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Abstract: Chlamydiae are obligate intracellular bacterial pathogens with an unusual biphasic lifecycle, which is underpinned by two bacterial forms of distinct structure and function. Bacterial entry and replication require a type III secretion system (T3SS), a widely conserved nanomachine responsible for the translocation of virulence effectors into host cells. Recent cell biology experiments supported by electron and cryo-electron tomography have provided fresh insights into Chlamydia-host interactions. In this review, we highlight some of the recent advances, particularly the in situ analysis of T3SSs in contact with host membranes during chlamydial entry and intracellular replication, and the role of the host rough endoplasmic reticulum at the recently described intracellular 'pathogen synapse'.

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complete with 4 Figures

Making connections: snapshots of chlamydial type III secretion systems in contact with host membranes

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Highlights

- *Chlamydia* are polarised, with distinct membrane and protein structures in opposite hemispheres.
- Type 3 secretion systems form a polar array contacting the host plasma membrane.
- Pathogen synapses connect bacterial, inclusion and host endoplasmic reticulum membranes.

Abstract

Chlamydiae **are obligate intracellular bacterial pathogens with an unusual biphasic lifecycle, which is underpinned by two bacterial forms of distinct structure and function. Bacterial entry and replication require a type III secretion system (T3SS), a widely conserved nanomachine responsible for the translocation of virulence effectors into host cells. Recent cell biology experiments supported by electron and cryo-electron tomography have provided fresh insights into *Chlamydia*-host interactions. In this review, we highlight some of the recent advances, particularly the *in situ* analysis of T3SSs in contact with host membranes during chlamydial entry and intracellular replication, and the role of the host rough endoplasmic reticulum at the recently described intracellular 'pathogen synapse'.**

Introduction

Although initially believed to be a virus [1], Gram-negative *Chlamydiae* were the first obligate intracellular bacteria with a biphasic lifecycle to be described [2]. *Chlamydiae* cause disease in humans and other animals, and in particular *Chlamydia trachomatis* remains the leading bacterial agent of sexually transmitted disease worldwide, while ocular infections cause blinding trachoma, which is designated as a neglected tropical disease by the World Health Organisation [3]. Studying *Chlamydiae* remains a challenge, since the bacteria cannot be cultured outside eukaryotic cells, and although transformation has recently been reported [4,5,6], there remains no routine methodology for directed mutagenesis. Many of the tools

that have driven the substantial advances in understanding the cellular microbiology of other bacterial pathogens still therefore remain inaccessible for *Chlamydiae*. Nevertheless, the developmental cycle can be reconstituted in the laboratory using cultured mammalian cells [7].

During the early stages of infection, extracellular infectious but metabolically inactive elementary bodies (EBs) adhere to the plasma membrane of the host cell and induce their own actin-dependent uptake into endocytic vacuoles. These early vacuoles coalesce and traffic to the microtubule-organising centre, forming a specialised membrane-bound compartment termed an inclusion. Within the inclusion, EBs differentiate into non-infectious but metabolically active reticulate bodies (RBs). RBs undergo a series of cell divisions before converting back into EBs, which are subsequently released from the cell by inclusion extrusion, or upon cell lysis [8].

EBs and RBs are not only distinct in function, but also in morphology. While both forms of the bacterium are coccoid, they differ significantly in size; EBs are 0.3-0.4µm in diameter in comparison to RBs at 1µm. Substantial changes in bacterial architecture therefore occur during EB-RB and RB-EB inter-conversion, which remain incompletely understood [8]. The most obvious distinguishing structural characteristic is the outer membrane, which is almost twice the thickness in EBs [9]. This is attributed to a disulphide-cross-linked network of major outer membrane proteins that confer the osmotic stability and rigidity of EBs [10]. By contrast, the disulphide bonds are reduced in RBs, allowing for greater membrane flexibility to

facilitate cell division [11]. Both EBs and RBs harbour type III secretion systems (T3SSs), nanomachines conserved among diverse Gram-negative bacterial pathogens. T3SSs translocate virulence effector proteins directly into host cells, where they subvert cellular processes to promote pathogen entry, survival or replication [12]. In this review, we will explore the relationship between the EB and RB T3SSs, their supramolecular organisation in contact with host membranes, and their contribution to sustaining the chlamydial lifecycle.

The chlamydial T3SS: the exception or the rule?

T3SSs are macromolecular complexes that span the bacterial envelope [13], first observed in *Salmonella* [14]. Subsequent single particle analysis of core complexes isolated from the membranes of diverse Gram-negative bacterial pathogens has revealed a conserved structure comprising oligomeric rings embedded in the inner and outer membranes connected by a cylindrical *trans*-periplasmic tube, enabling effector secretion without periplasmic intermediates. A helical 'needle' consisting of a single polymerised subunit connects proximally to the outer membrane ring and at the distal end to a translocon complex proposed to interact with a third membrane from the host [13]. The structure of this translocon and the nature of its interaction with the host membrane remain unknown, as it is never co-isolated with the detergent-solubilised core T3SS complex. *Chlamydiae* encode homologues of core complex components [15, 16, 17], yet in comparison to other pathogens in which the genes encoding T3SSs are grouped together on pathogenicity islands, T3SS-related genes are distributed across the genome in four distinct clusters composed of at

least ten separate operons [18]. Unusually, *Chlamydiae* also possess two copies of putative translocon components (CT578/CT579 and CT860/CT861) identified by primary sequence similarity to the *Yersinia* YopB and YopD translocon proteins [19], although the significance of this remains unresolved. Nevertheless, it is clear that the chlamydial T3SS is pivotal to virulence as T3SS inhibitors arrest the bacterial lifecycle [20, 21].

Polar organisation of T3SS arrays in *Chlamydia* EBs

Seminal early electron microscopy studies by Matsumoto identified surface projections and protein complexes termed 'rosettes' on the surface of *Chlamydia* EBs in the absence of host cells [*e.g.* 22]. These structures, observed well in advance of the identification of any T3SSs in bacteria, were only later proposed as T3SSs [23]. Indeed, the rosettes have also since been suggested to represent outer membrane protein complexes [24]. An elegant study by Peterson [25], also describes structures apparently connecting RBs to the inclusion membrane in chemically fixed sections by electron microscopy. These structures were similarly proposed to be T3SSs but had never been experimentally identified or examined in detail [23]. Recently, we applied cryo-electron tomography to examine EB structure in greater detail (**Figure 1A**) [26]. This revealed that EBs are polarised, whereby one hemisphere is characterised by pronounced expansion of the periplasmic space (~29nm compared to ~14nm on the opposite pole), which accommodates an array of 14-20 T3SSs, definitively identified by immunogold labelling. While the EB outer membrane remains rigid, each T3SS complex originates at a specific concave deformation of the inner membrane [26]. The opposite pole with the narrower periplasmic space

contains additional complexes of distinct morphology and as yet unknown composition, in addition to an invagination of the inner membrane [26], reminiscent of the complex and atypical membrane structures present in other members of the *Planctomycetes-Verrucomicrobia-Chlamydiae* [27].

