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REVIEW

SPECIAL ISSUE: CELL BIOLOGY OF HOST–PATHOGEN INTERACTIONS

Vacuolar escape of foodborne bacterial pathogens

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ABSTRACT

The intracellular pathogens *Listeria monocytogenes*, *Salmonella enterica*, *Shigella* spp. and *Staphylococcus aureus* are major causes of foodborne illnesses. Following the ingestion of contaminated food or beverages, pathogens can invade epithelial cells, immune cells and other cell types. Pathogens survive and proliferate intracellularly via two main strategies. First, the pathogens can remain in membrane-bound vacuoles and tailor organellar trafficking to evade host-cell defenses and gain access to nutrients. Second, pathogens can rupture the vacuolar membrane and proliferate within the nutrient-rich cytosol of the host cell. Although this virulence strategy of vacuolar escape is well known for *L. monocytogenes* and *Shigella* spp., it has recently become clear that *S. aureus* and *Salmonella* spp. also gain access to the cytosol, and that this is important for their survival and growth. In this Review, we discuss the molecular mechanisms of how these intracellular pathogens rupture the vacuolar membrane by secreting a combination of proteins that lyse the membranes or that remodel the lipids of the vacuolar membrane, such as phospholipases. In addition, we also propose that oxidation of the vacuolar membrane also contributes to cytosolic pathogen escape. Understanding these escape mechanisms could aid in the identification of new therapeutic approaches to combat foodborne pathogens.

KEY WORDS: Foodborne pathogen, Phagocytosis, Phagosomal escape, Vacuolar escape

Introduction

The USA Center for Disease Control and Prevention (CDC) estimates that 48 million people get sick from a foodborne illness in the USA annually, leading to over a hundred thousand hospitalizations and deaths caused by the presence of intracellular pathogens in raw or undercooked foods. Known foodborne intracellular pathogens include *Listeria monocytogenes*, *Salmonella enterica*, *Shigella* spp. and *Staphylococcus aureus* (Havelaar et al., 2012; Hoffmann et al., 2012; Horn et al., 2018; Scallan et al., 2011). All these bacteria evolved mechanisms to overcome innate host defense factors, such as saliva, gastric acid and the intestinal mucous layer, and invade intestinal epithelial cells, macrophages and other host cells (Case and Samuel, 2016; Castanheira and García-del Portillo, 2017; Flieger et al., 2018; Mitchell et al., 2016). Within these infected host cells, intracellular pathogens multiply and subsequently transit to other host cells, propagating the infection. They exit the cells via activating host-cell programmed cell death, active breaching of cell membranes or an exocytosis-like mechanism (Flieger et al., 2018).

Intracellular pathogens can invade both immune phagocytes (macrophages, dendritic cells and neutrophils) and non-phagocytic cells (e.g. epithelial cells) (Case and Samuel, 2016; Castanheira and García-del Portillo, 2017; Cossart and Helenius, 2014; Mitchell et al., 2016; Pucciarelli and García-del Portillo, 2017). Although the entry mechanism differs between these cell types, in both cases, after uptake, the bacterium initially localizes in the lumen of a membrane-bound compartment, called a vacuole, which are more bactericidal in phagocytes than in non-phagocytic cells (Günther and Seyfert, 2018) (Box 1; Fig. 1). Intracellular pathogens have evolved two mechanisms to subvert these cellular bactericidal conditions in order to survive and propagate within the host cell (Sprenger et al., 2018). First, some pathogens can actively create a less-bactericidal vacuolar niche to destabilize host defense responses by arresting phagolysosomal maturation (e.g. prevent lysosome fusion) and/or redirecting host organellar trafficking, as reviewed elsewhere (Case and Samuel, 2016; Creasey and Isberg, 2014; Sprenger et al., 2018). Second, pathogens can disrupt the vacuolar membrane to escape into the nutrient-rich cytosol, which allows them to avoid the harsh conditions in endolysosomal compartments (Case and Samuel, 2016; Creasey and Isberg, 2014; Mitchell et al., 2016).

Historically, intracellular pathogens are classified according to their virulence strategies into vacuolar (or phagolysosomal) and cytosolic. Of the intracellular foodborne bacterial pathogens, *Salmonella* spp. (Jantsch et al., 2003), *Campylobacter* spp. (Watson and Galán, 2008), *Brucella* spp. (Celli, 2015), *S. aureus* (Perskvist et al., 2002; Schröder et al., 2006) and *Yersinia enterocolitica* (Connor et al., 2018) have been considered to be vacuolar, whereas *L. monocytogenes* (Bierne et al., 2018) and *Shigella* spp. (Schroeder and Hilbi, 2008) were considered cytosolic pathogens. For *Cronobacter sakazakii*, which causes systemic infections and thus must penetrate the intestinal epithelial cell barrier and can also invade epithelial cell lines *in vitro* (Kim and Loessner, 2008; Mohan Nair and Venkitanarayanan, 2007), the intracellular localization is not well characterized, but electron microscopy analysis has shown that it resides in membrane-bound vacuoles in human brain microvascular endothelial cells (de Mey et al., 1995). However, the boundaries between vacuolar and cytosolic pathogens are fading, and cytosolic pathogens, such as *L. monocytogenes*, have been found to build vacuolar niches in certain cell types (Birmingham et al., 2008; Kortebi et al., 2017). Conversely, it is increasingly clear from electron microscopy and fluorescence approaches (Creasey and Isberg, 2014; Quereda et al., 2016) that the vacuolar pathogens *Campylobacter* spp. (Nemelka et al., 2009), *S. aureus* (Giese et al., 2011) and *Salmonella* spp. (Birmingham and Brumell, 2006; Knodler et al., 2014; Thurston et al., 2016) can also gain access to the cytosol. Thus, the concept is emerging that all foodborne intracellular pathogens can escape to the cytosol and that this is important for their virulence. With this Review, we provide an overview of the molecular mechanisms that

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Box 1. Pathogen invasion of immune phagocytes and non-phagocytic cells

