Chapter 8
Exit Strategies of *Ehrlichia*

Sunil Thomas

1 Introduction

The entry and exit of intracellular pathogens from host cells are crucial steps in the infectious cycle; however, the mechanism is not clearly understood in many intracellular pathogens. It has recently emerged that microbial exit is a process that can be directed by organisms from within the cell, and is not simply a consequence of the physical or metabolic burden that is imposed on the host cell (Hybiske and Stephens 2007, 2008).

Ehrlichia are obligately intracellular bacterium that thrives in the monocytes and macrophages and are transmitted to vertebrates through tick bites. Human monocytotropic ehrlichiosis (HME) was first reported in 1987 (Maeda et al. 1987). Since then, development of murine models of persistent and lethal ehrlichiosis has greatly facilitated understanding of the pathogenesis and mechanisms of host defenses against ehrlichial infections. In general, microorganisms can disseminate after host cell lysis via necrotic or apoptotic cell death, or by spreading from cell to cell (Hagedorn et al. 2009). Until recently, the mechanism by which *Ehrlichia* are released from host cells or how they gain entry into cells was not demonstrated (Li and Winslow 2003; Maender and Tyring 2004; Rikihisa 2010a, b).

Recently, in a mouse model of monocytotropic ehrlichiosis, we demonstrated by eastern blotting that the heat shock protein 60 (Hsp60/GroEL) is highly post-translationally modified in the non-virulent *E. muris*, compared to the highly virulent strain IOE (*Ixodes ovatus* ehrlichia) (Thomas et al. 2009). Based on this observation we generated an anti-*Ehrlichia*-specific Hsp60 antibody and used it to observe *E. chaffeensis*, *E. muris*, or IOE in cell culture. This chapter explains the exit strategies of the intracellular bacterium *Ehrlichia*.

S. Thomas (✉)
Lankenau Institute for Medical Research, Wynnewood, PA 19096, USA
e-mail: suntom2@gmail.com

S. Thomas (ed.), *Rickettsiales*, DOI 10.1007/978-3-319-46859-4_8

2 Morphology of *Ehrlichia*

Before delving to the exit strategies of *Ehrlichia*, it is imperative to know the morphology of the pathogen. *Ehrlichia* are Gram-negative bacteria, usually round or ovoid, non-motile bacteria that reside and grow in cytoplasmic vacuoles derived from an early endosome, forming loose to condensed aggregates of bacteria termed morula (morula = mulberry-like structure) in monocytes and macrophages (Paddock and Childs 2003) (Fig. 8.1). The bacteria stain dark blue to purple with Romanovsky-type stains (Fig. 8.2). Though most of the strains of *Ehrlichia* are observed in monocytes and macrophages, *Ehrlichia ewingii* infects neutrophils. Infection with *E. ewingii* may delay neutrophil apoptosis (Xiong et al. 2008). The key to the successful survival inside the host cell is by preventing fusion of the phagosome and lysosome (Barnewall et al. 1997). Another factor that contributes to the successful intracellular survival of *Ehrlichia* is inhibition of host cell apoptosis. *Ehrlichia* morulae also interact with mitochondria and inhibit mitochondrial metabolism (Liu et al. 2011).

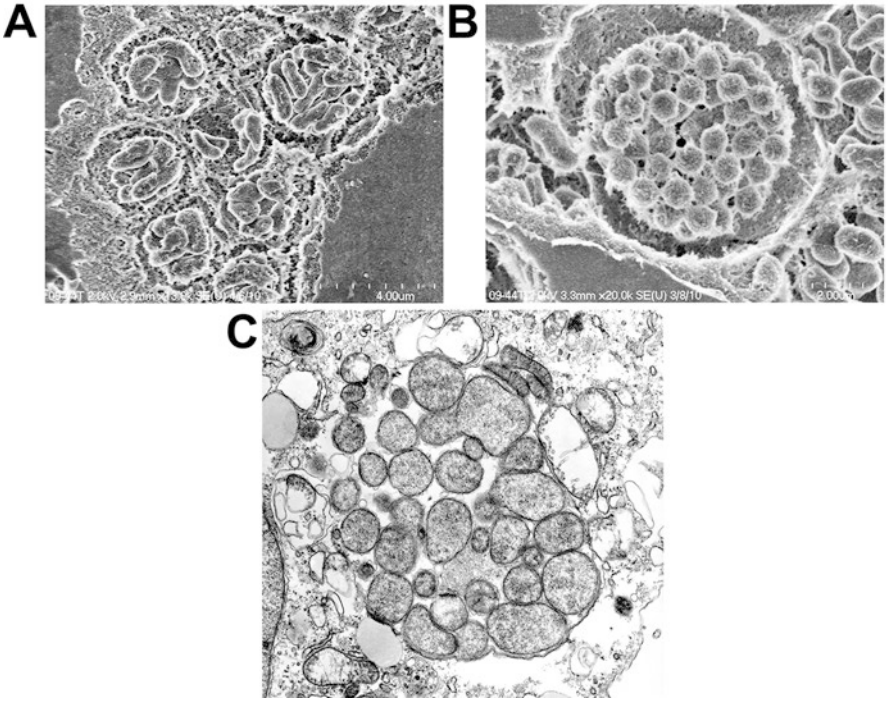

Fig. 8.1 Morula of *Ehrlichia muris*. Scanning electron micrograph of *E. muris* morula in DH82 host cells (**a**) reticulate cells and (**b**) dense cored cells. (**c**) Transmission electron micrograph of *E. muris* morula (reticulate cells) (Original image: magnification X32,000)

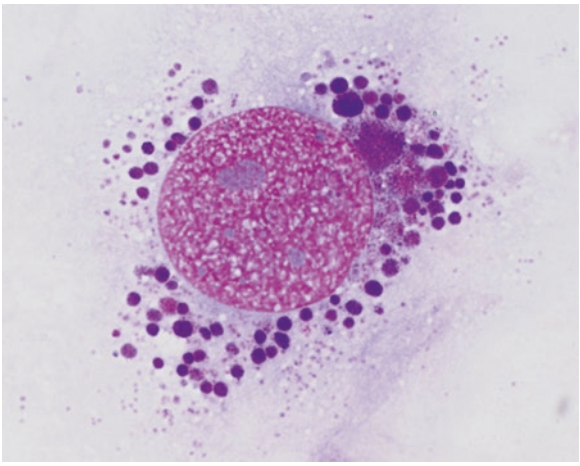

Fig. 8.2 *E. muris*-infected DH82 cell stained with Diff-Quik stain. The purple-colored *E. muris* morulae surround the host nucleus

Two distinct morphologic forms of *Ehrlichia* are identified in host cells, reticulate and dense-cored cells. The reticulate cells measure 0.7–1.9 μm, are of pleomorphic morphology with DNA and ribosomes distributed throughout the bacterial cytoplasm (Fig. 8.3). Whereas, the infectious dense-cored cells are predominantly coccoid bacteria and measure 0.4–0.6 μm in diameter, characterized by concentration of ribosomes and chromatin, and these predominate at early and late time points of infection (Fig. 8.4). Both cell types replicate by binary fission, and both demonstrate a Gram-negative-type cell wall, characterized by a smooth-contoured cytoplasmic membrane and a generally ruffled outer membrane, separated by a periplasmic space. The infectious dense-cored form of *Ehrlichia* transforms into reticulate forms 2–4 days post-infection, and later they undergo binary fission for about 2 days. Intermediate forms are seen 4–5 days post-infection and large numbers of dense-cored cells are observed 5–6 days after infection. The host cells rupture after 6–7 days of infection. Morulae range from 1.0 to 6.0 μm in width and contain 1 to >40 organisms of uniform or mixed cell types (Paddock and Childs 2003; Zhang et al. 2007). The intramorular space may contain a fine, striated fibrillar matrix that may be fibrillar ehrlichial antigen apparently shed from the surface of the cell wall (Fig. 8.5) (Popov et al. 1995; Paddock et al. 1997). The cellular morphology of *Ehrlichia* at several stages of its growth is shown in Fig. 8.6. In cell culture and infected human cells, host cell mitochondria are frequently apposed to the margins of morulae (Paddock and Childs 2003). Unlike other Gram-negative bacteria, *E. chaffeensis* lacks the genes for the biosynthesis of lipopolysaccharide (LPS) and most genes for the biosynthesis of peptidoglycan; thus, it does not produce LPS or peptidoglycan (Lin and Rikihisa 2003).