In the presence of host cells, EBs universally orient with their T3SS array facing the target cell plasma membrane with which they engage (**Figure 1B**) [26]. This co-ordinated alignment might be determined by prior engagement of host receptors or polysaccharides by polymorphic membrane proteins or outer membrane proteins such as OmcB [28, 29], which may also be similarly polarised on the EB surface. The membrane-engaged battery of T3SSs would enable the rapid coordinated delivery of a high local effector dose to trigger bacterial entry. Snapshots of the chlamydial entry process, captured by cryo-electron tomography, revealed an unexpected diversity of early host structures engaging EBs ranging from phagocytic cups, to filopodial capture events and complex ruffle-like plasma membrane invaginations (**Figure 2**) [26]. These cellular structures are compatible with a role for Rac1- and Arf6-dependent GTPase signalling events [30, 31], stimulated in part by the translocated effectors CT166 and Tarp [32, 33], although whether these captured intermediates represent sequential assemblies in a single pathway or denote multiple independent entry mechanisms requires further investigation by live imaging approaches. While the resulting membrane invaginations that remain accessible to the extracellular milieu frequently contain multiple EBs, it is striking that the majority of closed early vacuoles only encapsulate individual EBs [26]. In the first few hours after internalisation this apparent sorting is also accompanied by

reorganisation of both the bacterial and host vacuolar membranes. The vacuole membrane that initially loosely encloses the EB and co-envelopes host material transitions to form a tight structure proximal to the EB surface. During this time, the EBs lose their polarity, with an associated reduction of the pronounced periplasmic widening and a decrease in assembled T3SSs (**Figure 2**) [26].

Pathogen synapses: ordered connections between the T3SS, the inclusion membrane and the host endoplasmic reticulum

Following internalisation, the inclusion must be diverted from the cellular endocytic system to prevent degradation, yet nutrients must be selectively scavenged from the host cell and efficiently transported across the inclusion membrane to enable bacterial differentiation into RBs and subsequent replication [8]. *Chlamydiae* reassemble their T3SSs to control inclusion biogenesis, by delivering effectors that are integrated into the inclusion membrane or delivered beyond into the host cell cytosol and nucleus [8]. In particular, hydrophobic inclusion proteins (Incs) are a family of T3SS substrates that localise to the inclusion membrane during infection [34]. Although most of their underlying effector mechanisms remain undefined, they are likely involved in the active hijack of host components and organelles including lipid droplets [35], Golgi-derived vesicles [36], multivesicular bodies [37], cytoskeletal components [38], and the rough endoplasmic reticulum (rER) at the inclusion membrane [39, 40, 41]. Indeed, IncD indirectly recruits rER to the inclusion membrane [39], while additional Incs engage key mediators of intracellular trafficking and apoptosis [42, 43, 44].

This phase of nutrient acquisition and effector translocation commences in the mid-stage of the lifecycle, and coincides with rER recruitment to the inclusion membrane [41]. Multiple host proteins located in the rER lumen or membrane are enriched in patches at the inclusion periphery and a subset are present within the inclusion lumen. Disruption of the rER using aerolysin toxin at timepoints prior to its recruitment stalls inclusion biogenesis, whereas later treatment, at timepoints when association is normally observed, bursts the inclusion [41]. Mature chlamydial inclusions therefore gain sufficient ER-like character to render them susceptible to the toxin. Electron tomography revealed intimate and extensive apposition of the ribosome-studded rER and the cytoplasmic face of the inclusion membrane. These regions of contact, tethered by 'pin-like' complexes of as yet unknown composition, appear so tight that host ribosomes are always partitioned onto the inclusion distal side of the rER tubules. Tomograms of RBs at the inclusion periphery also revealed a polar array of 20-100 T3SSs in contact with the luminal face of the inclusion membrane, specifically formed at sites coincident with rER recruitment on the cytoplasmic face of the inclusion (**Figure 3**). These structures bridging the rER in the host cytosol to the RB envelope through the inclusion membrane are termed 'pathogen synapses' [41]. Intriguingly, as with EBs at the plasma membrane, RBs also engage host membranes with an ordered supramolecular array of T3SSs. Indeed, it is possible that the RB pathogen synapse establishes a template for the polar array of T3SSs present in EBs following redifferentiation.

Host rER: a membrane source for inclusion growth and receiver for hydrophobic T3SS substrates?

Although only recently recognised for *Chlamydiae* [39, 40, 41], incorporation of rER membranes into pathogen-containing vacuoles is not without precedent. It is likely that chlamydial acquisition of rER-derived membrane contributes to the progressive expansion of the inclusion as the RBs within divide, and that the conferred lipid composition of the inclusion membrane may also regulate the association of hydrophilic chlamydial or host proteins [45], as with *Legionella* and *Brucella* generated compartments [46, 47]. In addition to this more structural role, *Chlamydiae* scavenge lipids including sphingomyelin and cholesterol from the secretory pathway for metabolism [36], in part by harnessing ER-localised CERT-VAT lipid transporters engaged by IncD from the inclusion membrane [39, 40]. *Chlamydiae* may also modulate key rER functions including the ER stress response and ER-associated protein degradation (ERAD), possibly to prevent host alarm signals and suppress antigen presentation (**Figure 4**).

