability in eBCVs observed in *in vitro* models, they may appear counter-intuitive with regard to *Brucella* intracellular survival. This has led to the speculation that eBCVs that undergo full lysosomal maturation are not those permissive for bacterial survival and represent dead-end paths for the bacteria, while a small fraction of BCVs that do not mature along the endocytic pathway are those that generate rBCVs and allow for proliferation of the surviving bacteria. The following observations are however inconsistent with this possibility. First, LAMP1-positive eBCVs interact with ER cisternae and acquire ER markers, arguing that they undergo conversion into rBCVs (8). Second, eBCV acidification to a pH range of terminal lysosomes is necessary for rBCV biogenesis and bacterial replication (14–16), as inhibition of lysosomal acidification prevents eBCV to rBCV conversion and bacterial replication (14–16). Third, inhibition of Rab7 activity, which controls fusion with lysosomes, also prevents eBCV conversion into rBCVs and bacterial replication (16), demonstrating that a functional late endocytic pathway and lysosomal fusion are required for *Brucella* to undergo its intracellular cycle. Fourth, eBCV acidification is essential for the induction of the *virB* operon (15, 16), which rapidly occurs post uptake in maturing eBCVs, peaking at 4 h post-infection (16, 35). Taken together, these findings rather support a model in which the eBCV stage is a necessary step in the *Brucella* intracellular cycle, which provides intravacuolar cues, including a lysosomal pH, that are necessary for the induction of the VirB T4SS and the resulting conversion of eBCVs into rBCVs.

In addition to its role in promoting *Brucella* T4SS competency, the eBCV may provide cues that initiate intracellular bacterial growth prior to completion of the replication-permissive rBCV. Degehlt *et al.* established using fluorescence microscopy methods that monitor chromosomal replication in intracellular *Brucella* that bacteria within LAMP1-positive eBCVs initiate chromosomal replication by 8 h post infection, *i.e.* during the eBCV to rBCV conversion stage, while the infectious form are in G1-arrested phase prior to 6 h post infection (36). Whether this cell cycle change is triggered by the endosomal conditions of the eBCV, or by intravacuolar alterations during conversion to rBCV remains to be

determined. Yet, these findings indicate that *Brucella* extensively responds to its intravacuolar environment and initiate growth at the eBCV stage, further emphasizing the importance of the endosomal stage in the *Brucella* intracellular cycle.

THE REPLICATIVE BCV

Role Of The ER In rBCV Biogenesis.

Biogenesis of the rBCV is a hallmark of the *Brucella* intracellular cycle, as it consists of the conversion of the original endosomal bacterial vacuole into a specialized organelle derived from the ER that promotes bacterial proliferation. Consistently, the underlying mechanisms, host functions and bacterial effectors that mediate this process have been the subject of extensive research. The nature of the rBCV was initially discovered via its ultrastructural characterization, showing fusion with ER cisternae and studding with ribosomes, and was further confirmed by the detection of ER-associated proteins on its membrane by immunofluorescence microscopy, intravacuolar detection of glucose-6 phosphatase activity, an ER luminal enzyme, and sensitivity to the ER-vacuolating toxin Aerolysin (7, 8). The first clues about how a bacterial vacuole interacting with late endocytic and lysosomal compartments could convert into an ER-derived organelle came from the demonstration that ER exit sites (ERES), an ER sub-compartment where secretory transport is initiated through the formation of COPII-coated cargo vesicles (37), are functionally important for rBCV biogenesis (22). eBCVs undergo T4SS-dependent, sustained interactions with COPII coat-positive ERES structures during the eBCV to rBCV conversion stage (Fig. 3) and inhibition of the small GTPase Sar1, which controls COPII coat assembly, ERES formation and function, inhibits rBCV biogenesis and bacterial replication (22, 38). Consistent with these observations, *Brucella* infection upregulates production of Sar1 and the COPII components Sec23 and Sec24D by an unknown mechanism (39), suggesting that the bacterium upregulates Sar1- and COPII-mediated vesicular trafficking to promote rBCV biogenesis. Hence, *Brucella* may modulate ERES functions to promote rBCV biogenesis, by enhancing production of secretory vesicles that might fuse with the eBCV to initiate its conversion into a vacuole with ER fusogenic properties (Fig. 3), possibly creating a direct port of entry to the ER for the bacteria, while bypassing classical endosome-to-Golgi-to-ER retrograde trafficking processes.