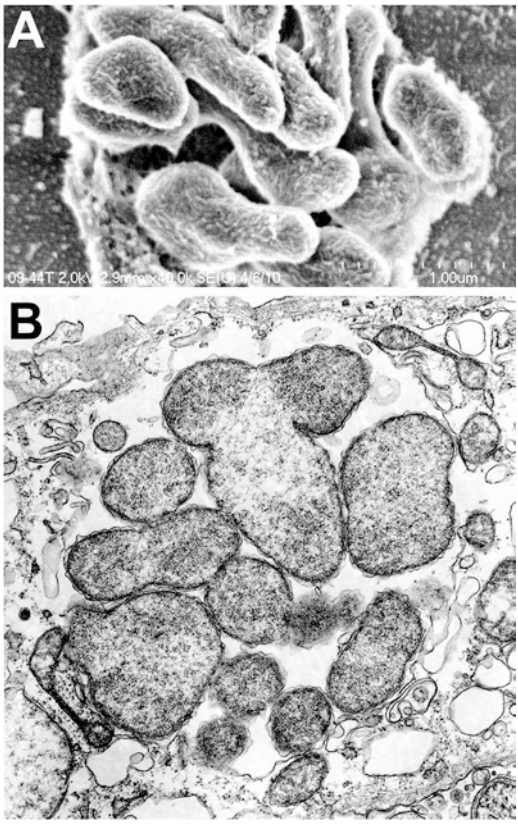

Fig. 8.3 Reticulate cells of *Ehrlichia muris*. (**a**) Scanning electron micrograph of *E. muris* reticulate cells and (**b**) Transmission electron micrograph of *E. muris* reticulate cells (Original image: magnification X47,000)

3 *Ehrlichia* Induce Filopodia in Infected DH82 Cells

The intracellular pathogens *E. chaffeensis* and *E. muris* are maintained in vitro in the DH82 monocyte cell line. Usually *Ehrlichia* are observed under a microscope after infection of uninfected DH82 cells with the bacterial pathogen. The 60 % confluent uninfected DH82 cells are seeded with *Ehrlichia*-infected DH82 cells and incubated for a further 3–4 days, when the DH82 cells achieve high confluence (no void between host cells), and all of them infected with *Ehrlichia. Ehrlichia* was observed after seeding around 1000 infected DH82 cells per slide so that they were separated from one another after 16 h of culture. After 16 h culture, the *E. muris*- and *E. chaffeensis*-infected DH82 cells were probed with the *Ehrlichia* Hsp60 antibody. By 16 h filopodia were observed in 30 % of DH82 cells infected with *Ehrlichia* (3 % in uninfected DH82 cells; $p < 0.0001$). Filopodia were extended from the polar ends of spindle-shaped *Ehrlichia*-infected host cells (*E. chaffeensis*: Fig. 8.7a; *E. muris*: Fig. 8.8a–d; uninfected DH82 cell: Figs. 8.7d and 8.8e; *E. muris*-infected

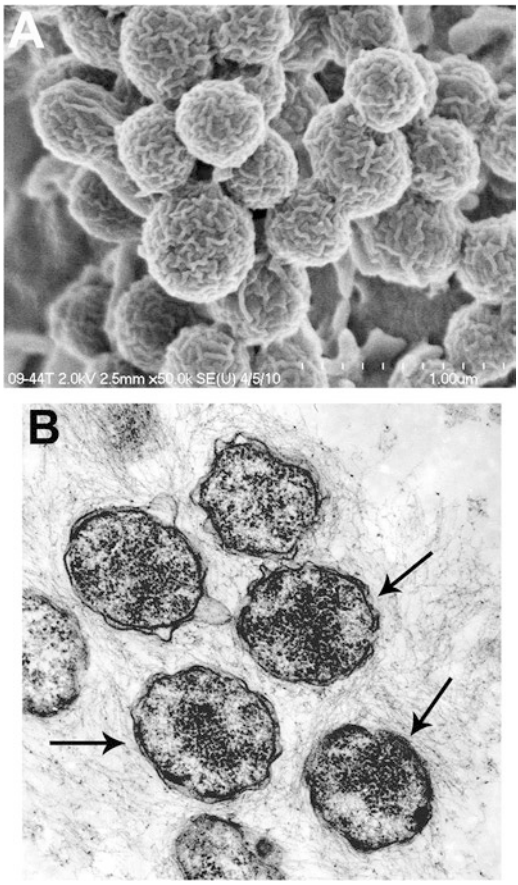

Fig. 8.4 Dense cored cells of *Ehrlichia*. (**a**) Scanning electron micrograph of *E. muris* dense cored cells and (**b**) Transmission electron micrograph of IOE dense cored cells (Original image: magnification X47,000)

DH82 cell without primary antibody: Fig. 8.7f) or from the non-polar sides of the cells when they contained many bacteria (*E. chaffeensis*: Fig. 8.7b). Filopodia of infected cells extended to the neighboring host cell (*E. chaffeensis*: Fig. 8.7b, *E. muris*: Fig. 8.8d). If host cells were not present in the vicinity of an infected cell, the leading edge of the filopodia of *Ehrlichia*-infected cells formed a flattened fan-shaped structure where the *Ehrlichia* cell cluster were contained (Fig. 8.7c). The bacterial cell cluster containing fan-shaped structure further developed its own filopodium (not shown). It is not known how the bacterial cell cluster-filled fan-shaped structure disintegrates and the contents released. Nevertheless, we have observed cluster of bacterial cells in vitro lying on host cells which we speculate are released on disintegration of the fan-shaped structure at the end of filopodium (Fig. 8.9).

Macrophage filopodia contain a meshwork of actin filaments and surround foreign organisms during phagocytosis (Hartwig et al. 1977); and cytochalasin D is known to inhibit actin polymerization (Rosania and Swanson 1996). Phalloidin has

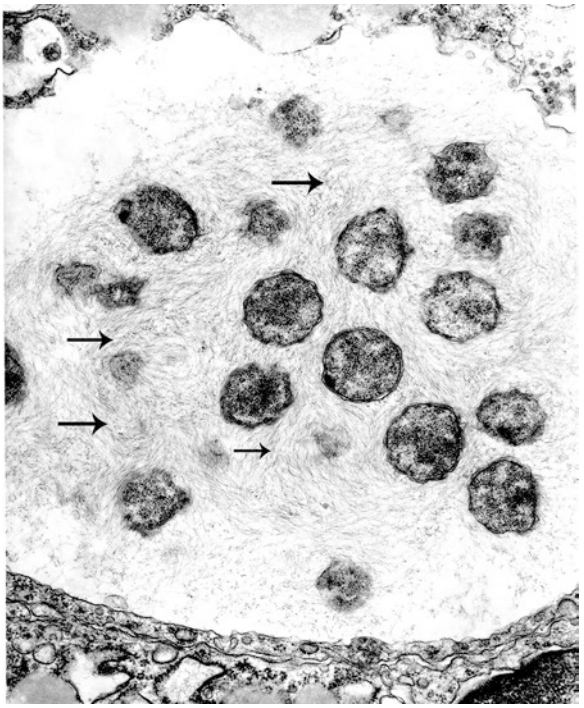

Fig. 8.5 Fine striated fibrilles are visualized in the morular matrix of IOE (*Arrows*) (Original image: magnification X47,000)

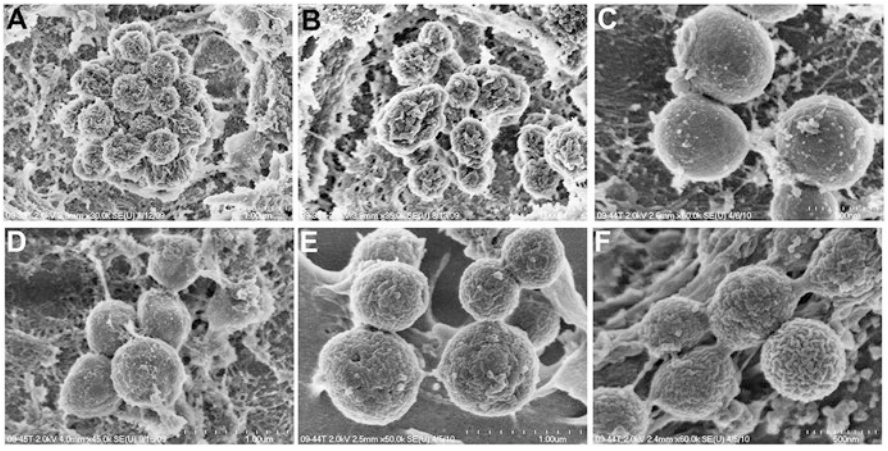

Fig. 8.6 Scanning electron micrographic collage of the morphology of *Ehrlichia*. (**a**–**c**) *E. chaffeensis*. (**d**–**f**) *E. muris*. Note the cell wall of the bacteria during various stages of division