The rER at pathogen synapses could also perform a more direct role. The plethora of hydrophobic Incs encoded by *Chlamydiae* prompts the question how these atypical T3SS substrates fold and insert into the inclusion membrane following their translocation. The rER contains both the Sec translocon and the Get complex [48], responsible for the insertion of eukaryotic transmembrane proteins, together with luminal chaperones required for protein folding [49]. It is tempting to speculate that the rER might act as a 'receiver membrane' for the incoming hydrophobic substrates

from the T3SS array, and that eukaryotic machinery might be co-opted to catalyse their folding and insertion (**Figure 4**). Furthermore, subsequent local trafficking of rER membrane from synapses into the inclusion may drive the incorporation of Incs into the inclusion membrane, although this seems not to involve COPII-dependent transport from rER exit sites [40, 41], which are subverted by *Legionella* [50]. Many Incs remain resident within ER membranes when exogenously expressed in cultured cells [51]. This is unusual, as most membrane proteins would transit into the secretory pathway by default. Differential residency within ER-like membrane could therefore potentially influence the lateral positioning of Incs within the inclusion membrane, in turn regulating Inc-Inc or Inc-host target interactions. Indeed, some Incs do apparently partition into microdomains [52], whereas others are distributed around the entire circumference of the inclusion membrane.

Conclusions

Recent studies of *Chlamydiae* have provided intriguing new insights into the supramolecular architecture of T3SSs and the nature of their interaction with host membranes, which act as critical interfaces between pathogen and host. These studies complement and extend the earlier pioneering work of Matsumoto and Peterson, and confirm the presence of T3SSs [22, 23, 25]. Imaging of EBs and RBs by electron tomography has allowed large numbers of assembled T3SSs to be captured in association with host membranes for the first time in any infection system [26, 41]. This provides an opportunity to visualise the T3SS translocon within the host membrane and other details of assembled T3SS structure *in situ*. Sub-tomogram

averaging of T3SSs in *Yersinia* in the absence of host cells has already revealed subtle alterations in T3SS interaction with the bacterial envelope when compared to the *in vitro* isolated core complexes [53]. Further work is now required to understand how EB polarity and orientation is determined and the location of other proteins, particularly adhesins, in relation to the T3SS array and inner membrane invagination. The identification of the pathogen synapse [41] raises intriguing questions about the role of the host rER in chlamydial infection and potentially in the insertion and folding of the hydrophobic Inc substrates of the T3SS. Although historically difficult to study, there is clearly much more to learn from the enigmatic *Chlamydiae*.

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Figure Legends

Figure 1. Polarised structure of the *Chlamydia trachomatis* EB

A. Left panel: *xy* tomographic slice (0.71 nm thick) from a denoised cryo-electron tomogram of a representative *C.trachomatis* EB. Scale bar, 100 nm. Right panel: Three-dimensional surface representation of the EB generated from segmentation of

a cryo-electron tomogram. Outer membrane (green), inner membrane (cyan and blue for the inner membrane invagination), T3SS (red), nucleoid (yellow), additional periplasmic complexes (brown) and ribosomes (purple) are shown.

B. Left panel: *xy* tomographic slice (0.71 nm thick) from a denoised cryo-electron tomogram showing a representative *C.trachomatis* EB in contact with a host cell. Scale bar, 110 nm. Right panel: Three-dimensional surface representation of the EB generated from segmentation of the cryo-electron tomogram. Cellular plasma membrane (yellow), bacterial outer membrane (green), inner membrane (cyan), inner membrane invagination (blue) and T3SS (red) are shown.

Figure 2. Early interactions between *Chlamydia trachomatis* EBs and host cells identified by cryo-electron tomography.

Schematic representation of the early stages of *Chlamydia trachomatis* entry into mammalian cells. Actin accumulation is shown in orange. Corresponding tomogram slices are shown beneath.

Figure 3. The pathogen synapse – a structure bridging the host rER to the RB envelope through the inclusion membrane.

Left panel: a single z-section from structured illumination high-resolution confocal microscopy (SIM) showing *Chlamydia trachomatis* RBs (green) at the inclusion periphery (indicated with a dotted yellow line). The sample is co-stained with an antibody against the needle component of the T3SS (red). Upper inset shows indicated RB at higher magnification. Lower inset shows a three-dimensional SIM reconstruction illustrating the T3SS polarised to the hemisphere of the RB facing the

inclusion periphery. Scale bar, 1 µm. Centre panel: shows a tomogram (average of 10 z-sections after reconstruction, alignment and de-noising) of a pathogen synapse. T3SS are evident traversing the chlamydial inner (IM) and outer (OM) membrane at a site where the rER contacts the cytoplasmic face of the inclusion membrane (IncM). Scale bar, 50 nm. Right panel: shows a mesh representation of the densities in the entire tomogram. T3SS core complexes from *Salmonella typhimurium* are fitted (red) and also shown in periplasmic cross section.

Figure 4. Possible roles for the host rER in *Chlamydia* inclusion biogenesis.

Schematic representation of a pathogen synapse (left) and additional rER contacts with the inclusion membrane (right) illustrating possible roles for the rER (dark green). T3SS (red) substrates in the RB are delivered across the bacterial inner (IM) and outer (OM) membrane via the T3SS translocon (grey) of unknown structure. Hydrophobic substrates, the inclusion proteins (Incs, purple), could be inserted into the rER directly, or via the eukaryotic Sec translocon (blue) / signal recognition particle receptor (pink) or Get complex (light green). Incs and rER-derived membrane are transported to the inclusion membrane independently of ER exit sites. Additional bacterial factors may influence the host ER stress (controlled via IRE-1 and ATF6) and ERAD responses. Lipid transfer, mediated by IncD binding to CERT-VAT occurs at additional sites on the inclusion membrane, where intimate contact is mediated by unidentified 'pin-like' complexes (grey), excluding the ribosomes that partition on the distal face of the apposed rER tubules.