Role Of The Unfolded Protein Response In The *Brucella* Intracellular Cycle.

Another ER-mediated function associated with the *Brucella* intracellular cycle is the Unfolded Protein Response (UPR). Upon ER stress triggered by accumulation of misfolded proteins within the ER, signaling pathways controlled by the ER-localized UPR receptors IRE1α, PERK and ATF6 are triggered and lead to a substantial reorganization of ER functions towards increased ER folding capacity, inhibition of protein synthesis and activation of autophagy, all aimed to resolve ER stress (40). Although there have been discrepancies about its extent, there is consensual agreement that *Brucella* infection induces the UPR in macrophages or HeLa cells via at least the IRE1α pathway (39, 41, 42). Moreover, IRE1α is required for *Brucella* replication (43) and pharmacological compensation of protein misfolding using Tauroursodeoxycholic acid (TUDCA) impairs replication of *B. melitensis* (41), which supports the idea that the UPR is beneficial to the

bacterium's intracellular cycle. Yet, whether and how this stress response promotes rBCV biogenesis and bacterial replication remains unclear. Taguchi *et al.* provided a link between UPR induction, ERES and rBCV biogenesis, by showing that IRE1α activation is via the ERES-localized protein Yip1A, which controls formation of large vacuoles in a manner dependent on the autophagy-associated proteins Atg9 and WIPI1, and is also required for rBCV biogenesis (39). Hence, these findings support a model in which *Brucella* actively causes UPR induction via activation of the IRE1α pathway to promote an activation cascade leading to formation at ERES of vacuoles of possible autophagic nature (43), which ultimately contributes to rBCV biogenesis (Fig. 3).

Is Autophagy Involved In rBCV Biogenesis?

Autophagy is a membrane-based process that normally captures intracellular content, whether cytosolic, damaged organelles or microorganisms, into double membrane vesicles called autophagosomes, to deliver it for degradation to the lysosomal compartment. While it can act as an innate immune antibacterial mechanism, it can also be beneficial to some pathogens and serve their intents (44, 45). Based on the roles of IRE1α and Yip1A in rBCV biogenesis and *Brucella* replication, autophagosome formation at ERES may provide ER-derived membranes that may contribute to eBCV to rBCV conversion, yet whether the canonical autophagic cascade is involved in rBCV biogenesis remains controversial. While a classical autophagic process was originally proposed as a mechanism for rBCV biogenesis in HeLa cells, based on the observation of multi-membrane structures on BCVs and accumulation of the lysomotropic compound monodansyl cadaverine (MDC) (7, 34), such structures are not seen on eBCVs in macrophages (8), nor does the autophagic marker LC3 accumulate on eBCVs, arguing against a canonical autophagic process. Furthermore, inhibition of autophagy via siRNA-mediated depletion of specific autophagy components involved in the autophagosome nucleation and elongation steps, namely ULK1, Beclin1, Atg5, Atg7, ATG16L and LC3B, does not prevent rBCV biogenesis and bacterial replication (17). Hence, there is no substantial evidence to support a role of the canonical autophagic cascade in rBCV biogenesis. However, the demonstrated roles of the autophagosome nucleation protein WIPI1 and of the autophagy protein Atg9 in rBCV biogenesis (39) clearly invoke specific autophagy-associated machineries in this process, suggesting that rBCV biogenesis requires a subset of cellular machineries associated with autophagosome formation at ERES and Atg9-mediated delivery of membranes. Further studies are actually needed to clarify how these autophagy-related events actually contribute to rBCV biogenesis.

Role Of The Secretory Pathway In rBCV Biogenesis.