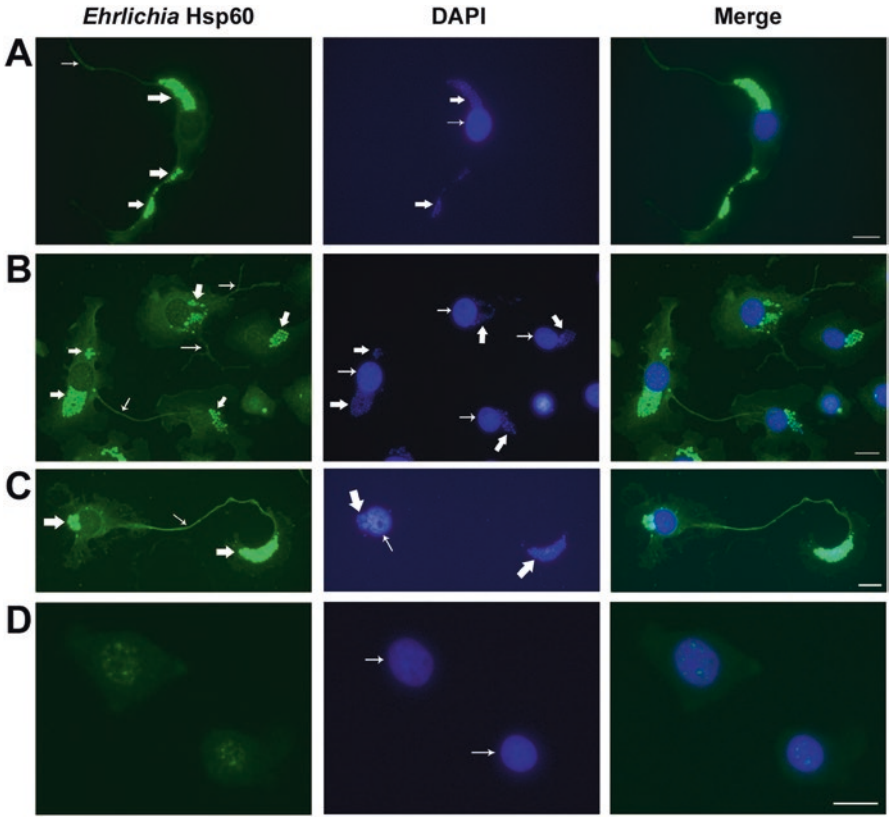

Fig. 8.7 *Ehrlichia* are contained in the filopodia of DH82 cells. (**a**) Filopodia extended from the polar ends of the *E. chaffeensis*-infected DH82 cell. *Left*: *E. chaffeensis*-infected DH82 cell probed with anti-Hsp60 antibody. *Thick arrow* indicates *E. chaffeensis* intracellular colonies and *thin arrow* indicates filopodium. *Middle*: *E. chaffeensis*-infected cell stained with DAPI. *Thick arrow* indicates morulae of *E. chaffeensis* stained with DAPI and *thin arrow* indicates host nucleus. *Right*: Merged figure. Scale bar, 25 μm. (**b**) Filopodia of *E. chaffeensis*-infected DH82 cells extended to neighboring cells. (**c**) When host cells were not in the immediate vicinity, the leading edge of an *E. chaffeensis*-infected DH82 cell formed a flattened fan-shaped structure filled with the pathogen. (**d**) Uninfected DH82 cell

a high affinity for actin, and phalloidin conjugated to Alexa 594 are used to detect actin in the filopodia. Filopodia stained with phalloidin-Alexa 594 were intensely red, whereas DAPI stained the host nucleus as well as the DNA of *E. chaffeensis* (Fig. 8.10a–d, uninfected DH82 cell: Fig. 8.10e). Filopodia formation was observed within an hour after culturing the infected DH82 cells. By 24 h the average length of a filopodium in infected cells was 120 μm (Fig. 8.10f). Filopodia measuring more than 10 times longer than the diameter of the host cell are sometimes observed during *Ehrlichia* infection. The filopodium is normally used by the macrophages or monocytes to probe for microorganisms and engulf them. However, the infectious

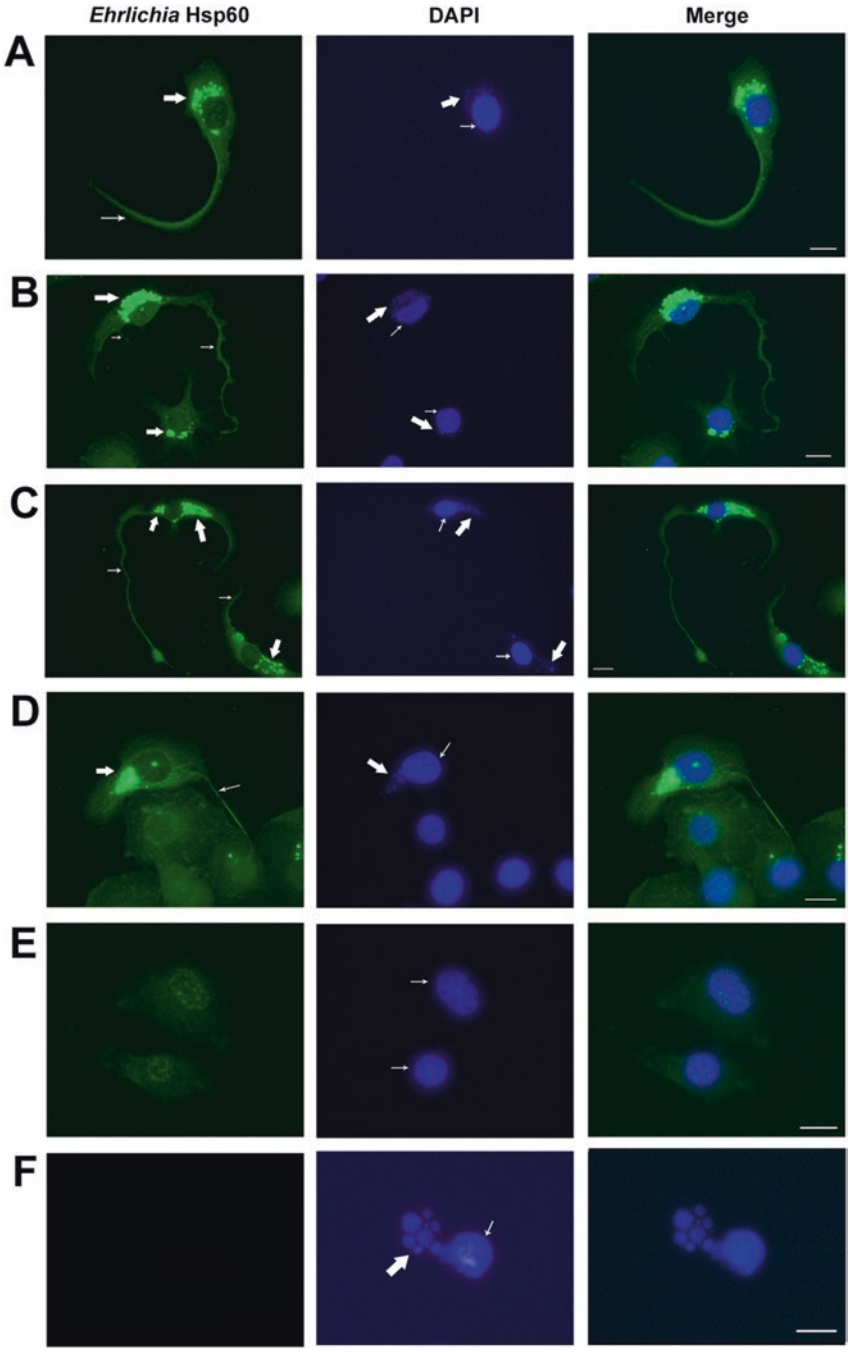

Fig. 8.8 *E. muris* is associated with the filopodia of DH82 cells. (**a**–**c**) Filopodium extending from the cell body of an *E. muris*-infected DH82 cell. *Left*: *E. muris*-infected DH82 cell probed with anti-*Ehrlichia* Hsp60 antibody. *Thick arrow* indicates *E. muris*, and *thin arrow* indicates filopodium.