Intracellular pathogens invade host cells through a variety of mechanisms (Cossart and Helenius, 2014). In professional phagocytes (macrophages, dendritic cells and neutrophils), pathogens can enter via host-driven phagocytosis following binding of the pathogen to pattern recognition receptors such as C-type lectins (Njiri et al., 2020). Moreover, antibodies and complement factors can bind to the pathogen in circulation, through so-called opsonization, allowing the uptake by Fc or complement receptors (Ernst, 1998). In non-phagocytic cells (e.g. epithelial cells), host-cell entry of pathogens is generally a bacterial-driven process where bacteria hijack host surface receptors and release factors into the host-cell cytosol, for instance by using the T3SS, that trigger cytoskeletal rearrangements and membrane remodeling (Cossart and Helenius, 2014). For instance, *S. aureus* enters epithelial cells via adhesins, which crosslink with the host α5β1 integrins via fibronectin bridges (Foster, 2016). As α5β1 integrin is normally only present at the basolateral side of epithelial cells, *S. aureus* infections occur after breaches of epithelial barriers; in Caco-2 epithelial cells, this disruption can be induced by the cytolytic α-toxin produced by *S. aureus* (Kwak et al., 2012), which triggers degradation of tight junction proteins or ectopic integrin expression at the apical side (Horn et al., 2018). The subsequent maturation of the bacteria-containing vacuoles also differs between immune phagocytes and non-phagocytic cells. First, in immune phagocytes, the NADPH oxidase 2 (NOX2) complex can produce high levels of ROS that can be converted into harmful nitrogen and chlorine radicals (Buvelot et al., 2019; Paardekooper et al., 2019). Second, in all cell types, the vacuolar-type H^+-ATPase (V-ATPase), present in the vacuolar membrane, transports large amounts of protons into the vacuolar lumen resulting in acidification (Haas, 2007). Third, a succession of fusion events with endosomes and lysosomes results in the delivery of hydrolytic enzymes and antimicrobial peptides into the vacuole lumen in all cell types (Haas, 2007). Fourth, especially in immune phagocytes, but also in other cell types, microorganisms can be detected by pattern recognition receptors located within the vacuolar membrane, such as Toll-like receptors, which results in activation of the immune system (Fig. 1) (Mitchell et al., 2016).

enable foodborne intracellular pathogenic bacteria to escape the vacuoles into the cytosol in macrophages and non-phagocytic cell types.

Invasion and survival mechanisms of foodborne pathogens

Listeria monocytogenes

Infection with the Gram-positive bacteria *L. monocytogenes* occurs mainly following the ingestion of contaminated raw dairy and seafoods (Radoshevich and Cossart, 2018; Schuppler and Loessner, 2010). Infections with *L. monocytogenes* are a cause of febrile gastroenteritis and can cause a severe invasive disease called listeriosis, hallmarked by blood poisoning and encephalomeningitis (Radoshevich and Cossart, 2018). Owing to the high mortality rate of listeriosis (20–30%), *L. monocytogenes* is one of the leading causes of death from a foodborne pathogen (de Noordhout et al., 2014). *L. monocytogenes* is an archetypal cytosolic pathogen that invades both macrophages and non-phagocytic cells, particularly epithelial cells of the gastrointestinal tract (Radoshevich and Cossart, 2018; Schuppler and Loessner, 2010). Macrophages are generally invaded by host-cell-driven phagocytosis, whereas entry of epithelial cells is a bacteria-driven process (Flieger et al., 2018). In both cases, *Listeria* enters the first host cell in a so-called primary vacuole, which it escapes from within minutes after uptake, to propagate within the cytosol (Bierne et al., 2018; Radoshevich and Cossart, 2018; Schuppler and Loessner, 2010). *Listeria* express the actin assembly-inducing protein ActA, which promotes Arp2/3-mediated formation of filamentous (F-)actin. This drives the formation of so-called comet-tails that propel the bacteria through the cell and allow egress of *Listeria* within cell bodies that are detached from the host cell (Bierne et al., 2018). This not only enables infection of neighboring epithelial cells, but also leads to the infection of macrophages, which ingest these cell bodies through efferocytosis (phagocytosis of apoptotic cells) (Czuczman et al., 2014). The *L. monocytogenes* bacteria enter these cells in secondary vacuoles that have two membranes: one from the original host cell and one from the recipient cell (Radoshevich and Cossart, 2018).

In both macrophages and epithelial cells, *L. monocytogenes* can escape from primary and secondary vacuoles using the same well-established mechanism (Nguyen et al., 2019). Its primary component is the pore-forming toxin listeriolysin O (LLO), which together with the phosphatidylinositol-specific phospholipase C (PI-PLC or PlcA) and the broad-range phospholipase C (PC-PLC or PlcB) disrupts the vacuolar membrane, resulting in the escape of the bacteria into the cytosol (Bierne et al., 2018). Expression of LLO, PI-PLC and PC-PLC is controlled by the transcription factor positive regulatory factor A (PrfA), which also regulates transcription of ActA and other virulence genes (de las Heras et al., 2011). LLO promotes *L. monocytogenes* infection not only via disruption of the vacuolar membrane; in macrophage-like cells, LLO also causes a Ca^{2+} influx that prevents the fusion of late endosomes and/or lysosomes to the pathogen-containing vacuoles (Kühbacher et al., 2018; Shaughnessy et al., 2006). In HeLa cells, these compartments are instead redirected to the plasma membrane, resulting in altered exposure of glycoproteins associated with infection on the host-cell surface (Kühbacher et al., 2018).

The roles of PI-PLC and PC-PLC in vacuolar escape are still unclear. It is possible that the destabilization of the vacuolar membrane is a direct effect of PLC-mediated cleavage of vacuolar lipids, promoting membrane fusion and thereby directly supplementing the activity of LLO, which results in the escape of *L. monocytogenes* from the vacuole (Alberti-Segui et al., 2007; Montes et al., 2004; Sibelius et al., 1996). A second, not mutually exclusive, possibility is that the effect of PI-PLC and PC-PLC is more complex and involves host-cell signaling; it has been shown in macrophages that PI-PLC promotes vacuolar escape by activating the host-cell protein kinase C-β (PKC-β) via a poorly understood mechanism, which involves translocation of PKC-β to early endosomes (Poussin et al., 2009; Wadsworth and Goldfine, 2002). A third possibility is that the action of PC-PLC might be inhibitory; phosphocholine, produced by PC-PLC from hydrolysis of the phospholipid phosphatidylcholine, directly binds to LLO in HeLa cells and inhibits its activity (La Pietra et al., 2020). This inhibition has been proposed to prevent the premature destruction of the plasma membrane of the host cell (La Pietra et al., 2020). Moreover, the off-target disruption of cellular membranes by LLO is prevented by its acidic pH optimum, which restricts LLO activity to the acidic lumen of the vacuoles (Glomski et al., 2002; Shaughnessy et al., 2006). In macrophages, γ-interferon-inducible lysosomal thiol reductase (GILT; also known as IFI30) and cystic fibrosis transmembrane conductance regulator (CFTR) are two host proteins that are also involved in the regulation of LLO activity (Radtke et al., 2011; Singh et al., 2008). GILT is a lysosomal protein present in the lumen of *L. monocytogenes*-containing vacuoles in murine bone marrow-derived macrophages that activates LLO by reducing one of its disulfide bonds (Singh et al., 2008). CFTR is a Cl^- channel localized on *L. monocytogenes*-containing vacuoles in macrophage-like cells and might potentiate LLO activity by increasing the vacuolar chloride concentration (Radtke et al.,