Based on the ER nature of the rBCV, the host secretory pathway certainly plays an essential role in the *Brucella* intracellular cycle. Whether secretory compartments other than the ER, such as the ER-to-Golgi Intermediate Compartment (ERGIC), the Golgi apparatus and Trans-Golgi Network (TGN), are important to the bacterium has been a longstanding question, warranted by examples of other intracellular pathogens that exploit these compartments (46). These include *Legionella pneumophila*, which intercepts Arf1 and Rab1-dependent secretory traffic to acquire ER-derived membranes (47, 48), or *Chlamydia trachomatis*, which redirects Golgi-derived lipid trafficking pathways for acquisition of

sphingolipids (49, 50). Cellular approaches based on altering specific functions along the secretory pathway using either pharmacological inhibition of host secretion, expression of dominant inactive alleles of small GTPases, or siRNA-mediated depletions, have revealed that ER-Golgi secretory traffic is important for rBCV biogenesis and bacterial replication (22, 38). Inhibition of Arf1-dependent secretory traffic using either Brefeldin A or via siRNA treatment impairs rBCV biogenesis to similar levels as depletion of Sar1 (22, 38). Additionally, depletion of the small GTPases Rab1a and Rab2, which control anterograde and retrograde vesicular traffic between the ER and Golgi apparatus (51), negatively affect replication of *B. abortus* in macrophages (38), further indicating a complex role of vesicular traffic between these compartments in promoting *Brucella* proliferation, possibly through acquisition by the eBCV of secretory membranes during their conversion process into the rBCV. Consistent with *Brucella* exploiting these pathways, host secretory traffic is altered in *Brucella*-infected cells in a T4SS-dependent manner (30), which argues that the bacterium actively modulates secretory functions via delivery of effectors to promote its intracellular cycle.

Bacterial Effectors Of rBCV Biogenesis.

The recent discoveries of several VirB T4SS effectors (27–32) and the characterization of the mode of action of some of them (Table 1) has provided opportunities to start deciphering at the molecular level the bacterial mechanisms of rBCV biogenesis and bacterial replication. VceC, identified as co-regulated with the VirB T4SS, binds the ER chaperone BiP/GRP78 and induces the UPR, which triggers an inflammatory response (42), yet whether it plays a role in rBCV biogenesis has not been elucidated. TcpB/BtpA, a TIR-domain-containing effector that down-modulates pro-inflammatory responses (52–58), also induces the UPR and promotes bacterial proliferation once rBCVs are formed (41), suggesting that it may not contribute to rBCV biogenesis. SepA, by contrast, is possibly involved in rBCV biogenesis, as a Δ*sepA* mutant is retained in eBCVs (32), yet its mode of action remains unknown.

The T4SS effectors BspA, BspB and BspF, which inhibit host protein secretion and promote bacterial replication (30, 38), support the hypothesis that *Brucella* specifically targets various host secretory functions, possibly for rBCV biogenesis purposes. While the modes of action of BspA and BspF are unknown, that of BspB has been elucidated. BspB is required for rBCV biogenesis and optimal bacterial replication in macrophages (38). This effector is delivered into host cells and traffics to the Golgi apparatus where it interacts with the conserved oligomeric Golgi (COG) complex (38) (Fig. 3), a CATCHR-family multi-subunit tethering complex (MTC) that serves as an interaction hub on Golgi membranes for secretory Rab GTPases, Golgi tethers and SNAREs, and that regulates intra-Golgi and retrograde vesicular traffic along the secretory pathway (59). COG functions are important for rBCV biogenesis and bacterial replication (38), implicating Golgi-associated functions in the *Brucella* intracellular cycle. By mechanisms to remain to be defined, BspB-COG interactions alter COG functions and lead to redirection of COG-dependent Golgi retrograde vesicular traffic to BCVs and acquisition of Golgi-derived membranes (38), demonstrating that *Brucella* likely recruits membranes from this secretory compartment during rBCV biogenesis, in addition to ER-derived membranes (Fig. 3). Interestingly, inhibition of Rab2-

dependent Golgi-to-ER retrograde traffic via depletion of Rab2 suppresses the replication defect of a Δ*bpsB* mutant (38), suggesting that BspB may affect retrograde secretory traffic to redirect COG-dependent Golgi vesicular traffic to the BCV. Interestingly, the T4SS effector RicA is involved in controlling rBCV biogenesis and binds the guanosine-di-phosphate-(GDP)-bound form of Rab2 (28) (Fig. 3). Although its mode of action is unknown, as it does not show any GEF activity (28), this suggests that *Brucella* delivers several effectors that may coordinately act to modulate Rab2-dependent vesicular trafficking and promote rBCV biogenesis.