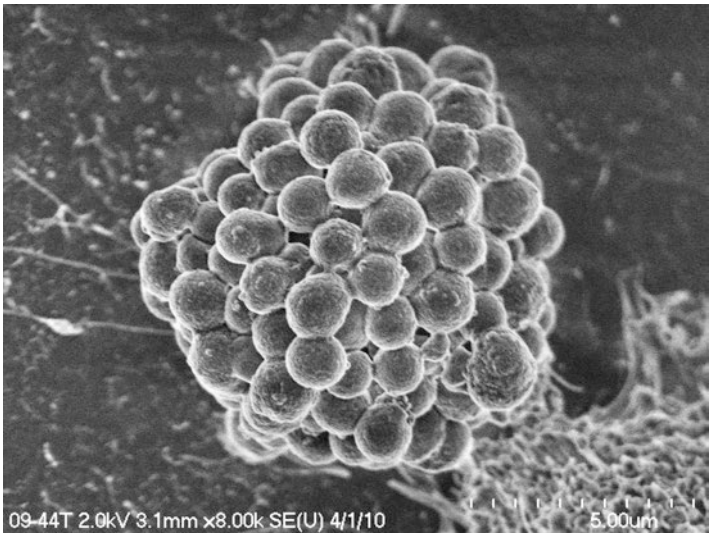

Fig. 8.9 SEM of a cluster of *E. chaffeensis* cells observed in vitro on DH82 cells. The fan-shaped structure at the end of the filopodium may disintegrate thereby releasing the cluster of bacterial cells

pathogens may use the filopodia to enter into host cells. The ultimate goal of any organism is to replicate and disseminate its progeny; microorganisms including bacteria are no exception. *Ehrlichia* bacteria use the filpodia as a vehicle to attach to neighboring cells. The major advantage of using the filopodia is non-recognition of the pathogens by the host immune system, thus evading death.

It is not possible to view the inside contents of a cell by scanning electron microscope (SEM). Hence, Scotch tape is used to mechanically break open the host cells and to observe the *Ehrlichia* inside DH82 cells by SEM. SEM of the mechanically opened cells demonstrated the presence of *Ehrlichia* in the filopodia of the DH82 host cells (Fig. 8.11a–f). On contact with a new host cell, the pathogens from the fan-shaped flattened structure (observed at the tip of the filopodium) were in a location where they can pass to the neighboring cell (Fig. 8.11e, f). Further, observation of cell membranes deformed from within by intracellular ehrlichiae revealed the opportunity for bacterial intrusion into the adjacent cells (Fig. 8.11g–i). These observations suggested that *Ehrlichia* passed from one host cell to another without entering the extracellular space.

Fig. 8.8 (continued) *Middle*: *E. muris*-infected cell stained with DAPI. *Thick arrow* indicates DNA of *E. muris* stained with DAPI, and *thin arrow* indicates host nucleus. *Right*: Merged figure. Scale bar, 25 μm. (**d**) Filopodium of an *E. muris*-infected DH82 cell extended to a neighboring cell. (**e**) Uninfected DH82 cells. (**f**) Absence of *Ehrlichia* Hsp60 primary antibody resulted in absence of staining *E. muris* in infected DH82 cells, but DAPI stained the *E. muris* DNA and DH82 nucleus

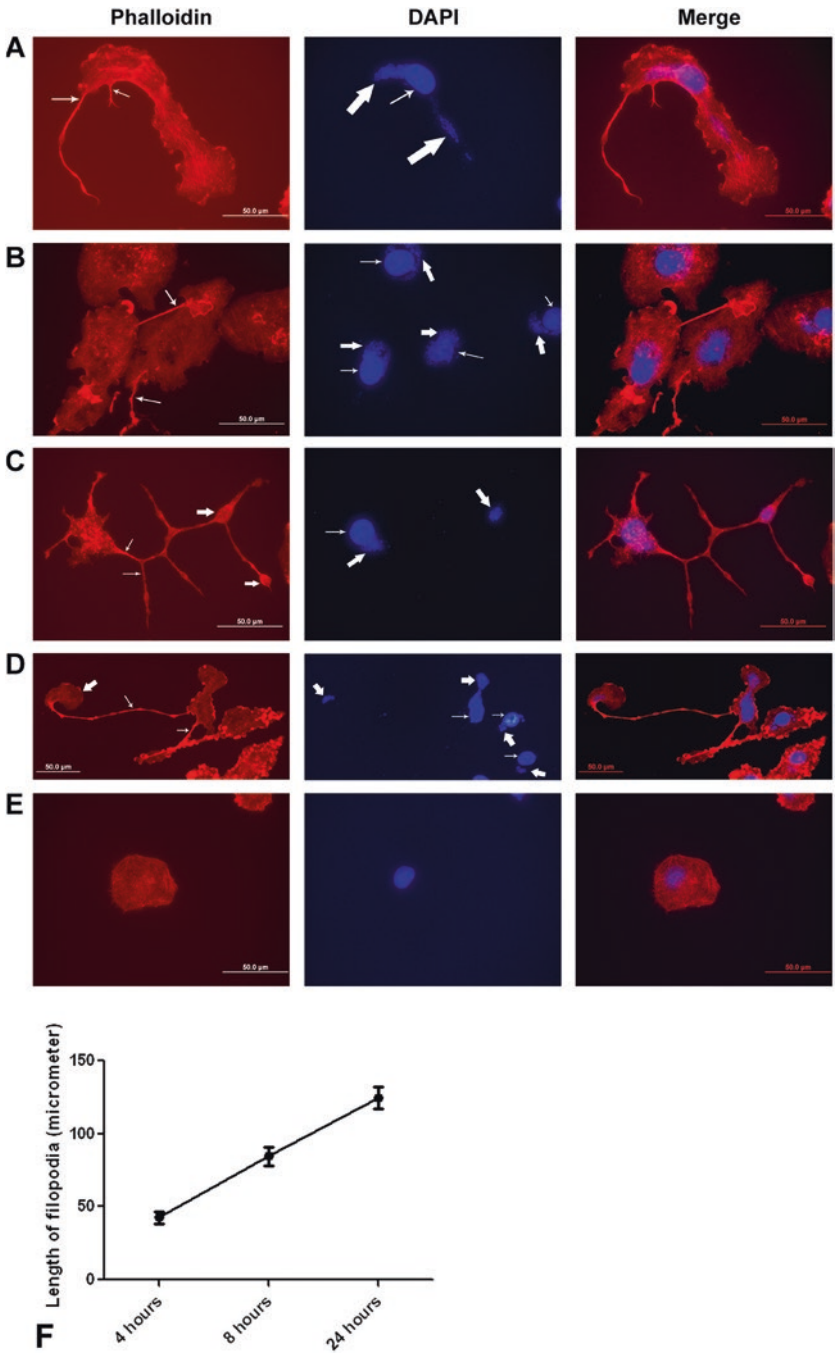

Fig. 8.10 Actin is a major protein of filopodia induced during *Ehrlichia chaffeensis* infection. *Left*: *E. chaffeensis*-infected DH82 cell probed with phalloidin. *Thin arrows* indicate filopodia. *Middle*: *E. chaffeensis*-infected cell stained with DAPI. *Thick arrow* indicates morulae of *E. chaffeensis* stained with DAPI, and *thin arrow* indicates host nucleus. *Right*: Merged figure. Scale bar, 50 μm.