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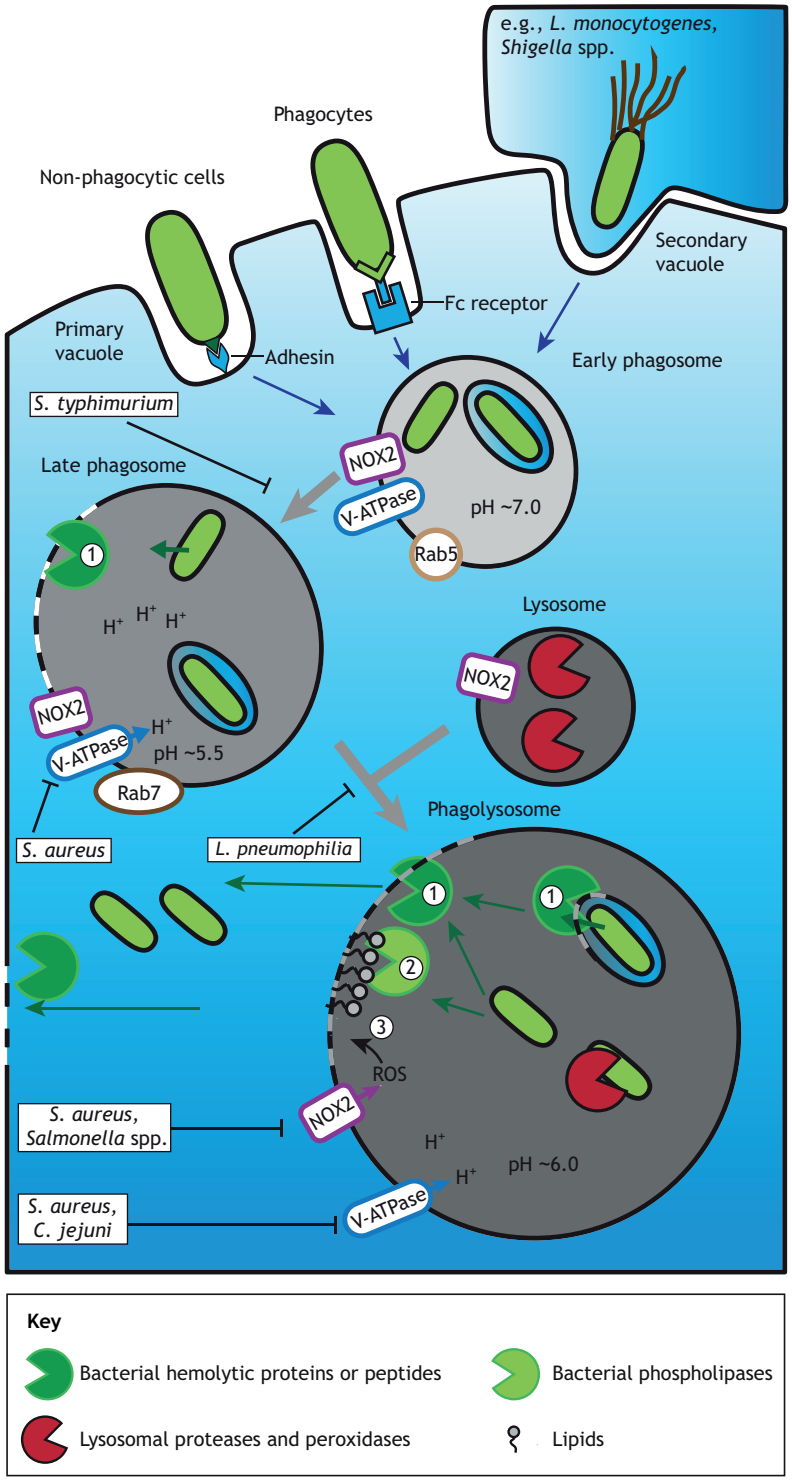

Fig. 1. Bacterial invasion, persistence and escape mechanisms in host cells. Pathogens actively invade host cells either via induced phagocytosis (e.g. via adhesins for non-phagocytic cells), via receptor-mediated uptake (e.g. using Fc receptors for professional phagocytes) or via secondary vacuoles formed following transfer from a neighboring host cell. Vacuoles mature from Rab5-positive early phagosomes into Rab7-positive late phagosomes and eventually to phagolysosomes. The V-ATPase pumps protons from the cytosol into the lumen of the vacuoles, reducing the pH of the vacuole. The NADPH-oxidase NOX2 produces reactive oxygen species (ROS) in the lumen of the vacuole. Fusion with lysosomes results in delivery of lysosomal proteases and peroxidases. This makes the environment of the vacuole lumen progressively more acidic, oxidative, proteolytic and bactericidal. To prevent this, some intracellular pathogens disrupt this phagosomal maturation (e.g. *S.* Typhimurium and *L. pneumophilia*). Others endure the complete maturation cycle (e.g. *S. aureus* and *C. jejuni*), but produce antagonists that block lysosomal proteases, prevent lysosomal acidification and neutralize ROS. Escape from primary or secondary vacuoles is achieved after formation of pores or weakening of the vacuolar membrane by (1) hemolytic proteins or peptides, which can also act on the plasma membrane to allow host-cell exit, (2) vacuolar lipid modification and (3) potentially lipid oxidation of the vacuolar membrane by NOX2-produced ROS.

2011). Conversely, host factors can also inhibit the vacuolar escape; it has been shown that heat-shock protein 70 (Hsp70) family proteins may limit damage to vacuolar membranes in murine bone-marrow derived macrophages by making them more resistant to pressure, potentially via activation of lysosomal acid sphingomyelinase, which alters their membrane composition (Davis et al., 2012).

In epithelial cell lines, but not in macrophages, the escape of *L. monocytogenes* from vacuoles is enhanced by peptide pheromone from lipoprotein A (pPplA), which is produced as a result of cleavage by signal peptidase II and the protease Eep, and might indicate to a *L. monocytogenes* bacterium whether the pathogen is present in a vacuole (Xayarath et al., 2015). In the confined vacuolar lumen, the secreted pPplA pheromone readily increases to high levels, which facilitates its re-uptake via the cysteine transport-associated protein (CtaP), thereby signaling in an autocrine fashion. This accumulation of pPplA in *L. monocytogenes* eventually promotes its vacuolar escape via an unknown mechanism, possibly by upregulating expression of a factor that stabilizes LLO-generated membrane pores (Xayarath et al., 2015).

For a long time, *L. monocytogenes* was thought to always escape to the cytosol following cellular invasion. However, it is now clear that there is also a population of bacteria residing in vacuoles in both macrophages and epithelial cells (Birmingham et al., 2008; Kortebi et al., 2017). These vacuolar bacteria are in a quiescent non- (or slow-)growing state and can be less susceptible to antibiotics and immune clearance (Birmingham et al., 2008; Kortebi et al., 2017). Moreover, *Listeria* also avoids escape from the entry vacuole in intestinal goblet cells by transcytosis, which allows its transmission to a neighboring cell without passing through the cytosol (Nikitas et al., 2011). This means that *L. monocytogenes* can survive the harsh conditions within the vacuoles. Although not studied in this context, it is interesting that *L. monocytogenes* possesses an acid-tolerance response (ATR) system that allows it to survive the low pH conditions in food or in the stomach (Smith et al., 2013), which might also be responsible for its survival in acidic vacuoles. In line with this hypothesis, it has been reported that exposure of *L. monocytogenes* to acid stress, which upregulates the ATR system, increases invasion of epithelial cells and bacterial proliferation in macrophage-like cells (Conte et al., 2000). However, the resistance to acid stress is also beneficial for the cytosolic population of bacteria, because, as mentioned above, LLO has a low pH optimum and the vacuole is already acidified prior to bacterial escape (Glomski et al., 2002; Shaughnessy et al., 2006).