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Figure 1

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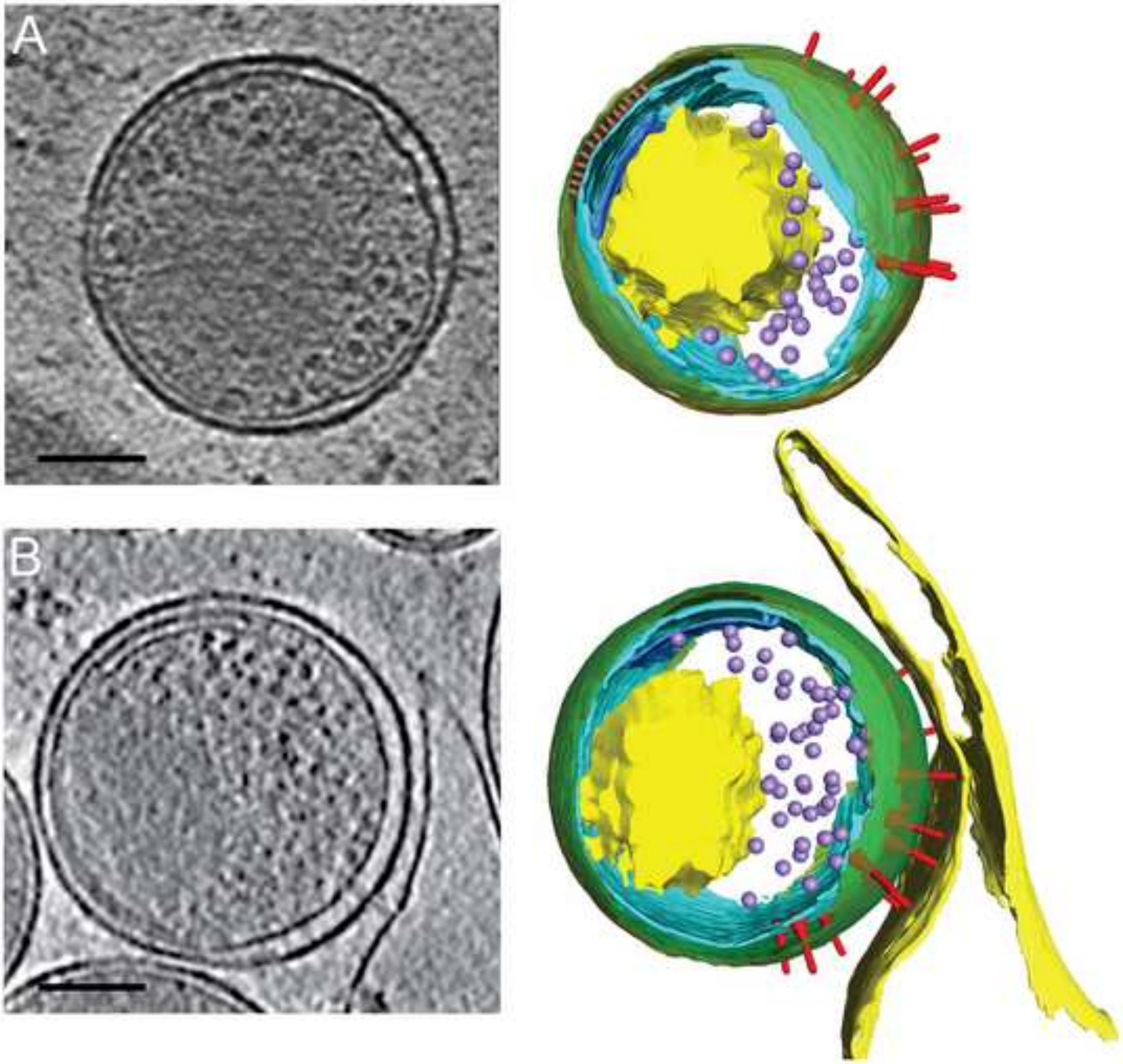

Figure 1

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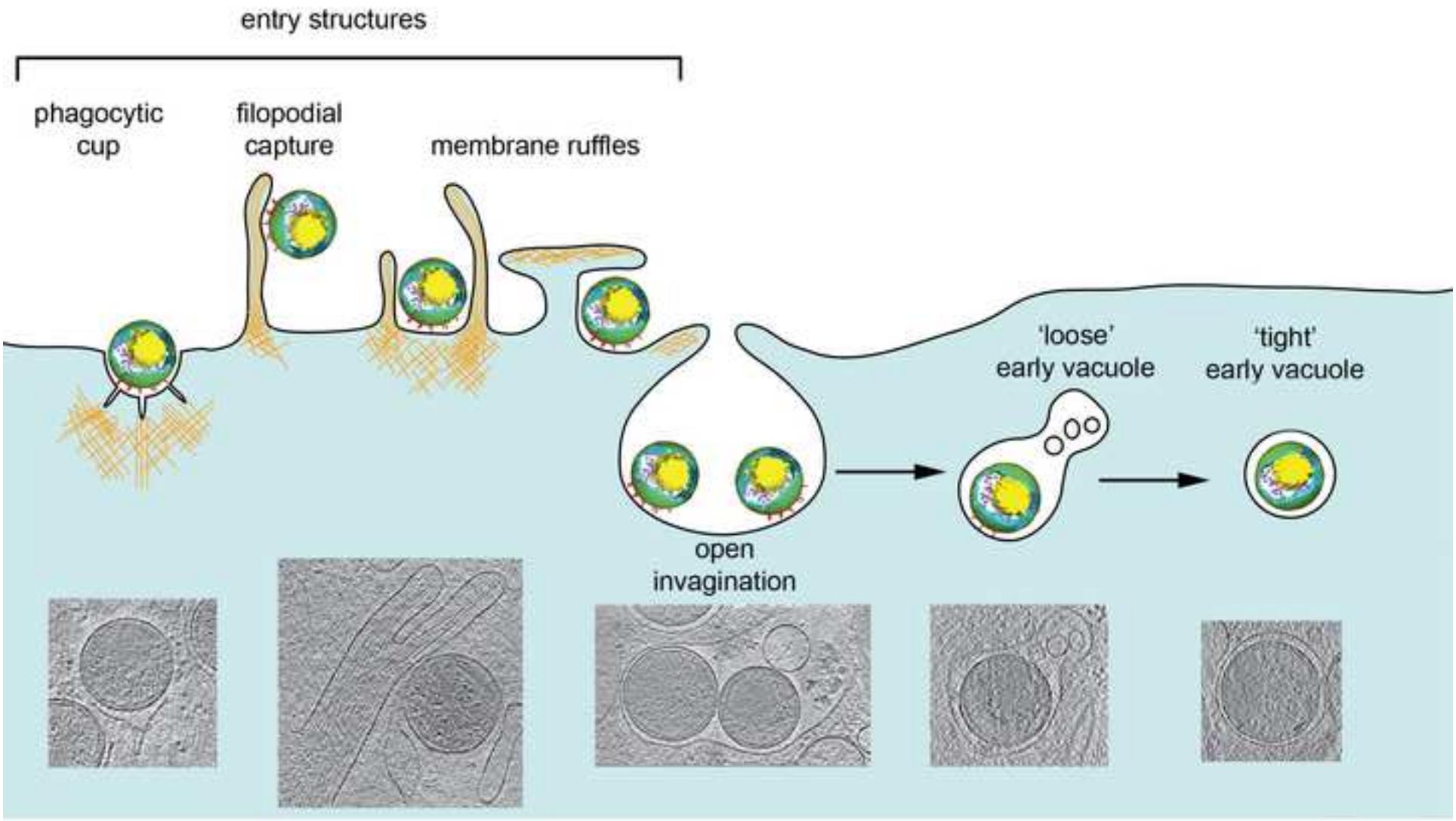