Altogether, the knowledge gained by studying VirB T4SS effectors that target the host secretory pathway has revealed yet another facet of *Brucella* interactions with this intracellular compartment and emphasize how the identification and characterization of these effectors are key to a comprehensive understanding of how this bacterium subverts host secretory functions.

THE AUTOPHAGIC BCV

While replication in ER-associated rBCVs is a key step in the pathogenesis of *Brucella*, how the bacterium completes its intracellular cycle following this proliferation stage has remained unknown for many decades. Unlike many pathogens that cause cell death to exit the cells in which they have replicated, *Brucella* prevents cell death programs to occur (60, 61), therefore preserving its intracellular niche. Starr *et al.* instead observed that following proliferation in rBCVs, by 48 h post infection and afterwards, the *Brucella* intracellular niche was converted from an ER-derived organelle to large vacuoles harboring features of late endosomal and lysosomal compartments, such as accumulation of LAMP1 and acidification (17). From an ultrastructural standpoint, these vacuoles are surrounded by multiple membranes and originate from the capture of rBCVs by crescent-shaped membrane structures reminiscent of autophagosomes, despite the lack of accumulation of the canonical autophagosome marker LC3 (17) (Fig. 2). The formation of these vacuoles, named aBCVs for autophagic BCVs, requires functions of the canonical autophagy nucleation, but not elongation complexes, as depletion or deletion of Beclin1, ULK1 and Atg14L, but not that of Atg5, Atg7, Atg4 or Atg16L, blocked their formation (17). aBCV formation therefore seem to require a subset of autophagy-associated molecular machineries, which may typify an alternate autophagic process or indicate that the bacterium actively exploits discrete functions of the canonical autophagy pathway to generate aBCVs.

Importantly, aBCV formation is tightly linked to bacterial egress, as new infection events of adjacent cells occur during the aBCV formation stage and are reduced upon inhibition of aBCV formation (17). Whether bacteria are released free or contained within a membrane-bound vacuole remains to be established, but the maintenance of the originally-infected cells argues that bacterial release is a non-lytic event, suggesting an exocytic process (Fig. 1). Based on the autophagic nature of the aBCV, a tempting hypothesis to explain aBCV-dependent bacterial release is that the bacterium takes advantage of the secretory functions of the autophagy pathway (62) to deliver aBCV-enclosed bacteria to the extracellular milieu.

By mediating the final step of the *Brucella* intracellular cycle, aBCV formation is likely controlled by the bacterium, potentially via the VirB T4SS. Testing this hypothesis is challenging, as the preceding step of rBCV formation is T4SS-dependent, precluding the classical use of reverse genetics and VirB T4SS-deficient mutants. Transient intracellular production of the VirB11 ATPase via a tightly controlled promoter, which energizes the T4SS, allows rBCV biogenesis and bacterial replication prior to T4SS intracellular inactivation and showed a requirement for a functional VirB apparatus in aBCV formation and bacterial egress, indicating that this final step of the *Brucella* intracellular cycle is likely controlled by T4SS effectors (63). Their future identification and characterization will likely shed light on the molecular mechanisms of aBCV formation and bacterial egress.

CONCLUDING REMARKS

Bacteria of the genus *Brucella* belong to a phylogenetic group closely associated with eukaryotic hosts. In this context, *Brucella*'s long-standing co-evolution with mammalian hosts has shaped the bacterium's complex intracellular cycle into the sequential exploitation of intracellular compartments of the endocytic, secretory and autophagy pathways. *Brucella* uses an array of T4SS-delivered effectors, and other virulence factors, to modulate discrete functions of these compartments, modifying its original phagosome into an ER-associated replication-permissive organelle that subsequently co-opts autophagic functions for completion of the bacterium's intracellular cycle, therefore ensuring its survival, proliferation and egress. While a few T4SS effectors have been identified, much remains to be understood about their modes of action and contributions to *Brucella's* intracellular cycle. Future characterization of these proteins will not only reveal unsuspected aspects of *Brucella* intracellular strategies, it will also teach us a great deal about host cell functions and their roles in many aspects of bacterial pathogenesis.