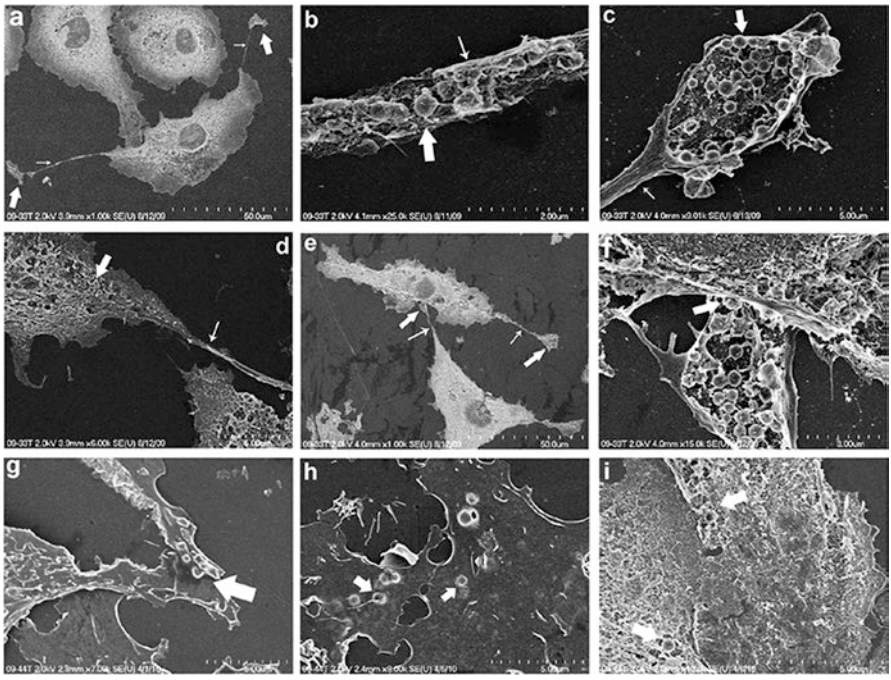

Fig. 8.11 *Ehrlichia* is transported through the filopodia of the host cells as observed under a scanning electron microscope. Scanning electron micrographs of DH82 cells infected with *E. chaffeensis*. (**a**) *E. chaffeensis* are observed in the filopodia of DH82 cells. The *thick arrow* indicates the flattened fan-shaped structure at the leading edge of the filopodium (indicated by *thin arrows*). (**b**) *Ehrlichia* bacteria in a filopodium from which the cell membrane has been removed. The *thick arrow* indicates an *Ehrlichia*. (**c**) A flattened fan-shaped structure filled with *Ehrlichia* from which the cell membrane had been removed. The *thick arrow* indicates *Ehrlichia*, and the *thin arrow* indicates a filopodium. (**d**) A filopodium that extended from an *Ehrlichia*-infected DH82 cell. (**e**) Low magnification of an *Ehrlichia*-infected host cell filopodium in contact with a neighboring cell. The *thick arrows* indicate the flattened fan-shaped structures, and the *thin arrows* indicate the filopodia. (**f**) High magnification of a flattened fan-shaped structure from which the cell membrane has been removed at the leading edge of an *Ehrlichia*-infected cell (depicted in figure **e**) in contact with the neighboring host cell. The *thick arrow* indicates an *Ehrlichia*. (**g**) Intracellular *Ehrlichia* deforming the overlying cell membrane at the junction of a neighboring cell. (**h**) Localization of *Ehrlichia* (*thick arrow*) deforming the overlying cell membrane of adjacent cells. (**i**) *Ehrlichia* seen in adjacent cells of a cracked open DH82 host cell

Fig. 8.10 (continued) (**a**) Filopodia extended from an *E. chaffeensis*-infected DH82 cell. (**b**) Filopodium of *E. chaffeensis*-infected DH82 cell extended to a neighboring cell. (**c**, **d**) *Ehrlichia* are contained in a long filopodium that had a flattened fan-shaped structure with no host cells in the immediate vicinity. *Thick arrow* indicates the flattened fan-shaped structure at the leading edge of the filopodium. (**e**) Uninfected DH82 cell. (**f**) Lengths of filopodia of DH82 cells infected with *E. chaffeensis* ($n = 25$)

4 Inhibition of Actin Polymerization in Host Cells Infected with *Ehrlichia* Prevents Filopodia Formation and Localizes the Pathogen in the Periphery

Actin and microtubules are involved in the formation of filopodia; hence, we determined the effect of the actin inhibitor, cytochalasin D on the transport of the pathogen in *Ehrlichia*-infected monocytes. Cytochalasin D inhibited filopodium formation in both *E. chaffeensis*- (Fig. 8.12a) and *E. muris*-infected cells (Fig. 8.12c, d, f, g). Ehrlichiae were confined to the periphery of the macrophages. *Ehrlichia* are transported through the filopodia so as to avoid the host immune system while the pathogen passed from cell to cell. The actin inhibitors latrunculin B, wiskostatin, and blebbistatin inhibited filopodium formation; whereas treatment of *E. muris*-infected DH82 cells with nocodazole (microtubule inhibitor) did not inhibit filopodium formation (Thomas et al. 2010).

To confirm indeed that filopodium is required for intercellular transport of *Ehrlichia*, the *E. muris*-infected DH82 cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) followed by seeding with non-labeled uninfected DH82. Alternately, the uninfected DH82 cells can also be labeled with CFSE and seeded with DH82 cells infected with *E. muris*. The filopodia/pseudopodia from the infected DH82 cells were in close proximity to the neighboring uninfected DH82 cells and *Ehrlichia* was also observed in the uninfected DH82 cells after 16 h of culture (Fig. 8.13a–c). In the presence of cytochalasin D there was absence of any filopodia in the *Ehrlichia*-infected DH82 cells, and there was also no infection in the neighboring uninfected cells (Fig. 8.13d–e).

The amount of *Ehrlichia* increases if it is transferred intracellularly through the filopodium where it could infect neighboring uninfected cells, thereby multiplying in these cells. Whereas, inhibition of filopodia formation could decrease the pathogen and these could be quantitated by quantitative real-time polymerase chain reaction (RT-PCR). The uninfected DH82 cells were seeded with *E. muris*-infected DH82 cells (in the presence and absence of cytochalasin D) and after 24 h the bacterial load was quantitated by RT-PCR. When the uninfected DH82 cells were seeded with *E. muris*-infected DH82 cells in the presence of cytochalasin D, the bacterial load decreased, whereas in the absence of cytochalasin D the bacterial load increased (Fig. 8.13g). The data confirmed that induction of filopodia formation in host cells by *Ehrlichia* is indeed an exit strategy of *Ehrlichia*.

Fig. 8.12 (continued) (**e**) Uninfected DH82 cells treated with cytochalasin D and probed with *Ehrlichia* Hsp60 antibody. (**f**) Scanning electron micrograph of *E. muris*-infected DH82 cells treated with cytochalasin D from which the cell membrane had been removed. (**g**) Transmission electron micrograph of *E. muris*-infected DH82 cell treated with cytochalasin D. *Thick arrows* indicate *Ehrlichia* morulae, N, nucleus. Scale bar, 1 μm. (**h**) A single IOE cell in mouse spleen. *Arrows* indicate actin filaments