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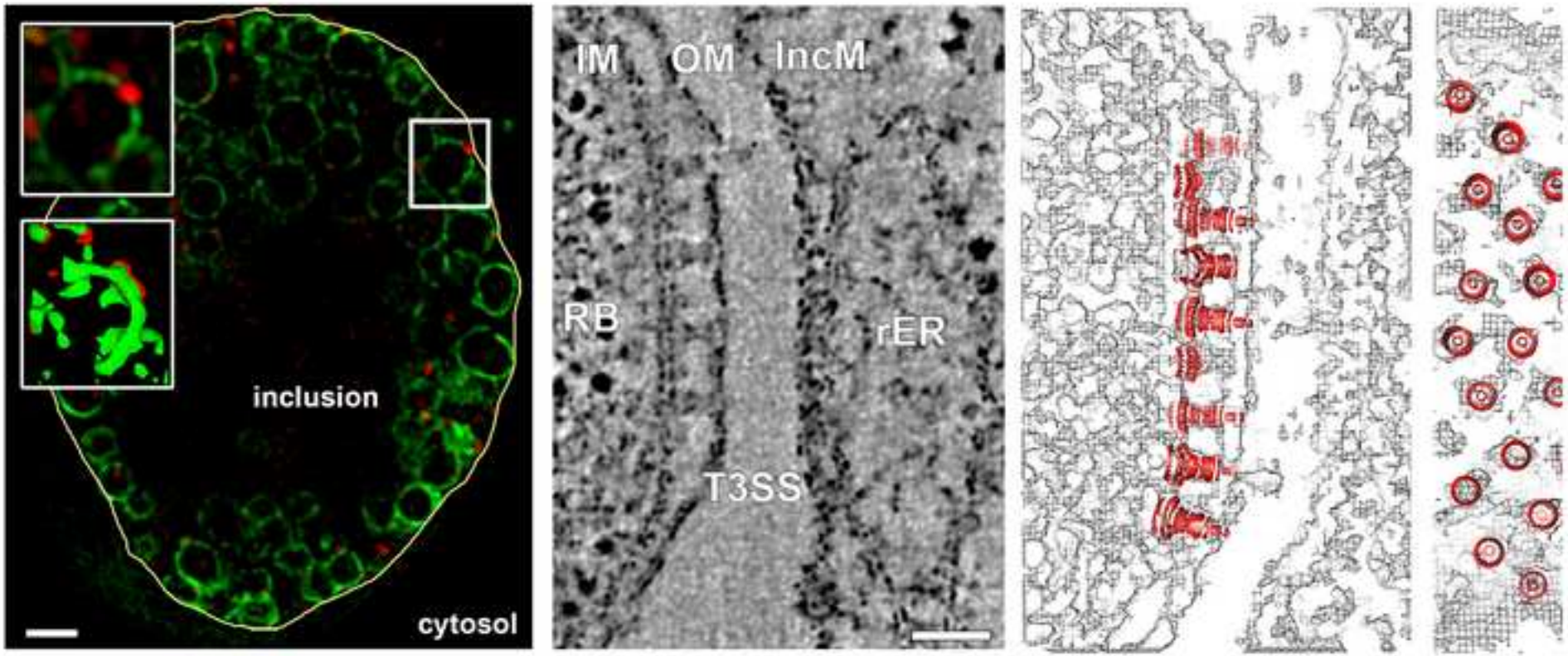

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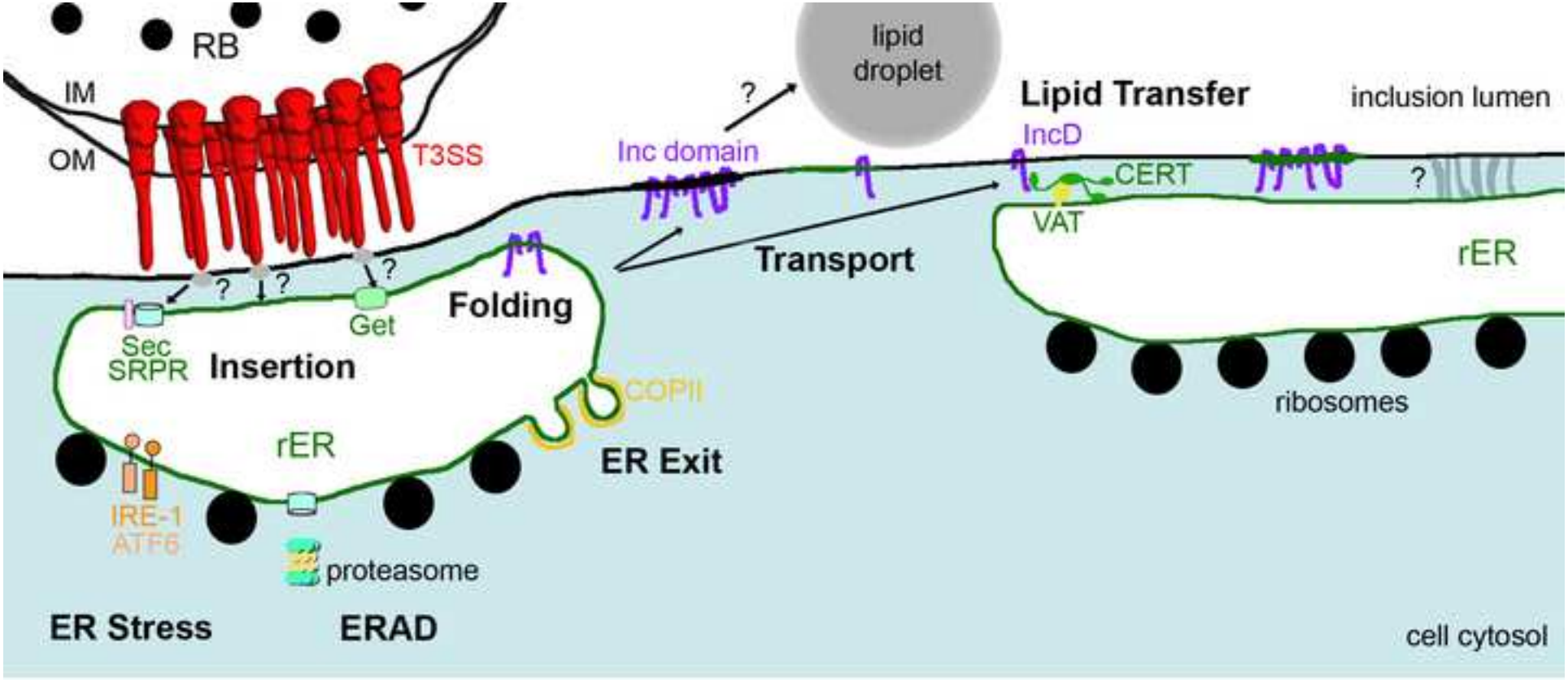