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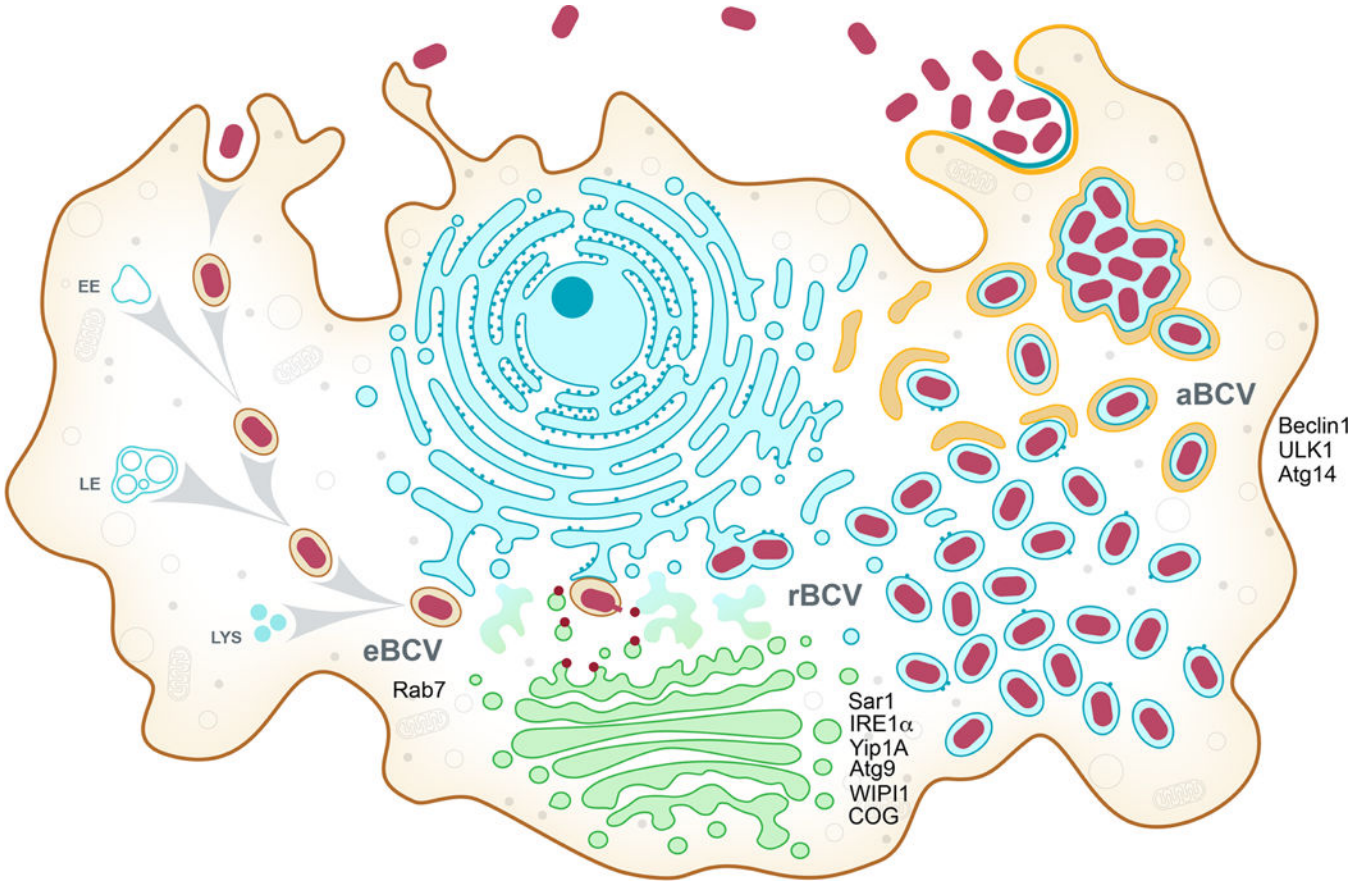

Figure 1. Model of the *Brucella* intracellular cycle in macrophages.