Shigella spp.

The gram-negative bacteria *S. flexneri* and *S. dysenteriae* are major causes of bacillary dysentery and outbreaks in developed countries occur mainly through contaminated food (Niyogi, 2005). *S. dysenteriae* type I produces potent poisons known as Shiga toxins, which cause hemolytic uremic syndrome (HUS), a severe kidney complication (Tesh, 2012). Although neutrophils and monocytes can effectively kill ingested *S. flexneri* (Hathaway et al., 2002; Weinrauch et al., 2002), the bacteria can escape from phagosomes into the cytosol in macrophages (Schroeder and Hilbi, 2008). However, macrophages are probably not the primary cell type targeted by *Shigella* spp., as their infection induces macrophage cell death (Schroeder and Hilbi, 2008). Instead, macrophage infection might serve to traverse the epithelial barrier, as bacteria released from the dying macrophages can invade nearby epithelial cells through the basolateral membrane (Ashida et al., 2015). In both macrophages and epithelial cells, ingested bacteria can disrupt the vacuolar membrane and propagate in the cytosol (Ashida et al., 2015; Carayol and Tran Van Nhieu, 2013) and this can happen shortly after invasion (within 10 min in HeLa cells) (Mellouk et al., 2014). The entry of the bacterium in non-phagocytic cells and the subsequent lysis of the vacuole are promoted by effector proteins that are injected into the cytosol of the host cell by a type III secretion system (T3SS) (Ashida et al., 2015; Kuehl et al., 2014). Similar to what is seen for *L. monocytogenes*, the bacteria propel within the host cells due to actin polymerization on one pole of the bacteria, using the membrane protein IcsA (also known as VirG), which also promotes Arp2/3-mediated formation of F-actin, despite having no sequence similarity to ActA of *Listeria* (Kocks et al., 1995); this allows infection of neighboring epithelial cells via secondary vacuoles (Ashida et al., 2015).

Lysis of the vacuolar membrane has been assigned to the T3SS effector invasion plasmid antigen (Ipa) proteins IpaB, IpaC and IpaD and the invasion plasmid gene D (IpgD) (Mellouk et al., 2014; Picking and Picking, 2016; Ramel et al., 2011). IpaB and IpaC form a complex in the vacuolar membrane that binds cholesterol, resulting in membrane degradation, and this is regulated by IpaD (Picking and Picking, 2016). IpgD is a phosphoinositide phosphatase that converts phosphatidylinositol (4,5)-biphosphate (PIP_2) into phosphatidylinositol 5-phosphate (PI5P), and, in infected HeLa cells, this results in the recruitment of Rab11a, a small GTPase of recycling endosomes, to the bacteria-containing vacuole (Mellouk et al., 2014). The subversion of the intracellular trafficking enables the disruption of the vacuolar membrane leading to bacterial dissemination (Mellouk et al., 2014); this process involves the formation of Rab11a-containing macropinosomes at the bacterial invasion site in HeLa cells (Weiner et al., 2016). The production of PI5P by IpgD at the bacterial entry sites also causes the mislocalization of the epidermal growth factor receptor (EGFR) to early endosomes, which prevents termination of host-cell survival signaling via phosphoinositide 3-kinase and protein kinase B (PI3K-Akt) and enables prolonged bacterial colonization (Ramel et al., 2011). In addition to Rab11a, a small interfering RNA (siRNA) screen, also performed in HeLa cells, revealed other host proteins involved in vacuolar rupture, including factors regulating early endosomal trafficking, such as Rab4a, Rab5 (forms Rab5a–c), early endosome antigen 1 (EEA1), synaptojanin 1 and 2, and the actin cytoskeleton, including the Rho-GTPase Cdc42 and several subunits of the Arp2/3 complex (Mellouk et al., 2014).

In various epithelial cell lines, the disruption of the vacuolar membrane allows the diffusion of the cytosolic lectin galectin-8 into the lumen of the disrupted phagosome, where it binds to glycoproteins and glycolipids and triggers danger-associated molecular pattern (DAMP) signaling (Thurston et al., 2012). In epithelial cell lines and mouse embryonic fibroblasts, remnants of the disrupted phagosome are also ubiquitylated, which triggers the recruitment of the LC3 autophagy machinery already prior to full bacterial escape into the cytosol (Campbell-Valois et al., 2015; Dupont et al., 2009; Thurston et al., 2012). LC3 family protein recruitment likely functions to repair the damaged vacuole and prevent bacterial escape, but *Shigella* counteracts this with the release of the bacterial virulence proteins IcsB and VirA (Campbell-Valois et al., 2015). IcsB is a cholesterol-binding protein and has been proposed to act as a protease or acyltransferase (Baxt and Goldberg, 2014; Kayath et al., 2010), whereas VirA is a GTPase-activating protein (GAP) for the small-GTPase Rab1 (Dong et al., 2012). The precise roles of IcsB and VirA in preventing LC3 recruitment are still unclear, but their activity is required for bacterial escape from the vacuole in epithelial cells (Campbell-Valois et al., 2015).

Staphylococcus aureus

The gram-positive bacterium *S. aureus* is one of the most common foodborne pathogens worldwide (Scallan et al., 2011). Infections by *S. aureus* lead to abdominal cramps, nausea, vomiting and diarrhea, and have a high morbidity and mortality rate (Ho et al., 2008). For a long time, *S. aureus* was considered as an extracellular pathogen and staphylococcal foodborne disease was mainly related to heat-resistant enterotoxins produced by this pathogen (Argudín et al., 2010). However, although direct proof of intestinal cell invasion is lacking, it is now clear that *S. aureus* is in fact a facultative intracellular pathogen in both macrophages and non-phagocytic cells, and intracellular *S. aureus* bacteria have been detected in various tissues (Horn et al., 2018; Moldovan and Fraunholz, 2019).