Figure 4

Highlights

- *Chlamydia* are polarised, with distinct membrane and protein structures in opposite hemispheres.
- Type 3 secretion systems form a polar array contacting the host plasma membrane.
- Pathogen synapses connect bacterial, inclusion and host endoplasmic reticulum membranes.

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Making connections: snapshots of chlamydial type III secretion systems in contact with host membranes

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Highlights

- *Chlamydia* are polarised, with distinct membrane and protein structures in opposite hemispheres.
- Type 3 secretion systems form a polar array contacting the host plasma membrane.
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Abstract

Chlamydiae **are obligate intracellular bacterial pathogens with an unusual biphasic lifecycle, which is underpinned by two bacterial forms of distinct structure and function. Bacterial entry and replication require a type III secretion system (T3SS), a widely conserved nanomachine responsible for the translocation of virulence effectors into host cells. Recent cell biology experiments supported by electron and cryo-electron tomography have provided fresh insights into *Chlamydia*-host interactions. In this review, we highlight some of the recent advances, particularly the *in situ* analysis of T3SSs in contact with host membranes during chlamydial entry and intracellular replication, and the role of the host rough endoplasmic reticulum at the recently described intracellular 'pathogen synapse'.**

Introduction

Although initially believed to be a virus [1], Gram-negative *Chlamydiae* were the first obligate intracellular bacteria with a biphasic lifecycle to be described [2]. *Chlamydiae* cause disease in humans and other animals, and in particular *Chlamydia trachomatis* remains the leading bacterial agent of sexually transmitted disease worldwide, while ocular infections cause blinding trachoma, which is designated as a neglected tropical disease by the World Health Organisation [3]. Studying *Chlamydiae* remains a challenge, since the bacteria cannot be cultured outside eukaryotic cells, and although transformation has recently been reported [4,5,6], there remains no routine methodology for directed mutagenesis. Many of the tools

that have driven the substantial advances in understanding the cellular microbiology of other bacterial pathogens still therefore remain inaccessible for *Chlamydiae*. Nevertheless, the developmental cycle can be reconstituted in the laboratory using cultured mammalian cells [7].

During the early stages of infection, extracellular infectious but metabolically inactive elementary bodies (EBs) adhere to the plasma membrane of the host cell and induce their own actin-dependent uptake into endocytic vacuoles. These early vacuoles coalesce and traffic to the microtubule-organising centre, forming a specialised membrane-bound compartment termed an inclusion. Within the inclusion, EBs differentiate into non-infectious but metabolically active reticulate bodies (RBs). RBs undergo a series of cell divisions before converting back into EBs, which are subsequently released from the cell by inclusion extrusion, or upon cell lysis [8].

EBs and RBs are not only distinct in function, but also in morphology. While both forms of the bacterium are coccoid, they differ significantly in size; EBs are 0.3-0.4µm in diameter in comparison to RBs at 1µm. Substantial changes in bacterial architecture therefore occur during EB-RB and RB-EB inter-conversion, which remain incompletely understood [8]. The most obvious distinguishing structural characteristic is the outer membrane, which is almost twice the thickness in EBs [9]. This is attributed to a disulphide-cross-linked network of major outer membrane proteins that confer the osmotic stability and rigidity of EBs [10]. By contrast, the disulphide bonds are reduced in RBs, allowing for greater membrane flexibility to

facilitate cell division [11]. Both EBs and RBs harbour type III secretion systems (T3SSs), nanomachines conserved among diverse Gram-negative bacterial pathogens. T3SSs translocate virulence effector proteins directly into host cells, where they subvert cellular processes to promote pathogen entry, survival or replication [12]. In this review, we will explore the relationship between the EB and RB T3SSs, their supramolecular organisation in contact with host membranes, and their contribution to sustaining the chlamydial lifecycle.

The chlamydial T3SS: the exception or the rule?

T3SSs are macromolecular complexes that span the bacterial envelope [13], first observed in *Salmonella* [14]. Subsequent single particle analysis of core complexes isolated from the membranes of diverse Gram-negative bacterial pathogens has revealed a conserved structure comprising oligomeric rings embedded in the inner and outer membranes connected by a cylindrical *trans*-periplasmic tube, enabling effector secretion without periplasmic intermediates. A helical 'needle' consisting of a single polymerised subunit connects proximally to the outer membrane ring and at the distal end to a translocon complex proposed to interact with a third membrane from the host [13]. The structure of this translocon and the nature of its interaction with the host membrane remain unknown, as it is never co-isolated with the detergent-solubilised core T3SS complex. *Chlamydiae* encode homologues of core complex components [15, 16, 17], yet in comparison to other pathogens in which the genes encoding T3SSs are grouped together on pathogenicity islands, T3SS-related genes are distributed across the genome in four distinct clusters composed of at

least ten separate operons [18]. Unusually, *Chlamydiae* also possess two copies of putative translocon components (CT578/CT579 and CT860/CT861) identified by primary sequence similarity to the *Yersinia* YopB and YopD translocon proteins [19], although the significance of this remains unresolved. Nevertheless, it is clear that the chlamydial T3SS is pivotal to virulence as T3SS inhibitors arrest the bacterial lifecycle [20, 21].