Following phagocytic uptake by macrophages, *Brucella* spp. reside in the first 8 to 12 h post infection within a membrane bound vacuole that undergoes endosomal maturation via sequential interactions with early (EE) and late (LE) endosomes and lysosomes (LYS) to become an acidified, endosomal *Brucella*-containing vacuole (eBCV). The host small GTPase Rab7 contributes to eBCV maturation, which provides physicochemical cues promoting expression of the VirB Type IV secretion system (T4SS), which translocates effector proteins (red) that mediate eBCV interactions with ER exit site and acquisition of ER and Golgi-derived membranes. These events lead to the biogenesis of replication permissive, ER-derived BCVs, called replicative BCVs (rBCVs). The host proteins Sar1, IRE1α, Yip1A, Atg9, WIPI1 and the COG complex contribute to rBCV biogenesis. Bacteria then undergo extensive replication in rBCVs between 12 and 48 h pi, after which rBCVs are captured within autophagosome-like structures in a VirB T4SS-dependent manner, to become autophagic BCVs (aBCVs). aBCV formation requires the host autophagy proteins Beclin1, ULK1 and Atg14. aBCVs harbors features of autolysosomes and are required for bacterial egress and new cycles of intracellular infections.

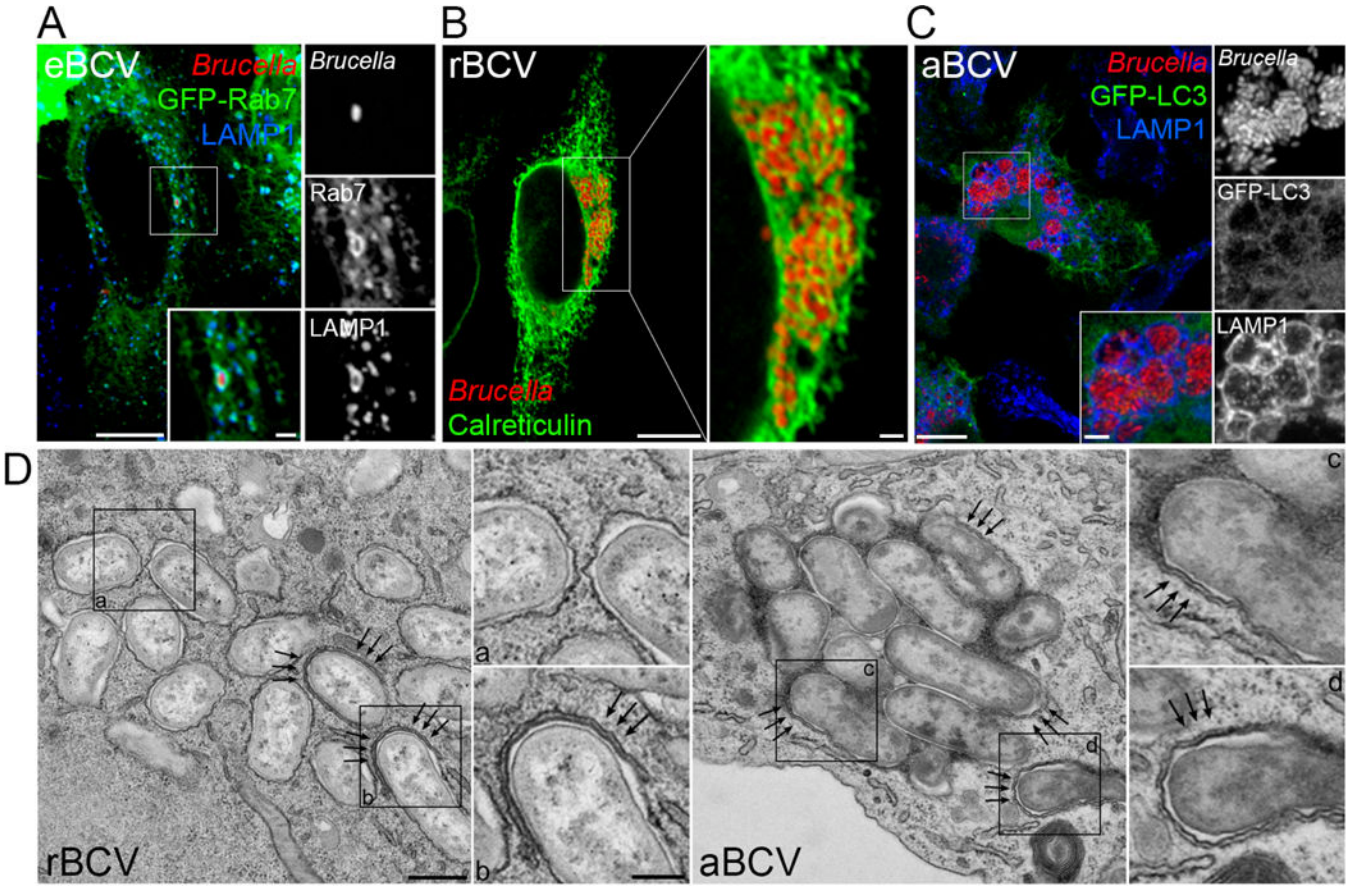

Figure 2. Structure and membrane composition of BCVs during the *Brucella* intracellular cycle.