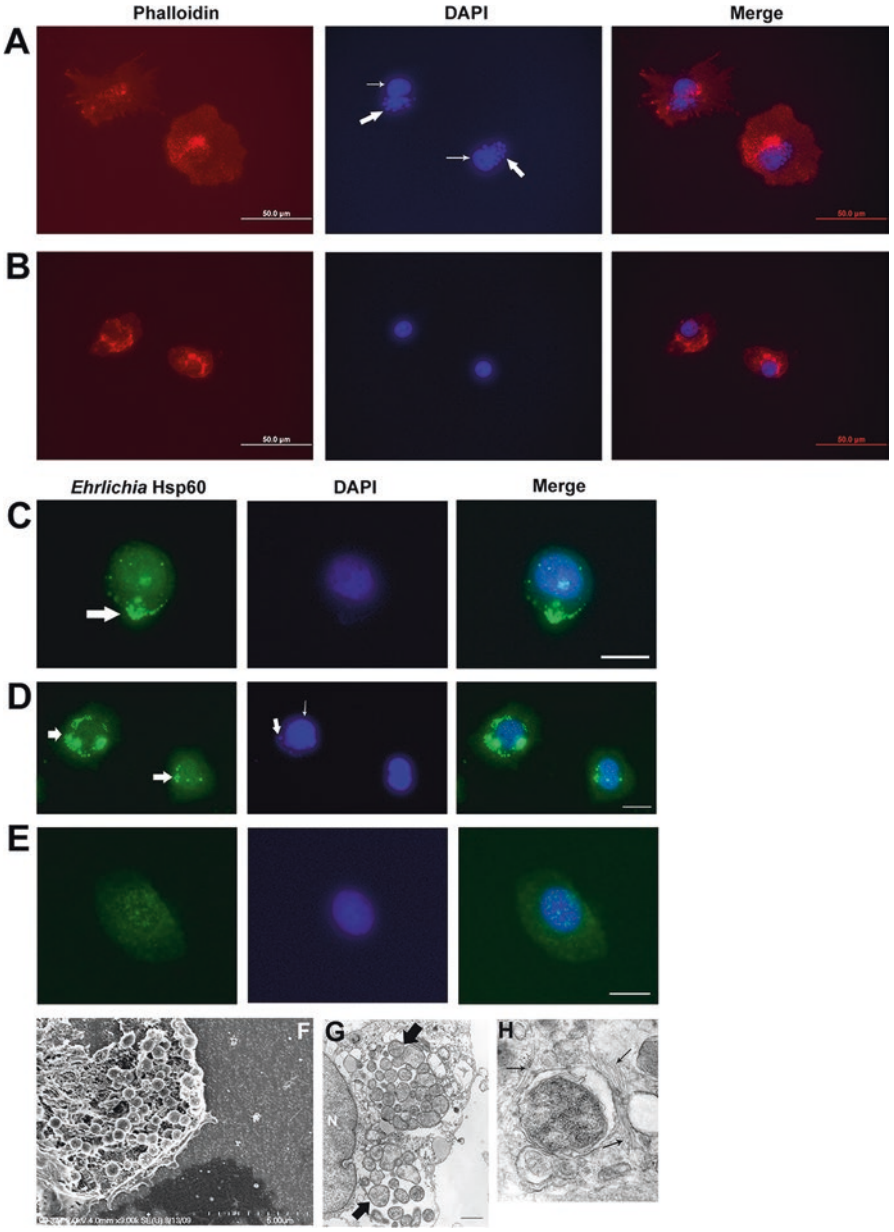

Fig. 8.12 Cytochalasin D inhibited filopodium formation in *Ehrlichia*-infected cells. (**a**) E. chaffeensis-infected DH82 cells treated with cytochalasin D and stained with phalloidin (*left*), DAPI (*middle*) (*thick arrows* indicate *Ehrlichia* morulae and *thin arrows* indicate host nuclei), and merged figure (*right*). (**b**) Uninfected DH82 cells treated with cytochalasin D and stained with phalloidin. (**c**, **d**) E. muris-infected DH82 cells treated with cytochalasin D and probed with *Ehrlichia* Hsp60 antibody (*left*) (*thick arrow* indicates *Ehrlichia*), DAPI (*middle*) (*thick arrow* indicates *Ehrlichia* DNA, and *thin arrow* indicates host cell nuclei), and merged figure (*right*).

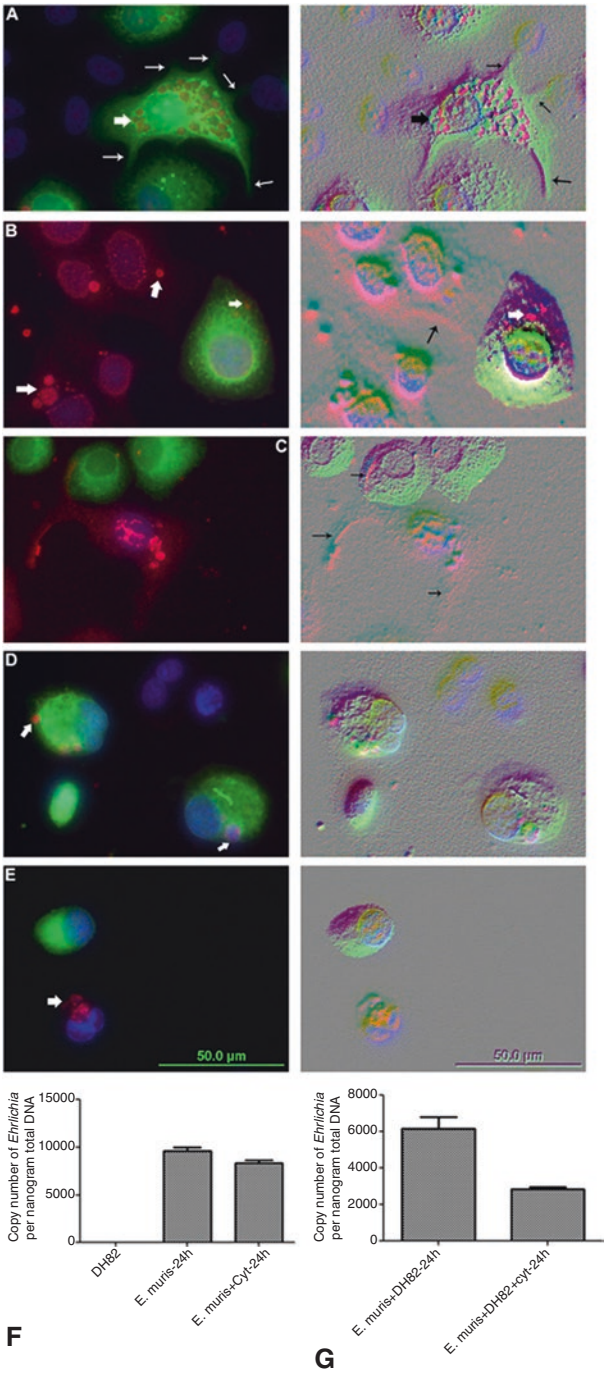

Fig. 8.13 Inhibition of filopodium formation prevented *Ehrlichia* intercellular transport. (**a**) *Ehrlichia*-infected DH82 cells were treated with CFSE and seeded with uninfected non-treated DH82 cells for 24 h. *Thick arrow* indicates *E. muris* (probed with *Ehrlichia* Hsp60), whereas the *thin arrows* indicate filopodia/pseudopodia of infected cells. DAPI stains the nucleus of both the

5 *Ehrlichia*-Induced Filopodia in Infected Mouse Macrophages

It is difficult to observe *Ehrlichia*-induced filopodium formation in monocytes/macrophages in vivo. Hence, splenocytes of mice that had been infected with *Ehrlichia* were cultured for 7 days. The cytoplasm of the macrophages from *E. muris*-infected mice harbored few pathogens on days 1–3 in cell culture, whereas by day 5 the macrophages were highly populated with *E. muris* (Fig. 8.14a, c–f, m). *E. muris*-infected mouse macrophages had filopodia that contained the pathogen similar to those observed in the infected DH82 cells. Similar results were observed when macrophages from the highly lethal *Ixodes ovatus* Ehrlichia (IOE)-infected mice (infected for 7 days prior to harvesting) were cultured for 5 days in vitro. IOE were also observed in the filopodia of infected mouse macrophages (Fig. 8.14b, g, h).

6 In Late Stages of Infection the *Ehrlichia* Ruptured the Overlying Host Cell Membrane

When DH82 cells infected with *E. muris* were cultured for 60 h, the morula was found to be enlarged probably due to fusion of adjacent morulae (Fig. 8.15b; Morulae of *E. muris* at 24 h: Fig. 8.15a). The cell membrane of *E. muris*-infected DH82 cell ruptured at 60 h (Fig. 8.15c) and the bacteria were released through the pores on the host cell membrane (Fig. 8.15d). The pathogens released after membrane rupture were observed attached to the filopodium of neighboring cells (Fig. 8.15e). Attached ehrlichiae were observed in association with ruffled cell membrane characteristic of entry by endocytosis (Fig. 8.15f). TEM of IOE-infected

Fig. 8.13 (continued) uninfected and infected cells. The adjacent figure is the Nomarski image, which clearly showed the filopodia/pseudopodia of infected cells. (**b**, **c**) DH82 cells were treated with CFSE and seeded with infected non-treated DH82 cells for 24 h. *Thick arrow* indicates *E. muris* (probed with *Ehrlichia* Hsp60) whereas the *thin arrows* indicate filopodia/pseudopodia of infected cells. DAPI stains the nuclei of both uninfected and infected cells. The adjacent figure is the Nomarski image which showed clearly the filopodia/pseudopodia of infected cells. (**d**) Ehrlichia-infected DH82 cells were treated with CFSE and seeded with uninfected non-treated DH82 cells for 24 h in the presence of cytochalasin D. *Thick arrow* indicates *E. muris* (the adjacent figure is the Nomarski image). (**e**) Uninfected DH82 cells were treated with CFSE and seeded with infected non-treated DH82 cells for 24 h in the presence of cytochalasin D. *Thick arrow* indicates *E. muris* (the adjacent figure is the Nomarski image). (**f**). Quantitative real-time PCR of bacterial loads of *E. muris*-infected DH82 cells to evaluate cytotoxicity in the presence of cytochalasin D ($n = 3$ per group). (**g**). Quantitative real-time PCR of bacterial load of *E. muris*-infected DH82 cells seeded with uninfected DH82 cells in the presence and absence of cytochalasin D ($n = 3$ per group)