S. aureus can survive killing by macrophages and neutrophils (Blättner et al., 2016; Flannagan et al., 2016), even though *S. aureus*-containing phagosomes readily mature into phagolysosomes in those immune phagocytes (Hagiwara et al., 2014; Schröder et al., 2006). It is unclear whether *S. aureus*-containing phagolysosomes are fully

acidified (Flannagan et al., 2016; Giese et al., 2009; Lâm et al., 2010) or whether acidification is reduced (Jubrail et al., 2016). Regardless, *S. aureus* has developed defense mechanisms to resist the bactericidal endolysosomal compartment and to replicate inside as a vacuolar pathogen (Tranchemontagne et al., 2016). The acidic phagolysosomal environment is essential for this, as it triggers expression of the quorum-sensing accessory gene regulator (*agr*) locus which encodes several Agr virulence factors of *S. aureus* (Tranchemontagne et al., 2016). Other factors also play a role in vacuolar survival, including sigma-B, catalase, superoxide dismutase and peroxiredoxins for resistance against acidic and oxidative stress (Clauditz et al., 2006; Cosgrove et al., 2007; Karavolos et al., 2003; Liu et al., 2005; Richardson et al., 2008), as well as specific inhibitory proteins against vacuolar enzymes, such as serine proteases and myeloperoxidase (de Jong et al., 2017; Stapels et al., 2014). Modification of the bacterial membrane and the peptidoglycan layer by the lipid-modifying enzyme multiple peptide resistance factor (MrpF), and the cell-wall modifying enzymes D-alanine-D-alanyl carrier protein ligase (DltA) and O-acetyltransferase (OatA) contribute to resistance against antimicrobial peptides, such as defensins and lysozyme released from neutrophilic and cytoplasmic granules (Peschel et al., 1999, 2001). Eventually, *S. aureus* replicates within professional phagocytes and egresses from the cells, persisting in a continuous cycle of phagocytosis, host-cell death and bacterial release (Moldovan and Fraunholz, 2019).

Following infection of various epithelial cell lines and a keratinocyte cell line (Box 1), some strains of *S. aureus* escape from the vacuole and proliferate in the cytosol (Chi et al., 2014; Giese et al., 2011; Strobel et al., 2016) and these strains are more lethal than those that only reside in the vacuoles (Strobel et al., 2016). In fibroblasts and keratinocyte-like cells, replication of *S. aureus* was observed in the cytosol after escape from autophagosomes (Neumann et al., 2016). In addition, ubiquitylation of *S. aureus* was detected as taking place in the cytosol (Neumann et al., 2016). Cytosolic localization of *S. aureus* has also been detected in macrophage-like cells (Münzenmayer et al., 2016).

The key player in vacuolar escape in both professional and nonprofessional phagocytes are Agr virulence proteins; *S. aureus* mutants without a functional *agr* locus cannot escape from vacuoles (Münzenmayer et al., 2016; Neumann et al., 2016; Shompole et al., 2003). The exact mechanism by which Agr virulence proteins induce phagosomal escape is still unknown, but this might involve upregulation of the lytic proteins α-haemolysin (Giese et al., 2009; Jarry et al., 2008; Lâm et al., 2010) and δ-toxin together with the sphingomyelinase β-toxin (Giese et al., 2011). The *agr* locus also controls expression of phenol-soluble modulins (PSMs) (Münzenmayer et al., 2016), which are small amphipathic peptides with cytolytic activity that are upregulated in methicillin-resistant *S. aureus* (MRSA) strains (Queck et al., 2008; Wang et al., 2007). The deletion of the ABC transporter Pmt, which exports PSMs from the bacteria, disrupts vacuolar escape of *S. aureus* in epithelial cell lines (Blättner et al., 2016). Additionally, the nonribosomal peptide synthetase (NRPS) complex AusAB is involved in phagosomal escape, but its mode of action is still unclear (Blättner et al., 2016).

Salmonella spp.

According to the CDC, species of the gram-negative bacteria genus *Salmonella* cause 1.35 million infections annually in the USA, and especially *S. enterica* serovar Typhimurium leads to hundreds of deaths. *S.* Typhimurium invades both macrophages and non-phagocytic cells and was previously considered a vacuolar pathogen that resides exclusively in membrane-enclosed vacuoles within the host cell, called *Salmonella*-containing vacuoles (SCVs) (Jantsch et al., 2003). Work in macrophages and non-phagocytic cell lines showed that the pathogen establishes these replication vacuoles by rearranging the actin cytoskeleton and redirecting intracellular trafficking to prevent fusion with bactericidal lysosomes (Castanheira and García-del Portillo, 2017).

However, it recently has become clear that a fraction (10–20% in epithelial cells; 2–6% in macrophages) of *S.* Typhimurium cells traverses into the cytosol (Knodler et al., 2010; Malik-Kale et al., 2012). Despite these fractions being small, they are important for pathogenesis, because the cytosolic population of *S.* Typhimurium replicates more rapidly than the vacuolar population, possibly due to the higher availability of nutrients, and most *Salmonella* replication in epithelial cells occurs within the cytosol (Brumell et al., 2002; Knodler et al., 2010; Malik-Kale et al., 2012).

The invasion of *Salmonella* spp. involves many effector molecules that are injected in the host cell by type III secretion systems 1 and 2 (T3SS1 and T3SS2) (Bayer-Santos et al., 2016; McSorley, 2014). *S.* Typhimurium residing in the cytosol of various epithelial cell lines primarily expresses T3SS1, whereas the vacuolar population also expresses T3SS2 (Knodler et al., 2010). Although effector molecules injected by T3SS1 are thought to mediate the disruption of the vacuolar membrane for escape of the bacteria (Birmingham and Brumell, 2006; Miki et al., 2004), T3SS2-translocated effectors also play a role in this process, especially *Salmonella*-induced filament A (SifA) and *Salmonella*-secreted effector SseJ (Beuzón et al., 2000; Dumont et al., 2010; Lossi et al., 2008). SseJ exhibits phospholipase A and cholesterol transferase activities, which destabilize the vacuolar membrane in both epithelial cells and macrophages (Lossi et al., 2008; Ohlson et al., 2005). At least in HeLa cells, host factors are also important for vacuolar lysis of *Salmonella*-containing phagosomes, including Rab5 and Rab7a, and coat protein complex II (COP-II) (Santos et al., 2015), but the underlying mechanism is still unclear.

The disruption of the vacuolar membranes can trigger the host-cell defenses via macroautophagy in both macrophages and epithelial cell lines (Birmingham and Brumell, 2006; Thurston et al., 2016), which in HeLa cells and mouse embryonic fibroblasts can lead to membrane repair (Kreibich et al., 2015). Electron microscopy showed that this macroautophagy process requires the ubiquitylation of proteins of the ruptured *Salmonella*-containing vacuole, similar to what was described for *Shigella* spp. above (Kishi-Itakura et al., 2020). The survival and replication of the bacteria in the cytosol of infected mouse embryonic fibroblasts depend on the *Salmonella* invasion protein A (SipA), a multi-function effector protein secreted by both vacuolar and cytosolic *Salmonella* via T3SS1 (Chong et al., 2019; Klein et al., 2017). SipA prevents macroautophagy by an unknown mechanism (Chong et al., 2019), possibly by diverting organellar trafficking through effects on the cytoskeleton, as SipA also has a role in cell invasion by promoting F-actin formation and by subverting microtubule-dependent motors (Brawn et al., 2007; Chong et al., 2019). Cytosolic bacteria also secrete the T3SS1-effector SopB in infected epithelial and macrophage cell lines (Finn et al., 2017; Klein et al., 2017). SopB delays cell death in epithelial cells containing hyper-proliferating cytosolic *Salmonella*, presumably to enable longer replication times (Finn et al., 2017). A genetic screen in epithelial cell lines recently identified several other *S.* Typhimurium proteins required for efficient cytosolic proliferation, such as the transcription modulator YdgT and the DNA recombinase RecA, as well as the Mg^{2+} transport protein CorA (Wrande et al., 2016).