(A) Confocal fluorescence micrograph of a HeLa cells expressing GFP-Rab7 and infected with DsRed$_m$-expressing *B. abortus* strain 2308 for 6 h. The inset shows an eBCV typified by the accumulation of the late endosomal/lysosomal markers Rab7 and LAMP1. Scale bars, 10 and 2 μm. (B) Confocal fluorescence micrograph of a HeLa cells infected with DsRed$_m$-expressing *B. abortus* strain 2308 for 24 h and stained for the endoplasmic reticulum (ER) marker Calreticulin. The inset shows a cluster of Calreticulin-positive rBCVs containing replicating bacteria and associated with the ER network. Scale bars, 10 and 1 μm. (C) Confocal fluorescence micrograph of a primary murine bone marrow-derived macrophage (BMM) expressing the autophagy marker GFP-LC3 and infected with DsRed$_m$-expressing *B. abortus* strain 2308 for 72 h. The inset shows a group of aBCVs typified by the accumulation of the late endosomal/lysosomal LAMP1, but not LC3. Scale bars, 10 and 2 μm. (D) Transmission electron micrographs of BMMs infected with *B. abortus* strain 2308 for 72 h and showing the ultrastructures of rBCVs (left-hand image, single membrane-bound vacuoles, inset a), of forming aBCVs (inset b and arrows) and of completed double membrane-bound aBCVs (right-hand image, insets c and d and arrows). Scale bars, 500 and 200 nm. Images reprinted from Cell Host&Microbe (Starr 2012) with permission of the publisher.