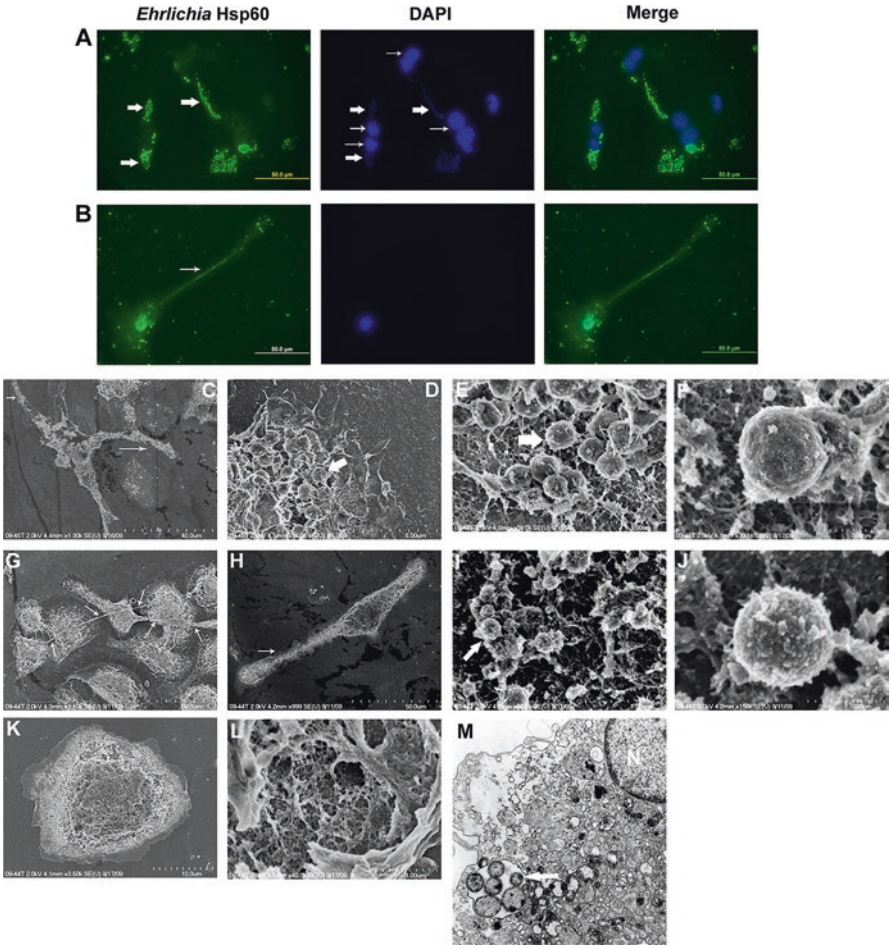

Fig. 8.14 *Ehrlichia* are observed in the filopodia of mouse macrophages. (**a**) *E. muris*-infected mouse macrophages probed with *Ehrlichia* Hsp60 antibody (*left*), DAPI (*middle*) (*thick arrows* indicate DNA of *E. muris*, and *thin arrows* indicate mouse macrophage nuclei), and merged figure (*right*). (**b**) IOE-infected mouse macrophage probed with *Ehrlichia* Hsp60 antibody (*left*) (*thin arrow* indicates filopodium), DAPI (*middle*), and merged figure (*right*). (**c**) Scanning electron micrograph of *E. muris*-induced filopodium in a mouse macrophage; *thin arrow* indicates the filopodium. (**d**) The interior of a mouse macrophage from which the cell membrane has been removed contained *E. muris*. (**e**) Higher magnification of *E. muris* in a mouse macrophage. (**f**) Scanning electron micrograph of an *E. muris* bacterium. (**g**, **h**) Scanning electron micrograph of IOE-induced filopodia in mouse macrophages; *thin arrows* indicate the filopodia. (**i**) IOE microorganisms in a mouse macrophage. (**j**) Scanning electron micrograph of a single IOE bacterium. (**k**) Uninfected mouse macrophage. (**l**) High magnification of an opened uninfected mouse macrophage. (**m**) Transmission electron micrograph of a mouse macrophage that contained an *E. muris* morula (*thick arrow*), N, nucleus. Scale bar, 1 μm

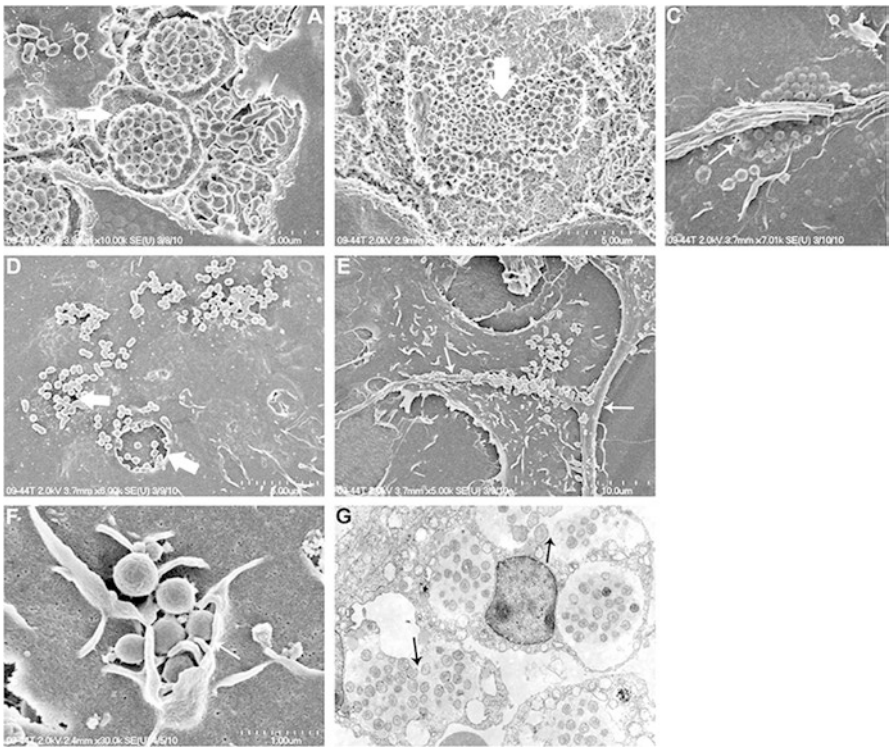

Fig. 8.15 *Ehrlichia* morulae inside a DH82 cell with an overlying ruptured host cell membrane. (**a**) Different stages of *E. muris* in a mechanically opened DH82 cell. *Thin arrow* indicates dividing ehrlichiae; *thick arrow* indicates mature cells. (**b**) Mature ehrlichiae cells in a large morula (*thick arrow*). (**c**) Pore formation on a DH82 host cell containing many ehrlichiae that have deformed the overlying cell membrane (*thin arrows*) (intact DH82 cell). (**d**) Host cell membrane ruptured at the location of ehrlichial exit from the cell (intact DH82 cell). (**e**) Extracellular *Ehrlichia* attached with high affinity to the filopodium of neighboring host cells (*thin arrows*). (**f**) Ehrlichiae attached to the DH82 cell membrane adjacent to a cell membrane ruffle (*thin arrow*) (intact DH82 cell). (**g**) TEM of an IOE-infected spleen (*arrows* indicate fused morula)

spleen confirmed morula fusion (Fig. 8.15g). Thus at a later time of infection, when the host cells are filled with the pathogenic bacteria, the *Ehrlichia* could disrupt the host cell membrane and exit the host cells.

7 Summary

Ehrlichia are obligatory intracellular bacterium which infects macrophages and monocytes. Using in vitro cell culture systems, the exit mechanism of *Ehrlichia* was described recently. The schematic diagram of *Ehrlichia* exit is shown in Fig. 8.16. *Ehrlichia* survive and replicate exclusively within inclusions in

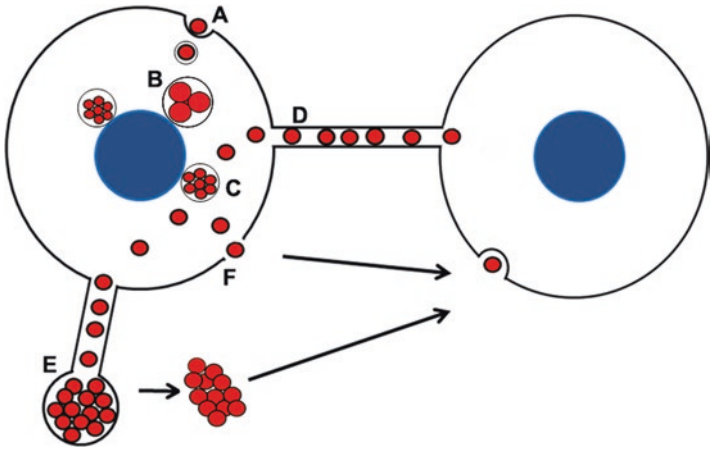

Fig. 8.16 Schematic diagram of *Ehrlichia* exit. *Ehrlichia* replicate and survive in host cell membrane-bound endosome compartment (A). The single cell *Ehrlichia* divides and form large pleomorphic reticulate cells (B). The reticulate cells divide and form the dense cored cells (C). The dense cored cells induce filopodium formation in host cells. The filopodium attach to neighboring cells and the pathogen pass through the filopodium to the neighboring cells (D). If there are no neighboring cells in the immediate vicinity, the end of the filopodium forms a fan shaped structure filled with *Ehrlichia* (E). These fan-shaped structures could detach and the cluster of ehrlichial cells could infect neighboring host cells. When the host cells are filled with the ehrlichial pathogens, they could break open the host cell membrane and the bacterial cells released (F)