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Thus, *S.* Typhimurium seems to have evolved two parallel strategies to survive and proliferate in host cells – not only can it survive in vacuoles but it can also escape to the cytosol using a variety of mechanisms. This feature is shared in common with most other foodborne pathogenic bacteria described in this Review (*L. monocytogenes, Campylobacter* spp. and *S. aureus*). Only *Shigella* spp. has been exclusively described as a cytosolic pathogen, but, since all other pathogens have now been found in both vacuoles and the cytosol, our prediction is this species might also be able to persist in vacuoles.

Common strategies for vacuolar escape

For all bacteria described in this Review, vacuolar escape is observed in both infected macrophages and non-professional phagocytes (Cossart and Helenius, 2014; Haas, 2007) (Box 1). To escape from vacuoles, all these foodborne intracellular pathogens use a combination of three different effector proteins to escape from the vacuole (Figs 1 and 2). The first are hemolytic proteins or peptides that form pores and/or mechanically rupture membranes, such as LLO of *L. monocytogenes* (Bierne et al., 2018) and PSMs of *S. aureus* (Münzenmayer et al., 2016). The second category of proteins modifies phospholipids and thereby presumably weakens the integrity of the vacuolar membrane, such as PC-PLC of *L. monocytogenes* (Alberti-Segui et al., 2007). The third category of proteins promotes vacuolar escape by modifying host-cell mechanisms, such as PI-PLC of *L. monocytogenes* (Poussin et al., 2009) and IpgD of *Shigella* spp. (Mellouk et al., 2014). These three strategies are not exclusive features of foodborne pathogenic bacteria, as many non-foodborne intracellular pathogens use similar mechanisms to escape to the cytosol (Box 2). Thus, most, if not all, intracellular pathogens seem to have evolved three different mechanisms to actively disrupt the vacuolar membrane. Nevertheless, especially in macrophages and other immune phagocytes, evidence suggests that disruption of vacuolar membranes might actually be a common outcome of phagosomal maturation processes, and intracellular pathogens need to counteract this to prevent their vacuolar escape.

Lipid peroxidation in vacuolar escape

Several vacuolar pathogens might not be directly responsible for the rupture of the vacuolar membrane, but rather be involved in the maintenance of its integrity (Creasey and Isberg, 2014). For *Legionella pneumophila*, another canonical vacuolar pathogen, this maintenance requires effector proteins injected by a type 4

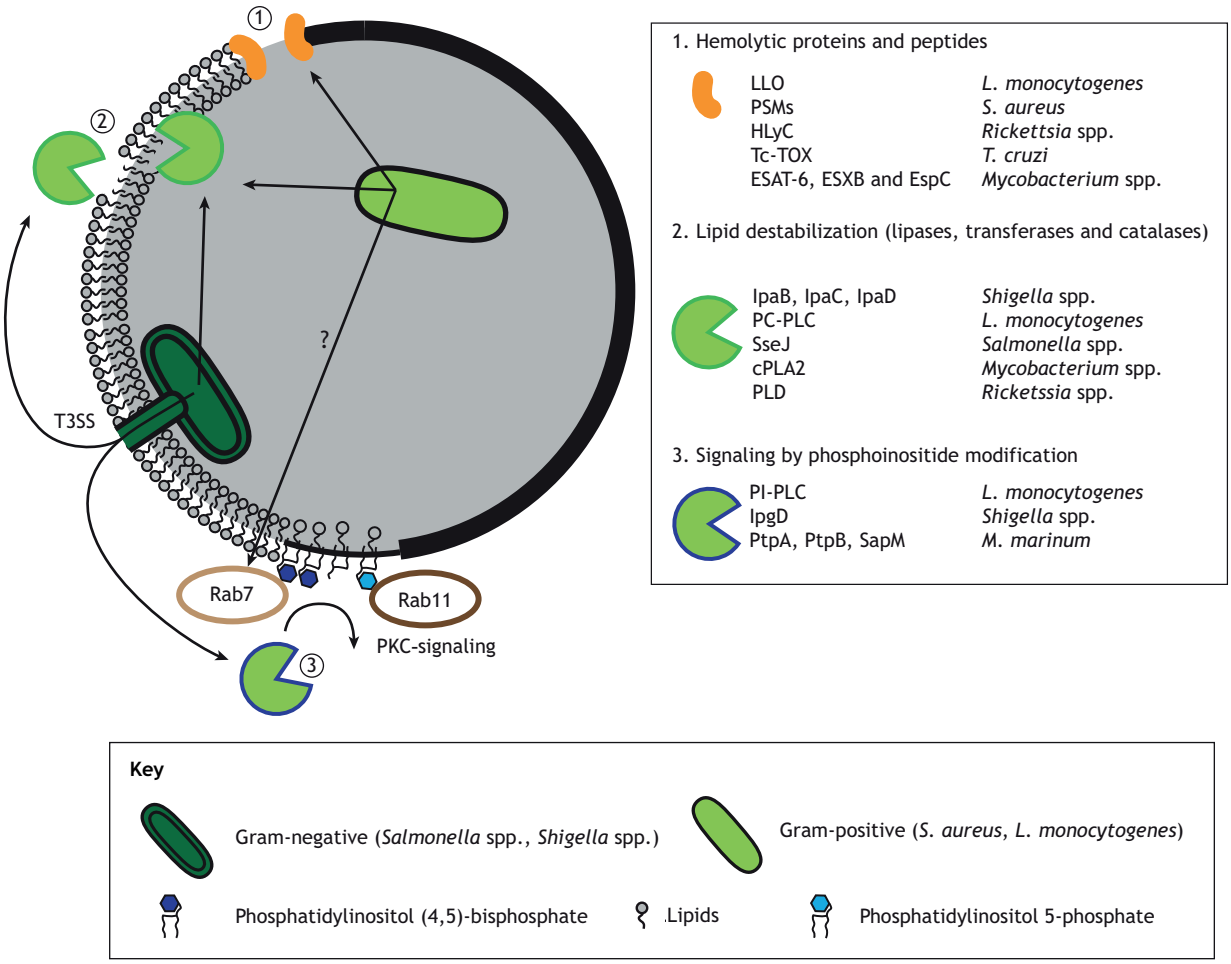

Fig. 2. Main vacuolar escape mechanisms of intracellular pathogens. Bacteria employ three main strategies for vacuolar escape, all resulting in the destabilization of the vacuolar membrane. (1) Hemolytic proteins and peptides integrate in the vacuolar membrane, creating pores. (2) Lipid-destabilizing enzymes, such as phospholipases, phospho- and sterol-transferases, degrade or change lipids and thereby rupture membranes. (3) Proteins changing the phagolysosomal identity and altering trafficking, for instance via depletion or alteration of phosphoinositide lipids by phosphatases and lipases, resulting in change of Rab GTPase and protein kinase C (PKC) signaling. Effector molecules are either secreted in the vacuolar lumen by both Gram-positive and Gram-negative bacteria or directly into the cytosol via type 3 and 4 secretion systems (T3SS and T4SS) by some Gram-negative bacteria.