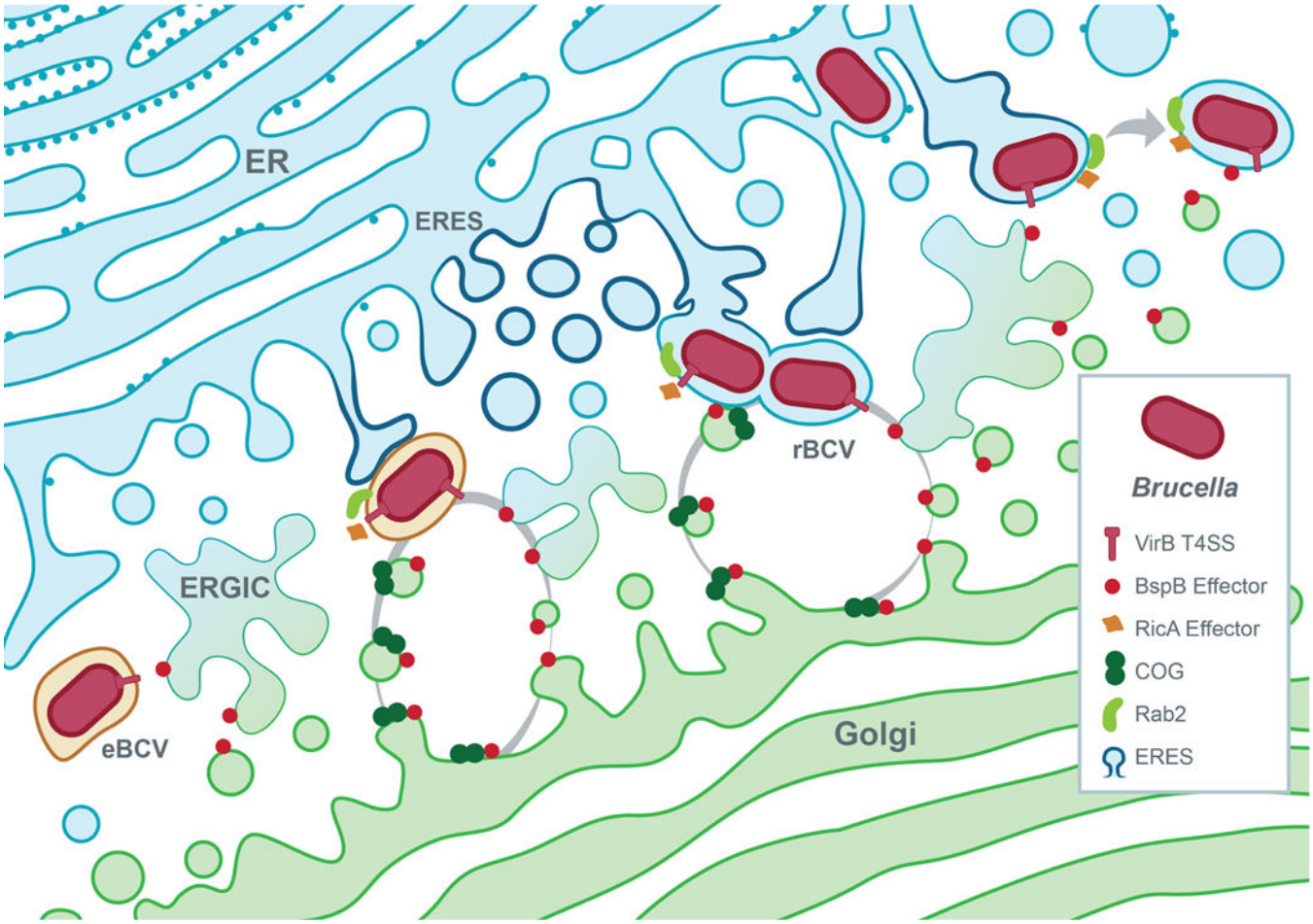

Figure 3. Model of VirB T4SS-dependent biogenesis of the rBCV.

Bacteria in eBCVs induce expression of the VirB T4SS, which delivers effector proteins into the host cell. Among these, BspB traffics to Golgi membranes via the ER-to-Golgi Intermediate Compartment (ERGIC) and binds to the Conserved Oligomeric Golgi (COG) complex to promote redirection of Golgi-derived vesicular traffic to BCVs. RicA binds the small host secretory GTPase Rab2, which contributes to its recruitment on maturing eBCVs and role in rBCV biogenesis. Additionally, eBCVs interaction with ER exit sites (ERES) is accompanied with the upregulation of COPII coat components, induction of IRE1α and Yip1A-dependent formation of ER-derived vesicles that are also thought to contribute to rBCV biogenesis. T4SS-dependent acquisition of ER- and Golgi-derived secretory membranes to BCVs is thought to mediate eBCV to rBCV conversion.

Table 1.

Brucella T4SS effectors

Name	ORF in *B. abortus*	Host target	Function	References
RicA	BAB1_1279	Rab2	modulates rBCV biogenesis	(28)
VceA	BAB1_1652	unknown	unknown	(27)
VceC	BAB1_1058	Grp78/BiP	UPR activation	(27, 42)
BspA	BAB1_0678	unknown	intracellular replication	(30)
BspB	BAB1_0712	COG	redirects Golgi vesicular traffic; rBCV biogenesis; intracellular replication	(30, 38)
BspC	BAB1_0847	unknown	unknown	(30)
BspE	BAB1_1675	unknown	unknown	(30)
BspF	BAB1_1948	unknown	intracellular replication	(30)
BtpA/TcpB	BAB1_0279	MAL	Inhibition of TLR signaling; UPR induction	(52)
BtpB	BAB1_0756	unknown	unknown	(31)
SepA	BAB1_1492	unknown	eBCV trafficking	(32)
BPE005	BAB1_2005	unknown	Unknown	(29)
BPE043	BAB1_1043	unknown	Unknown	(29)
BPE275	BAB1_1275	unknown	Unknown	(29)
BPE123	BAB1_0123	unknown	Unknown	(29)