Polar organisation of T3SS arrays in *Chlamydia* EBs

Seminal early electron microscopy studies by Matsumoto identified surface projections and protein complexes termed 'rosettes' on the surface of *Chlamydia* EBs in the absence of host cells [*e.g.* 22]. These structures, observed well in advance of the identification of any T3SSs in bacteria, were only later proposed as T3SSs [23]. Indeed, the rosettes have also since been suggested to represent outer membrane protein complexes [24]. An elegant study by Peterson [25], also describes structures apparently connecting RBs to the inclusion membrane in chemically fixed sections by electron microscopy. These structures were similarly proposed to be T3SSs but had never been experimentally identified or examined in detail [23]. [Early freeze-fracture electron microscopy by Matsumoto suggested that *Chlamydia* EBs and RBs both exhibited macromolecular surface projections [*e.g.* 22, 23], although these structures and any relationship between them were not identified at that time] Recently, we applied cryo-electron tomography to examine EB structure in greater detail (**Figure 1A**) [26]. This revealed that EBs are polarised, whereby one hemisphere is characterised by pronounced expansion of the periplasmic space (~29nm compared to ~14nm on the opposite pole), which

accommodates an array of 14-20 T3SSs, definitively identified by immunogold labelling. While the EB outer membrane remains rigid, each T3SS complex originates at a specific concave deformation of the inner membrane [26]. The opposite pole with the narrower periplasmic space contains additional complexes of distinct morphology and as yet unknown composition, in addition to an invagination of the inner membrane [26], reminiscent of the complex and atypical membrane structures present in other members of the *Planctomycetes-Verrucomicrobia-Chlamydiae* [27].

In the presence of host cells, EBs universally orient with their T3SS array facing the target cell plasma membrane with which they engage (**Figure 1B**) [26]. This co-ordinated alignment might be determined by prior engagement of host receptors or polysaccharides by polymorphic membrane proteins or outer membrane proteins such as OmcB [28, 29], which may also be similarly polarised on the EB surface. The membrane-engaged battery of T3SSs would enable the rapid coordinated delivery of a high local effector dose to trigger bacterial entry. Snapshots of the chlamydial entry process, captured by cryo-electron tomography, revealed an unexpected diversity of early host structures engaging EBs ranging from phagocytic cups, to filopodial capture events and complex ruffle-like plasma membrane invaginations (**Figure 2**) [26]. These cellular structures are compatible with a role for Rac1- and Arf6-dependent GTPase signalling events [30, 31], stimulated in part by the translocated effectors CT166 and Tarp [32, 33], although whether these captured intermediates represent sequential assemblies in a single pathway or denote multiple independent entry mechanisms requires further investigation by live imaging approaches. While the resulting membrane invaginations that remain

accessible to the extracellular milieu frequently contain multiple EBs, it is striking that the majority of closed early vacuoles only encapsulate individual EBs [26]. In the first few hours after internalisation this apparent sorting is also accompanied by reorganisation of both the bacterial and host vacuolar membranes. The vacuole membrane that initially loosely encloses the EB and co-envelopes host material transitions to form a tight structure proximal to the EB surface. During this time, the EBs lose their polarity, with an associated reduction of the pronounced periplasmic widening and a decrease in assembled T3SSs (**Figure 2**) [26].

Pathogen synapses: ordered connections between the T3SS, the inclusion membrane and the host endoplasmic reticulum

Following internalisation [deleted text], the inclusion must be diverted from the cellular endocytic system to prevent degradation, yet nutrients must be selectively scavenged from the host cell and efficiently transported across the inclusion membrane to enable bacterial differentiation into RBs and subsequent replication [8]. *Chlamydiae* reassemble their T3SSs to control inclusion biogenesis, by delivering effectors that are integrated into the inclusion membrane or delivered beyond into the host cell cytosol and nucleus [8]. In particular, hydrophobic inclusion proteins (Incs) are a family of T3SS substrates that localise to the inclusion membrane during infection [34]. Although most of their underlying effector mechanisms remain undefined, they are likely involved in the active hijack of host components and organelles including lipid droplets [35], Golgi-derived vesicles [36], multivesicular bodies [37], cytoskeletal components [38], and the rough endoplasmic reticulum

(rER) at the inclusion membrane [39, 40, 41]. Indeed, IncD indirectly recruits rER to the inclusion membrane [39], while additional Incs engage key mediators of intracellular trafficking and apoptosis [42, 43, 44].

This phase of nutrient acquisition and effector translocation commences in the mid-stage of the lifecycle, and coincides with rER recruitment to the inclusion membrane [41]. Multiple host proteins located in the rER lumen or membrane are enriched in patches at the inclusion periphery and a subset are present within the inclusion lumen. Disruption of the rER using aerolysin toxin at timepoints prior to its recruitment stalls inclusion biogenesis, whereas later treatment, at timepoints when association is normally observed, bursts the inclusion [41]. Mature chlamydial inclusions therefore gain sufficient ER-like character to render them susceptible to the toxin. Electron tomography revealed intimate and extensive apposition of the ribosome-studded rER and the cytoplasmic face of the inclusion membrane. These regions of contact, tethered by 'pin-like' complexes of as yet unknown composition, appear so tight that host ribosomes are always partitioned onto the inclusion distal side of the rER tubules. Tomograms of RBs at the inclusion periphery also revealed a polar array of 20-100 T3SSs in contact with the luminal face of the inclusion membrane, specifically formed at sites coincident with rER recruitment on the cytoplasmic face of the inclusion (**Figure 3**). These structures bridging the rER in the host cytosol to the RB envelope through the inclusion membrane are termed 'pathogen synapses' [41]. Intriguingly, as with EBs at the plasma membrane, RBs also engage host membranes with an ordered supramolecular array of T3SSs. Indeed, it is

possible that the RB pathogen synapse establishes a template for the polar array of T3SSs present in EBs following redifferentiation.

Host rER: a membrane source for inclusion growth and [sophisticated] receiver for hydrophobic T3SS substrates?

Although only recently recognised for *Chlamydiae* [39, 40, 41], incorporation of rER membranes into pathogen-containing vacuoles is not without precedent. It is likely that chlamydial acquisition of rER-derived membrane contributes to the progressive expansion of the inclusion as the RBs within divide, and that the conferred lipid composition of the inclusion membrane may also regulate the association of hydrophilic chlamydial or host proteins [with the inclusion membrane] [45], as with *Legionella* and *Brucella* generated compartments [46, 47]. In addition to this more structural role, *Chlamydiae* scavenge lipids including sphingomyelin and cholesterol from the secretory pathway for metabolism [36], in part by harnessing ER-localised CERT-VAT lipid transporters engaged by IncD from the inclusion membrane [39, 40]. *Chlamydiae* may also modulate key rER functions including the ER stress response and ER-associated protein degradation (ERAD), possibly to prevent host alarm signals and suppress antigen presentation (**Figure 4**).