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Box 2. Vacuolar escape by non-foodborne pathogens

Although *Mycobacterium* is mainly a vacuolar pathogen, some strains of *M. tuberculosis*, *M. marinum* and *M. abscessus* can escape from vacuoles into the cytosol (Jamwal et al., 2016; Kim et al., 2019; Koliwer-Brandl et al., 2019; Simeone et al., 2015) by secreting hemolytic proteins, including the early secretory antigenic protein 6 (ESAT-6), the ESAT-like protein EsxB (CFP-10) and the ESX-1 secretion-associated protein EspC (Hiller et al., 2018; Lou et al., 2017). *Mycobacterium* also secretes proteins that modify the phospholipids of the vacuole, for instance cytoplasmic phospholipase A2 (cPLA$_2$) (Jamwal et al., 2016; Lee et al., 2011) and, for *M. marinum*, the protein tyrosine phosphatases PtpA and PtpB and the secretory acid phosphatase SapM, all of which target phosphoinositides (Koliwer-Brandl et al., 2019). Mycobacteria thus rupture vacuolar membranes via both lytic proteins and phospholipid-modifying enzymes. However, *M. tuberculosis* also employs a third strategy for vacuolar escape based on phthiocerol dimycocerosates (PDIMs), which are methyl-branched long-chain fatty acids that are non-covalently associated with the cell wall of *M. tuberculosis*, and these somehow affect the integrity of the vacuolar membrane (Lerner et al., 2018; Quigley et al., 2017). *Legionella pneumophila*, another canonical vacuolar pathogen, ruptures the vacuolar membrane using the metalloprotease ProA and the chitinase ChiA (Truchan et al., 2017). The typhus group of gram-negative bacteria *Rickettsia* spp. (*R. prowazekii* and *R. typhi*) lyses the vacuolar membrane with the lytic protein hemolysin C (HlyC) (Whitworth et al., 2005) and phospholipase D (PLD) (Renesto et al., 2003). In the case of *Trypanosoma cruzi*, the secreted hemolytic toxin Tc-TOX is activated by the acidic pH in the lumen of the vacuole (Andrews et al., 1990); this pathogen also uses a trans-sialidase on its surface that removes sialic acid from vacuolar membrane proteins and makes it more prone to Tc-TOX-mediated lysis (Freire-de-Lima et al., 2016; Hall et al., 1992; Rubin-de-Celis et al., 2006). Intracellular pathogens thus, in general, employ a combination of membrane-destabilizing proteins (or lipids) and enzymes that actively remodel vacuolar lipid and protein constituents for their vacuolar escape.

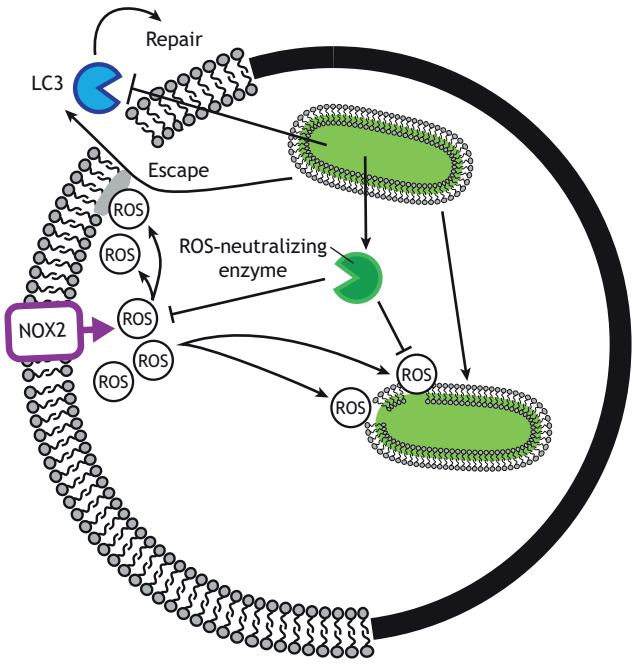

Fig. 3. Model of vacuolar escape via lipid oxidation. A proposed mechanism for vacuolar escape is the destabilization of the unsaturated phospholipids via ROS. The NADPH-oxidase NOX2 produces ROS in the lumen of the vacuole, eventually resulting in the rupture of the vacuolar membrane. To maintain the integrity of the vacuolar membrane, autophagy proteins, such as those from the LC3 family, are recruited by the infected host cell. Bacterial pathogens resist high levels of bactericidal ROS by producing neutralizing enzymes, such as catalase, superoxide dismutase and peroxiredoxins, targeting the increased ROS levels. Furthermore, pathogens (e.g. *Shigella* spp.) excrete virulence proteins preventing LC3 recruitment and thereby promoting destabilization of the vacuolar membrane and escape into the cytosol.

secretion system (T4SS) (Creasey and Isberg, 2012), because *Legionella* mutants with a dysfunctional T4SS are unable to maintain the integrity of their vacuoles in both infected macrophage-like cells and epithelial cells (Molmeret et al., 2007). One candidate for this maintenance of vacuolar membrane integrity is the T4SS-secreted protective factor SdhA, which counteracts the activity of the T2SS-secreted phospholipase A2 (Pla2) in macrophages and macrophage-like cells (Creasey and Isberg, 2012). Pla2 destabilizes the vacuolar membrane to allow *L. pneumophila* escape upon host-cell exit (Hiller et al., 2018); however, since it recently has become clear that a fraction of *L. pneumophila* escapes into the cytosol in human macrophage cell lines (Truchan et al., 2017), Pla2 might also mediate vacuolar escape for the cytosolic population. Evidence suggests that *S.* Typhimurium also actively maintains the host-cell vacuolar membrane to prevent its escape into the cytosol; in infected HeLa cells, the *Salmonella* T3SS2 effector protein SifA counteracts the membrane-destabilizing activity of SseJ and thereby stabilizes the vacuolar membrane, possibly by binding to SifA, the kinesin-interacting protein (SKIP) and the microtubule motor kinesin-1 (Boucrot et al., 2005; Dumont et al., 2010). This stabilization of *Salmonella*-containing vacuoles might be essential to prevent bacterial escape, as the switch of Rab5 to Rab7 can result in disruption of the vacuolar membrane and escape of *S.* Typhimurium into the cytosol (Brumell et al., 2002).