monocytes and macrophages, which are primary effector cells of antimicrobial defense. Therefore, ehrlichiae must convert the hostile inclusion environment to a hospitable environment conducive to their survival and replication. Once the pathogenic bacteria are taken up, *Ehrlichia* replicate and survive in host cell membrane-bound endosome compartment (A). The *Ehrlichia* containing endosome does not fuse with the lysosome, thereby escaping destruction (Barnewall et al. 1997). The single cell *Ehrlichia* divides and form large pleomorphic reticulate cells (B). The reticulate cells divide and form the dense cored cells (C). Once the dense cored cells leave the morula, they could induce filopodium formation in host cells. The filopodium attach to neighboring cells and the pathogen pass through the filopodium to the neighboring cells (D). If there are no neighboring cells in the immediate vicinity, the end of the filopodium forms a fan-shaped structure filled with *Ehrlichia* (E). These fan-shaped structures could detach later and the cluster of ehrlichial cells could infect neighboring host cells. When the host cells are filled with the ehrlichial pathogens, they could break open the host cell membrane and the bacterial cells released (F).

8 Note to Readers

How the exit strategy of *Ehrlichia* was observed for the first time

Every scientific project is like a Russian doll (matryoshka doll); there is a hidden component lurking somewhere in the project that the investigator has to discover. Most often, the new discovery or phenomenon (the hidden component(s)) will turn out to be greater or groundbreaking than the planned main project. The important characters required for the investigator to discover something novel includes: a keen sense of observation, creativity, and imagination.

While working on *Ehrlichia* at the University of Texas Medical Branch, Galveston, Texas, my major goal was to develop a vaccine for *Ehrlichia*. The first question I had to address was why *Ehrlichia muris* is non-virulent compared to the highly virulent *Ixodes ovatus* ehrlichia (IOE). Both the strains had the same protein profile when characterized by SDS-PAGE (1D or 2D gels) or after probing with *Ehrlichia* antibodies by Western blotting. Prior to working on *Ehrlichia*, I used to work on lipid rafts at Mount Sinai School of Medicine, New York. We used to detect lipoproteins on T cells with Cholera toxin B (CTB); hence, I wondered whether it was possible to detect the lipoproteins of *Ehrlichia* by CTB on a blot. When the transferred proteins of *Ehrlichia* were probed with CTB, I observed some of the antigenic proteins in *E. muris* to be more lipoylated. Subsequently, I asked whether it was possible to observe glycosylated proteins. Since lectins bind to glucose moieties, I used wheat germ agglutinin (WGA) and concanavalin A (ConA) as probes. Though WGA was not a good probe, nevertheless, ConA could detect the glycosylated antigenic proteins of *Ehrlichia*. Finally, phosphomolybdate was used to detect phosphorylated proteins of *Ehrlichia*. The antigenic proteins of *E. muris* (GroEL/*Ehrlichia* Hsp60 and P28-19) had more post-translational protein modifications compared to the antigenic proteins of IOE. I coined the term "Eastern Blotting" for the detection of post-translational protein modifications.

Using *in silico* analyses, we determined the epitopes of *Ehrlichia* Hsp60 and P28-19. We generated peptides to these epitopes and they were injected in mice and the antibody generated was used to diagnose *E. muris* in DH82 cells.

When culturing *Ehrlichia*, the procedure involves seeding *E. muris*-infected DH82 cells on 60–70 % confluent uninfected DH82 cells. After 1 week of culturing, the infection rate is determined by light microscopy (Diff-quik staining). At that period the DH82 cells are fully confluent (cells closely packed) and it is difficult to observe any single cells. As I was not interested in highly infected DH82 cells, I seeded around 1000 DH82 cells infected with various amounts of *E. muris* on a culture slide and after 16 h stained with the newly generated antibodies. After 16 h of culture, the DH82 cells were far apart, and to my amazement I could observe tail like extensions (filopodia) on the host cells; some of the filopodia were seen attached to the neighboring cells. On staining with the *Ehrlichia* Hsp60 antibody, I could observe *E. muris* in the cells (in the cytoplasm as well as in the filopodia). Later, Dr. Vsevolod Popov showed me how to "peel open" the DH82 cells with Scotch tape and he asked his technician Julie Wen to help me with the staining for scanning

electron microscope (SEM). Since the laboratory was busy, Dr. Popov encouraged me to work on the SEM. While working on the SEM, I observed that after *Ehrlichia* are over-populated inside host cells, they break open the host cell membrane and exit cells. These exited cells could return and infect the neighboring cells. The findings were very well received by the scientific community.

References

Barnewall RE, Rikihisa Y, Lee EH (1997) *Ehrlichia chaffeensis* inclusions are early endosomes which selectively accumulate transferrin receptor. Infect Immun. 65:1455–1461.

Hagedorn M, Rohde KH, Russell DG, Soldati T (2009) Infection by tubercular mycobacteria is spread by nonlytic ejection from their amoeba hosts. Science 323: 1729–1733.

Hartwig JH, Davies WA, Stossel TP (1977) Evidence for contractile protein translocation in macrophage spreading, phagocytosis, and phagolysosome formation. J Cell Biol 75: 956–967.

Hybiske K, Stephens RS (2007) Mechanisms of host cell exit by the intracellular bacterium Chlamydia. Proc Natl Acad Sci U S A. 104:11430-11435.

Hybiske K, Stephens RS (2008) Exit strategies of intracellular pathogens. Nat Rev Microbiol. 6:99-110.

Li JS, Winslow GM (2003) Survival, replication, and antibody susceptibility of *Ehrlichia chaffeensis* outside of host cells. Infect Immun 71: 4229–4237.

Lin M, Rikihisa Y (2003) Ehrlichia chaffeensis and Anaplasma phagocytophilum lack genes for lipid A biosynthesis and incorporate cholesterol for their survival. Infect Immun. 71: 5324-5331.

Liu Y, Zhang Z, Jiang Y, Zhang L, Popov VL, Zhang J, Walker DH, Yu XJ (2011) Obligate intracellular bacterium Ehrlichia inhibiting mitochondrial activity. Microbes Infect. 13:232–238.

Maeda K, Markowitz N, Hawley RC, Ristic M, Cox D, McDade JE. (1987). Human infection with *Ehrlichia canis*, a leukocytic Rickettsia. N Engl J Med. 316:853-856.

Maender JL, Tyring SK (2004) Treatment and prevention of rickettsial and ehrlichial infections. Dermatol Ther 17: 499–504.

Paddock CD, Childs JE (2003) *Ehrlichia chaffeensis*: a prototypical emerging pathogen. Clinical Microbiol Rev 16: 37–64.

Paddock CD, Sumner JW, Shore GM, Bartley DC, Elie RC, McQuade JG, Martin CR, Goldsmith CS, Childs JE (1997) Isolation and characterization of *Ehrlichia chaffeensis* strains from patients with fatal ehrlichiosis. J Clin Microbiol. 35:2496-2502.

Popov VL, Chen SM, Feng HM, Walker DH. (1995). Ultrastructural variation of cultured *Ehrlichia chaffeensis*. J Med Microbiol. 43:411-421.

Rikihisa Y (2010a) Molecular events involved in cellular invasion by *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum*. Vet Parasitol 167: 155–166.

Rikihisa Y (2010b) *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*: subversive manipulators of host cells. Nat Rev Microbiol 8: 328–339.

Rosania GR, Swanson JA (1996) Microtubules can modulate pseudopod activity from a distance inside macrophages. Cell Motil Cytoskeleton 34: 230–245.

Thomas S, Popov VL, Walker DH (2010) Exit mechanisms of the intracellular bacterium *Ehrlichia*. PLoS One 5(12): e15775.

Thomas S, Thirumalapura NR, Crossley EC, Ismail N, Walker DH (2009) Antigenic protein modifications in *Ehrlichia*. Parasite Immunol 31: 296–303.

Xiong Q, Bao W, Ge Y, Rikihisa Y (2008) *Ehrlichia ewingii* infection delays spontaneous neutrophil apoptosis through stabilization of mitochondria. J Infect Dis. 197:1110-1118.

Zhang JZ, Popov VL, Gao S, Walker DH, Yu XJ (2007) The developmental cycle of *Ehrlichia chaffeensis* in vertebrate cells. Cell Microbiol. 9:610-618.