The rER at pathogen synapses could also perform a more direct role. The plethora of hydrophobic Incs encoded by *Chlamydiae* prompts the question how these atypical T3SS substrates fold and insert into the inclusion membrane following their translocation. The rER contains both the Sec translocon and the Get complex [48],

responsible for the insertion of eukaryotic transmembrane proteins, together with luminal chaperones required for protein folding [49]. It is tempting to speculate that the rER might act as a 'receiver membrane' for the incoming hydrophobic substrates from the T3SS array, and that eukaryotic machinery might be co-opted to catalyse their folding and insertion (**Figure 4**). Furthermore, subsequent local trafficking of rER membrane from synapses into the inclusion may drive the incorporation of Incs into the inclusion membrane, although this seems not to involve COPII-dependent transport from rER exit sites [40, 41], which are subverted by *Legionella* [50]. Many Incs remain resident within ER membranes when exogenously expressed in cultured cells [51]. This is unusual, as most membrane proteins would transit into the secretory pathway by default. Differential residency within ER-like membrane could therefore potentially influence the lateral positioning of Incs within the inclusion membrane, in turn regulating Inc-Inc or Inc-host target interactions. Indeed, some Incs do apparently partition into microdomains [52], whereas others are distributed around the entire circumference of the inclusion membrane.

Conclusions

Recent studies of *Chlamydiae* have provided intriguing new insights into the supramolecular architecture of T3SSs and the nature of their interaction with host membranes, which act as critical interfaces between pathogen and host. **These studies complement and extend the earlier pioneering work of Matsumoto and Peterson, and confirm the presence of T3SSs [22, 23, 25].** Imaging of EBs and RBs by electron tomography has allowed large numbers of assembled T3SSs to be captured

in association with host membranes for the first time in any infection system [26, 41]. This provides an opportunity to visualise the T3SS translocon within the host membrane and other details of assembled T3SS structure *in situ*. Sub-tomogram averaging of T3SSs in *Yersinia* in the absence of host cells has already revealed subtle alterations in T3SS interaction with the bacterial envelope when compared to the *in vitro* isolated core complexes [53]. Further work is now required to understand how EB polarity and orientation is determined and the location of other proteins, particularly adhesins, in relation to the T3SS array and inner membrane invagination. The identification of the pathogen synapse [41] raises intriguing questions about the role of the host rER in chlamydial infection and potentially in the insertion and folding of the hydrophobic Inc substrates of the T3SS. Although historically difficult to study, there is clearly much more to learn from the enigmatic *Chlamydiae*.

Acknowledgements

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Figure Legends

Figure 1. Polarised structure of the *Chlamydia trachomatis* EB

A. Left panel: *xy* tomographic slice (0.71 nm thick) from a denoised cryo-electron tomogram of a representative *C.trachomatis* EB. Scale bar, 100 nm. Right panel: Three-dimensional surface representation of the EB generated from segmentation of a cryo-electron tomogram. Outer membrane (green), inner membrane (cyan and blue for the inner membrane invagination), T3SS (red), nucleoid (yellow), additional periplasmic complexes (brown) and ribosomes (purple) are shown.

B. Left panel: *xy* tomographic slice (0.71 nm thick) from a denoised cryo-electron tomogram showing a representative *C.trachomatis* EB in contact with a host cell. Scale bar, 110 nm. Right panel: Three-dimensional surface representation of the EB generated from segmentation of the cryo-electron tomogram. Cellular plasma membrane (yellow), bacterial outer membrane (green), inner membrane (cyan), inner membrane invagination (blue) and T3SS (red) are shown.

Figure 2. Early interactions between *Chlamydia trachomatis* EBs and host cells identified by cryo-electron tomography.

Schematic representation of the early stages of *Chlamydia trachomatis* entry into mammalian cells. Actin accumulation is shown in orange. Corresponding tomogram slices are shown beneath.

Figure 3. The pathogen synapse – a structure bridging the host rER to the RB envelope through the inclusion membrane.

Left panel: a single z-section from structured illumination high-resolution confocal microscopy (SIM) showing *Chlamydia trachomatis* RBs (green) at the inclusion periphery (indicated with a dotted yellow line). The sample is co-stained with an

antibody against the needle component of the T3SS (red). Upper inset shows indicated RB at higher magnification. Lower inset shows a three-dimensional SIM reconstruction illustrating the T3SS polarised to the hemisphere of the RB facing the inclusion periphery. Scale bar, 1 µm. Centre panel: shows a tomogram (average of 10 z-sections after reconstruction, alignment and de-noising) of a pathogen synapse. T3SS are evident traversing the chlamydial inner (IM) and outer (OM) membrane at a site where the rER contacts the cytoplasmic face of the inclusion membrane (IncM). Scale bar, 50 nm. Right panel: shows a mesh representation of the densities in the entire tomogram. T3SS core complexes from *Salmonella typhimurium* are fitted (red) and also shown in periplasmic cross section.

Figure 4. Possible roles for the host rER in *Chlamydia* inclusion biogenesis.

Schematic representation of a pathogen synapse (left) and additional rER contacts with the inclusion membrane (right) illustrating possible roles for the rER (dark green). T3SS (red) substrates in the RB are delivered across the bacterial inner (IM) and outer (OM) membrane via the T3SS translocon (grey) of unknown structure. Hydrophobic substrates, the inclusion proteins (Incs, purple), could be inserted into the rER directly, or via the eukaryotic Sec translocon (blue) / signal recognition particle receptor (pink) or Get complex (light green). Incs and rER-derived membrane are transported to the inclusion membrane independently of ER exit sites. Additional bacterial factors may influence the host ER stress (controlled via IRE-1 and ATF6) and ERAD responses. Lipid transfer, mediated by IncD binding to CERT-VAT occurs at additional sites on the inclusion membrane, where intimate contact is mediated by

unidentified ‘pin-like’ complexes (grey), excluding the ribosomes that partition on the distal face of the apposed rER tubules.

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