A potential mechanism for the nonspecific rupture of the vacuolar membrane is mediated by reactive oxygen species (ROS) produced by phagosomal NADPH oxidases and other cellular sources (Fig. 3) (Davis et al., 2012). ROS are highly reactive and indiscriminately oxidize lipids and membrane proteins (Girotti, 1998), which leads to the disruption of the integrity of biological membranes (Cwiklik and Jungwirth, 2010; Wong-Ekkabut et al., 2007). Phagosomal membrane components could be particularly vulnerable to oxidation, as NOX2 (a multi-protein complex composed of CYBA, CYBB, NCF1, NCF2 and NCF4) locates to their membranes (Buvelot et al., 2019). In macrophages, the rupture of phagosomal membranes is commonly observed when these cells ingest micrometer-sized inorganic particles (Davis et al., 2012), indicating that this can occur independently of pathogen-driven processes and that host-cell responses suffice to disrupt phagosomal membranes. Similarly in apoptotic cells, vacuolar cathepsins have been shown to be released into the cytosol due to vacuolar rupture (Johansson et al., 2010; Terman et al., 2006). This rupture might well be the result of oxidation of the phagosomal membrane, because the production of ROS by NOX2 can result in lipid peroxidation and permeabilization of the phagosomal membrane in phagocytic cells (Dingjan et al., 2016, 2017). This loss of integrity of phagosomal membranes can be prevented by the lipophilic radical scavenger vitamin E and is not observed in patients suffering from chronic granulomatous disease (CGD), a genetic disease resulting in dysfunctional NOX2 (Dingjan et al., 2016, 2017), further supporting the idea that NOX2-produced ROS can disrupt the integrity of membranes. These findings show that the loss of membrane integrity by ROS-induced oxidation is a common process that might contribute to the vacuolar escape of intracellular pathogens.

Indeed, several studies provide direct evidence that ROS play a role in the escape of intracellular pathogens into the cytosol. First, in macrophages derived from mice lacking NOX2, the escape of *L. monocytogenes* from vacuoles is inhibited (Davis et al., 2012). Second, ROS might play a role in the rupture of the vacuolar membrane by the basidiomycetous fungus *Cryptococcus neoformans* (Tucker and Casadevall, 2002). *C. neoformans* is mainly a vacuolar intracellular pathogen, but it was also recently observed to reach the cytosol in macrophages (Coelho et al., 2014). The mechanism of vacuolar rupture is unclear, but in murine alveolar macrophages, lipid peroxidation was observed upon *C. neoformans* infection and this could be attributed to ROS production by the macrophage and not the pathogen (Gross et al., 2000). Moreover, in order to survive in the hostile intracellular environment of the macrophage, *C. neoformans* within infected macrophage-like cells upregulates expression of genes involved in responses to oxidative stress (Fan et al., 2005), including SOD2 (Giles et al., 2005). Finally, as previously mentioned, *S. aureus* also expresses ROS-neutralizing enzymes in order for it to persist in the vacuole.

Thus, ROS production by infected host cells, particularly immune phagocytes, might well contribute to, and in some cases even suffice for, the rupturing of the vacuolar membrane and escape of intracellular pathogens into the cytosol. Intracellular pathogens seem to have adapted to tolerate the high levels of ROS, which actually facilitate the vacuolar escape of the pathogen. Thereby, intracellular pathogens might not only have evolved a variety of mechanisms to disrupt the vacuolar membrane themselves, but also to hijack host defense responses for propagation in the cytosol.

Concluding remarks

It has become apparent that the classical distinction between vacuolar and cytosolic intracellular pathogens does not hold. Thus, the paradigm is changing, and evidence shows that many vacuolar pathogens in fact reach the cytosol; many canonical vacuolar pathogens, such as *Mycobacterium* spp. (Jamwal et al., 2016), *Salmonella* spp. (Birmingham and Brumell, 2006; Knodler et al., 2014; Thurston et al., 2016), *Campylobacter* spp. (Nemelka et al., 2009) and *S. aureus* (Giese et al., 2011; Strobel et al., 2016) also reside in the cytosol. Accessing the nutrient-rich cytosol therefore appears to be a general characteristic for intracellular pathogens; our prediction is that other foodborne vacuolar pathogens not addressed in this Review, such as *Y. enterocolitica* (Connor et al., 2018), *Brucella* spp. (Celli, 2015) and *C. sakazakii* (de Mey et al., 1995; Mohan Nair and Venkitanarayanan, 2007), might also gain access to the cytosol at some point during their life cycle. The vacuolar and cytosolic populations might each have unique contributions to the disease pathology, as described for *S.* Typhimurium (Brumell et al., 2002; Knodler et al., 2010; Malik-Kale et al., 2012). It is therefore important to delineate the impact of the different stages in term of immune responses, namely how they contribute to bacterial hyper-replication in acute infections or to bacterial dormancy for long-term colonization in chronic infections, and resistance to antibiotic treatment.

A key open question is which conditions drive pathogens to become cytosolic or remain vacuolar and how this depends on both microbial and host-cell factors. One prediction that follows from this Review is that the vacuolar escape might be more efficient in dendritic cells than macrophages; both mouse and human dendritic cells have higher activity of NOX2 than macrophages (Mantegazza et al., 2008; Savina et al., 2006), raising the possibility that vacuolar escape by NOX2-mediated oxidation of vacuolar membranes might be more efficient in dendritic cells. However, the process and contribution of peroxidation to vacuolar escape are incompletely understood, and more research is needed to determine if and to what extent this contributes to the vacuolar escape of each pathogen.

Another limitation is that most studies have been performed *in vitro* with model cell lines, often cancerous and derived from organs other than the gut. As enterocytes and intestinal macrophages are often the main entry point for foodborne pathogens (Case and Samuel, 2016; Castanheira and García-del Portillo, 2017; Cossart and Helenius, 2014; Flieger et al., 2018; Mitchell et al., 2016; Pucciarelli and García-del Portillo, 2017), it is important to validate findings with relevant cell lines. For instance, although LLO is important for bacterial escape from the primary vacuoles (Nguyen et al., 2019), it is not always strictly essential, since vacuolar escape has been observed in various epithelial cell lines for *L. monocytogenes* mutants lacking LLO production (Marquis et al., 1995; Mueller and Freitag, 2005), although the physiological relevance of this finding is unclear. Moreover, although cytosolic populations have been observed *in vitro* for many pathogens that were previously believed to be exclusively vacuolar, this has to be confirmed *in vivo* and it has to be established in which tissue this phenomenon occurs.

The escape of pathogens from vacuoles of infected host cells can have therapeutic relevance. It is clear that although pathogens have evolved highly complex and unique vacuolar escape mechanisms, the main processes are similar across species – most (but not all) intracellular pathogens rely on a combination of lytic pore-forming proteins and enzymes that modify the vacuolar membranes, such as phospholipases. In addition, ROS production by immune phagocytes might also contribute to their escape, as argued in this Review. Understanding the fundamental features of how these mechanisms enable the escape of pathogens from vacuoles, as well as the differences and similarities between different pathogenic species, aids in development of new therapeutic approaches for the treatment of both foodborne and other intracellular pathogens.

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Competing interests

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